Métabolisme du carbone et virulence chez Neisseria meningitidis

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Résumé

Neisseria meningitidis possède un PTS incomplet. Constitué des composants générales EI et HPr et de deux EIIAs ($EIIA^{Ntr}$ et $EIIA^{Man}$), ce système ne permet pas le transport des sucres chez cette bactérie. Cependant, nous avons confirmé que la cascade de phosphorylation (EI → HPr → $EIIA^{Ntr}$) est fonctionnelle et que HPr est aussi phosphorylée sur sa Ser-46 par une HprK/P.

Dans l'objectif d'étudier l'effet de HPr sur la virulence de *N. meningitidis*, nous avons construit un mutant Δ*ptsH* chez *N. meningitidis* 2C4-3. Le mutant Δ*ptsH* a montré une faible survie chez la souris, une faible production de la capsule, une meilleure adhérence aux cellules épithéliales et un niveau élevé de cellules apoptotiques, par rapport à la souche sauvage. HPr semble intervenir dans la virulence de *N. meningitidis* en interagissant avec la protéine CrgA. L'interaction HPr/CrgA est plus forte quand HPr est phosphorylée sur sa Ser-46 par HprK/P.

N. meningitidis utilise le glucose et le maltose comme seuls sucres. Nous avons identifié une perméase à glucose (GlcP) et une perméase à maltose (MalY), responsables du transport de ces sucres.

Une perméase putative à gluconate (GntP) a été également identifiée chez *N. meningitidis* 2C4-3. Cette perméase n'assure pas le transport du gluconate, dans les conditions testées. La délétion de *gntP* chez *N. meningitidis* 2C4-3 induit une meilleure croissance sur glucose et une bonne survie du mutant Δ*gntP* chez la souris, par rapport à la souche sauvage. La fonction réelle de la perméase GntP chez *N. meningitidis* reste inconnue et suscite des études ultérieures.

Mots clés : PTS, HPr, *Neisseria meningitidis*, glucose, maltose, gluconate, virulence.

Carbon metabolism and virulence in *Neisseria meningitidis*

Abstract

Neisseria meningitidis has an incomplete PTS composed of general proteins EI and HPr and two EIIAs ($EIIA^{Ntr}$ and $EIIA^{Man}$). This system does not allow the transport of sugars in this bacterium. However, we confirmed that the phosphorylation cascade ($EI \rightarrow HPr \rightarrow EIIA^{Ntr}$) is functional and HPr is also phosphorylated at its Ser-46 by an HprK/P.

In order to study the effect of HPr on meningococcal virulence, we constructed a Δ*ptsH* mutant in *N. meningitidis* 2C4-3. Compared to the wild-type strain, the Δ*ptsH* mutant showed poor survival in mice, low production of capsule, better adherence to epithelial cells and high levels of apoptotic cells. HPr appears to be involved in the virulence of *N. meningitidis* by interacting with CrgA protein. The HPr/CrgA interaction is stronger when HPr is phosphorylated at its Ser-46 by HprK/P.

N. meningitidis uses glucose and maltose as the only sugars. We identified permeases for glucose (GlcP) and maltose (MalY), which catalyze the uptake of these sugars.

A putative gluconate permease (GntP) has also been identified in *N. meningitidis* 2C4-3. Under the conditions tested, this permease did not catalyze the transport of gluconate. Compared to the wild-type strain, deletion of *gntP* in *N. meningitidis* 2C4-3 induced faster growth on glucose and a better survival of the mutant in mice. To understand the function of the GntP permease in *N. meningitidis* further studies will have to be carried out.

Key words: HPr, *Neisseria meningitidis*, glucose, maltose, gluconate, virulence.

Table de matières

Liste des abréviations

ABC : ATP binding cassette

ADN : Acide désoxyribonucléique

ADP : Adénosine diphosphate

AMPc : Adénosine monophosphate cyclique

ARN : Acide ribonucléique

ATP : Adénosine triphosphate

CcpA : Catabolite control protein A

***cre* :** Catabolite responsive element

CREN : Contact regulatory element of Neisseria

Crp : cAMP receptor protein

Dha-P : Dihydroxyacétone-P

DO : Densité optique

DTT : Dithiothreitol

ED : Entner-Douderoff

Eda : 2-keto-3-deoxy-6-P gluconate aldolase

Edd: Gluconate-6-P déhydratase

EDTA : Ethylène di-amine tétra-acide acétique

EI : Enzyme I

EII : Enzyme II

ELISA : Enzyme Linked ImmunoSorbent Assay

EMP : Embden-Meyerhof-Parnas

FbaA : Fructose-1,6-bisphosphate aldolase

FBP : Fructose-1,6-bisphosphate

GalM : Aldose 1-epimerase

GAP : Glycéraldéhyde-3-P

Glc : Glucose

GlcP : Perméase à glucose

Glk : Glucokinase

Gnd : 6-Phosphogluconate déshydrogénase

GntK : Gluconate kinase

GntP : Perméase à gluconate

HPr : *Histidine containing protein*

HprK/P : HPr kinase/phosphorylase

IgA : Immunoglobulines A

IgG : Immunoglobulines G

IPTG : Isopropyl β-D-thiogalactopyranoside

kDa : kilo Dalton

KDPG : 2-keto-3-deoxy-6-P gluconate

LCR : liquide céphalorachidien

LPS : Lipopolysaccharide

MalP : Maltose phosphorylase

MalY : Perméase à maltose

NAD : Nicotinamide adénine dinucléotide

NADP : Nicotinamide adénine dinucléotide phosphate

Ni-NTA : Nickel-nitrilotriacetic acid-agarose

P~His-HPr : HPr phosphorylée sur son histidine 15

PAGE : Electrophorèse en gel de polyacrylamide

PBS : phosphate buffered saline

PEP : Phosphoénol pyruvate

Pfk : fructose-6-P 1-kinase

PgcM : α-Phosphoglucomutase

Pgi1 : glucose-6-phosphate isomérase

Pi : Phosphate inorganique

PP : Pentoses phosphates

PPi : Pyrophosphate inorganique

PRD : PTS regulation domain

P-Ser-HPr : Forme phosphorylée d'HPr sur la sérine 46

PTS : Phosphoenolpyruvate (PEP):-carbohydrate phosphotransferase system

RCC : Répression catabolique carbonnée

Rpe : Ribulose-5-P 3-épimérase

RpiA : Ribose-5-P isomérase A

SDS : Sodium dodécyl sulfate

Tal : Transaldolase

Tkt : Transketolase

TpiA : Triose-phosphate isomérase

Zwf : Glucose-6-phosphate 1-déshydrogénase

Liste des figures

Liste des tableaux

INTRODUCTION BIBLIOGRAPHIQUE

I. *Neisseria meningitidis*

Neisseria meningitidis, également connu sous le nom de méningocoque, est une bactérie à Gram négatif, appartenant au genre *Neisseria*. Ce genre doit son nom au bactériologiste allemand Albert Neisser, qui en 1879 fit la découverte de son premier représentant pathogène, *Neisseria gonorrhoeae* responsable de la gonorrhée, une maladie humaine sexuellement transmissible. Quelques années plus tard, en 1887, *N. meningitidis* fut découverte par le médecin autrichien Anton Weichselbaum qui l'a isolé à partir du liquide céphalo-rachidien d'un sujet atteint d'une méningite purulente.

N. meningitidis est présente sous forme asymptomatique chez 5 à 30% de la population humaine (Brandtzaeg et van Deuren, 2012). Il s'agit d'un pathogène strictement d'origine humaine. Le rhinopharynx représente l'habitat naturel pour cette bactérie ; aucun réservoir animal n'a été détecté à ce jour. Pour des raisons inconnues, *N. meningitidis* peut submerger les défenses immunitaires de l'organisme et engendrer des maladies graves parfois fatales telles que la méningite. *N. meningitidis* appartient à la classe des β-protéobactéries ; elle se présente sous forme de diplocoque à face aplatie « grain de café », asporulé, non mobile, encapsulé et non acidorésistant (Ryan, 2004 ; Ala'Aldeen et Turner, 2006). Il s'agit d'une bactérie très fragile craignant le froid et les variations du pH, sa croissance exige un environnement aérobie contenant 5% de CO_2 (Ryan, 2004).

I. 1. Interaction de *N. meningitidis* avec les cellules de l'hôte

I. 1. 1. Transmission

La transmission de *N. meningitidis* s'effectue de personne à personne par des gouttelettes de sécrétions respiratoires ou pharyngées ; cette transmission peut être transitoire conduisant à un portage asymptomatique comme elle peut déclencher une maladie invasive (Yazdankhah et Caugant, 2004 ; Mueller *et al*., 2006). Une fois avoir traversé la barrière mucosale des voies respiratoires et atteint les cellules épithéliales, la bactérie provoque la formation d'une couche de micro-colonies qui envahit la paroi épithéliale (Stephens *et al*., 1983) (Figure 1).

La transmission de *N. meningitidis* est favorisée par plusieurs facteurs tels que : l'âge, les contacts étroits et prolongés, la promiscuité avec une personne infectée, le tabagisme actif et passif et la présence d'autres infections bactériennes ou virales (Tzeng et Stephens, 2000 ; Yazdankhah et Caugant, 2004 ; MacLennan *et al*., 2006).

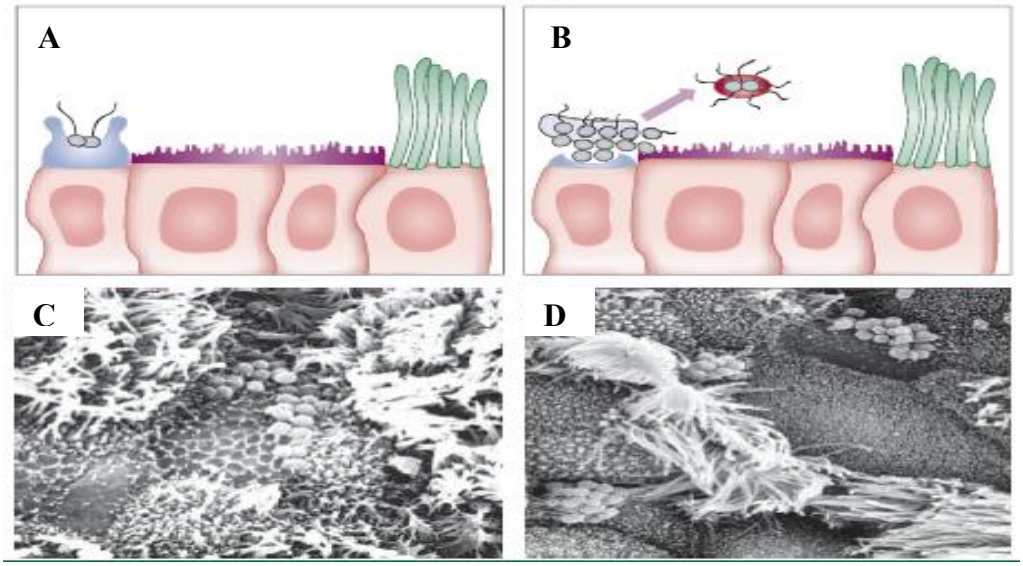


Figure 1: Représentation schématique de la colonisation des muqueuses du rhinopharynx par *N. meningitidis* (Stephens *et al*., 2007).

(A): Attachement de *N. meningitides*, **(B):** Formation de micro-colonies, **(C et D) :** Images microscopiques de la colonisation des muqueuses du rhinopharynx.

I. 1. 2. Pathogénie

La pathogénie de *N. meningitidis* commence dès sa présence dans la circulation sanguine. Cette bactérie provoque un large éventail de manifestations cliniques, qui vont d'un léger mal de gorge passager, à une méningite (37-49%), un choc septique ou méningococcémie (10-18%) ou une combinaison des deux (7-12%) (Brandtzaeg et van Deuren, 2012). D'autres manifestations moins courantes telles que : la pneumonie, l'arthrite septique, la péricardite, la conjonctivite, l'urétrite, la pharyngite, la bronchite, la myocardite et l'endocardite, peuvent être aussi liées à une infection méningococcique (Rosenstein *et al*., 2001 ; Ala'Aldeen et Turner, 2006 ; Brigham et Sandora, 2009).

I. 1. 2. 1. Méningite

La méningite est une maladie infectieuse qui se caractérise par l'inflammation des membranes qui protègent le système nerveux central « les méninges ». Il s'agit de la forme la plus fréquente des infections méningococciques ; elle peut survenir à tout âge, cependant la susceptibilité à ce genre d'infection est maximale aux deux extrêmes de la vie. La méningite peut également survenir chez les personnes ayant été en contact avec un patient atteint de méningite, les patients avec une infection oto-rhino-laryngologique (ORL) de voisinage, ceux avec une endocardite bactérienne, les toxicomanes intraveineux, les patients avec des

antécédents neurochirurgicaux et les patients immunodéprimés (asplénie, déficit de la cascade du complément, diabète, alcoolisme, VIH, etc…) (Mace, 2008).

Les symptômes de la méningite sont regroupés sous le terme de "syndrome méningé". Ce syndrome associe le plus souvent : une fièvre, des maux de tête violents, une raideur de la nuque, des vomissements, un bombement de la fontanelle (chez le nourrisson), une irritabilité et des convulsions. Une diminution du niveau de conscience et un coma peuvent également survenir (Ala'Aldeen et Turner, 2006 ; Brigham et Sandora, 2009).

I. 1. 2. 2. Méningococcémie ou « méningite fulminante »

La méningococcémie est une septicémie due à la dissémination du méningocoque dans le sang. Cette infection peut compliquer une méningite cérébro-spinale comme elle peut survenir sans atteinte des méninges. La méningococcémie se manifeste en général par les symptômes suivants : fièvre d'apparition rapide, vomissements, photophobie, convulsions, éruption cutanée, léthargie, irritabilité, somnolence, diarrhées, douleurs musculaires, arthralgie et, rarement, douleurs abdominales aiguës (Ala'Aldeen et Turner, 2006). L'éruption cutanée représente le symptôme de septicémie méningococcique le plus spécifique et le plus facile à détecter. La méningococcémie peut également induire de graves séquelles allant jusqu'à la perte de doigts ou de membres due à la coagulation intravasculaire disséminée (Ryan, 2004 ; Ala'Aldeen et Turner, 2006 ; Brigham et Sandora, 2009).

Dans des cas plus graves, la méningococcémie peut causer un choc septique qui entraîne une insuffisance respiratoire, une insuffisance rénale, un coma et parfois la mort dans les 24 heures qui suivent l'apparition des symptômes. La méningococcémie représente la forme la plus mortelle des infections liées à *N. meningitidis* avec un taux de mortalité de 16 à 52% (Ala'Aldeen et Turner, 2006 ; Brigham et Sandora, 2009 ; Brandtzaeg et van Deuren, 2012).

I. 2. Epidémiologie

La surveillance des infections invasives à méningocoques repose sur la déclaration obligatoire, ce qui permet notamment de détecter les situations épidémiques, les augmentations d'incidence et de décrire l'évolution annuelle de la maladie. En France, le Centre national de référence (CNR) des méningocoques contribue à la surveillance des clones épidémiques potentiels par les typages moléculaires de *N. meningitidis* (http://www.pasteur.fr/fr/sante/centres-nationaux-reference/les-cnr/meningocoques).

À l'échelle mondiale, on estime que *N. meningitidis* infecte plus de 500.000 personnes annuellement et qu'au moins 10% de ces cas conduisent à des décès (Brigham et Sandora, 2009 ; Rouphael et Stephens, 2012). Les sérogroupes A, B et C sont responsables de 90% des cas de méningite à méningocoques dans le monde (Figure 2). Le taux d'incidence le plus élevé est associé au sérogroupe A qui cause d'importantes épidémies dans la « ceinture de la méningite cérébrospinale» qui s'étend du Sénégal à l'ouest jusqu'à l'Éthiopie à l'est. On y dénombre environ 1000 cas pour 100.000 habitants et un taux de mortalité d'environ 75% chez les sujets de moins de 15 ans (au moment de l'épidémie) (Stephens *et al.*, 2007 ; Brigham et Sandora, 2009). Au Niger, 479 personnes sont décédées des suites de la méningite et près de 7500 personnes auraient été touchées par la maladie entre janvier et mai 2015 (http://www.ouest-france.fr/la-meningite-fait-479-depuis-le-debut-de-lannee-3431826).

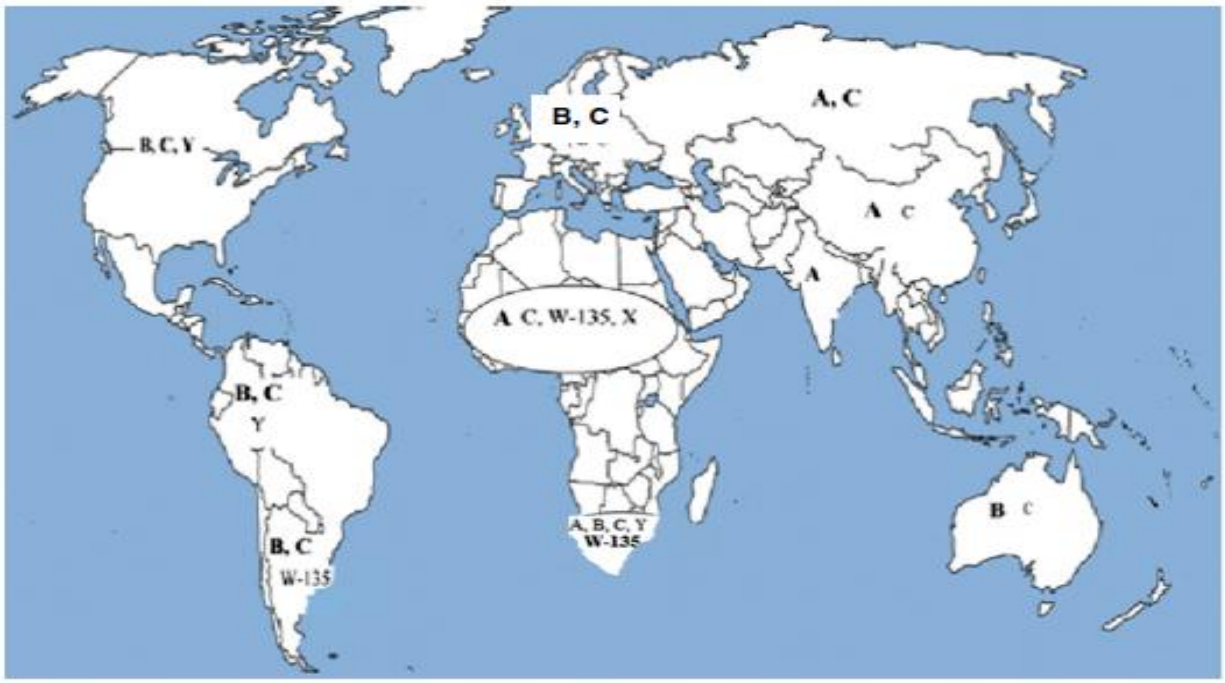


Figure 2 : Répartition des sérogroupes de *N. meningitidis* par région (Jafri *et al*., 2013).

La ceinture de la méningite est indiquée avec un cercle, les sérogroupes prédominants sont écrits en gras.

En France, le nombre des cas d'infection invasive à méningocoque (IIM) déclaré en 2014 à l'institut de veille sanitaire (InVS) était de 417 cas en France métropolitaine et de 9 cas dans les départements d'outre-mer ; avec un taux d'incidence de 0,72 cas pour 100.000 habitants. Les sérogroupes B, C, Y et W ont été responsables de 56%, 30%, 10% et 4,5% des cas, respectivement. Un seul cas dû au sérogroupe X a été rapporté (Nicand, 2015). Aux États-Unis, les sérogroupes B, C et Y sont responsables de l'infection d'environ 1400 à 2800 cas

par année pour 100.000 habitants. La fréquence la plus élevée a été observée chez les nourrissons et également chez les adolescents et les jeunes adultes (Brigham et Sandora, 2009). Les sérogroupes B et C ont également causé la majorité des cas de maladies endémiques au Canada ; tandis que le taux d'incidence de la méningite du sérogroupe Y est resté relativement stable ; cette dernière touchait généralement des adultes plus âgés (45 ans). Pour le sérogroupe W135, une éclosion de méningite est survenue en 2000 et en 2001 chez les pèlerins revenant du pèlerinage islamique annuel en Arabie saoudite et leur entourage. Le taux d'infection était de 25 cas pour 100.000 pèlerins (Wilder-Smith *et al.*, 2003).

I. 3. Structure génétique des méningocoques

Le génome de *N. meningitidis* contient approximativement 2,1 Mb codant pour environ 2000 gènes (Raymond, 2012). Une des caractéristiques principales de cette bactérie est sa capacité de transformation lui permettant des échanges génétiques avec des *Neisseria* commensales ou d'autres bactéries. Contrairement à d'autres bactéries naturellement compétentes, la transformation chez les *Neisseria* pathogènes n'est pas soumise à une régulation, ces dernières restent compétentes pendant tout leur cycle de vie (Lorenz et Wackernagel, 1994). La transformation dépend de la présence dans l'ADN exogène d'une séquence spécifique de 10 pb dite DUS (*DNA uptake sequence*) « 5'-GCCGTCTGAA- 3' ». Cette séquence représente l'élément le plus répété dans le génome du méningocoque, en effet, environ 2000 copies de DUS ont été identifiées chez *N. meningitidis* indiquant l'intégration de molécules d'ADN exogènes dans le génome de cette espèce (Kroll *et al.*, 1998 ; Smith *et al.*, 1999).

En plus de sa compétence naturelle pour la transformation, le méningocoque à une structure génomique très variable incluant les variations de phase (Hammerschmidt *et al.*, 1996a ; van der Ende *et al.*, 1999), la transposition d'éléments mobiles (Hammerschmidt *et al.*, 1996b) et aussi la présence d'éléments IS (*Insertion Sequence*) et de répétitions d'éléments et de nucléotides de tailles différentes (Davidsen et Tonjum, 2006 ; Schoen *et al.*, 2007 ; Bentley *et al.*, 2007). Cette variabilité génétique contribue à la génération de nouveaux variants qui peuvent moduler leur virulence et/ou leur transmissibilité.

I. 4. Facteurs de virulence

Plusieurs facteurs contribuent à la virulence de *N. meningitidis*. La capsule, les lipopolysaccharides (LPS), les pili et les protéines de la membrane externe constituent tous une barrière contre les peptides antimicrobiens.

I. 4. 1. Capsule

Le potentiel épidémique de *N. meningitidis* est directement lié à sa capacité d'exprimer et de modifier sa capsule. Selon ses propriétés sérologiques, 12 sérogroupes ont été décrits : A, B, C, 29E, H, I, K, L, W135, X, Y et Z ; parmi ces derniers, les groupes A, B, C, Y, W135 et X sont associés à la plupart des infections de méningites recensées dans le monde (Stephens *et al.*, 2007 ; Boisier *et al.*, 2007).

Les gènes codant les protéines de la capsule sont localisés sur un ilôt de pathogénicité de 24 kb nommé : *cps* (*capsule gene complex*) (Frosch *et al.*, 1989). Ce dernier est réparti en trois régions différentes: la région (A) code pour des protéines impliquées dans la synthèse et l'élongation des polymères de capsule. Les régions (B) et (C) codent pour le complexe d'export des polymères de capsule à travers les membranes cellulaires, par un mécanisme ATP dépendant (Tzeng *et al*., 2005) (Figure 3). Cette organisation génétique est similaire à celle d'*Haemophilus influenzae* et certaines espèces pathogènes d'*E. coli* (Roberts, 1996 ; Whitfield et Roberts, 1999).

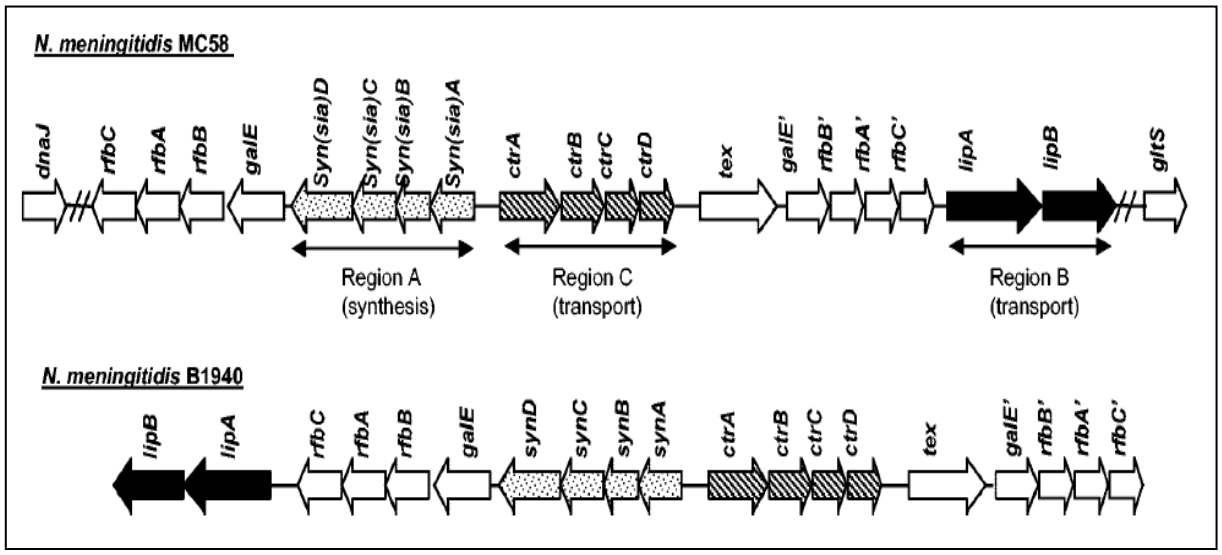


Figure 3: Organisation génétique des gènes de la capsule chez quelques souches de *N. meningitidis* (Tzeng *et al*., 2005).

La région A est composée des gènes codant pour les polymères de capsule (*siaABCD* ou nommé aussi *synABCD*). Les régions B (*lipA* et *lipB*) et C (*ctrABCD*) codent pour les transporteurs des polymères de capsule à travers les membranes cellulaires (Tzeng et *al*., 2005).

***dnaJ* :** molecular chaperone DnaJ, ***rfbC* :** dTDP-4-dehydrorhamnose 3,5-epimerase, ***rfbA* :** glucose-1-phosphate thymidylyltransferase, ***rfbB* :** dTDP-D-glucose 4,6-dehydratase, ***galE* :** UDP-glucose 4-epimerase, ***tex* :** transcriptional accessory protein Tex, ***gltS* :** sodium/glutamate symport carrier protein.

La capsule joue un rôle très important dans la pathogénie de *N. meningitidis* ; elle protège la bactérie contre les anticorps et les cellules phagocytaires, facilitant ainsi sa propagation dans le sang (Jarvis et Vedros, 1987 ; Stephens *et al.*, 2007). Des études ont rapporté que 16 à 20% des isolats de méningocoques retrouvés dans le rhinopharynx de porteurs sains ne possèdent pas les gènes de synthèse et/ou de transport des polymères de capsule (Claus *et al.*, 2002 ; Dolan-Livengood *et al.*, 2003 ; Weber *et al.*, 2006 ; Caugant *et al.*, 2007). Bien que l'expression de la capsule soit nécessaire à la virulence de la bactérie, quelques cas de méningites causées par des méningocoques non capsulés ont été également rapportés chez des patients immunocompétents (Vogel *et al.*, 2004 ; Hoang *et al.*, 2005 ; Findlow *et al.*, 2007). Les méningocoques ont également développé au cours de leur évolution la capacité de commuter leur phénotype capsulaire par le transfert horizontal de gènes (Swartley *et al.*, 1997 ; Alcala *et al.*, 2002 ; Tsang *et al.*, 2005). Ceci leur permettrait de se protéger vis-à-vis de l'immunité induite par un vaccin ou acquise naturellement (Swartley *et al.,* 1997).

À l'exception des sérogroupes A et X dont la capsule est composée des unités de N-acetyl-mannosamine-1-phosphate et N-acetyl-D-glucosamine-1-phosphate, respectivement (Liu *et al.*, 1971a ; Bundle *et al.*, 1974), la capsule des méningocoques associés aux maladies invasives est composée de plusieurs unités d'acide sialique. Les capsules de type B et C contiennent des unités d'acide poly-sialique liées en (α2→8) et (α2→9), respectivement (Liu *et al.*, 1971b), tandis que les capsules de type Y et W135 sont composées des unités d'acide sialique alternées par des unités de D-glucose et D-galactose, respectivement (Bhattacharjee *et al.*, 1976).

I. 4. 2. Lipopolysaccharides (LPS)

Les lipopolysaccharides sont des composants majeurs de la membrane externe du méningocoque. Ils sont constitués d'une partie hydrophobe ancrée dans la membrane externe (le lipide A, encore nommé endotoxine) et d'une extension oligosaccharidique hydrophile (Wright *et al.*, 2006) (Figure 4). Les LPS jouent un rôle important dans l'adhérence, la colonisation et la pathogénie de la bactérie avec une stimulation excessive du système immunitaire de l'hôte (Stephens *et al.*, 2007). Le lipide A est reconnu par le système immunitaire de l'hôte (Palsson-McDermott et O'Neill, 2004). Les études ont montré qu'environ 9% des isolats, issus de patients infectés par *N. meningitidis*, possèdent un lipide A modifié avec une des chaînes d'acide gras en moins. Ces souches présentant un lipide A modifié échappent davantage au système immunitaire de l'hôte (Fransen *et al*., 2010). Des formes modifiées de LPS ont été proposées comme de potentiels adjuvants de vaccins

méningococciques (Baldridge et Crane, 1999 ; Arigita *et al.*, 2005 ; Fransen *et al.*, 2007 ; de Vries *et al.*, 2009 ; Nagaputra *et al.*, 2014).

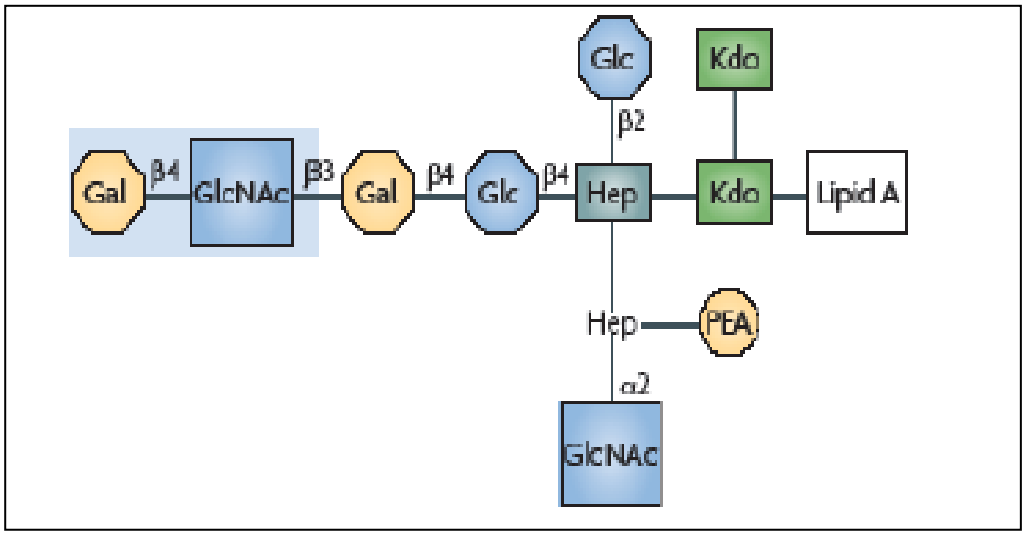


Figure 4 : Structure du LPS de *N. meningitidis* (Vasta, 2009).

Gal : Galactose, **GlcNAc :** N-acétylglucosamine , **Glc :** Glucose, , **Hep :** Heptose, **Kdo :** 2-kéto-3-déoxyoctonate, **PEA :** phosphoéthanolamine.

I. 4. 3. Protéines de la membrane externe

I. 4. 3. 1. Pili

Les pili, aussi connus sous le nom de fimbriae, sont des appendices filamenteux présents chez plusieurs bactéries à Gram négatif (Wolfgang *et al.*, 2000). Les pili de type IV (Tfp) sont les plus répandus chez les bactéries (Mattick, 2002 ; Pelicic, 2008), il s'agit de filaments flexibles et minces (5 à 8 nm) de plusieurs micromètres de longueur. Les Tfp jouent un rôle très important dans l'adhérence aux cellules épithéliales et endothéliales. Chez les méningocoques capsulés, ils semblent être le composant principal impliqué dans l'initiation de l'interaction de la bactérie avec les cellules de l'hôte (Nassif *et al.*, 1994). Les pili sont également impliqués dans l'agrégation bactérienne, la transformation et la motilité (Mattick, 2002 ; Carbonnelle *et al.*, 2006 ; Pelicic, 2008).

Une machinerie complexe d'une vingtaine de protéines est requise pour l'assemblage correct des Tfp fonctionnels (Brown *et al.*, 2010) (Figure 5). Parmi ces protéines, les protéines PilE et PilC jouent un rôle fondamental. La protéine PilE ou « piline » représente la principale sous-unité du pilus. Codée par le gène *pilE*, elle est synthétisée sous forme de pré-piline puis clivée

et N-méthylée par la peptidase PilD (Lory et Strom, 1997). La protéine PilE subit ensuite plusieurs modifications des résidus sérine en position 63 et 68 qui conduisent à sa maturation (Forest *et al.*, 1999 ; Hegge *et al.*, 2004). Des copies tronquées du gène *pilE* ont été également retrouvées dans le chromosome de *N. meningitidis*. Ces allèles incomplets, nommées *pilS* (silent) permettent, par recombinaison génétique, la synthèse de nouvelles protéines PilE.

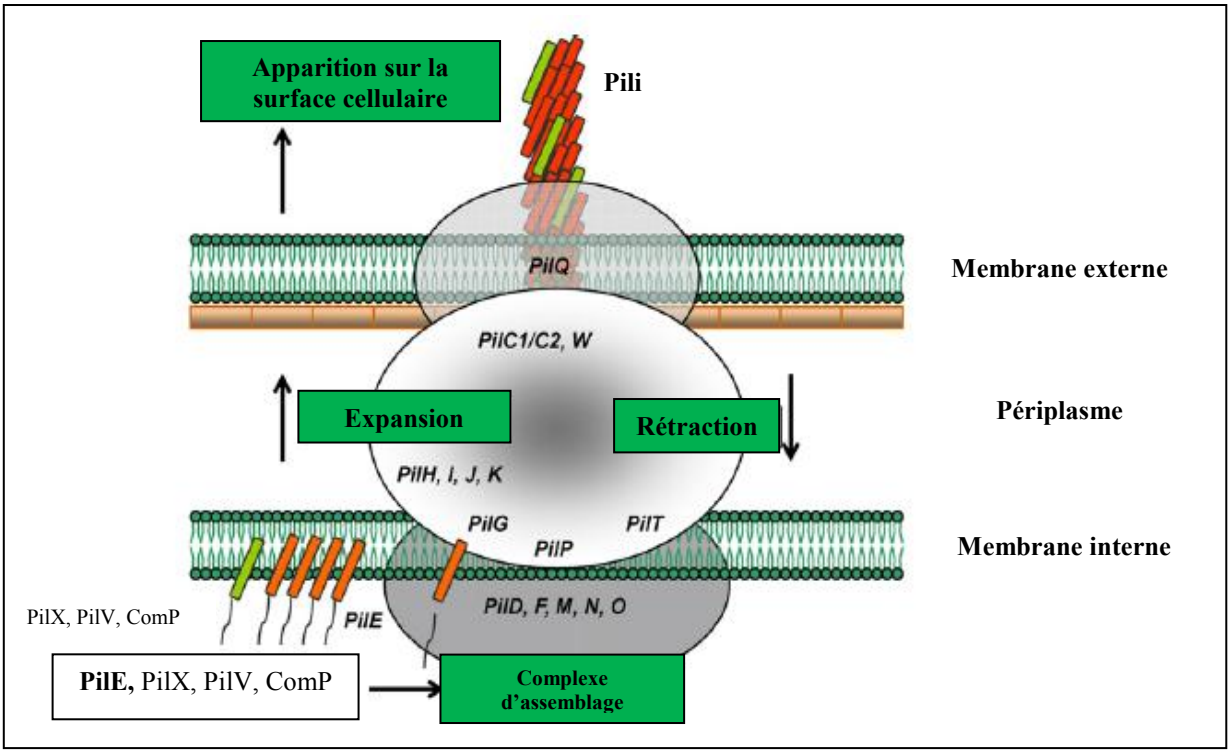


Figure 5: Représentation schématique des différentes protéines impliquées dans la biosynthèse des pili de type IV (Carbonnelle *et al*., 2009).

L'assemblage de la piline PilE est réalisé par le complexe protéique PilD, PilF, PilM, PilN, PilO, PilP dans l'interface membrane interne/périplasme. La piline est ensuite transportée vers la surface de la cellule via la sécrétine pilQ. L'élongation et la rétraction du pilus sont assurées par PilF et PilT, respectivement. Les protéines PilC1/C2, PilG, PilH, PilI, PilJ, PilK et PilW sont nécessaires pour l'expansion du pilus. Les protéines PilC1, PilW, PilI, PilJ, PilK, PilX, PilV et ComP sont importantes pour la fonction du pilus. Parmi les composants mineurs (PilH, PilI, PilJ, PilV, PilX et ComP), les protéines PilV, X et ComP sont incorporées dans la fibre du pilus.

PilC est une protéine de 110 kDa, localisée à l'extrémité du fimbriae et qui possède un domaine de fixation aux cellules de l'hôte (Rudel *et al.*, 1995). Plusieurs souches de méningocoques possèdent un ou deux loci *pilC* permettant l'expression des protéines PilC1 et PilC2. Chez *N. meningitidis* l'adhérence est assurée par l'adhésine PilC1 (Nassif *et al.*, 1994 ;

Nassif *et al.*, 1997 ; Morand *et al.*, 2004). En effet, *pilC1* est surexprimé au cours de l'adhérence initiale du méningocoque avec les cellules de l'hôte, ensuite, son expression diminue à un niveau basal après 9 h d'adhérence (Taha *et al*., 1998). L'expression du gène *pilC1* est contrôlée par une séquence de 150 pb localisée en amont de ce dernier. Cette séquence a été désignée CREN (*Contact Regulatory Element of Neisseria*) (Taha *et al.*, 1998). Au total, 16 gènes sont contrôlés par cette séquence nommée aussi Rep2 (Parkhill *et al.*, 2000). Parmi ces derniers, 14 gènes (dont *pilC1*) sont surexprimés pendant le contact initial du méningocoque avec les cellules épithéliales de l'hôte (Morelle *et al.*, 2003).

I. 4. 3. 2. Porines A et B

Les porines A et B sont les protéines les plus abondantes chez le méningocoque. Elles représentent plus de 70 % de l'ensemble des protéines de la membrane externe produites par cette bactérie. Il s'agit de protéines d'une structure trimérique (Derrick *et al.*, 1999), la protéine PorA est codée par le gène *porA* tandis que la protéine PorB est codée par deux allèles du gène *porB*. La fonction principale des porines est l'échange de cations (PorA) et d'anions (PorB) entre la bactérie et son environnement; elles permettent en parallèle la diffusion de quelques nutriments hydrophiles vers la cellule (Blake et Gotschlich, 1987 ; Tommassen *et al.*, 1990). Les porines interviennent également dans l'interaction avec les cellules de l'hôte et représentent d'importantes cibles pour les anticorps bactéricides (Tzeng et Stephens, 2000).

I. 4. 3. 3. Protéines d'opacité

Les protéines d'opacité Opa et Opc jouent un rôle important dans l'adhérence du méningocoque aux cellules épithéliales. Il s'agit de protéines de la membrane externe ayant un poids moléculaire de 28 kDa. Elles interviennent dans l'interaction entre les méningocoques non capsulés et les cellules de l'hôte. Les protéines Opa et Opc sont fortement inhibées par la sialylation des LPS (Virji *et al.*, 1992 ; Virji *et al.*, 1993 ; De Vries *et al.*, 1998). Les protéines Opa se fixent sur les protéines de la famille des CD66 favorisant ainsi l'adhérence et l'invasion des cellules épithéliales (Virji *et al.*, 1994 ; Virji *et al.*, 1996). L'expression des protéines Opc est limitée à certaines souches de méningocoques ; lorsqu'elles sont synthétisées en grandes quantités, elles augmentent considérablement l'adhérence des bactéries et l'extension de l'invasion aux cellules épithéliales et aux cellules endothéliales de la veine ombilicale humaine (Virji *et al.*, 1992 ; Sarkari *et al.*, 1994).

I. 4. 3. 4. Autotransporteurs NhhA, App, NadA et IgA1 protéase

Bien que les pili et les protéines d'opacité Opc et Opa représentent les adhésines les plus importantes et les mieux étudiées chez les méningocoques, d'autres protéines ont été également décrites comme étant essentielles à l'adhérence bactérienne aux cellules de l'hôte. Parmi ces dernières, on trouve les protéines : NhhA (*Neisseria hia/hsf homologue*), App (*Adhesion and penetration protein*), NadA (*Neisseria adhesin A*) et l'IgA1 protéase, appartenant à la famille des autotransporteurs. Les autotransporteurs sont une famille de protéines présentes chez les bactéries à Gram négatif et qui jouent un rôle important dans l'invasion (Capecchi *et al.* 2005), l'adhérence (Bullard *et al.*, 2007 ; Lipski *et al.*, 2007), la survie dans les cellules eucaryotes (Fexby *et al.*, 2007), la motilité intracellulaire (Stevens *et al.*, 2005), l'agrégation (Klemm *et al.*, 2004 ; Heras *et al.*, 2014) et la formation de biofilms (Sherlock *et al.*, 2004 ; Valle *et al.*, 2008).

Les protéines NhhA et App sont des homologues aux adhésines d'*H. influenzae* (Hadi *et al.*, 2001 ; Serruto *et al.*, 2003). Ces protéines facilitent l'adhérence du méningocoque aux cellules épithéliales de l'hôte (Serruto *et al.*, 2003 ; Scarselli *et al.*, 2006) , mais elles interviennent également dans la protection de la bactérie contre la phagocytose et les protéines du système du complément (Sjolinder *et al.*, 2008).

La protéine NadA est un autotransporteur se présentant sous forme de trimère appartenant à la famille des transporteurs Oca (*oligomeric coiled-coil adhesins*) (Comanducci *et al.*, 2002). Le gène codant la protéine NadA a été retrouvé chez 30% des isolats pathogènes de méningocoques, il est principalement associé à des souches hypervirulentes du sérogroupe B (Comanducci *et al.*, 2004; De Filippis *et al.*, 2012). La protéine NadA forme des trimères stables à la surface bactérienne, favorisant l'adhérence des bactéries aux cellules épithéliales de l'hôte (Capecchi *et al.*, 2005). Parmi les adhésines identifiées à ce jour, la protéine NadA représente un candidat prometteur pour la fabrication de vaccins méningococciques vu sa capacité à induire un niveau élevé d'anticorps chez l'Homme.

Les IgA1 protéases sont des enzymes capables de cliver les immunoglobulines A1 (IgA1) au niveau des régions charnières, entrainant la dissociation de l'anticorps (Mulks et Shoberg, 1994). Les IgA1 jouent un rôle très important dans les défenses immunitaires au niveau des muqueuses (Childers *et al*., 1989 ; Kraehenbuhl et Neutra, 1992). Chez l'Homme, 90 % des immunoglobulines A (IgA) produites dans les voies respiratoires supérieures sont des IgA1 (Engström *et al*., 1990 ; Kirkeby *et al*., 2000). En synthétisant des IgA1 protéases, *N.*

meningitidis peut échapper davantage à l'action de ces anticorps considérés comme la première ligne de défenses immunitaires contre les agents infectieux.

I. 5. Le régulateur transcriptionnel « CrgA »

Bien que les processus d'infection par *N. meningitidis* soient accompagnés de plusieurs mécanismes de régulation, le génome de cette bactérie ne code que pour quelques régulateurs transcriptionnels (Dietrich *et al.*, 2003 ; Pareja *et al.*, 2006). Parmi ces derniers on trouve : AsnC qui contrôle la réponse au stress dans des conditions défavorables en nutriments (Ren *et al.*, 2007), Fur «*ferric uptake regulator* » impliqué dans l'utilisation du fer (Grifantini *et al.*, 2003 ; Delany *et al.*, 2004), FNR «*fumarate and nitrate reductase regulator* » pour l'adaptation à des environnements à faible teneur en oxygène (Bartolini *et al.*, 2006 ; Edwards *et al.*, 2010), NsrR qui agit comme un répresseur de gènes en absence de l'oxyde nitrique (Rock *et al.*, 2007 ; Heurlier *et al.*, 2008) et CrgA «*Contact regulated gene A* » qui intervient dans l'adhérence du méningocoque aux cellules de l'hôte (Deghmane *et al*., 2000 ; Deghmane *et al.*, 2002 ; Deghmane et Taha, 2003).

CrgA est une protéine de 33 kDa appartenant à la famille des LTTRs « *LysR-type transcriptional regulators* » (Henikoff *et al.*, 1988 ; Schell, 1993 ; Zaim et Kierzek, 2003). Les LTTRs sont des régulateurs transcriptionnels qui agissent en même temps comme activateur et répresseur de l'expression d'un gène ou d'un groupe de gènes, en se fixant sur un motif T-N11-A (Schell, 1993). Les LTTRs interviennent dans plusieurs fonctions dans la cellule, telles que la division cellulaire, la virulence, la motilité, la fixation de l'azote et la production de toxines (Maddocks et Oyston, 2008).

La protéine CrgA se présente sous forme d'un octamère, chaque sous-unité est composée d'un domaine N-terminal de fixation à l'ADN contenant un motif HTH (*Helix-turn-Helix*) et d'un domaine de régulation en C-terminal (résidu 89 à 300) ; les deux domaines sont connectés par un linker (LH). Le domaine C-terminal est composé de deux sous-domaines RDI (résidu 89 à 162 et 265 à 299) et d'un sous-domaine RDII (résidu 163 à 264). L'interface RDI/RDII permet la fixation d'un effecteur putatif, supposé être capable de moduler l'activité de CrgA (Figure 6) (Sainsbury *et al.*, 2009). Cette région a été identifiée dans le domaine C-terminal de tous les LTTRs dont la structure cristallographique a été étudiée (Maddocks et Oyston, 2008).

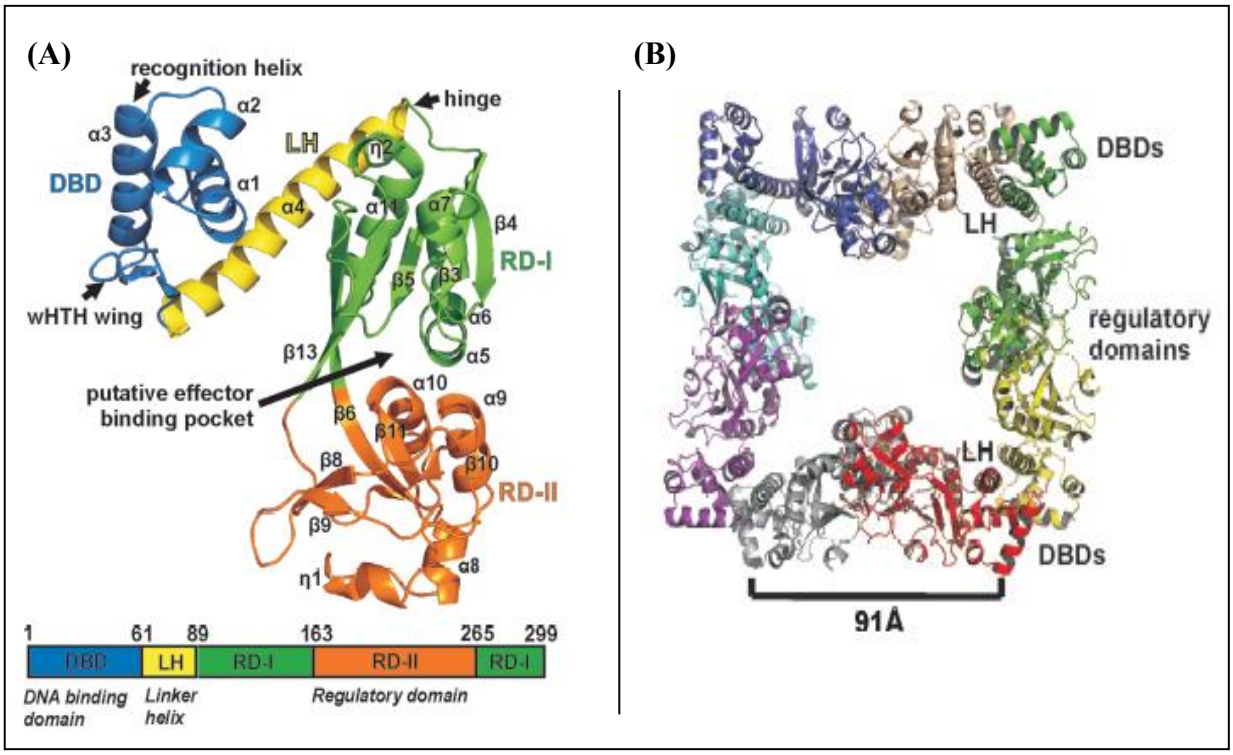


Figure 6 : Structure cristallographique du monomère (A) et de l'octamère (B) de la protéine CrgA avec ses différents domaines (Sainsbury *et al*., 2009).

(A) : forme monomérique de CrgA : la protéine CrgA est composée d'un domaine N-terminal de fixation à l'ADN (DBD) connecté via un linker (LH) au domaine C-terminal. Le domaine N-terminal contient un motif HTH (structures α1, α2, α3, β1 et β2). Le domaine C terminal est composé de deux sous-domaines RDI et d'un sous-domaine RDII. Le sous-domaine RDI forme une structure composée de 5 feuillets β (β3, β4, β5, β6 et β13) et 4 hélices α (α5, α6, α7, α11). Le sous-domaine RDII est composé de trois hélices α (α8, α9, α10) et une série de feuillets β (β7 à β12). La région de fixation de l'effecteur putatif est localisée dans l'interface RDI/RDII.

(B) : CrgA sous sa forme octamérique.

Le rôle de CrgA dans l'adhérence du méningocoque aux cellules de l'hôte a été confirmé par plusieurs études (Deghmane *et al*., 2000 ; Deghmane *et al.*, 2002 ; Deghmane et Taha, 2003). L'adhérence du méningocoque aux cellules cibles passe par deux étapes, une adhérence initiale caractérisée par la présence des pili et de la capsule et une adhérence intime pendant laquelle la bactérie rétracte ses pili et résorbe sa capsule. Le passage de l'adhérence initiale à l'adhérence intime est assuré par la protéine CrgA qui régule l'expression des gènes codant des protéines de pili et de la capsule (Deghmane *et al*., 2002). Comme *pilC1*, *crgA* est également précédé par une séquence CREN (Deghmane *et al.*, 2000). La transcription de *crgA* se fait à partir de deux promoteurs P1 et P2, qui sont localisés en amont et dans la séquence

CREN, respectivement. L'expression de *crgA* est induite de manière CREN dépendante à partir du promoteur P2 quand le méningocoque rentre en contact avec les cellules cibles (Deghmane *et al*., 2000 ; 2002). En effet, en absence de cellules épithéliales, *crgA* est exprimé à un niveau basal uniquement à partir du promoteur P1 qui ne semble pas être réprimé par CrgA. La protéine CrgA réprime sa propre expression en bloquant l'ARN polymérase sur le promoteur P2 (Deghmane *et al.*, 2002 ; 2004). En présence de cellules épithéliales, CrgA se libère de l'ARN polymérase qui transcrit alors le gène *crgA* (Deghmane *et al.*, 2004). La protéine CrgA se fixe alors sur les régions promotrices des gènes *pilC1*, *pilE* et *sia* et réprime leur expression, favorisant ainsi l'adhérence du méningocoque aux cellules épithéliales (Deghmane *et al.*, 2002). Deghmane *et al*. (2000) ont montré que l'inactivation de *crgA* réduit fortement l'adhérence intime du méningocoque aux cellules épithéliales. En revanche, chez *N. gonorrhoeae*, CrgA induit l'expression de gènes de synthèse des pili et une répression du gène *crgA* a été observée au cours de l'adhérence du gonocoque aux cellules épithéliales (Matthias et Rest, 2014).

Les études transcriptionnelles ont montré que les promoteurs du gène *crgA* (P_{crgA}) et du gène *mdaB* (P_{mdaB}) codant une NADPH-quinone oxydoréductase se chevauchent. Ieva *et al.* (2005) ont montré que, en plus de réprimer sa propre expression comme cela a été décrit par Deghmane *et al.* (2002 ; 2004), CrgA agit également comme un activateur transcriptionnel du gène *mdaB*. L'auto-répression et l'activation exercée par CrgA sur P_{crgA} et P_{mdaB}, respectivement, est augmentée en présence de l'α-méthylène-γ-butyrolactone (MBL), un inducteur de la NADPH-quinone oxydoréductase. En revanche, ces auteurs n'ont observé aucun effet concernant la régulation par CrgA ou MBL des gènes codant pour la synthèse des pili et de la capsule.

I. 6. Métabolisme et virulence chez *N. meningitidis*

En plus de la capsule et des composants de la membrane externe (pili, LPS, protéines d'opacité,…) qui jouent un rôle important dans la virulence du méningocoque, plusieurs autres protéines impliquées dans différentes voies métaboliques sont liées à la virulence de cette bactérie (Sun *et al*., 2000).

La colonisation du rhinopharynx représente la première étape du cycle de vie du méningocoque chez l'Homme. Hey *et al.* (2013) ont montré un changement du niveau d'expression de plus de 200 gènes codant des protéines à fonctions métaboliques au cours de la colonisation du rhinopharynx. Parmi ces derniers, figurent des gènes impliqués dans le

métabolisme des acides aminés et dans le transport et le métabolisme des ions inorganiques. Exley *et al.* (2005a) ont également montré une faible colonisation du rhinopharynx par des mutants Δ*lctP* déficients de la perméase à lactate, par rapport à la souche sauvage ; aucun changement n'a été détecté concernant l'expression des gènes codant les adhésines (pili et protéines d'opacité) chez ces mutants.

D'autres études ont également montré l'importance du sulfure pour la colonisation du rhinopharynx par le méningocoque. Les voies métaboliques requises pour la réduction du sulfate en sulfure sont complètes chez *N. meningitidis* et *Neisseria lactamica* qui colonisent le rhinopharynx contrairement à *N. gonorrhoeae* qui colonise les voies urogénitales (Rusniok *et al*., 2009). Les études ont également montré l'expression de plusieurs gènes codant des enzymes impliquées dans le transport du sulfate au cours de l'adhérence du méningocoque aux cellules épithéliales (Grifantini *et al*., 2002 ; Dietrich *et al*., 2003 ; Joseph *et al*., 2010).

La présence du méningocoque dans le sang est également caractérisée par l'expression de plusieurs gènes codant des enzymes impliquées dans différentes fonctions métaboliques (Echenique-Rivera *et al*., 2011 ; Hedman *et al*., 2012). Parmi ces derniers, on trouve des gènes codant des enzymes impliquées dans l'absorption du fer et dans le métabolisme du glucose, du pyruvate et du glutamate. Le glutamate joue un rôle important dans la protection du méningocoque contre les défenses immunitaires de l'hôte. Une fois transporté à la cellule via un ABC transporteur (GltT), le L-glutamate est transformé en glutathion (γ-L-glutamyl-L-cystéinylglycine, GSH) par la glutamate-cystéine ligase (GshA) et la glutathion synthétase (GshB). Le glutathion protège la bactérie contre les dérivés réactifs de l'oxygène (*Reactive Oxygen Species* (ROS)) produits par les granulocytes neutrophiles (Tala *et al*., 2011).

Le métabolisme du lactate permet également la protection du méningocoque contre le système immunitaire de l'hôte. Lo *et al*. (2009) ont montré que les mutants Δ*lctP*, incapables de transporter le lactate sont plus sensibles au système du complément de l'hôte, que la souche sauvage. En effet, les intermédiaires du catabolisme du lactate sont impliqués dans la synthèse de l'acide sialique (Exley *et al*., 2005b). Ce dernier, permet à la bactérie d'échapper au système immunitaire de l'hôte en empêchant la déposition de la molécule C3 du système du complément sur la bactérie. La sialylation des lipooligosaccharides réduit également la phagocytose des méningocoques par les cellules dendritiques (Unkmeir *et al*., 2002).

Le liquide céphalorachidien (LCR) représente un autre environnement assez différent du sang et dans lequel le méningocoque se multiplie et cause une méningite. Les études ont montré

que le lactate et le L-glutamate sont également indispensables pour la survie du méningocoque dans le LCR (Exley *et al*., 2005b ; Colicchio *et al*., 2009).

II. Le PTS (*phosphoenolpyruvate (PEP): carbohydrate phosphotransferase system*)

Le PTS, ou *phosphoenolpyruvate (PEP): carbohydrate phosphotransferase system* est le principal système de transport des sucres chez plusieurs bactéries. Il s'agit d'un complexe multi-enzymatique qui permet le transport et la phosphorylation concomitante de nombreux sucres dans la cellule. Le PTS a été décrit pour la première fois chez *E. coli* comme un simple système de transport des hydrates de carbone (Kundig *et al.*, 1964). Depuis sa découverte, le PTS a été mis en évidence chez beaucoup d'autres espèces bactériennes comprenant : firmicutes, actinobactéries, entérobactéries et également des archaea (Postma *et al.*, 1993 ; Saier et Reizer, 1994 ; Barabote et Saier, 2005 ; Bräsen *et al.*, 2014). Ce système a fait l'objet de plusieurs études montrant les différentes fonctions qu'il peut assurer chez la bactérie (Deutscher *et al.*, 2006 ; Deutscher *et al.*, 2014a).

II. 1. Composants du PTS

Le PTS est composé de deux protéines cytoplasmiques communes à tous les PTS : l'enzyme I (EI) et l'HPr (*histidine-containing protein*) et de trois ou quatre protéines spécifiques au sucre transporté : les enzymes II (EII) A, B, C et parfois D (Postma *et al.*, 1993).

II. 1. 1. Enzyme I (EI)

L'EI est la première enzyme qui intervient dans la cascade de phosphorylation du PTS. Il s'agit d'une enzyme d'environ 63 kDa codée par le gène *ptsI*. Cette enzyme est hautement conservée chez les bactéries à Gram positif et Gram négatif, notamment autour du résidu histidine phosphorylé : His-190 dans l'EI d'*Enterococcus faecalis* (Alpert *et al.*, 1985) et His-189 dans l'EI d'*E. coli* (Postma *et al.*, 1993).

L'autophosphorylation de l'EI par le PEP s'effectue en présence de cations divalents tels que le Mg^{2+} ou le Mn^{2+} et nécessite la forme dimérique de la protéine (Weigel *et al.*, 1982a). Le site actif « l'histidine phosphorylable » est localisée dans le domaine N-terminal, dans le motif conservé (GALIVMF)-X4-G-(GA)-X-(TN)-(SC)-H-(STA)-X-(LIVM)2-(STA)-R qui caractérise les enzymes utilisant le PEP (le X représente n'importe quel acide aminé) (Pocalyko *et al.*, 1990 ; Niersbach *et al.*, 1992 ; Reizer *et al.*, 1996). Le domaine C terminal est nécessaire à la dimérisation de la protéine et contient le site de fixation du PEP (Chauvin *et al.*, 1996, Seok *et al.*, 1996).

II. 1. 2. HPr (*histidine-containing protein*)

HPr est une protéine monomérique de 9 à 10 kDa (14 kDa chez certaines α-protéobactéries) codée par le gène *ptsH*. Outre son rôle dans la cascade de phosphorylation du PTS lors de l'importation des sucres, l'HPr est considérée comme un régulateur central du métabolisme carboné chez les firmicutes. En plus de sa phosphorylation sur l'His-15 par P~EI, l'HPr est également phosphorylée sur la Ser-46 par une protéine kinase ATP dépendante, l'HprK/P (HPr kinase/phosphorylase) (Deutscher et Saier, 1983 ; Deutscher *et al.*, 1986) (à voir en détails dans la partie « Fonctions régulatrices du PTS »). La protéine HPr a été purifiée chez plusieurs espèces (Postma *et al.*, 1993). Des similarités de séquences ont été retrouvées essentiellement autour du site de phosphorylation His-15 chez la plupart des entérobactéries et des firmicutes (Weigel *et al.*, 1982b ; Powers et Roseman, 1984 ; Deutscher *et al.*, 1986). Les structures cristallographiques et par résonance magnétique nucléaire (RMN) de l'HPr de plusieurs espèces ont été résolues (Van Nuland *et al.*, 1994 ; Waygood, 1998 ; Azuaga *et al.*, 2005). Cette dernière forme une structure en β-sandwich à face ouverte consistant en quatre brins antiparallèles formant des feuillets β couverts d'un côté par une courte et deux longues hélices α (Figure 7).

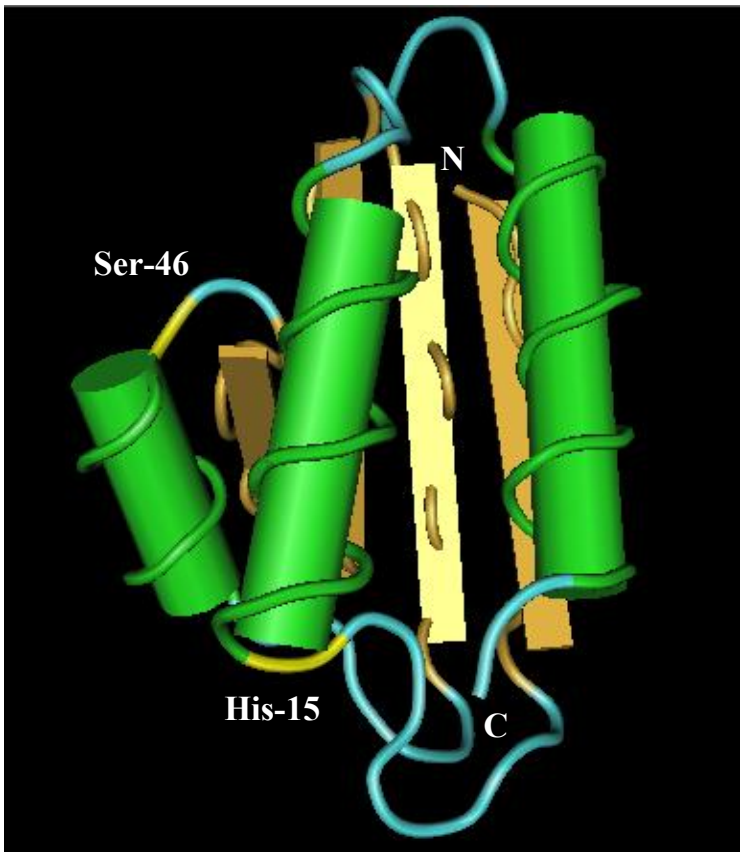


Figure 7 : Structure cristallographique de l'HPr d'*E. coli* (Van Nuland *et al.*, 1994).

Les domaines N- et C- terminale ainsi que l'histidine en position 15 et la sérine en position 46 sont indiqués. En vert : hélices α ; en jaune : feuillets β.

II. 1. 3. Enzyme II (EII)

La spécificité du PTS vis-à-vis des sucres PTS est due aux EIIs. Il s'agit d'un complexe multi-enzymatique composé de deux protéines (ou domaines) cytoplasmiques hydrophiles (EIIA et EIIB) de tailles similaires et d'une ou deux protéines (ou domaines) transmembranaires (EIIC, et parfois EIID). Ces protéines peuvent être fusionnées pour former deux, trois ou une seule protéine à plusieurs domaines. Le nombre ainsi que l'ordre des domaines sont très variables, les fusions EIIBC ou EIICB sont les plus répandues, mais on trouve aussi IIABC (EII spécifique au fructose) ou EIICBA (EII spécifique au glucose) et EIIBCA (EII spécifique aux β-glucosides). Les protéines peuvent être également séparées comme c'est le cas de l'EII du PTS lichenan/cellobiose de *Bacillus subtilis* (Tobisch *et al.*, 1997).

Selon leur spécificité de substrat, l'organisation des différents domaines protéiques et l'homologie de séquence des complexes EII, 7 familles de PTS ont été identifiées: la famille du glucose (Glc), fructose (Fru), lactose (Lac), glucitol (Gut), galactitol (Gat), mannose (Man) et de l'ascorbate (Asc) (Barabote et Saier, 2005).

Les gènes codant les EIIs appartenant au même PTS sont en général rassemblés au sein d'un même opéron. Il est en outre fréquent de retrouver au sein de ce dernier un ou plusieurs gènes impliqués dans le métabolisme du sucre transporté par le PTS, ainsi qu'un gène codant un régulateur transcriptionnel. Ce régulateur est généralement capable d'activer l'expression de l'opéron uniquement en présence de son substrat (Deutscher *et al.*, 2002).

II. 2. Cascade de phosphorylation des protéines du PTS

La cascade de phosphorylation est assurée par le phosphoenolpyruvate (PEP) comme donneur de phosphate (Kaback, 1968). L'EI va d'abord s'autophosphoryler sur l'azote ε2 d'un résidu histidine (Alpert *et al.*, 1985) en utilisant le PEP comme donneur de phosphate. Le relais phospho-protéique continue ensuite avec la phosphorylation de la protéine HPr par la P~EI sur l'azote δ1 de l'histidine en position 15. La P~His-HPr assure la transmission du phosphate sur l'azote ε2 d'une histidine de l'EIIA qui le transmet ensuite à l'EIIB. Le site de phosphorylation de l'EIIB est soit une cystéine, soit l'azote δ1 d'une histidine (PTS mannose). La P~EIIB transfère ensuite son groupement phosphate à l'OH du C-6 du sucre fixé à l'EIIC (à l'exception du fructose qui est phosphorylé sur le C-1) (Begley *et al.*, 1982). Le sucre phosphorylé est alors libéré dans le cytoplasme de la cellule (Postma *et al.*, 1993) (Figure 8).

Tous les sucres transportés par le PTS sont phosphorylés au cours de leur passage par l'EIIC, à l'exception du fucosyl-α-1,3-*N*-acetylglucosamine qui est transporté par le PTS de *Lactobacillus casei* sans être phosphorylé (Rodriguez-Diaz *et al.*, 2012).

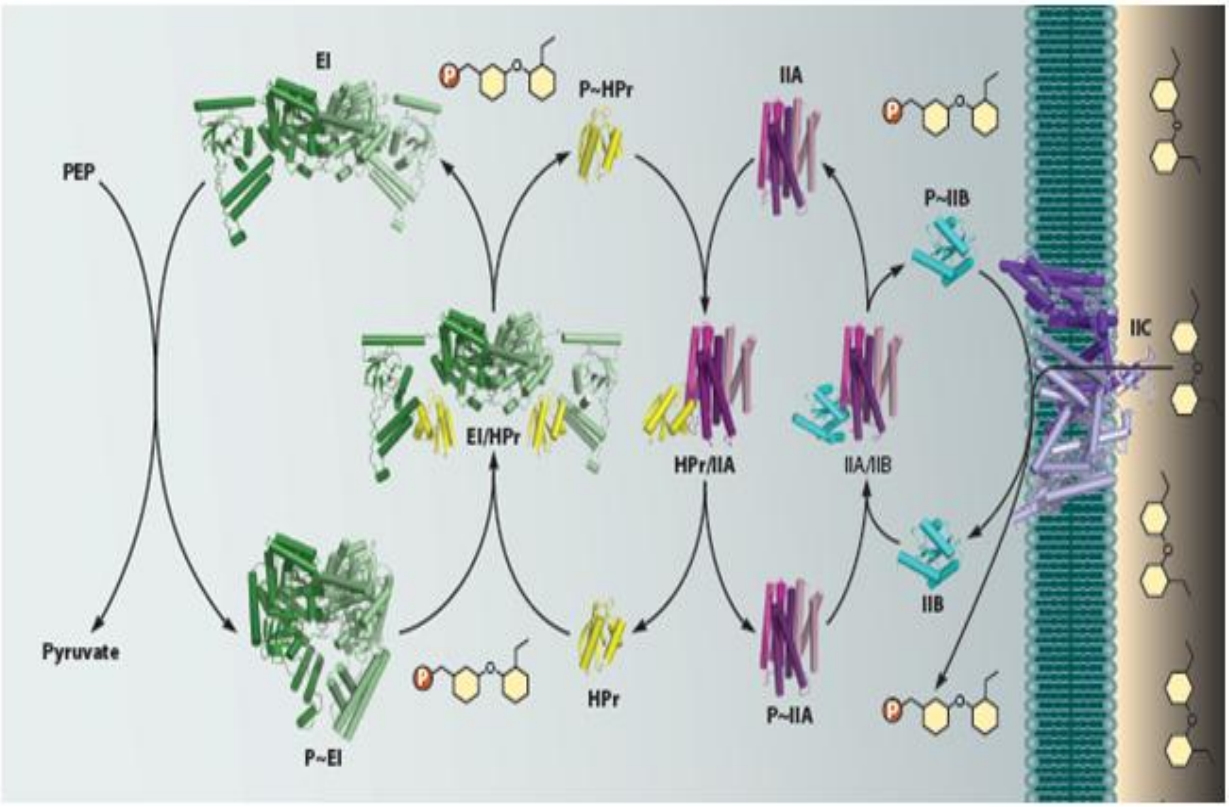


Figure 8 : Cascade de phosphorylation des protéines du PTS spécifique pour le transport du N, N'-diacetylchitobiose chez *E. coli* (Deutscher *et al*., 2014a).

II. 3. Fonctions régulatrices du PTS

Les protéines du PTS, sous formes phosphorylées ou non, exercent plusieurs fonctions régulatrices dans la cellule (Deutscher *et al.*, 2006, Görke et Stülke, 2008 ; Gabor *et al.*, 2011 ; Deutscher *et al.*, 2014a). Nous détaillons dans cette partie les effets liés à l'état de phosphorylation des protéines PTS (HPr et $EIIA^{Glc}$) et des protéines non PTS et leurs conséquences sur la régulation du métabolisme carboné chez les bactéries.

II. 3. 1. HPr et fonctions régulatrices

Chez de nombreuses bactéries, la présence dans le milieu de culture d'une source de carbone rapidement assimilable telle que le glucose, entraîne une réduction de l'expression de nombreux gènes ou opérons impliqués dans le transport ou le métabolisme d'une source de

carbone alternative. Ce phénomène est appelé répression catabolique carbonée (RCC) (Contesse *et al.*, 1969).

Chez les firmicutes, le mécanisme qui régit la RCC repose essentiellement sur la protéine HPr. En plus de sa phosphorylation par l'EI sur l'His-15 aux dépens du PEP, l'HPr est également phosphorylée sur la Ser-46 par une enzyme bifonctionnelle, l'HPr kinase/phosphorylase (HprK/P), avec l'ATP comme donneur de phosphate (Deutscher et Saier, 1983, Kravanja *et al.*, 1999). La P-Ser-HPr interagit avec la protéine CcpA (*Catabolite control protein A*) qui est un répresseur de type LacI (Henkin *et al.*, 1991, Deutscher *et al.*, 1995). Le complexe P-Ser-HPr/CcpA se fixe à l'ADN au niveau de sites spécifiques nommés *cre* (*catabolite response elements*) (Fujita *et al.*, 1995). Chez les firmicutes, ces sites sont localisés à l'intérieur ou en amont de nombreux gènes ou opérons dont ils inhibent la transcription après liaison avec le complexe P-Ser-HPr/CcpA (Figure 9).

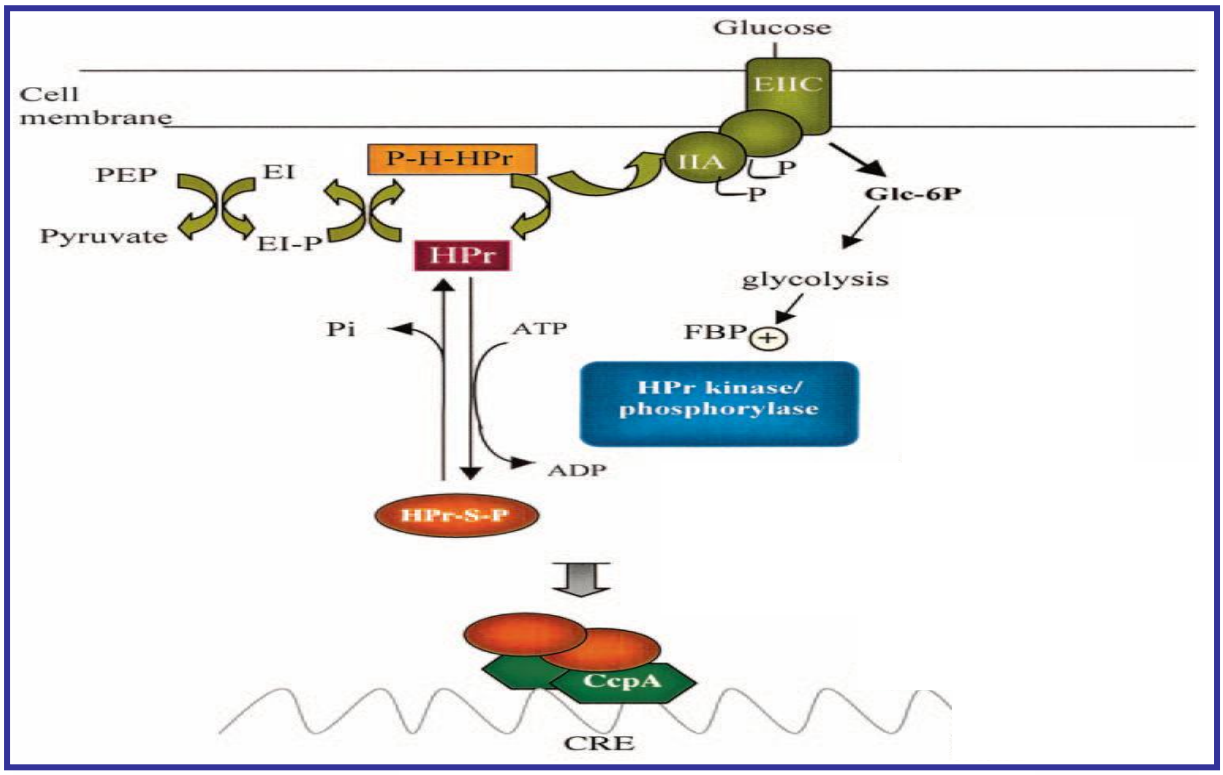


Figure 9 : Répression catabolique chez les firmicutes (Deutscher *et al.*, 2002).

La protéine HPr est phosphorylée sur la Ser-46 par l'HprK/P avec l'ATP comme donneur de phosphate. La P-Ser-HPr interagit avec la protéine CcpA et forme un complexe qui se fixe sur des sites spécifiques nommés *cre*. Ces sites sont localisés à l'intérieur ou en amont de nombreux gènes ou opérons dont ils inhibent la transcription après liaison avec le complexe P-Ser-HPr/CcpA.

L'activité kinase/phosphorylase de l'HprK/P est contrôlée par les concentrations intracellulaires en phosphate inorganique (Pi), pyrophosphate inorganique (PPi), ATP, ADP et également par la présence de certains intermédiaires glycolytiques tels que le fructose-1,6-bisphosphate (FBP) (Jault *et al.*, 2000). Le métabolisme du glucose ou d'autres sucres rapidement métabolisables induit l'augmentation des concentrations en ATP et FBP qui stimulent l'activité kinase de l'HprK/P. La capacité de l'HprK/P à déphosphoryler la P-Ser-HPr a été mise en évidence après son activité kinase (Kravanja *et al.,* 1999). Cette activité requiert la présence du Pi comme substrat avec la production de l'HPr et du PPi (Mijakovic *et al.*, 2002). Le Pi résulte de la croissance en présence de sources de carbone moins favorables. A forte concentration, il rentre en compétition avec l'ATP et le PPi pour leurs site de fixation sur l'HprK/P (Fieulaine *et al.*, 2001).

Contrairement à la P-Ser-HPr, la régulation par la P~His-HPr n'a été rapportée que dans une seule étude ou elle stimule l'activité de YesS, un activateur transcriptionnel des gènes codant les enzymes du métabolisme de la pectine et du galacturonate chez *B. subtilis* (Poncet *et al.*, 2009a).

II. 3. 2. EIIA^{Glc} et fonctions régulatrices

L'EIIA^{Glc}, nommée aussi Crr (*catabolite repression resistance*) chez les enterobactéries, joue un rôle très important dans les mécanismes de régulation chez ces organismes. En effet, ce groupe de bactéries est dépourvu de l'HprK/P et la RCC ne peut être assurée par la P-Ser-HPr. Toutefois le rôle de cette dernière est assuré par l'EIIA^{Glc} qui permet la régulation de la RCC par exclusion d'inducteur (Inada *et al.*, 1996). En effet, l'état de phosphorylation de l'EIIA^{Glc} dépend du ratio PEP/pyruvate. En présence du glucose ou d'autres sources de carbones rapidement métabolisables, le ratio PEP/pyruvate est faible et l'EIIA^{Glc} se trouve essentiellement sous forme non phosphorylée (Hogema *et al.*, 1998). L'EIIA^{Glc} non phosphorylée inhibe l'activité des perméases non PTS telles que la perméase à lactose (LacY), la protéine MalK du système ABC transporteur du maltose et certaines enzymes du métabolisme carboné telles que la glycérol kinase (GlpK) (Deutscher *et al.*, 2006 ; Chen *et al.*, 2013 ; Deutscher *et al.*, 2014a) (Figure 10).

Sous sa forme phosphorylée, l'EIIA^{Glc} intervient dans l'activation de l'adénylate cyclase (Cya). L'adénylate cyclase augmente la formation de l'adénosine monophosphate cyclique (AMPc) à partir de l'ATP (Park *et al.*, 2006). L'AMPc forme un complexe avec la protéine

Crp (*cAMP receptor protein*) qui se fixe alors sur des sites opérateurs en amont de plusieurs opérons cataboliques stimulant ainsi leurs expressions (Deutscher *et al.*, 2006) (Figure 10).

En plus de son interaction avec des transporteurs non PTS et l'adénylate cyclase, l'EIIA^{Glc}, sous forme phosphorylée ou non, interagit avec plusieurs autres protéines et régule leurs activités. Chez *Vibrio vulnificus*, l'EIIA^{Glc} non phosphorylée, interagit avec vIDE (*Vibrio insulin-degrading enzyme*), une protéine homologue des insulinases des mammifères, et stimule son activité (Kim *et al.*, 2010). Chez *E. coli*, l'EIIA^{Glc} phosphorylée est impliquée dans la régulation de l'expression du gène *rpoS* (Ueguchi *et al.*, 2001). Ce gène code pour le facteur σ^{38} qui est impliqué dans le contrôle d'une série de gènes dans les conditions de stress, telles que le manque en nutriments dans le milieu.

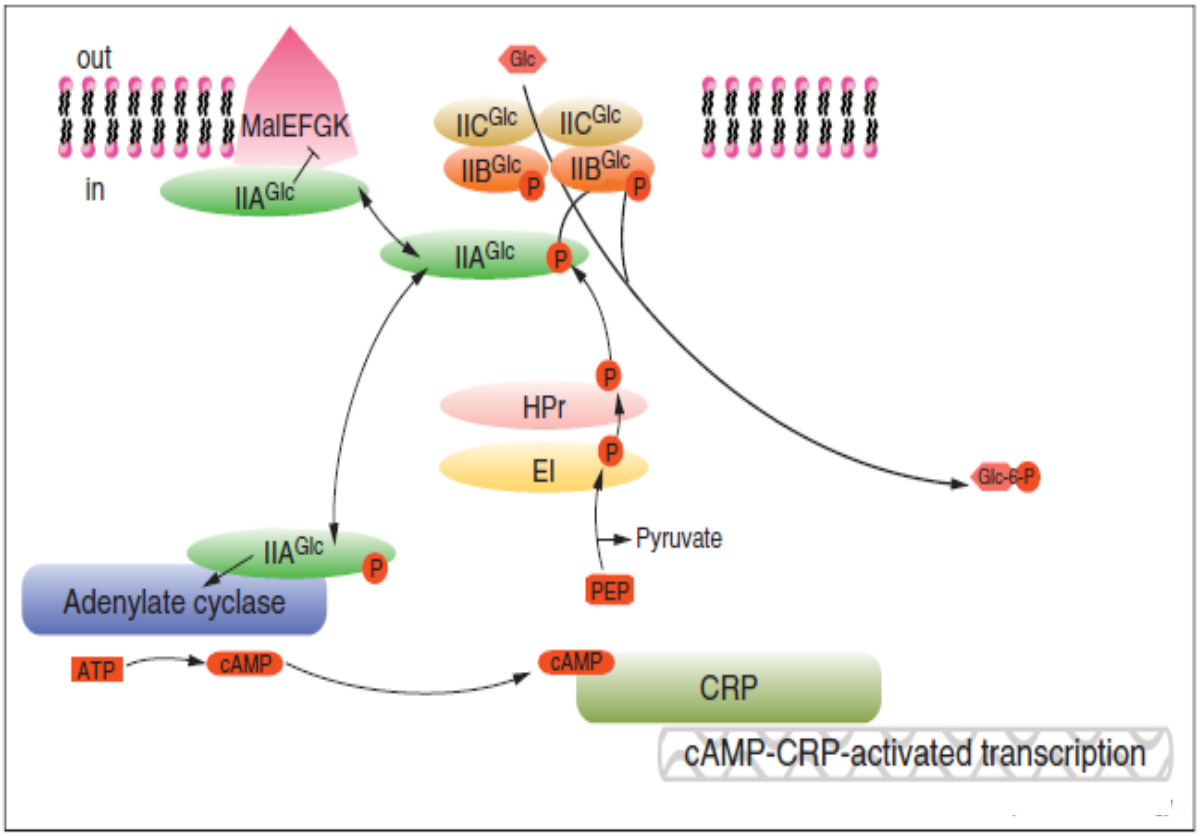


Figure 10 : Rôle de l'EIIA^{Glc} dans l'exclusion d'inducteur et la formation de l'AMPc chez *E. coli* (Vastermark et Saier, 2014).

En présence du glucose, l'EIIA^{Glc} non phosphorylée inhibe le transport des sucres non PTS tels que le maltose. L'EIIA^{Glc} interagit avec la protéine MalK du système ABC transporteur MalEFGK. D'un autre coté l'EIIA^{Glc} phosphorylée active l'adénylate cyclase qui transforme l'ATP en AMPc. L'AMPc se fixe sur le récepteur Crp et stimule l'expression de plusieurs opérons cataboliques.

II. 3. 3. Phosphorylation des protéines non PTS et régulation

En plus des fonctions régulatrices exercées par les protéines du PTS, ce système exerce également ses fonctions à travers la phosphorylation de protéines non PTS ou de leurs cofacteurs. Ces protéines possèdent, à leurs extrémités N- ou C-terminal, un ou plusieurs domaines PTS ayant conservé leurs sites de phosphorylation (histidine ou cystéine). Les domaines PTS sont en général les protéines EIIA, EIIB et EIIC de plusieurs classes de PTS (mannose, mannitol, galactitol, glucose….) mais peuvent être également des fragments de ces protéines. Dans d'autres protéines, comme HPrR de *Clostridium acetobutylicum*, on trouve aussi des domaines HPr-like (Reizer *et al.*, 1999a). La phosphorylation ou la déphosphorylation sur les domaines PTS peut engendrer des changements de structure de la protéine cible qui peuvent stimuler ou inhiber son activité. Parmi les protéines renfermant des domaines PTS on trouve les transporteurs non PTS (Poolman *et al.*, 1989), les activateurs transcriptionnels (Henstra *et al.*, 1999, Deutscher *et al.*, 2002) et plusieurs autres protéines de fonction inconnue (Barabote et Saier, 2005 ; Deutscher *et al.*, 2014a) (Figure 11).

Les protéines non PTS peuvent également renfermer des domaines spécifiques appelés PRD (*PTS regulation domain*). Ces domaines sont composés de 170 acides aminés qui se présentent sous forme de dimères (Van Tilbeurgh *et al.*, 2001 ; Graille *et al.*, 2005). Les PRDs sont souvent retrouvés dans des activateurs de transcription ou des antiterminateurs. Ils sont en général en nombre de deux, renfermant chacun 1 à 2 sites de phosphorylations potentielles par les protéines du PTS (Débarbouillé *et al.*, 1991 ; Lindner *et al.*, 1999 ; Van Tilbeurgh *et al.*, 2001 ; Deutscher *et al.*, 2006) (Figure 11). Les PRDs sont abondants chez les firmicutes et les actinobactéries et absents chez les α-, β- et δ-protéobactéries. Ces domaines sont phosphorylés soit par la P~His-HPr soit par la P~EIIB. Dans la plupart des antiterminateurs, la P~His-HPr phosphoryle une ou deux histidines dans le PRD2 (Lindner *et al.*, 1999) ce qui stimule leurs activités, tandis que la P~EIIB phosphoryle une ou deux histidines dans le PRD1, ce qui inhibe leurs activités (Tortosa *et al.*, 2001). La phosphorylation des PRDs des activateurs transcriptionnels est très variable ; Chez les activateurs LevR-like (Zébré *et al.*, 2015) et chez MtlR de *B. subtilis* (Joyet *et al.*, 2010 ; Heravi et Altenbuchner, 2014), seul le PRD2 est phosphorylé par la P~His-HPr. En revanche, chez LicR de *B. subtilis* les quatre histidines conservées dans les deux PRDs sont phosphorylées et le remplacement d'une des quatre histidines par une alanine provoque l'inactivation de ce régulateur (Tobisch *et al.*, 1999).

En plus des protéines renfermant les domaines PTS ou PRD, d'autres protéines ont également acquis des sites de phosphorylation reconnus par les protéines du PTS. La glycérol kinase « GlpK » des firmicutes représente le seul exemple étudié de ce genre de protéines (Deutscher et Sauerwald, 1986). Cette protéine possède un résidu histidyl au milieu d'une séquence de trois acides aminés aromatiques (Phe ou Tyr) (Yeh *et al.*, 2004) ; un tel site de phosphorylation n'a pas été identifié chez les protéines du PTS. Ce résidu est phosphorylé par la P~His-HPr (Deutscher et Sauerwald, 1986 ; Charrier *et al.*, 1997) et est présent uniquement chez les GlpK des firmicutes. La phosphorylation par la P~His-HPr augmente l'activité de GlpK d'un facteur de 10 à 15.

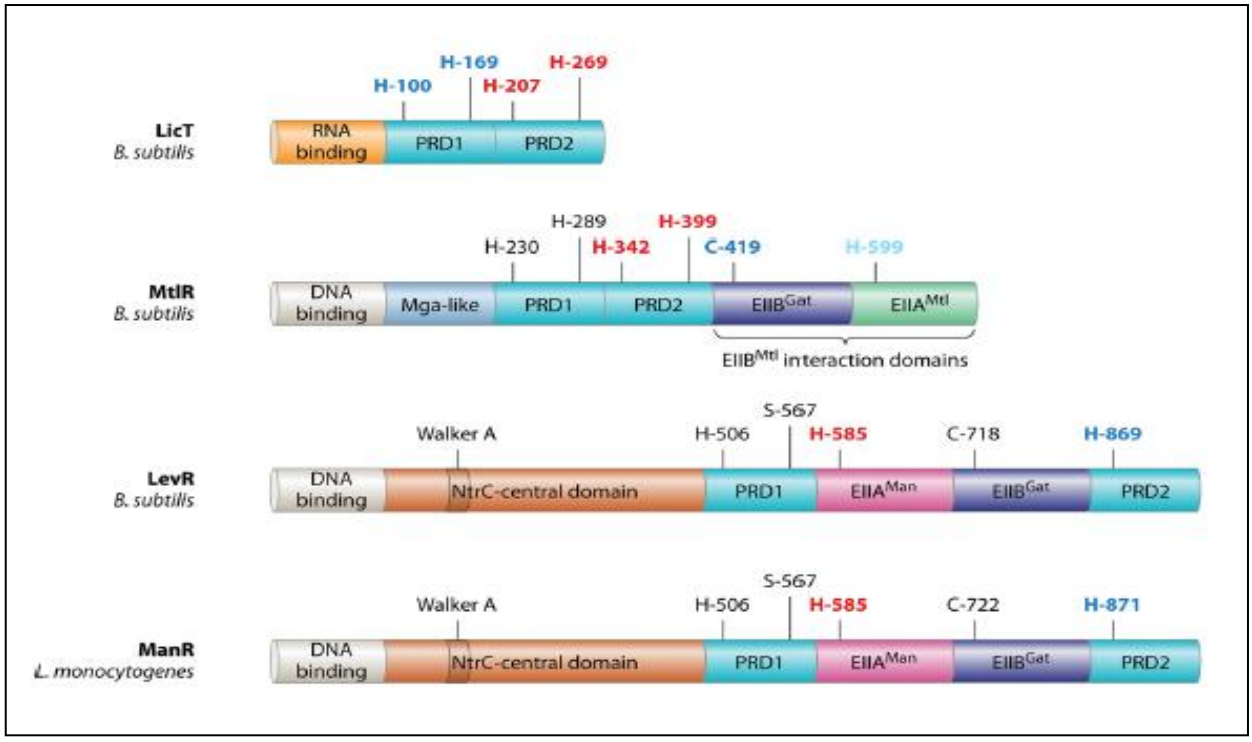


Figure 11: Représentation schématique de quelques régulateurs transcriptionnels (MtlR, LevR et ManR) avec leurs domaines PTS ($EIIB^{Gat}$, $EIIA^{Mtl}$ et $EIIA^{Man}$) et PRDs et d'un antiterminateur (LicT) avec ses PRDs (Deutscher *et al*., 2014a).

Les sites de phosphorylation par la P~His-HPr sont écrits en rouge. Les sites de phosphorylation par la P~EIIA ou la P~EIIB sont écrits en bleu (le bleu clair de H-599 de MtlR indique une faible phosphorylation).

H : histidine, **C :** cystéine, **S :** sérine, **Gat :** galactitol, **Mtl :** mannitol, **Man :** mannose.

II. 4. Le PTS^{Ntr} et ses fonctions régulatrices

Le génome de plusieurs bactéries, y compris *N. meningitidis*, renferme des gènes codant pour un PTS incomplet. Ce système, appelé PTS^{Ntr}, a été identifié pour la première fois chez *E. coli* (Powell *et al.*, 1995 ; Reizer *et al.*, 1996 ; Rabus *et al.*, 1999 ; Tchieu *et al.*, 2001). Il est composé des enzymes EI^{Ntr} (paralogue de l'EI), NPr (paralogue de HPr), EIIA^{Ntr} (paralogue de EIIA^{Fru} et EIIA^{Mtl}) et EIIA^{Man}. Ces enzymes sont codées par les gènes *ptsP*, *ptsO*, *ptsN* et *ptsM* respectivement (Reizer *et al.*, 1996 ; Rabus *et al.*, 1999). Dépourvu des enzymes EIIB et EIIC, ce système ne permet pas le transport des sucres chez les bactéries. Les gènes codant les protéines du PTS^{Ntr} sont en général à proximité du gène *rpoN* qui est impliqué dans le métabolisme de l'azote, d'où l'appellation PTS^{Ntr} (Powell *et al.*, 1995 ; Reizer *et al.*, 1996 ; Reizer et *al*., 1999b ; King et O'Brian, 2001). En plus des entérobactéries, le PTS^{Ntr} est également présent chez la plupart des α-, β- et γ-protéobactéries (*N. meningitidis*), les spirochètes (*Treponema denticola, Treponema pallidium*, *Leptospira interrogans*) et chez les chlorobi (*Chlorobium tepidum*, *Chlorobium ferrooxidans*) (Boël *et al*., 2003 ; Barabote et Saier, 2005 ; Stonestrom *et al*., 2005). Chez ces organismes, les protéines du PTS^{Ntr} sont aussi nommées EI, HPr, EIIA^{Ntr} (ou EIIA^{Fru}) et EIIA^{Man}.

II. 4. 1. Phosphorylation des protéines du PTS^{Ntr}

Comme les protéines des PTSs transportant des hydrates de carbone, les protéines du PTS^{Ntr} forment également une cascade de phosphorylation, avec le PEP comme donneur de phosphate et l'EIIA^{Ntr} comme accepteur final (Krausse *et al*., 2009 ; Pflüger-Grau et Görke, 2010). Cependant, chez certaines espèces, aucune phosphorylation n'a été observée *in vitro* pour l'EIIA^{Man} bien que le résidu histidyl soit conservé dans cette protéine (Krausse *et al*., 2009) (Figure 12). L'état de phosphorylation des protéines du PTS^{Ntr} est lié au rapport PEP/pyruvate dans le milieu (Hogema *et al*., 1998) mais aussi au rapport α-kétoglutarate/glutamine (Lee *et al*., 2013). En effet, chez *E. coli*, l'α-kétoglutarate et la glutamine se fixent sur l'EI^{Ntr} et exercent des effets antagonistes sur son activité. Par contre, chez *Sinorhizobium meliloti*, seule la glutamine se fixe sur l'EI^{Ntr} et stimule son autophosphorylation (Goodwin et Gage, 2014).

Contrairement aux entérobactéries, les autres bactéries ayant un PTS^{Ntr} possèdent également une HprK/P et la région autour de la Ser-46 (site de phosphorylation de HPr par HprK/P) à une forte similarité à celle des firmicutes. L'HprK/P permet la phosphorylation de NPr sur la Ser-46 en utilisant l'ATP comme donneur de phosphate (Krausse *et al*., 2009) (Figure 12). La

phosphorylation de NPr sur la Ser-46 semble affecter la cascade de phosphorylation des protéines du PTS^{Ntr}; en effet, la délétion du gène *hprK* chez *Brucella melitensis* conduit à une augmentation significative de la phosphorylation de l'$EIIA^{Ntr}$ (Dozot *et al*., 2010). L'activité phosphatase de l'HprK/P est très faible comme cela a été démontré chez *Agrobacterium tumefaciens* (Mijakovic *et al*., résultats non publiés) et *B. melitensis* (Dozot *et al*., 2010). Cette faible activité pourrait être due à l'absence, dans l'HprK/P, de la région C-terminale nécessaire à la déphosphorylation de la P-Ser-HPr (Fieulaine *et al*., 2001 ; Monedero *et al*., 2001 ; Poncet *et al*., 2004). À l'exception des entérobactéries qui sont dépourvus de l'HprK/P, les gènes codant les enzymes du PTS^{Ntr} et l'HprK/P sont généralement organisés en deux opérons (Krausse *et al*., 2009 ; Dozot *et al*., 2010). Un opéron dont les gènes codent l'$EIIA^{Man}$, EI^{Ntr} et NPr et un opéron codant l'HprK/P et l'$EIIA^{Ntr}$ (Krausse *et al*., 2009).

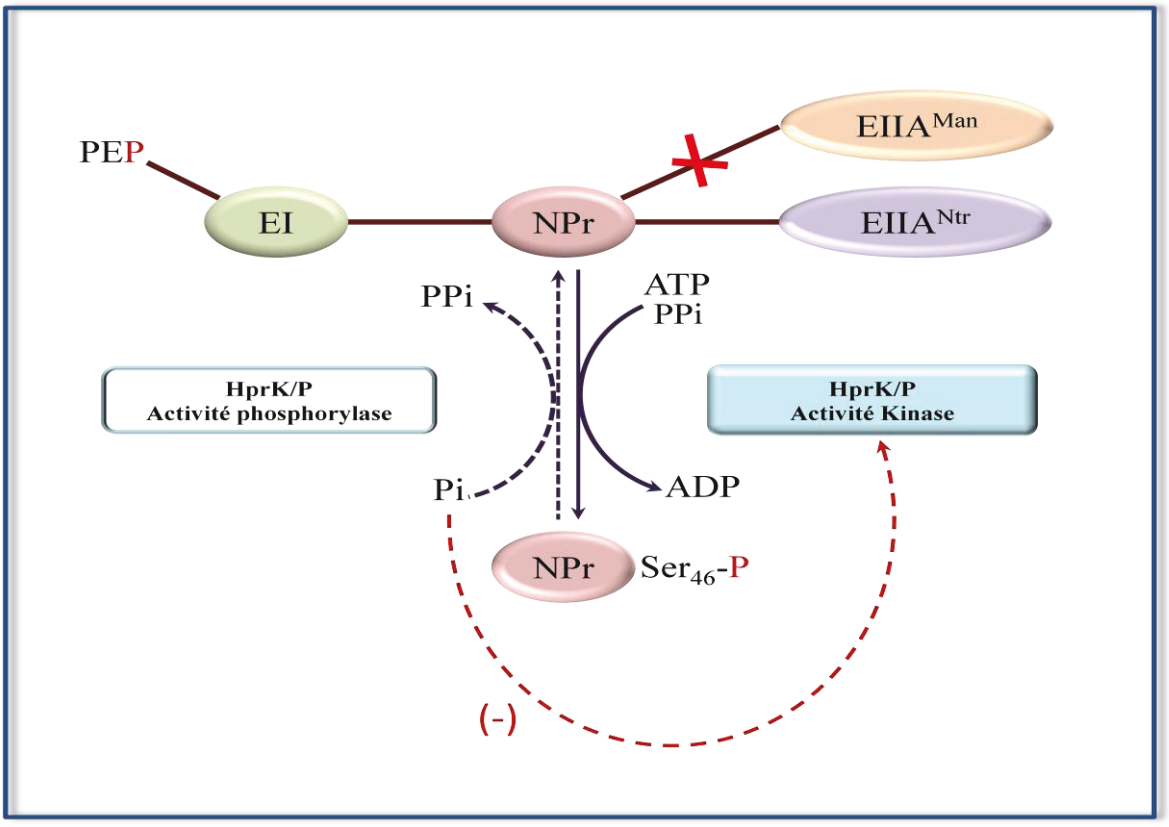


Figure 12 : Cascade de phosphorylation des protéines du PTS^{Ntr}

L'EI s'autophosphoryle en utilisant le phosphoénolpyruvate (PEP) comme donneur de phosphate. La P~EI phosphoryle ensuite l'NPr sur l'His-15 qui phosphoryle à son tour l'$EIIA^{Ntr}$ et non pas $EIIA^{Man}$. NPr est également phosphorylée sur la Ser-46 par l'HprK/P en utilisant l'ATP ou le PPi comme donneur de phosphate. Cependant la déphosphorylation de la P-Ser-NPr par l'HprK/P est très faible. Le phosphate inorganique (Pi) rentre en compétition avec l'ATP et le PPi pour leur site actif sur l'HprK/P ; à forte concentration il empêche la phosphorylation de l'NPr par l'HprK/P.

II. 4. 2. Fonctions régulatrices du PTS^{Ntr}

Le PTS^{Ntr} ne permet pas le transport des hydrates de carbone dans la cellule, mais cela n'exclut pas qu'il exerce des fonctions régulatrices (King et O'Brian, 2001 ; Lee *et al*., 2005 ; Lee *et al*., 2007; Mao *et al*., 2007 ; Noguez *et al*., 2008). En plus de son implication dans le métabolisme de l'azote et du carbone chez plusieurs bactéries (Pflüger-Grau et Görke, 2010), ce système est également impliqué dans la régulation du taux de K^+ dans la cellule. En effet, chez *E. coli*, l'$EIIA^{Ntr}$ régule le transport du K^+ en interagissant avec TrkA, une protéine soluble requise pour le transport du K^+ via le transporteur à faible affinité TrkH (Lee *et al*., 2007, Cao *et al*., 2011). L'$EIIA^{Ntr}$ interagit aussi avec la kinase KdpD d'un système à deux composants, qui contrôle l'expression de l'opéron *kdpFABC* codant un ABC transporteur de très forte affinité pour le K^+ chez *E. coli* (Lüttmann *et al*., 2009). La répression catabolique du xylène et la dégradation du toluène chez *P. putida* sont aussi contrôlées par l'$EIIA^{Ntr}$ (Cases *et al*., 1999 ; Del Castillo et Ramos, 2007).

Le PTS^{Ntr} est aussi impliqué dans la régulation de la synthèse du poly-β-hydroxybutyrate et la fixation de l'azote chez *Azotobacter vinelandii* (Segura et Espin, 1998 ; Noguez *et al*., 2008). Chez les *Rhizobia*, le PTS^{Ntr} est associé à la synthèse de la mélanine, la fixation de l'azote et la régulation de l'activité des systèmes ABC transporteurs (Michiels *et al*., 1998 ; Prell *et al*., 2012 ; Untiet *et al*., 2013).

Les protéines du PTS^{Ntr} interviennent également dans les mécanismes de virulence de certaines bactéries pathogènes, comme *Legionella pneumophila* (Higa et Edelstein, 2001) et *Salmonella enterica* serovar Typhimurium (Choi *et al*., 2010).

II. 5. PTS et virulence

Le PTS n'intervient pas seulement dans le transport des hydrates de carbone mais il est également impliqué dans plusieurs autres processus cellulaires, tels que la formation de biofilm, la chimiotaxie, la répression catabolique carbonée et la virulence (Postma *et al*., 1993 ; Chen *et al*., 1998 ; Weaver *et al*., 2000 ; Abranches *et al*., 2003 ; Loo *et al*., 2003 ; Lun et Willson, 2005).

Le lien entre les protéines du PTS et la virulence a été étudié depuis longtemps et a été établie chez plusieurs espèces pathogènes. Les premières études remontent à 1969 quand Morse et ses collaborateurs ont montré que l'entérotoxine B produite par *Staphylococcus aureus* est soumise à la répression catabolique carbonée. Depuis, plusieurs études ont été réalisées chez d'autres espèces telles que : *Clostridium perfringens, Streptococcus pyogenes*, *Vibrio*

cholerae et *Listeria monocytogenes* (Forget *et al*., 1969 ; Pine et Reeves, 1978 ; Moorthy et Watnick, 2004 ; Herro *et al*., 2005 ; Houot et Watnick, 2008 ; Bowden *et al*., 2009 ; Poncet *et al*., 2009b).

Les études réalisées sur la virulence de *L. monocytogenes*, agent responsable de la listériose, ont montré que la présence dans le milieu, de sources de carbone rapidement métabolisables et transportées par le PTS telles que le glucose, le fructose, le mannose et le cellobiose, aurait un effet inhibiteur sur PrfA, l'activateur transcriptionnel qui régule l'expression de plusieurs gènes de virulence. Cet effet n'a pas été observé en présence de sucres moins favorables et non transportés par le PTS, tels que le galactose, le glycérol et le maltose (Park et Kroll, 1993; Milenbachs *et al*., 1997; Deutscher *et al*., 2014b). Behari et Youngman (1998) ont montré que l'inactivation du gène *ccpA* n'empêche pas l'inhibition de PrfA en présence de sources de carbones rapidement métabolisables. En 2005, Herro et ses collaborateurs, ont montré un effet inhibiteur de la P-Ser-HPr sur l'activité de PrfA. Cependant, cette inhibition est indirecte et elle n'est pas due à la RCC exercée par le complexe P-Ser-HPr/CcpA. De plus, les études d'Aké *et al*. (2011) et Zébré *et al*. (2015) ont montré qu'aucun composant du système principal de glucose (le $PTS^{Man/Glc}$) n'est impliqué dans la régulation de l'expression des gènes de virulence chez *L. monocytogenes*.

La virulence chez *S. pyogenes* est aussi liée à la présence des protéines PTS. En effet, le gène codant l'activateur transcriptionnel MgA, nécessaire pour l'expression de plusieurs gènes de virulence (Hondorp et McIver, 2007) est précédé par des séquences *cre* suggérant que ce régulateur est soumis à la RCC par le complexe P-Ser-HPr/CcpA (Deutscher *et al*., 2005). Mga possède également deux domaines PRDs contenant chacun un résidu histidine conservé. La présence de ces domaines suggère une régulation de cet activateur par les protéines du PTS (Deutscher *et al*., 2005). Effectivement, Hondorp *et al*. (2013) ont montré que Mga est phosphorylé par la P~His-HPr et que cette phosphorylation module l'expression des gènes de virulence chez *S. pyogenes*.

Le PTS joue également un rôle dans la virulence et la formation des biofilms chez plusieurs organismes (Loo *et al*., 2003 ; Abranches *et al*., 2006 ; Rouquet *et al*., 2009 ; Houot *et al*., 2010). Chez *V. cholerae,* un lien direct a été établi entre la présence de sucres transportés par le PTS et l'activation de la transcription des gènes *vps* (*vibrio polysaccharide*) codant pour la formation de biofilms (Moorthy et Watnick, 2004).

Le lien entre les protéines du PTS et la virulence a été également étudié chez *Salmonella. enterica* serovar Typhimurium, agent responsable des gastroentérites. Bowden *et al*. (2009)

ont montré que l'inactivation de l'EI et de l'HPr empêcherait la réplication de cette bactérie dans les macrophages. De plus, la délétion du gène *crr* codant l'EIIA^{Glc} diminue la virulence de cette espèce. En effet, Mazé *et al*. (2014) ont montré que l'EIIA^{Glc} interagit avec des protéines d'un système de sécrétion de type III (SST3), nécessaire pour le transport des facteurs de virulence vers le cytoplasme des cellules cibles. De façon similaire, l'inactivation de l'EI (EI^{Ntr}) ou de l'HPr (NPr) de *B. melitensis* réduit significativement la synthèse des protéines VirB qui forment un système de sécrétion de type IV, un autre système nécessaire pour les échanges génétiques et la libération de toxines dans la cellule cible (Dozot *et al*., 2010).

Chez *N. meningitidis*, le lien entre les protéines du PTS et la virulence a été confirmé par les seuls travaux de Sun *et al*. (2000) qui ont montré l'implication de plusieurs gènes dans la virulence du méningocoque, y compris des gènes codant l'HPr et l'EI.

D'autres protéines comme CcpA et HprK/P jouent également un rôle non négligeable dans la virulence des bactéries. La protéine CcpA est nécessaire pour la sporulation, la production de la collagénase et la répression des pili de type IV chez *C. perfringens* (Varga *et al*., 2004 ; Mendez *et al*., 2008). Chez *Streptococcus pneumoniae*, l'inactivation de CcpA affecte la synthèse de la capsule et la virulence (Giammarinaro et Paton, 2002). CcpA contrôle également la synthèse de BgaA (*surface-attached β-galactosidase*), une protéine nécessaire pour la colonisation du rhinopharynx par cette bactérie (Kaufman et Yother, 2007). Chez *Clostridium difficile* CcpA inhibe l'expression des gènes codant pour les toxines en réponse à la disponibilité du sucre PTS dans le milieu (Antunes *et al*., 2011).

La protéine HprK/P est présente chez la plupart des bactéries possédant un PTS complet mais également chez des bactéries ayant des systèmes incomplets, comme *N. meningitidis*, *Treponema denticola* (Boël *et al*., 2003), *B. melitensis* (Dozot *et al*., 2010) et *A. tumefaciens* (Mijakovic *et al*., résultats non publiés). Chez ces bactéries, l'HprK/P phosphoryle l'HPr sur la Ser-46 mais joue également un rôle dans leur virulence. En effet, chez A. *tumefaciens* le gène *hprK* est organisé en opéron avec les gènes *chvI* et *chvG* (Boël *et al*., 2003). Ces gènes codent pour des régulateurs transcriptionnels impliqués dans la virulence chez cette espèce (Charles et Nester, 1993). Aussi l'inactivation du gène *hprK* chez *N. meningitidis* augmente la synthèse de la capsule polysaccharidique induisant une réduction significative de l'adhérence aux cellules de l'hôte (Boël *et al*., 2003).

But et organisation de la recherche

N. meningitidis est une bactérie pathogène exclusivement d'origine humaine. Cette bactérie est présente naturellement à l'état asymptomatique dans le rhinopharynx de l'Homme, mais pour des raisons inconnues elle devient pathogène et cause une méningite ou une septicémie mortelle.

L'objectif principal de ce travail est d'étudier le lien entre le métabolisme du carbone et la virulence chez *N. meningitidis*. Les différentes techniques utilisées sont décrites dans la partie Matériel et méthodes. Les résultats obtenus sont présentés sous forme de deux articles précédés chacun par une présentation synthétique des résultats. Le premier article est intitulé: « **The phosphocarrier protein HPr of *Neisseria meningitidis* interacts with the transcription regulator CrgA and its deletion affects capsule production, cell adhesion and virulence** ». Dans cet article, un lien entre la virulence de *N. meningitidis* et la protéine HPr (protéine clé du PTS) a été mis en évidence par différentes techniques *in vivo* et *in vitro*. La protéine HPr exerce son effet sur la virulence du méningocoque en interagissant avec le régulateur transcriptionnel CrgA qui régule l'expression des gènes codant les protéines des pili et de la capsule. Dans cet article, on présente également les résultats des tests de phosphorylation des protéines PTS de *N. meningitidis* (EI, HPr, $EIIA^{Ntr}$ et $EIIA^{Man}$) en présence du PEP comme donneur de phosphate. Les résultats de la phosphorylation de HPr (sur la sérine 46) par la protéine HprK/P en présence de l'ATP ou du PPi comme donneurs de phosphate sont également présentés. La phosphorylation de l'HPr sur sa Ser-46 semble augmenter son interaction avec la protéine CrgA.

Le deuxième article est intitulé : « **Transport and catabolism of carbohydrates by *Neisseria meningitidis* ** ». Une partie de ce travail a été consacrée à l'identification des perméases à glucose et à maltose chez *N. meningitidis*. Nous avons également identifié une nouvelle perméase putative à gluconate chez le méningocoque. Le rôle de cette perméase dans le transport du gluconate chez le méningocoque n'a pas été démontré dans les conditions expérimentales testées. En revanche, il semble que la perméase à gluconate soit impliquée dans la virulence de cette bactérie. Dans cet article, nous avons aussi décrit les principales enzymes impliquées dans le métabolisme du glucose et du maltose chez *N. meningitidis*. Ces enzymes ont été purifiées et leurs activités ont été confirmées par des tests *in vitro*.

Le métabolisme du glucose et du maltose chez *N. meningitidis* conduit à la production de plusieurs métabolites, entre autres : le fructose 1,6 biphosphate (FBP), l'ATP et le

phosphoénol pyruvate (PEP). Le PEP est utilisé comme donneur de phosphate au cours de la cascade de phosphorylation des protéines PTS. En présence du PEP, l'EI de *N. meningitidis* s'autophosphoryle et permet ensuite la phosphorylation de la protéine HPr sur son His-15. L'ATP est également utilisée comme source de phosphate par l'HprK/P qui phosphoryle l'HPr sur sa Ser-46 ; l'activité de l'HprK/P est stimulée par le FBP. L'état de phosphorylation de l'HPr pourrait avoir des effets sur la virulence du méningocoque. Ces effets méritent d'êtres étudiés dans des travaux ultérieurs.

Les articles présentés dans ce travail sont suivis d'une partie « Résultats supplémentaires » et d'une partie « Discussion ». Une conclusion générale et des perspectives sont également présentées à la fin de ce manuscrit.

MATÉRIEL

ET

MÉTHODES

I. Souches utilisées

Ce travail a été réalisé sur *N. meningitidis* 2C4-3, un variant isolé à partir de la souche 8013 du sérogroupe C (Nassif *et al*., 1993), dont le génome a été entièrement séquencé (http://www.ncbi.nlm.nih.gov/nuccore/385323172?report=fasta). La souche *N. meningitidis* MC58 ayant une capsule du sérogoupe B a été également utilisée dans quelques expériences, comme contrôle négatif pour la production de la capsule du sérogroupe C (http://www.ncbi.nlm.nih.gov/nuccore/385323172?report=fasta).

Des souches d'*Escherichia coli* ont été utilisées afin de surproduire des protéines recombinantes. Les clonages des gènes d'intérêts ont été effectués dans les souches d'*E. coli* NM522 (Stratagene). Les purifications des protéines recombinantes ont été réalisées dans les souches NM522 [pREP4-GroES/EL] (Amrein *et al*., 1995) et BL21(DE3) (Merck Millipore).

II. Milieux de culture

Les différentes cultures de *N. meningitidis* sont réalisées en milieu GCB (*GC medium base*, Difco), avec ou sans antibiotique, additionné de supplément I (1%) et supplément II (0.1%) (Kellogg *et al*., 1963). L'incubation se fait à une température de 37°C dans une atmosphère contenant 5% de CO_2. D'autres milieux ont été également utilisés comme le RPMI (*Roswell Park Memorial Institute*, Life Technologies) et le CTA (*Cystine Trypticase Agar*, Difco). Ces milieux sont dépourvus de sources de carbone et nécessite leurs complémentations afin de faire les tests voulus. Les cultures d'*E. coli* ont été réalisées sur LB (*Luria Bertani*, Difco) en présence ou en absence d'antibiotique.

III. Méthodes concernant l'ADN

III. 1. Extraction de l'ADN génomique de *N. meningitidis*

III. 1. 1. Par choc thermique

Le principe de cette technique est basé sur la lyse des membranes cellulaires et la libération de l'ADN suite à un changement brusque de température. Pour cela, une suspension bactérienne est préparée dans 100 µl d'eau distillée stérile, à partir de colonies isolées d'une culture de 18 h sur gélose GCB. La suspension est ensuite congelée pendant quelques minutes à -80°C et immédiatement chauffée pendant 10 min à 100°C. Cette étape est suivie d'une centrifugation de 10 min à 16000 rpm afin d'éliminer les débris cellulaires. L'ADN présent dans la phase aqueuse est alors récupéré et conservé à -20°C.

III. 1. 2. Par lyse enzymatique

Cette méthode est basée sur la lyse des membranes cellulaires sous l'action de la protéinase K. Une suspension bactérienne est alors incubée pendant 1 h à 65°C dans le tampon TE en présence de 0.05% SDS et 0.1 µg/µl de protéinase K. Un volume équivalent de phénol/chloroforme (1:1) est ensuite ajouté au mélange et l'ensemble est centrifugé pendant 10 min à 13000 rpm. La phase aqueuse est ensuite reprise dans un volume équivalent de chloroforme et la solution est centrifugée 10 min à 13000 rpm. L'ADN présent dans la phase aqueuse est ensuite précipité pendant 30 min à -20°C en présence de 0.6 volumes d'isopropanol. Après centrifugation de 30 min à 13000 rpm, le culot est lavé avec 1 ml d'éthanol à 70%, resuspendu dans 100 µl de tampon TE contenant 1 mg/ml de RNase A et incubé 1 h à 37°C.

III. 2. Amplifications PCR

Les gènes étudiés dans ce travail ont été amplifiés par PCR, en utilisant des couples d'amorces contenant dans leur région 5' un site de restriction permettant l'insertion du gène amplifié dans le vecteur correspondant (Tableau 1). Les sites de restriction ont été définis sur le site (http://nc2.neb.com/NEBcutter2/).

Les réactions de PCR ont été réalisées dans un volume final de 50 µl sur environ 270 ng d'ADN en présence de : tampon GoTaq 1X, 1 µM de chacune des deux amorces, 0.2 mM de dNTP et de 1.25 U de GoTaq DNA Polymerase (Promega). Les conditions d'amplification sont: 1 cycle de dénaturation initiale de 2 min à 95°C, 30 cycles comprenant : une dénaturation à 95°C pendant 30 s, une hybridation à 56°C pendant 40 s et une élongation à 72°C (1 min/kb). Un dernier cycle de 5 min à 72°C assure une élongation complète des produits d'amplification. La purification des produits de PCR est effectuée avec le Kit *Wizard SV Gel and PCR Clean-Up System* (Promega) suivant le protocole décrit par le fournisseur.

III. 3. Vecteurs de clonage

Les vecteurs d'expression pQE30 (QIAGEN), pET28b (Novagen) et pGP172 (Prof. J. Stülke, Göttingen) ont été utilisés pour la surproduction des protéines recombinantes (Figure 13).

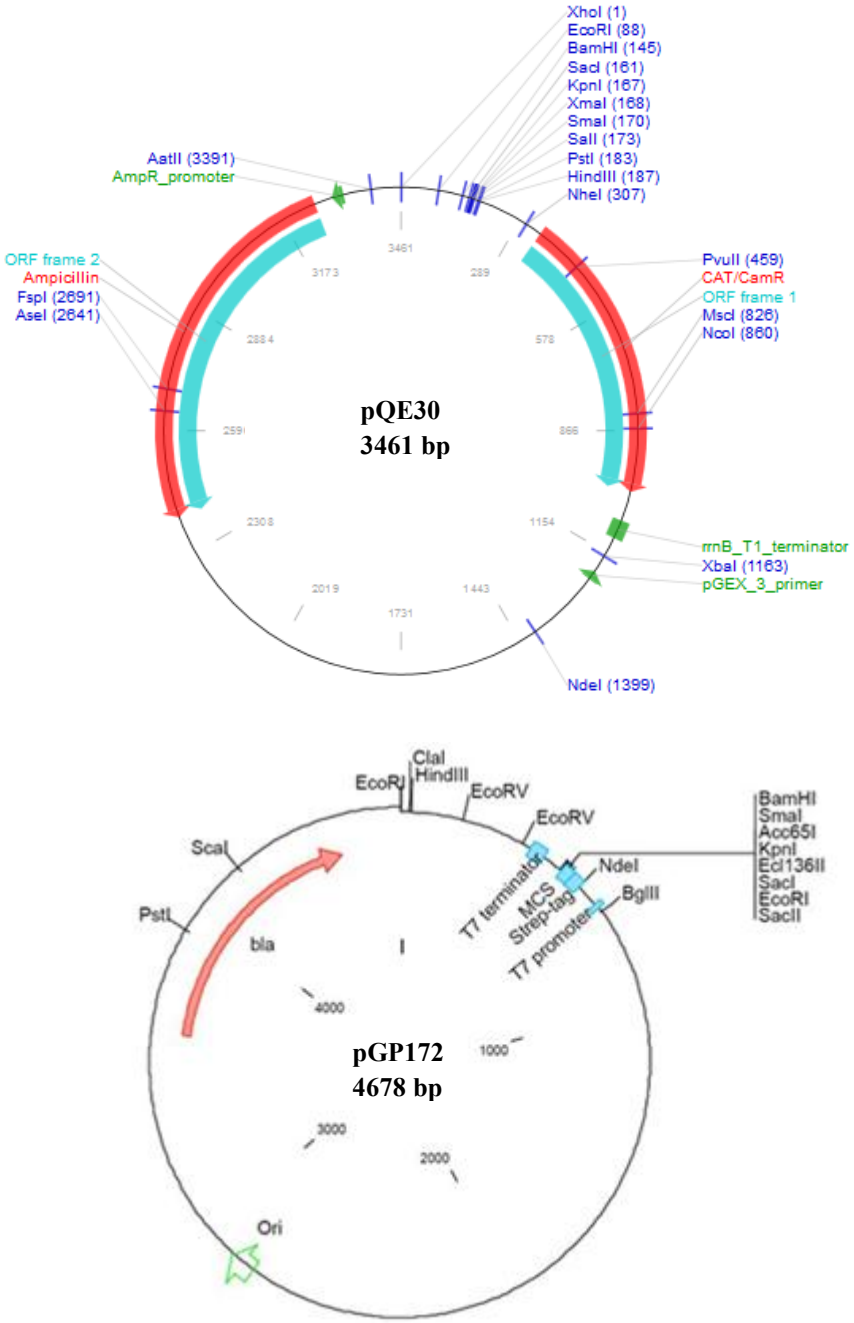
Tableau 1 : Couples d'amorces utilisées pour l'amplification des gènes d'intérêt

Gène/protéine	Amorce	Séquence	Enzyme de restriction	Plasmide
crgA/CrgA	CrgA2C43-BsaIF	GGG**GGTCTC**CCATGAAAACCAATTCAGAAGAAC[a]	BsaI	pET28b (His-tag)
	CrgA2C43-XhoI	GGGG**CTCGAG**TCCACAGAGATTGTTTCCC	XhoI	
ptsH/HPr	ptsHNmStrepForKpnI	GGG**GGTACC**AATGCTCAAACAATCCATCG	KpnI	pGP172 (Strep-tag)
	ptsHNmStrepRevBa	GGC**GGATCC**TTTGCCCGCCGCCACGCCG	BamHI	
glK/Glucokinase	GlkNmBaFor	GTGA**GGATCC**ATGTCTTCTACGCCGAAT	BamHI	pQE30 (His-tag)
	GlkNmHiRev	ATAT**AAGCTT**CGATAATGCAGCCCCGCTG	HindIII	
gnd/6-Phosphogluconate déshydrogenase	Gnd8013KpnIF	GCTT**GGTACC**ATGAAAGGCGATATTGGTG	KpnI	pQE30 (His-tag)
	Gnd8013HindR	CGGC**AAGCTT**ATCAAATATCGTAGG	HindIII	
rpiA/D-Ribose-5-P isomérase A	rpiA8013bamF	GGAC**GGATCC**ATGACGACACAAGACGAACTCAAGCG	BamHI	pQE30 (His-tag)
	rpiA8013HindR	GACGG**AAGCTT**AACGTCAGGATTACCCTTGGCAGGG	HindIII	
rpe/D-Ribulose-5-P 3-épimérase	Rpe8013BamF	GGA**GGATCC**ATGACCGCCTACCGTATCGCACCCAG	BamHI	pQE30 (His-tag)
	Rpe8013KpnIR	CTGAA**GGTACC**CTAATTTTCAGACGGCATTTCTATTTC	KpnI	
PgcM/α-Phosphoglucomutase	pgmB8013BamF	GGG**GGATCC**ATGACGTTTACCGCAGTGCTCTTCG	BamHI	pQE30 (His-tag)
	pgmB8013HindR	CTTA**AAGCTT**TATCTGACGCGTTTTACCTGCCC	HindIII	
edd/ Gluconate-6-P déhydratase	Edd8013BamF	GAGA**GGATCC**GTGAACACTACTCCTATCCACTCC	BamHI	pQE30 (His-tag)
	Edd8013HindR	TGAA**AAGCTT**GTCTGAAACGCGCATCAG	HindIII	

Tableau 1 (suite): Couples d'amorces utilisées pour l'amplification des gènes d'intérêt

Gène/protéine	Amorce	Séquence	Enzyme de restriction	Plasmide
fbA/ Fructose-1,6-bisphosphate aldolase	FbaNmBaHiFor	GGAG**GGATCC**ATGGCACTCGTATCCATGCG	BamHI	pQE30 (His-tag)
	FbaNmBaHiRev	AAGC**AAGCTT**TTATTTGACGATTTGGTTC	HindIII	
gntK/ Gluconate kinase	GntKNmBaHiFor	AGGA**GGATCC**ATGACTACGCATTTTGTCG	BamHI	pQE30 (His-tag)
	GntKNmBaHiRev	TGTC**AAGCTT**CAGACGGCATATTGCTTCAA	HindIII	
NMV_1007	NMV-1007-2CBamF	GGGG**GGATCC**ATGCCGTCTGAACGCCGACC	BamHI	pQE30 (His-tag)
	NMV-1007-2CKpnIR	GGG**GGTACC**TCAGAGCCGCTTTGGCAGTTC	KpnI	

[a] Les sites de restriction sont soulignés et écrits en gras



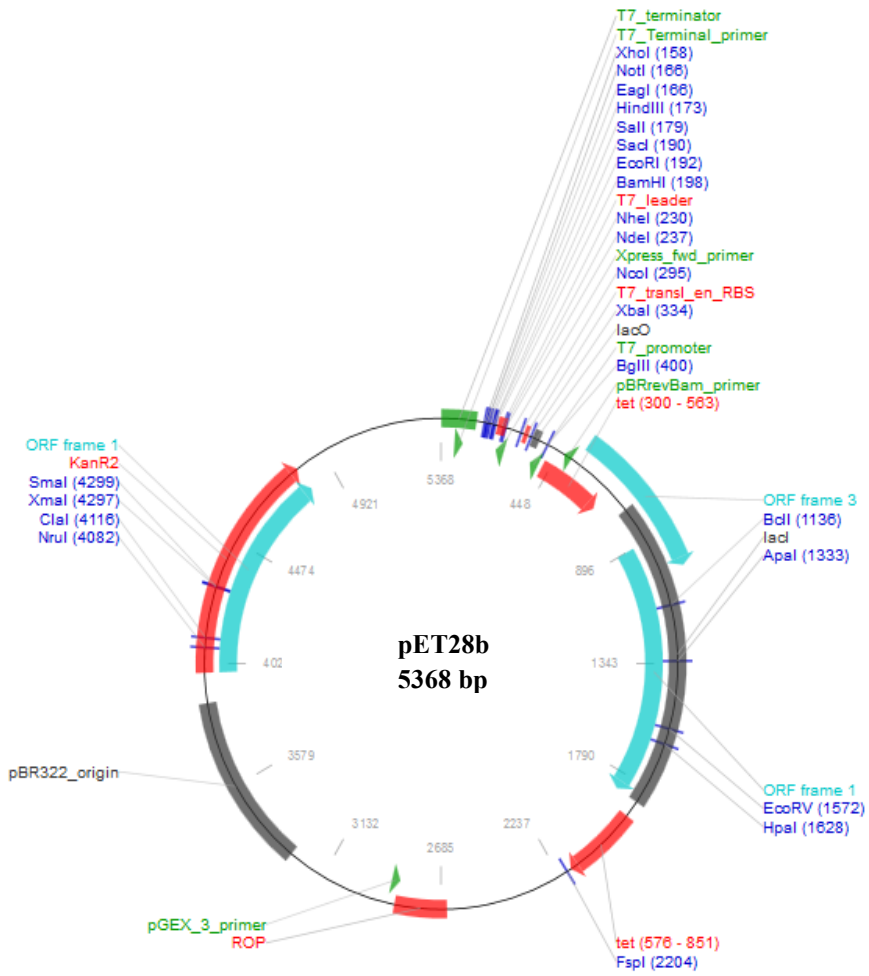


Figure 13 : vecteurs utilisés pour le clonage des gènes d'intérêt.

- Le vecteur **pQE30** permet, après clonage du gène d'intérêt dans le site multiple de clonage (MCS), la surproduction d'une protéine recombinante portant une étiquette poly-histidines fusionnée à son extrémité N-terminale. L'ORF insérée dans le MCS est transcrite à partir du promoteur T5 reconnu par l'ARN polymérase d'*E. coli* en association avec RpoD. L'expression est sous le contrôle de l'opérateur *lacO* et par conséquent inductible par l'IPTG. L'étiquette poly-histidines possède une très forte affinité pour les ions Ni^{2+}, ce qui permet la purification de la protéine par affinité sur colonne Ni-agarose (Ni-NTA, QIAGEN).

- Le vecteur **pGP172** permet l'expression de protéines recombinantes fusionnées dans leurs extrémités N-terminale à une étiquette Strep-tag. Les gènes clonés dans ce vecteur sont transcrits à partir du promoteur T7 reconnu par l'ARN polymérase du phage T7. Le gène codant cette polymérase est présent dans la souche BL21(DE3) et son expression est inductible par l'IPTG. L'étiquette Strep-tag est une séquence de 8 acides aminés (Trp-Ser-His-Pro-Gln-Phe-Glu-Lys) ayant une affinité à la résine Strep-Tactin (Novagen).

- Le vecteur **pET28b** permet la surexpression d'une protéine portant une étiquette poly-histidines à son extrémité N- ou C-terminale. Les gènes clonés dans ce vecteur sont également transcrits à partir du promoteur T7 dans la souche BL21(DE3).

III. 4. Digestion et ligature

Les différents vecteurs ainsi que les produits de PCR ont été digérés par deux enzymes de restriction appropriées. Chaque digestion est réalisée séquentiellement pendant 3 h à 37°C dans le tampon recommandé par le fournisseur (NEB). Après chaque étape, les produits de digestion sont purifiés sur colonne (*Wizard SV Gel and PCR Clean-Up System,* Proméga) et vérifiés sur gel d'agarose.

La ligature a été réalisée à 4°C pendant la nuit en présence de 3 U de T4 DNA ligase dans le tampon fourni (Invitrogen).

III. 5. Électroporation

L'électroporation est une technique qui permet d'introduire des molécules d'ADN exogène dans la cellule suite à une dépolarisation des membranes cellulaires par des impulsions électriques. 4 µl du mélange réactionnel de ligaturation sont ajoutés à 50 µl de cellules électrocompétentes d'*E. coli* , l'ensemble est ensuite transféré dans une cuvette d'électroporation. Le choc électrique est réalisé à l'aide du *Gene Pulser* (Bio-Rad) sous les paramètres suivants : 2.5 KV, 25 µF, 200 Ω. Après le choc électrique, les cellules sont

immédiatement resuspendues dans 1 ml de milieu LB, incubées à 37°C pendant 1 h puis étalées sur milieu LB+ antibiotique. Le criblage des clones positifs se fait par PCR.

Les cellules électrocompétentes d'*E. coli* ont été préparées stérilement à partir d'une culture dans 500 ml de milieu LB avec ou sans antibiotique, selon la souche. La culture est incubée à 37°C sous agitation jusqu'à une DO_{600} de 0,4 à 0,6. Les cellules sont ensuite centrifugées à 4°C pendant 15 min à 7000 rpm et subissent trois lavages successifs dans 500 ml d'eau froide et stérile. Un dernier lavage est effectué dans 250 ml de glycérol froid 10%. Après centrifugation, le culot cellulaire est repris dans 1 ml de glycérol 10%. Les cellules sont ensuite aliquotées et utilisées pour la transformation.

III. 6. Extraction de plasmides ou « miniprep »

L'extraction des plasmides a été réalisée avec le kit *QIAprep-Spin Miniprep* (QIAGEN) suivant les indications du fournisseur. Cette technique permet de purifier jusqu'à 20 µg d'ADN plasmidique à partir de 1 à 5 ml d'une culture d'*E. coli* en milieu liquide.

III. 7. Séquençage

Le séquençage des gènes clonés a été effectué par la société Beckman Coulter Genomics (Royaume Uni). Les amorces utilisées pour le séquençage des gènes étudiés sont indiquées dans le Tableau 2. Les séquences obtenues ont été ensuite visualisées à l'aide du logiciel FinchTV (http://www.geospiza.com/Products/finchtv.shtml) et alignées sur les séquences des souches de référence à l'aide du logiciel *multAlin* (http://multalin.toulouse.inra.fr/multalin/).

Tableau 2 : Couples d'amorces utilisées pour le séquençage des gènes d'intérêt

Plasmide	Amorce	Séquence
pQE30	FpqE	CGGATAACAATTTCACACAG
	DpqE	GTTCTGAGGTCATTACTGG
pET28b et pGP172	T7Pro	TAATACGACTCACTATAGGG
	T7Ter	GCTAGTTATTGCTCAGCGG

IV. Méthodes concernant les protéines

IV. 1. Purification des protéines recombinantes

La purification des protéines recombinantes a été réalisée à partir des souches BL21(DE3) ou NM522 [pREP4-GroES/EL] selon la solubilité des protéines à purifier et le type de promoteur permettant la transcription du gène d'intérêt (Tableau 3). La souche BL21(DE3) a été utilisée pour la purification des protéines dont les gènes sont transcrits à partir du promoteur T7. La souche NM522 [pREP4-GroES/EL] a été utilisée pour la purification de protéines insolubles. Cette souche permet l'expression des gènes contrôlés par le promoteur T5. Elle possède le plasmide pREP4 codant le répresseur LacI qui assure un bon contrôle du gène transcrit. La souche NM522 [pREP4-GroES/EL] permet également la production des protéines chaperons GroES/EL qui assurent un bon repliement des protéines surexprimées.

Tableau 3 : Souches d'*E. coli* utilisées pour la purification des protéines recombinantes

Protéines recombinantes	***E. coli***
CrgA	BL21(DE3)
HPr	
Glucokinase (Glk)	NM522 [pREP4-GroES/EL]
6-phosphogluconate déshydrogénase (Gnd)	
D-Ribose-5-P isomérase A (RpiA)	
D-Ribulose-5-P 3-épimérase (Rpe)	
α-Phosphoglucomutase (PgcM)	
Gluconate-6-P déhydratase (Edd)	
Fructose-1,6-bisphosphate aldolase (FbaA)	
Gluconate kinase (GntK)	
NMV-1007	

La purification des protéines a été réalisée dans 500 ml de milieu LB+antibiotique ensemencé avec une préculture de cellules (dilution 1/100ème). L'ensemble est incubé sous agitation à 37°C jusqu'à une DO_{600} de 0,4 à 0,6. L'IPTG est alors ajouté à une concentration finale de 100 µM et la culture est remise à 37°C pendant 3 h d'induction. Les cellules sont ensuite séparées du milieu par une centrifugation à 4°C pendant 15 min à 7000 rpm et resuspendues dans 20 ml de tampon de lyse (50 mM Tris-HCl pH 7.4, 1 mg/ml de lysozyme, 5 mM $MgCl_2$). Le mélange est ensuite incubé pendant 1 h à 37°C, ce qui permet de fragiliser la membrane

cellulaire grâce à l'action du lysozyme. La lyse complète des bactéries est ensuite obtenue par sonication (8 décharges de 30 s espacées de 30 s de repos dans la glace, Sonicateur de type *Branson Sonifier*). La suspension est ensuite incubée en présence de 5 µg/ml de DNAse I et 10 µg/ml de RNAse A pendant 20 min à température ambiante, puis centrifugée à 4°C pendant 15 min à 15000 rpm. À ce stade, les différentes étapes de la purification dépendent de la nature de l'étiquette utilisée (His-tag, Strep-tag). La Purification des protéines His-tag a été effectuée sur une colonne de Ni-NTA (Promega) grâce à l'interaction entre les ions Ni^{2+} et l'étiquette poly-histidines. La purification des protéines Strep-tag a été réalisée sur une colonne Strep-Tactin (Novagen) qui assure la liaison entre le Strep-tag et la desthiobiotine.

La quantité des protéines présentes dans les fractions d'élution est estimée par dosage de Bradford (décrit dans le paragraphe suivant). Les fractions d'élutions contenant les concentrations les plus élevées en protéines sont ensuite dialysées pendant 2 h à 4°C, dans un boudin de dialyse (SERVAPOR), contre 2 litres de tampon (50 mM Tris-HCl "pH 7,4", 0,1 mM DTT, 1 mM EDTA pH8). La dialyse est ensuite répétée contre 2 litres du même tampon pendant la nuit. La conservation des protéines est réalisée à -80°C en présence de 15% de glycérol.

IV. 2. Dosage des protéines par la méthode de Bradford

La méthode de Bradford (Bradford, 1976) est une méthode d'analyse spectroscopique utilisée pour mesurer la concentration des protéines en solution. Cette méthode est utilisée afin d'estimer la présence de protéines dans les fractions d'élution avant la dialyse. Pour cela des volumes connus des différentes fractions d'élution de la protéine d'intérêt, sont mélangées à 1 ml du réactif de Bradford 1X. La densité optique est ensuite mesurée à 595 nm et les échantillons contenant le maximum de protéines sont dialysés.

La méthode de Bradford est également utilisée comme une méthode de mesure quantitative pour déterminer la concentration de la protéine obtenue après dialyse. Pour cela, une gamme étalon est préparée avec des quantités croissantes et connues d'une protéine de référence « la BSA (*Bovine Serum Albumin*) » dans 1 ml du réactif de Bradford 1X. En parallèle, différents volumes de la protéine d'intérêt ont été préparés dans 1 ml du même réactif. L'absorbance des échantillons est ensuite mesurée à 595 nm. Les valeurs obtenues à partir des échantillons de la gamme étalon obtenue pour la BSA permettent de tracer une droite d'étalonnage : absorbance = f (quantité de protéine). Cette proportionnalité permet de déterminer la concentration de la protéine purifiée.

IV. 3. Electrophorèse en gel de polyacrylamide (PAGE)

IV. 3. 1. Electrophorèse dénaturante

La pureté des protéines est vérifiée en conditions dénaturantes (présence de SDS) sur un gel de polyacrylamide (Laemmli, 1970). La concentration en polyacrylamide utilisée dépend de la taille des protéines purifiées. Les protéines sont déposées sur le gel après suspension en tampon Laemmli 1X (0.05 M Tris-HCl "pH 6.8", 0.1 M DTT, 2.5% SDS, 10% glycérol et 0.01% bleu de bromophénol). L'électrophorèse se fait à 120 volts en tampon Tris-glycine-SDS pH 7.4 (25 mM Tris, 192 mM glycine, 0.1% SDS).

IV. 3. 2. Electrophorèse non dénaturante (ou native)

L'électrophorèse en gel de polyacrylamide, en conditions natives (absence de SDS), a été utilisée afin de séparer la forme phosphorylée de la forme non phosphorylée des protéines étudiées. Les gels non dénaturants permettent de séparer les protéines en fonction de leur repliement et de leur charge nette. L'électrophorèse a été réalisée dans un tampon Tris-glycine pH 7,4 en absence totale de SDS.

IV. 3. 3. Electrophorèse en gel de polyacrylamide-urée

L'électrophorèse en gel de polyacrylamide-urée correspond à une électrophorèse dénaturante sous l'action de l'urée (6 M dans cette étude). Cette méthode a été utilisée afin de séparer la P-Ser-HPr de l'HPr. L'électrophorèse est réalisée à 120 volts en tampon Tris-glycine-SDS pH 7,4.

IV. 4. Coloration des gels de polyacrylamides

Après migration, les gels de polyacrylamide sont colorés dans un tampon à 0.1% de bleu de Coomassie R-250, préparé dans 50% d'éthanol et 10% d'acide acétique. Les protéines sont visualisées après décoloration dans une solution d'éthanol 10% et acide acétique 7%.

IV. 5. Activité enzymatique des protéines

L'activité enzymatique des protéines recombinantes a été mesurée par spectrophotométrie à 340 nm (*UVIKON 9 X 3 W double beam UV/VIS spectrophotometer*) en utilisant des tests couplés à des déshydrogénases. Cette longueur d'onde permet de détecter la formation ou la consommation du NADH ou NADPH au cours des réactions d'oxydo-réduction. Les milieux réactionnels utilisés pour tester l'activité de chaque protéine sont indiqués dans le Tableau 4.

Tableau 4 : Milieux réactionnels utilisés pour faire les tests d'activité enzymatiques

Enzyme (gène)	Milieu réactionnel
Glucokinase (Glk)	Glucose: 10 Mm
	Glucose-6-phosphate 1-déshydrogénase (Zwf) (Sigma): 10 µg
	$MgCl_2$: 10 mM
	ATP: 1 mM
	NADP: 1 mM
	Glk: 8 µg
	Tris-HCl 50 mM pH 7.4: 500 µl
6-Phosphogluconate déshydrogénase (Gnd)	Gluconate 6P : 1 mM
	$MgCl_2$: 10 mM
	NADP: 1 mM
	Gnd : 10 µg
	Tris-HCl 50 mM pH 7.4: 500 µl
D-Ribose-5-P isomérase A (RpiA)	D-ribitol-5-P 2-déshydrogenase (*L. casei*)[1] : 18 µg
	Ribose-5-P: 1 mM
	$MgCl_2$: 10 mM
	NADH: 1 mM
	RpiA: 30 µg
	Tris-HCl 50 mM pH 7.4: 500 µl
D-Ribulose-5-P 3-épimérase (Rpe)	D-xylulose-5-P : 0.5 mM
	D-ribitol-5-P 2-déshydrogenase (*L. casei*)[1]: 18 µg
	$MgCl_2$: 10 mM
	NADH: 1mM
	Rpe: 10 µg
	Tris-HCl 50 mM pH 7.4: 500 µl
α-Phosphoglucomutase (PgcM)	Maltose: 10 mM
	Maltose phosphorylase (*E. faecalis*)[2]: 4 µg
	Glucose-6-phosphate 1-déshydrogénase (Zwf) : 10 µg
	$MgCl_2$: 10 mM
	NADP: 1 mM
	PgcM: 20 µg
	Tris-HCl 50 mM pH 7.4: 500 µl

Tableau 4 (suite)**:** Milieux réactionnels utilisés pour faire les tests d'activité enzymatiques

Enzyme	Milieu réactionnel
Gluconate-6-P déhydratase (Edd)	Gluconate-6-P : 1 mM
	2-Déhydro-3-deoxyphosphogluconate aldolase (*E. coli*) : 30 µg
	Glycérol-3-P déshydrogénase (Sigma) : 24 µg
	Triosephosphate isomérase (Sigma): 20 µg
	NADH: 1 mM
	$MgCl_2$: 10 mM
	Edd: 40 µg
	Tris-HCl 50 mM, pH 7.4 : 500 µl
Fructose-1,6-bisphosphate aldolase (FbaA)	Fructose-1,6-bisphosphate (FBP): 5 mM
	NADH: 1 mM
	$MgCl_2$: 10 mM
	Triosephosphate isomérase: 20 µg
	Glycérol-3-P déshydrogénase: 24 µg
	FbA : 5 µg
	Tris-HCl 50 mM, pH 7.4 : 500 µl
Gluconate kinase (GntK)	Gluconate de Na, pH 6.4 : 1 mM
	6-phosphogluconate déshydrogenase (Sigma): 25 µg
	$MgCl_2$: 10 mM
	NADP: 1 mM
	ATP: 1 mM
	GntK: 2 µg
	Tris-HCl 50 mM pH 7.4: 500 µl
NMV_1007	Fructose-6-P: 1 mM
	fructose-1,6-bisphosphate aldolase (Sigma): 1.4 U
	Triosephosphate isomérase+Glycérol-3-P déshydrogénase (Sigma): 2.8 U
	NADH: 1 mM
	$MgCl_2$: 10 mM
	ATP/PPi*: 1 mM
	NMV-1007: 14 µg
	Tris-HCl 50 mM pH 7.4: 500 µl

[1] : (Bourand *et al*., 2013)
[2] : (Mokhtari *et al*., 2013)
*** :** les tests ont été réalisés avec l'ATP et le PPi comme sources de phosphate.

V. Manipulation des ARNs

V. 1. Extraction des ARNs

L'extraction de l'ARN a été réalisée en suivant les recommandations du fournisseur « *RNeasy Mini Kit »* (QIAGEN). Un second protocole, basé sur l'utilisation du TRIzol, a été également utilisé. Pour cela, un culot de cellules préalablement lavées avec du PBS 1X a été resuspendu dans 1 ml de TRIzol. Après incubation de 5 min à température ambiante, 200 µl de chloroforme ont été ajoutés. L'ensemble est fortement agité pendant 15 s, incubé 5 min à température ambiante et centrifugé à 4°C pendant 15 min à 12000 rpm. L'ARN contenu dans la phase aqueuse est ensuite précipité avec 500 µl d'isopropanol et 1,2 M de NaCl (ou 0,8 M de citrate de sodium) pendant 10 min à température ambiante. Après centrifugation de 15 min à 4°C, l'ARN précipité dans le culot est lavé avec l'éthanol 70%, puis centrifugé 10 min à 4°C et séché 5 min à l'air ambiant. L'ARN est ensuite dissous dans 50 µl d'eau RNase/DNase-*free*, chauffé 3 min à 60°C et conservé à -80°C. La concentration des ARNs totaux est déterminée par la mesure de la densité optique à 260 nm avec un *NanoDrop ND 1000 spectrophotometer*. La qualité des ARN est vérifiée avec un *Bioanalyseur Agilent 2100*.

V. 2. Traitement des ARNs avec la DNase

Les ARNs sont incubés pendant 40 min à 37°C en présence de l'enzyme TURBO^{TM} DNase (2U pour 10 µg d'ARN) et du tampon 10X (0.1 volume). L'arrêt de la réaction est réalisé avec 0.1 volume de l'inactivateur (*DNase Inactivation Reagent*). L'ensemble est incubé 5 min à température ambiante et centrifugé 2 min à 10000 rpm. L'absence d'ADN contaminant est vérifiée par PCR quantitative en utilisant des amorces qui amplifient dans le gène *rpoB* (Tableau 5).

V. 3. Transcription inverse ou « *Reverse transcription* »

La transcription inverse permet de synthétiser un brin d'ADN complémentaire (ADNc) à partir de l'ARN. Cette réaction a été réalisée, selon les recommandations du fournisseur (Promega), sur 1 à 2 µg d'ARN en présence d'une transcriptase inverse AMV (*Avian Myeloblastosis Virus*) et avec pour amorce des héxanucléotides (1µg/µg d'ARN).

V. 4. PCR quantitative (qPCR)

La PCR quantitative, ou PCR en temps réel est une méthode précise et sensible pour quantifier les séquences d'ADN. Elle peut être réalisée à partir de l'ADN génomique ou de

l'ADNc obtenu par transcription inverse de l'ARN. La réaction de PCR s'effectue en utilisant des amorces spécifiques de la région à quantifier et des composés se liant à l'ADN et donnant un signal fluorescent proportionnel à la quantité d'ADN formé à chaque cycle de PCR.

La qPCR a été réalisée sur 80 ng d'ADNc en présence du Master Mix 1X (*FastStart Universal SYBR Green*, Rox, Roche) et de 0.2 µM de chacune des deux amorces. Le programme utilisé pour la qPCR est : 1 cycle de 2 min à 50°C, 1 cycle de 10 min à 95°C et 40 cycles de 15 s à 95°C et de 1 min à 60°C. Les séquences des couples d'amorces utilisées pour faire les qPCR sont indiquées dans le Tableau 5.

Tableau 5: Couples d'amorces utilisées pour la PCR quantitative

Gène	Amorce	Séquence
rpoB	RpoB-RTF	GGCAGCGGTAAGAAAGAAGA
	RpoB-RTR	CGAATACAGGAGAGGCGAAA
ptsH	ptsHRT-F3	ATGGGGCTGATGATGCTCGC
	ptsHRT-R3	GTAGCCGTTGATTAAGTCGG

VI. Construction des mutants Δ*ptsH* et Δ*gntP*

La construction des mutants Δ*ptsH* et Δ*gntP* chez *N. meningitidis* 2C4-3 a été réalisée par double crossing-over avec des vecteurs de délétion construits à partir du plasmide pBlueScript II KS+ chez *E. coli* NM522. La construction des vecteurs de délétion a été réalisée en clonant des fragments d'ADN d'environs 500 pb en amont et en aval du gène *ptsH* ou *gntP*, selon le cas, dans le plasmide pBlueScript II KS+. Ces fragments ont été amplifiés par PCR à partir de l'ADN génomique de *N. meningitidis* 2C4-3 (Figure 14). Entre ces deux fragments, un gène *aphA-3*, codant une cassette de résistance à la kanamycine dépourvue du terminateur, a été inséré. Le gène *aphA-3* a été amplifié à partir du plasmide pMGC20 (Nassif *et al*., 1991). Ce gène sert à remplacer le gène *ptsH* ou *gntP* après double crossing-over. La transformation chez *N. meningitidis* a été réalisée après linéarisation des vecteurs de délétion par digestion avec l'enzyme KpnI. La sélection des mutants a été réalisée sur GCB agar en présence de kanamycine (100 µg/ml). La délétion des gènes a été vérifiée par PCR et séquençage. Les séquences des couples d'amorces utilisées pour la construction et la vérification des mutants sont indiquées dans les Tableaux 6 et 7, respectivement.

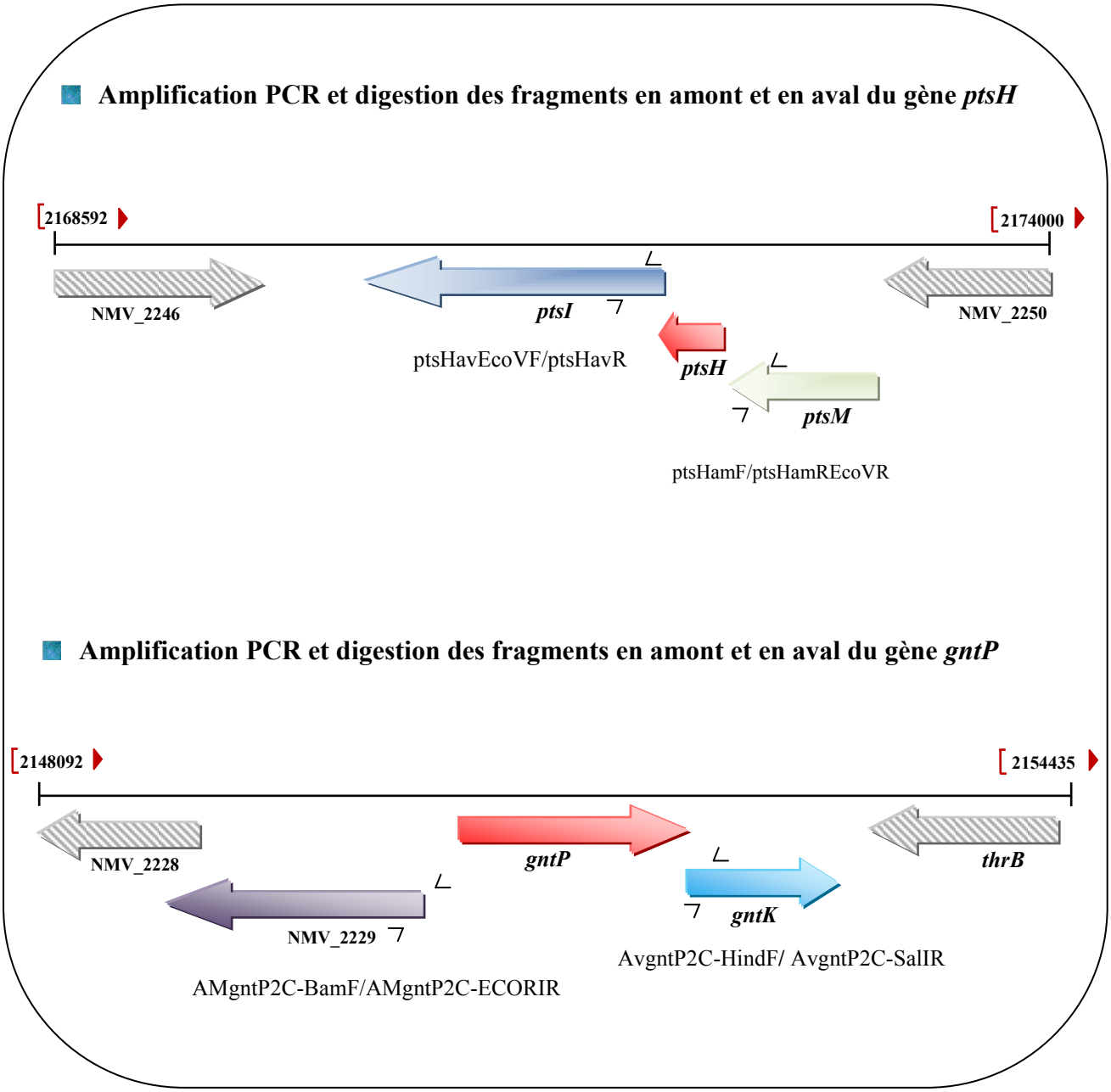


Figure 14 : Représentation schématique de la construction des mutants Δ*ptsH* et Δ*gntP* chez *N. meningitidis* 2C4-3.

Les produits de PCR des fragments d'ADN, en amont et en aval du gène *ptsH*, ont été digérés avec les enzymes BamHI/EcoRV et EcoRV/KpnI, respectivement.

Les produits de PCR des fragments d'ADN, en amont et en aval du gène *gntP*, ont été digérés avec les enzymes BamHI/EcoRI et HindIII/SalI, respectivement.

Tableau 6: Oligonucléotides utilisés pour la construction des mutants Δ*ptsH* et Δ*gntP*

mutant	Région amplifiée	Amorce	Séquence	Enzyme de restriction
Δ*ptsH*	En amont du gène *ptsH*	ptsHamF	CCGC**GGATCC**GCGTGCAACCGACGGAAGAC[a]	BamHI
		ptsHamREcoVR	TATG**GATATC**GGCAGCAGACGCCGTTTAAAATG	EcoRV
	Cassette kanamycine	kanaSmaHindF	ACGT**CCCGGGAAGCTT**CCCAGCGAACCATTTGAGG	SmaI-HindIII
		kanaSmaRev	TAAA**CCCGGG**TACTAAAACAATTCATCCAG	SmaI
	En aval du gène *ptsH*	ptsHavEcoVF	CGAC**GATATC**AACGGCTACTTCGGCGAG	EcoRV
		ptsHavR	CCG**GGTACC**CGTTCAGACGGCTTGCAGCATATCCTGC	KpnI
Δ*gntP*	En amont du gène *gntP*	AMgntP2C-BamF	GCAC**GGATCC**TGCCGCTGCCTGAAATACCGTCC	BamHI
		AMgntP2C-ECORIR	AAGC**GAATTC**CTGTAATAAAAGTACAAAATTGTCAGG	EcoRI
	Cassette kanamycine	KangntP2C-ECORIF	GGGGG**GAATTC**CCCAGCGAACCATTTGAGG	EcoRI
		kangntP2C-HindR	CTAAA**AAGCTT**TTCAGACGGCTACTAAAACAATTCATCCAG	HindIII
	En aval du gène *gntP*	AvgntP2C-HindF	CATC**AAGCTT**CAGACGGAAAGGATAGTAAATG	HindIII
		AvgntP2C-SalIR	GGG**GTCGAC**GGCTTCAGACGGCATATTGCTTC	SalI

[a] Les sites de restriction sont soulignés et écrits en gras.

Tableau 7: Amorces utilisées pour vérifier la construction des mutants Δ*ptsH* et Δ*gntP*

mutant	Amorce	Séquence
Δ*ptsH*	ptsM1-F	CATCACACACGAAACCATAGG
	ptsI4-R	CCACAAGCTCGATGCAGACC
Δ*gntP*	Deltagntp-F	GCCCGCCACCAACGTGGGCATC
	DeltagntP-F1	CTTGCACAAACCCCTG
	Deltagntp-R	GGTGCTTCAGCACCTTAGAGAATC
	KanF1	GATGTGGAACGGGAAAAGGACATGATG
	KanR1	TCTTCATACTCTTCCGAGCAAAGGACGCC

VII. Complémentation des mutants Δ*ptsH* et Δ*gntP*

La complémentation des mutants Δ*ptsH* et Δ*gntP* de *N. meningitidis* 2C4-3 a été réalisée dans une région intergénique située entre les gènes *NMV_0958* et *NMV_0959* codant respectivement une porine (PorA) et une métallopeptidase. Les vecteurs utilisés pour faire la complémentation des mutants Δ*ptsH* et Δ*gntP* ont été construits à partir du plasmide $pComP_{RBS}$ (Ieva *et al.*, 2005 ; Seib *et al.*, 2009). Ce plasmide permet une expression constitutive des gènes d'intérêt et code une cassette de résistance à la spectinomycine. Le plasmide $pComP_{RBS}$ contient également deux fragments d'une région intergénique, située entre les gènes *NMB1428* et *NMB1429* de *N. meningitidis* MC58 (*NMV_0959* et *NMV_0958*, respectivement, chez *N. meningitidis* 2C4-3). Ces fragments sont utilisés pour faire le double crossing-over avec la même région intergénique chez *N. meningitidis* 2C4-3 afin d'insrérer le gène *ptsH* ou *gntP* (Figure 15). La complémentation du mutant Δ*gntP* a été également réalisée en utilisant un autre vecteur construit à partir du plasmide $pComP_{ind}$ (Ieva *et al.*, 2005 ; Seib *et al.*, 2009). Le plasmide $pComP_{ind}$ contient, en plus des éléments du plasmide $pComP_{RBS}$, un gène codant le represseur LacI. Ce plasmide permet l'expression du gène d'intérêt après induction avec l'IPTG (Figure 15). Les vecteurs de complémentation ont été lignarisés par digestion avec XmaI. La complémentation des mutants Δ*ptsH* et Δ*gntP* avec les gènes *ptsH* et *gntP*, respectivement a été réalisée par double crossing-over (Figure 16). La sélection des mutants complémentés a été réalisée sur gélose GCB additionnée de kanamycine (100 µg/ml) et de spectinomycine (75 µg/ml). La vérification de la séquence des gènes *ptsH* et *gntP* a été réalisée par PCR et séquençage. Les oligonucléotides utilisés pour la complémentation et la

vérification de l'insertion correcte des gènes sont indiqués dans les Tableaux 8 et 9, respectivement.

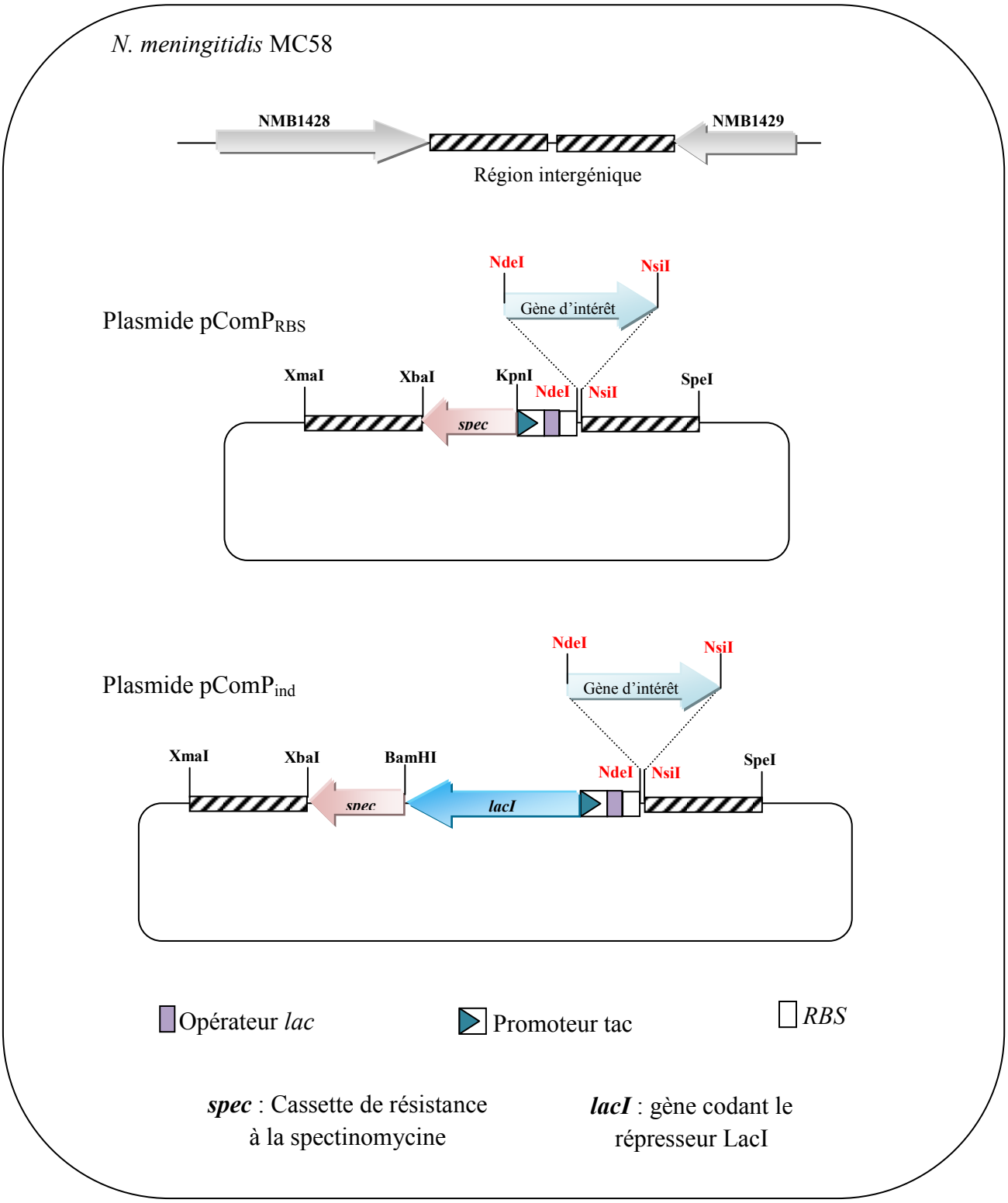


Figure 15 : Construction des vecteurs de complémentation à partir des plasmides pComP$_{RBS}$ et pComP$_{ind}$.

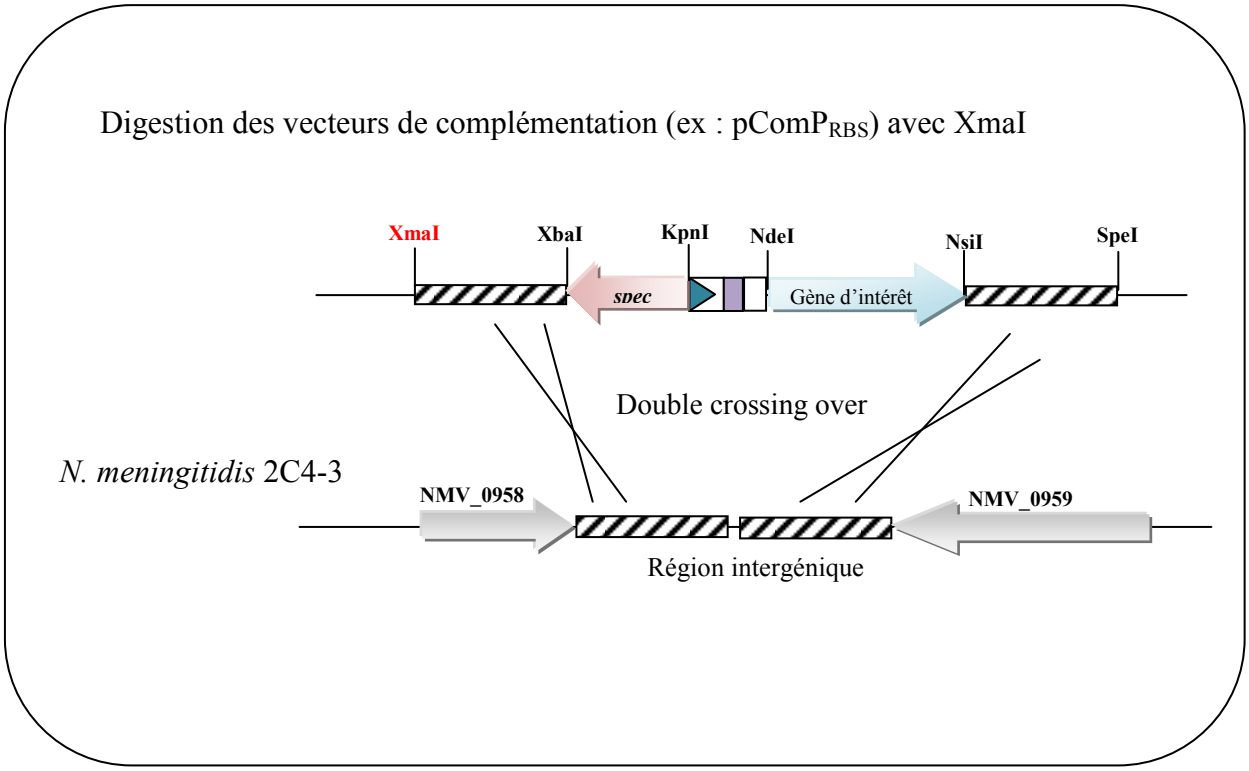


Figure 16 : Linéarisation des vecteurs de complémentation et transformation chez *N. meningitidis* 2C4-3.

Tableau 8 : Oligonucléotides utilisés pour faire la complémentation des mutants Δ*ptsH* et Δ*gntP*

Objectif	Amorce	séquence	Enzyme de restriction
Complémentation du mutant Δ*ptsH*	ptsH-F-NdeI	ATTCAG**CATATG**CTCAAACAATCCATCG AAATCATC[a]	NdeI
	ptsH-R-NsiI	ATTCAG**ATGCAT**TTATTCGCCCTCGCC GAAGTAGCCGTTG	NsiI
Complémentation du mutant Δ*gntP*	gntP2C4-3-NdeI-F	CTTTA**CATATG**GACGGCTGGACA CAGACGCTG	NdeI
	gntP2C4-3-NsiI-R	CTTTC**ATGCAT**TCAGACGATG GCGAACAGCAG	NsiI

[a] Les sites de restriction sont soulignés et écrits en gras

Tableau 9 : Amorces utilisées pour vérifier la complémentation des mutants Δ*ptsH* et Δ*gntP*

Mutant complémenté	Région	Amorce	Séquence
Δ*ptsH*/*ptsH* et Δ*gntP*/*gntP*	En dehors de la zone de recombinaison	COMCFW-NVD	CCTCGAGCCGCTGACCGAAGG
		COMCREV-NVD	ACCGGCATCGGCAACTACAC
	Cassette spectinomycine	aad3-XbaI	ATTCAGTCTAGAGCTTAGTGCATCTAACGCTTG
		aadA1	TGCCGTCACGCAACTGGTCCA
		aadA2	CAACTGATCTGCGCGCGAGGC
Δ*ptsH*/*ptsH*	Gène *ptsH*	ptsH-NdeIF	ATTCAGCATATGCTCAAACAATCCATCGAAATCATC
		ptsH-NsiIR	ATTCAGATGCATTTATTCGCCCTCGCCGAAGTAGCCGTTG
Δ*gntP*/*gntP*	Gène *gntP*	gntP2C4-3-NdeI-F	CTTTACATATGGACGGCTGGACACAGACGCTG
		gntP2C4-3-NsiI-R	CTTTCATGCATTCAGACGATGGCGAACAGCAG
		gntP-F1	GTTCCCGTTCCCGAACTGCTCAGCG
		gntP-R1	GGGAATCAGCATGATGGCGACGAC

VIII. Transformation chez *N. meningitidis*

N. meningitidis est une bactérie naturellement compétente pour la transformation. Pour cela, une suspension bactérienne est préparée dans 1 ml de GCB liquide, à partir d'une culture de 18 h en GCB agar. La suspension est ensuite ajustée à une DO_{600} = 2 et 100 µl de cette dernière sont mélangés avec 10 µg d'ADN plasmidique, préalablement linéarisés par restriction enzymatique. La transformation est réalisée dans 900 µl de GCB liquide additionné de 5 mM de $MgCl_2$. L'ensemble est incubé pendant 5 h à 37°C dans une atmosphère contenant 5% de CO_2. Les suspensions sont ensuite ensemencées sur gélose GCB en présence de l'antibiotique adéquat. Le criblage des clones positifs se fait par PCR et séquençage.

IX. Expérience du gel shift

Le retard sur gel est une technique rapide pour la détection des interactions protéine/ADN. Cette technique est basée sur la variation de migration de l'ADN en présence de protéines qui reconnaissent spécifiquement la séquence d'intérêt.

Les régions promotrices des gènes d'intérêt ont été amplifiées par PCR à l'aide des couples d'amorces indiquées dans le Tableau 10. L'ADN a été mis en solution avec des concentrations croissantes de protéines. Le mélange est ensuite déposé en conditions natives sur un gel de polyacrylamide 7%/Tris-glycine 1X/Glycérol 2,5%. L'électrophorèse est réalisée à 100 volts en tampon Tris-glycine pH 7,4 (25 mM Tris, 192 mM glycine). L'ADN est visualisé sous irradiation UV, après immersion du gel dans une solution de bromure d'éthidium (BET) à 1 µg/ml pendant 15 min.

Tableau 10: Couples d'amorces utilisées pour amplifier les régions promotrices des gènes *crgA, sia, pilC1* et *pilE*

Gène	Oligo	Séquence
crgA	CrgAPro98-2F	GATGAATGTATTGTGCCGTTTTAC
	CrgAPro98-3R	CACCACTTGAACAAATACGGTCAGTTCTTC
sia	SiaIGRFor	CATCAATCAGCTCCACTTCAGG
	SiaIGRRev	GCTCTGGTACCTGTAATGCAAAG
pilC1	PromPilC1-C1-152	CCTGCCCGACGGTATCCCGCGAAGCAAGAT
	PromPilC1-C1-8	CTGCCTTTTTAAAGTTTTATTCATCGT
pilE	PromPilE-F350	CACCCGATGCCCCATGCCAATAGAG
	PromPilE-Rev	GGGTAAAACCTTTTTGAAGGGTGTTC

X. Transfert de protéines sur membrane de nitrocellulose « Western blot »

Le transfert sur membrane, également appelé western blot est une technique de biologie moléculaire qui permet la détection et l'identification des protéines dans un échantillon donné. Cette technique nous a permis de détecter la forme phosphorylée de l'EIIA^{Ntr} chez la souche 2C4-3, le mutant Δ*ptsH* et le mutant complémenté Δ*ptsH*/*ptsH*. Pour cela plusieurs étapes ont été effectuées:

X. 1. Préparation de l'extrait protéique brut de *N. meningitidis*

L'extrait protéique a été préparé à partir d'une culture de 18h sur gélose GCB. Les cellules ont été ensuite resuspendues dans 3 ml de Tris 10 mM pH8 et la suspension a été lysée par sonication (3 décharges de 20 s dans la glace). Les protéines des extraits cellulaires ont été ensuite séparées sur un gel de polyacrylamide sans SDS (Laemmli, 1970). L'électrophorèse se fait à 120 volts en tampon Tris-glycine pH 7,4 (25 mM Tris, 192 mM glycine).

X. 2. Transfert sur membrane

Après migration les protéines sont transférées sur une membrane de nitrocellulose (*Protran Nitrocellulose Transfer Membrane.* Schleicher & Schuell, BioScience) préalablement trempée dans une solution de Tris-glycine-SDS pH 7,4 1X et éthanol 20%. Le transfert se fait pendant 1 h à 25 volts ; le courant électrique appliqué aux plaques de l'appareil (*Amersham BioSciences*) permet la migration des protéines du gel vers la membrane. Le transfert des protéines est vérifié par coloration de la membrane au rouge ponceau (0.1% ponceau S, 1% acide acétique).

X. 3. Saturation

Afin d'éviter les interactions non spécifiques entre les anticorps et la membrane, cette dernière a été trempée pendant 15 min à température ambiante, dans une solution de PBS 1X, 0.05% Tween-20 et 5% lait écrémé.

X. 4. Traitement avec les anticorps primaires

La détection de l'EIIA^{Ntr} a été réalisée avec un sérum d'anticorps polyclonaux de lapin dirigés contre la protéine EIIA^{Ntr} de *N. meningitidis*. Ces anticorps ont été dilués au 1/1000ème dans une solution de PBS 1X, 0,05% Tween-20 et 5% lait écrémé. La membrane a été incubée avec la solution d'anticorps, sous agitation modérée, pendant 1 h à température ambiante.

Trois lavages successifs de 10 min chacun ont été ensuite réalisés dans du tampon (PBS 1X, 0,05% Tween-20) afin d'éliminer les anticorps non fixés.

X. 5. Traitement avec les anticorps secondaires

Après lavage, la membrane est exposée à une solution d'anticorps II^{aires} (*Anti-Rabbit IgG (Fc), AP Conjugate 1 mg/ml,* Promega). Ces anticorps reconnaissent les IgG de lapin et sont couplés à la phosphatase alcaline ce qui permet de visualiser la protéine recherchée. Les anticorps II^{aires} ont été dilués au 1/5000ème dans une solution de PBS 1X, 0,05% Tween-20 et 5% lait écrémé. La membrane a été incubée avec la solution d'anticorps secondaires puis lavée trois fois en PBS 1X, 0,05% Tween-20.

X. 6. Détection des protéines sur membrane

La visualisation des protéines est réalisée en incubant la membrane en présence de 15 ml d'une solution de BCIP (5-bromo-4-chloro-3-indolyl-phosphate)/ NBT (Nitroblue Tetrazolium) (*BCIP/NBT Blue One Component AP membrane Substrat*, BioFX). La solution BCIP/NBT permet la détection colorimétrique des protéines transférées, suite à une réaction chimique catalysée par la phosphatase alcaline présente sur l'anticorps II^{aire}. La réaction est arrêtée par rinçage de la membrane à l'eau.

XI. Quantification de la production de capsule par la technique ELISA

L'ELISA (*Enzyme Linked ImmunoSorbent Assay*) est une technique immuno-enzymatique qui permet de détecter la présence et/ou doser la quantité d'un antigène (protéine, poly- ou oligosaccharide, acide aminé…etc) dans un échantillon donné. Le principe de cette méthode repose sur la fixation d'un anticorps spécifique sur l'antigène d'intérêt (polysaccharides capsulaires dans cette étude) puis à révéler la présence de cet anticorps grâce à un second anticorps anti-IgG couplé à la peroxydase.

La technique ELISA est réalisée sur des plaques maxisorp microtiter NUNC (VWRI réf 1322201) qui permettent une fixation électrostatique de l'antigène sur le fond des puits. Cette technique se réalise en 4 étapes :

XI. 1. Coating de l'antigène

Une suspension de $2{,}5.10^7$ bactéries/ml (0.5 unités Macfarland) préparée dans du tampon PBS est inactivée pendant 30 min à 56°C. 100 µl de cette suspension sont ajoutés dans les puits des plaques ELISA puis incubés pendant une nuit à 45°C. Après incubation, les

plaques sont lavées avec un tampon de lavage (PBS 1X, 0.1% Tween-80) et saturées pendant 30 min à 37°C en PBS 1X, 0.1% Tween-80 et 5% lait écrémé. Un autre lavage est réalisé afin d'éliminer les protéines non fixées.

XI. 2. Fixation des anticorps I^{aires} et II^{aires}

Les anticorps utilisés pour cette étude sont des anticorps monoclonaux synthétisés chez la souris et dirigés contre les polysaccharides de la capsule du sérogroupe C (*Anti-Meningococcal Serogroup C mAb, NIBSC*). Ces anticorps sont dilués au $1/1000^{ème}$ dans une solution de PBS 1X, 0,1 % Tween-80 et 5% lait écrémé. 100 µl de la dilution d'anticorps sont ensuite déposés par puits et incubés pendant 1 h à 37 °C. Les puits sont ensuite lavés avec le tampon de lavage afin d'éliminer les anticorps en excès.

La même opération a été réalisée avec 100 µl d'une solution d'anticorps II^{aires} diluée au $1/5000^{ème}$ en tampon PBS 1X, 0.1% Tween-80 et 5% lait écrémé. Les anticorps II^{aires} sont couplés à la péroxydase et se fixent spécifiquement sur les IgG de la souris (anticorps I^{aires}).

XI. 3. Révélation des anticorps II^{aires} fixés

La révélation des anticorps II^{aires} se fait grâce à une réaction colorée catalysée par la péroxydase. Pour cela 100 µl d'une solution de tampon citrate (0.1 M pH 5.5) additionnée du substrat ortho-phénylènediamine (OPD) et du péroxyde d'hydrogène sont déposés par puits. Les plaques sont ensuite maintenues à l'obscurité pendant 10 à 30 min à température ambiante. En présence de H_2O_2, la peroxydase catalyse la dimérisation et l'oxydation de l'OPD en 2,3-diaminophénazine, un produit orangé. La réaction est arrêtée en rajoutant 50 µl par puits d'acide sulfurique 2N. La lecture des plaques est réalisée à l'aide du lecteur Multiskan Ascent (*MTX LAB SYSTEMS*) à une longueur d'onde de 492 nm.

RÉSULTATS ET DISCUSSION

Article 01

The phosphocarrier protein HPr of *Neisseria meningitidis* interacts with the transcription regulator CrgA and its deletion affects capsule production, cell adhesion and virulence

I. Présentation générale des résultats

N. meningitidis, l'agent responsable de la méningite, possède un PTS incomplet, constitué de : EI, HPr, $EIIA^{Ntr}$ et $EIIA^{Man}$. Dépourvu des enzymes EIIB et EIIC, ce système ne permet pas le transport des hydrates de carbone à la cellule. Cependant, il a été montré que le PTS intervenait dans plusieurs fonctions régulatrices dont la virulence de certaines bactéries pathogènes (Loo *et al.*, 2003 ; Herro *et al.*, 2005 ; Abranches *et al.*, 2006). Chez *N. meningitidis*, les travaux de Sun *et al.* (2000), réalisés sur une série de 2850 mutants par insertion de transposons, ont montré que 73 gènes étaient impliqués dans la virulence de cette bactérie parmi lesquels ceux codant des facteurs de virulence connus tels que les lipopolysaccharides, la capsule et les transporteurs de Fer (Ala'Aldeen et Borriello, 1996 ; Plested *et al.*, 1999) mais également les protéines du PTS : EI et HPr.

La protéine HPr intervient dans plusieurs fonctions régulatrices chez les bactéries. Outre son rôle dans la cascade de phosphorylation du PTS au cours du transport des sucres, cette protéine controle la répression catabolique carbonée (ou RCC), qui permet à la cellule de hiérarchiser l'entrée et l'utilisation des sucres dans la cellule (Poncet *et al*., 2009b). La protéine HPr intervient également dans la virulence de certaines bactéries pathogènes comme *Listeria monocytogenes* (Herro *et al*., 2005) et *Salmonella enterica* serovar Typhimurium (Bowden *et al*., 2009). Dans le but d'étudier le rôle de la protéine HPr dans la virulence de *N. meningitidis*, nous avons construit un mutant Δ*ptsH* à partir de la souche 2C4-3 (clone isolé de la souche 8013), appartenant au sérogroupe C. La construction de ce mutant a été réalisée en remplaçant le gène *ptsH* par le gène *aphA-3* codant une cassette de résistance à la kanamycine. Le mutant Δ*ptsH* a été complémenté *en trans* par l'introduction d'un plasmide portant le gène *ptsH* placé sous le contrôle d'un promoteur permettant son expression constitutive dans la cellule. La souche 2C4-3, le mutant Δ*ptsH* et le mutant complémenté Δ*ptsH/ptsH* ont fait l'objet de plusieurs expériences *in vivo* et *in vitro*.

Le premier objectif de ce travail à été d'étudier les effets de la délétion du gène *ptsH* sur la virulence de *N. meningitidis* en analysant plus précisément: la survie du méningocoque chez la souris, la production de la capsule, l'adhérence cellulaire et l'apoptose.

La survie du méningocoque a été étudiée chez la souris par injection intrapéritonéale de la suspension bactérienne testée (souche sauvage 2C4-3, mutant Δ*ptsH* ou mutant complémenté Δ*ptsH/ptsH*) et dénombrements dans le sang après 2 h, 6 h et 24 h d'infection. Les résultats obtenus montrent que le nombre de bactéries qui survivent après 2 h et 6 h, chez les souris

infectées avec le mutant Δ*ptsH*, est plus faible que le nombre de bactéries chez les souris infectées par la souche sauvage et le mutant complémenté Δ*ptsH/ptsH*.

La capsule polysaccharidique joue un rôle important dans la virulence de *N. meningitidis* (Jarvis et Vedros, 1987 ; Stephens *et al.*, 2007). Afin de déterminer la quantité de capsule produite chez les trois souches étudiées, nous avons réalisé des tests ELISA, en utilisant des anticorps dirigés contre les polysaccharides de la capsule du sérogroupe C, au quel appartient la souche 2C4-3. Une plus faible production de capsule a été observée chez le mutant Δ*ptsH* par rapport à la souche sauvage et au mutant complémenté. Ces résultats ont été également confirmés par des tests de cytométrie en flux.

L'adhérence du méningocoque aux cellules de l'hôte représente une étape cruciale dans la pathogénie de la bactérie (Deghmane *et al.*, 2000). L'effet de la délétion du gène *ptsH* sur l'adhérence de *N. meningitidis* a été étudié en utilisant des cellules épithéliales humaines « Hec-1-B » comme cellules cibles. Le mutant Δ*ptsH* a montré un niveau d'adhérence plus élevé aux cellules « Hec-1-B » que celui de la souche sauvage et du mutant complémenté. Ce résultat est probablement dû à la faible production de la capsule favorisant ainsi le contact entre le mutant et les cellules cibles.

L'induction de l'apoptose des cellules cibles est utilisée par plusieurs bactéries pathogènes qui régulent ce mécanisme afin de moduler les défenses immunitaires de l'hôte (Guzman *et al.*, 1996 ; Gao et Kwaik, 2000). Les *Neisseriae* pathogènes induisent l'apoptose dans différents types de cellules (Maisey *et al.*, 2003 ; Schubert-Unkmeir *et al.*, 2007). L'effet de la délétion de *ptsH* sur ce mécanisme a été étudié sur les cellules épithéliales humaines « Hec-1-B » infectées par la souche sauvage 2C4-3, le mutant Δ*ptsH* ou le mutant complémenté Δ*ptsH/ptsH.* Un niveau élevé de cellules apoptotiques a été observé chez les cellules Hec-1-B infectées par le mutant Δ*ptsH* par rapport à celui observé chez les cellules infectées par la souche sauvage et le mutant complémenté. Un niveau élevé de cellules apoptotiques, comparable à celui causé par le mutant Δ*ptsH*, était également observé chez des cellules infectées par un mutant *ctrA*, incapable d'exprimer les gènes codant pour les transporteurs des polymères de capsule chez *N. meningitidis*.

Le deuxième objectif de ce travail a été de comprendre les mécanismes par lesquels HPr influence la virulence de *N. meningitidis* et plus précisément son rôle dans l'adhérence de cette bactérie aux cellules de l'hôte. Selon Deghmane *et al.* (2002 ; 2004), CrgA, un régulateur transcriptionnel de type LTTR, intervient dans l'adhérence du méningocoque aux cellules de l'hôte en modulant sa propre expression ainsi que l'expression des gènes *pilC1*,

pilE et *sia* codant l'adhésine, la piline et la capsule, respectivement. Afin de vérifier si le rôle de l'HPr dans l'adhérence aux cellules est dépendant de CrgA, nous avons réalisé des expériences du gel shift pour étudier l'effet de la protéine HPr sur l'affinité de CrgA aux régions promotrices des gènes qu'elle contrôle (*crgA*, *pilC1*, *pilE* et *sia*). Les résultats du gel shift ont montré que la présence de HPr augmentait l'affinité de CrgA aux régions promotrices des gènes *crgA, pilE* et probablement aussi *pilC1*. En revanche, aucun effet lié à la présence de HPr n'a été observé pour l'affinité de CrgA à la région promotrice des gènes *sia* (à voir en détails dans la partie « Résultats supplémentaires »). Les résultats des gels shift suggèrent une interaction entre les protéines HPr et CrgA. Cette interaction a été confirmée par des analyses de spectrométrie de masse qui ont révélé la présence simultanée de peptides de HPr et CrgA dans un échantillon de gel shift analysé. Des tests de co-immunoprecipitation *in vivo* réalisés sur les extraits cellulaires de la souche sauvage 2C4-3, du mutant Δ*ptsH*, du mutant complémenté Δ*ptsH*/*ptsH* ainsi que du mutant Δ*crgA*,ont permis de montrer la formation d'un complexe entre CrgA et HPr dans la souche sauvage et dans le mutant complémenté Δ*ptsH/ptsH* en précipitant ces protéines soit avec des anticorps anti-HPr ou anti-CrgA. Ce résultat confirme une interaction entre la protéine HPr et CrgA.

Dans ce travail nous avons également réalisé des tests de phosphorylation *in vitro* sur les protéines purifiées de *N. meningitidis* (HPr, $EIIA^{Ntr}$ et $EIIA^{Man}$) afin de savoir si la cascade de phosphorylation du PTS était fonctionnelle chez cette bactérie. Au vu des difficultés de purifier l'EI de *N. meningitidis* qui est insoluble et se retrouve dans des corps d'inclusion, nous avons utilisé l'EI de *B. subtilis*. Cette dernière phosphoryle parfaitement la protéine HPr de *N. meningitidis*. La P~His-HPr phosphoryle ensuite l'$EIIA^{Ntr}$. En revanche, aucune phosphorylation de l'$EIIA^{Man}$ n'a été observée dans les conditions testées. La phosphorylation de l'$EIIA^{Ntr}$ de *N. meningitidis* a été également vérifiée *in vivo* par Western blot. Pour cela, les protéines présentes dans les extraits cellulaires de la souche 2C4-3, du mutant Δ*ptsH* et du mutant complémenté Δ*ptsH/ptsH* ont été séparées par PAGE en conditions non-dénaturantes. Ces protéines ont été ensuite transférées sur membrane et traitées avec un sérum de lapin anti-$EIIA^{Ntr}$ de *N. meningitidis*. La P~$EIIA^{Ntr}$, migrant plus vite que la forme non-phosphorylée pendant l'électrophorèse, a été détectée dans l'extrait cellulaire de la souche sauvage 2C4-3 et du mutant complémenté Δ*ptsH*/*ptsH*. En revanche, en absence de HPr dans le mutant Δ*ptsH*, la bande correspondante à la P~$EIIA^{Ntr}$ n'a pas été détectée.

N. meningitidis possèdent aussi une protéine HprK/P (HPr kinase/phosphorylase) (Boël *et al.*, 2003). Dans le but de tester l'activité kinase de l'HprK/P de *N. meningitidis*, nous avons

réalisé des tests de phosphorylation de la protéine HPr par l'HprK/P, en présence de l'ATP et du PPi comme donneurs de phosphate et de concentrations croissantes en FBP et Pi. Les résultats ont montré une phosphorylation de HPr par l'HprK/P en utilisant l'ATP ou le PPi comme donneurs de phosphate. Cette phosphorylation est inhibée par de fortes concentrations en Pi et elle est activée par le FBP. Contrairement à l'activité kinase, l'activité phosphorylase de l'HprK/P de *N. meningitidis* a été très faible.

↳ Contribution aux travaux de l'article

- Construction du vecteur de délétion du gène *ptsH* de *N. meningitidis*.

- Construction du vecteur de complémentation du mutant Δ*ptsH* à partir du plasmide $pComP_{RBS}$.

- Complémentation du mutant Δ*ptsH* de *N. meningitidis* 2C4-3.

- Purification des protéines CrgA His-tag et HPr Strep-tag.

- Phosphorylation de l'HPr Strep-tag de *N. meningitidis* sur sa ser-46 par l'HprK/P(V267F) de *L. casei*.

- Expériences du gel shift.

- Quantification de la production de capsule par la technique ELISA.

- Western blot pour la recherche de l'$EIIA^{Ntr}$ phosphorylée dans les extraits cellulaires de : *N. meningitidis* 2C4-3, Δ*ptsH* et Δ*ptsH*/*ptsH*.

- Extraction de l'ARN de : *N. meningitidis* 2C4-3, Δ*ptsH* et Δ*ptsH*/*ptsH*.

- Transcription inverse et PCR quantitative.

The phosphocarrier protein HPr of *Neisseria meningitidis* interacts with the transcription regulator CrgA and its deletion affects capsule production, cell adhesion and virulence

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Short title: HPr affects virulence in *Neisseria meningitidis*

Summary

The bacterial phosphotransferase system (PTS) transports and phosphorylates sugars, but also carries out numerous regulatory functions. The β-proteobacterium *Neisseria meningitidis* possesses an incomplete PTS lacking any known membrane component and therefore cannot transport carbon sources. Nevertheless, the residual phosphorylation cascade is functional and the meningococcal PTS was therefore expected to carry out regulatory roles. Interestingly, a Δ*ptsH* mutant, which was constructed from strain 2C4-3 and which lacks the PTS protein HPr, exhibited reduced virulence in mice. After intraperitoneal challenge the Δ*ptsH* mutant was rapidly cleared from the bloodstream of BALB/c mice. The rapid clearance correlates with lower capsular polysaccharide production by the Δ*ptsH* mutant, which is probably also responsible for its increased adhesion to Hec-1-B epithelial cells. In addition, compared to the wild-type strain more apoptotic cells were detected when Hec-1-B cells were infected with the Δ*ptsH* strain. Co-immunoprecipitation experiments revealed an interaction of HPr with the LysR type transcription regulator CrgA, which among others controls the expression of its own gene, *crgA*. The presence of HPr during electrophoretic mobility shift assays enhanced the affinity of CrgA for its target site preceding the *crgA* gene. HPr therefore seems to affect virulence via CrgA-mediated autorepression.

Introduction

The β-proteobacterium *Neisseria meningitidis* is a commensal microorganism colonizing the human nasopharynx of about 15% of healthy individuals (Caugant and Maiden, 2009). However, for reasons not yet fully understood it can cross the epithelial barrier and cause severe infections leading to septicemia and meningitis with sometimes fatal outcome. Metabolic changes seem to play a role in the switch from commensal to invasive behaviour (Schoen *et al.*, 2014). In addition, the virulence of this organism was proposed to be related to the energetic state of the bacterium, because several genes, which were found to be essential for bacteremic infection by *N. meningitidis*, were related to carbon metabolism (Sun *et al.*, 2000), including EI and HPr, two components of the phosphoenolpyruvate (PEP):sugar phosphotransferase system (PTS).

In firmicutes, enterobacteriaceae, actinobacteria and also some archaea the PTS transports and phosphorylates numerous carbohydrates. The PTS was also reported to carry out numerous regulatory functions related among others to carbon, nitrogen and phosphate metabolism, ion transport and chemotaxis (Deutscher *et al.*, 2014). The PTS is usually composed of four proteins, which form a phosphorylation cascade, and an integral membrane protein that catalyzes the transport step (Deutscher *et al.*, 2006). Enzyme I (EI) autophosphorylates with PEP at a conserved histidine (Alpert *et al.*, 1985) and then transfers the phosphoryl group to His-15 in HPr. EI and HPr are the general PTS components. P~His-HPr phosphorylates the sugar-specif EIIA proteins (Deutscher *et al.*, 1982), which transfer the phosphoryl group to their cognate EIIBs. The P~EIIBs finally phosphorylate carbohydrates bound to the corresponding membrane-spanning EIICs. Similar to numerous proteobacteria, *N. meningitidis* possesses only an incomplete PTS, which is composed of EI, HPr, $EIIA^{Ntr}$ and an EIIA of the mannose PTS family (Fig. 1A). Meningococci lack any known EIIB and EIIC component. The PTS of *Neisseriae* therefore does not transport carbohydrates, but carries out regulatory functions some of which are related to virulence (Sun *et al.*, 2000). A library of insertional mutants was tested for systemic infection in an infant rat model and 73 meningococcal genes essential for bacteremic disease were identified, including *ptsI* and *ptsH*. Involvement of PTS components in bacterial virulence has been reported for other pathogens. For example, PTS-mediated carbohydrate transport inhibits virulence gene expression in *Listeria monocytogenes* (Aké *et al.*, 2011; Milenbachs *et al.*, 1997). Deletion of EI or HPr prevents the expression of the *virB* genes in *Brucella melitensis* (Dozot *et al.*,

2010). EIIA^{Ntr} inhibits the expression of numerous genes in the *Salmonella enterica* pathogenicity island PI-2 (Choi *et al.*, 2010), and EIIA^{Glc} activates virulence factor secretion in this organism (Mazé *et al.*, 2014). Finally, inactivation of the *ptsI*, *ptsH* or *crr* gene in *Vibrio choleare* lowered the production of the major intestinal colonization factor and of cholera toxin (Wang *et al.*, 2015).

N. meningitidis contains also a homologue of HprK, which phosphorylates HPr at Ser-46 (Deutscher and Engelman, 1984) and usually also catalyzes the Pi-dependent pyrophosphate-producing dephosphorylation of P-Ser-HPr (Mijakovic *et al.*, 2002). Similar to many other proteobacteria containing an incomplete PTS (Dozot *et al.*, 2010; Kraußе *et al.*, 2009), *N. meningitidis hprK* is preceded by the gene encoding EIIA^{Ntr} (*ptsN*) and followed by a gene encoding a homologue of *Escherichia coli* YhbJ (Fig. 1A), which is a key component in the GlmYZ sRNA regulatory cascade (Kalamorz *et al.*, 2007). The gene encoding HPr (*ptsH*) is located about 0.56 Mb further on the genome close to the origin of replication and is preceded by *ptsM* (encodes EIIA^{Man}) and followed by *ptsI* (encodes EI). In firmicutes, P-Ser-HPr plays an important role in carbon catabolite repression (Deutscher, 2008) and inducer exclusion (Monedero *et al.*, 2001a; Monedero *et al.*, 2008). In contrast, there is little known about the regulatory functions of P-Ser-HPr in proteobacteria (Kraußе *et al.*, 2009).

In order to study the observed role of PTS components in *N. meningitidis* virulence in more detail we deleted the *ptsH* gene, which encodes the central phosphocarrier protein of the PTS. As mentioned above, this protein is the substrate of two protein kinases, the PEP-dependent EI and the ATP-dependent HprK; its different forms carry out numerous regulatory functions (Deutscher *et al.*, 2014). We observed that deletion of *N. meningitidis ptsH* lowers capsule synthesis. Low capsule synthesis is important for *N. meningitidis* cell adhesion and allows the transition from loose to close host cell contact. Repression of capsule production in response to host cell contact was proposed to be controlled by CrgA (contact-regulated gene A), a LysR-type transcription repressor (LTTR) (Deghmane *et al.*, 2002). However, CrgA was later reported to control the expression of its upstream gene *mdaB*, but not of the pili and capsule genes (Ieva *et al.*, 2005). In contrast, in a recent study (Matthias and Rest, 2014) CrgA of *Neisseria gonorrhoeae* was found to stimulate the expression of *pilE* and to repress the *lst* gene (encodes a lipooligosaccharide sialyltransferase) (Shell *et al.*, 2002). Seen that both, CrgA and HPr, affect the synthesis of cell surface components we decided to study whether the HPr effects on meningococcal virulence are mediated via CrgA.

Results

Impact of the ptsH *deletion on meningococcal virulence*

In order to study the observed effects of HPr inactivation on meningococcal infection (Sun *et al.*, 2000) in more detail, we replaced the *ptsH* gene of *N. meningitidis* strain 2C4-3 (also known as strain 8013) with a kanamycin resistance cassette. To assess that the observed phenotypes were indeed due to the deletion of *ptsH* and not to polar effects caused by the insertion of the kanamycin resistance cassette, we complemented the Δ*ptsH* mutant by transforming it with plasmid pComP$_{RBS}$ptsH (see Experimental procedures and Fig. 1B), thus providing strain Δ*ptsH/ptsH*. qRT-PCR revealed that in the complemented strain the *ptsH* gene is expressed at a level similar to that in strain 2C4-3 (data not shown). The virulence of the *ΔptsH* mutant was compared to that of the wild-type and the complemented Δ*ptsH/ptsH* strain by using a murine model of bacteraemia (Zarantonelli *et al.*, 2006). Mice were infected intraperitonally and the amount of bacteria present in the blood was determined after 2 h, 6 h and 24 h post-infection. After 2 h of bacterial challenge the number of bacteria present in the blood of mice infected with the Δ*ptsH* mutant was more than 10-times lower than in mice infected with the wild-type strain ($P = 0.02$) (Fig. 2A). The difference increased to more than 100-fold after 6 h of bacterial challenge. Apparently, the Δ*ptsH* mutant was cleared from the bloodstream more rapidly than the wild-type strain. Complementation of Δ*ptsH* restored the wild-type phenotype.

Effect of ptsH *deletion on capsule production*

Meningococcal capsule is a major factor in survival in the bloodstream in experimental infection in mice (Alonso *et al.*, 2003). One possible explanation for the rapid clearance of the Δ*ptsH* mutant from infected mice was an alteration of its capsule production. We therefore carried out enzyme-linked immunosorbent assays (ELISA) with bacterial suspensions (2.5 x 10^7 bacteria/ml) of the wild-type strain 2C4-3, the Δ*ptsH* mutant and the complemented strain Δ*ptsH*/*ptsH*. In addition, we used mouse monoclonal antibodies directed specifically against the capsule of *N. meningitidis* serogroup C strains. The detected amount of capsule in the Δ*ptsH* mutant was only about half of that produced by the wild-type strain 2C4-3 (Fig. 3). Complementation with the *ptsH* allele restored capsule production in the Δ*ptsH* mutant to nearly wild-type level. Strain MC58, which belongs to serogroup B and therefore forms a different type of capsule, served as a negative control (Fig. 3).

To further assess the role of *ptsH* on capsule synthesis, flow cytometry was performed with a serogroup C capsule-specific rabbit polyclonal antibody (pAb) and the wild type strain 2C4-3, the Δ*ptsH* mutant and the complemented strain Δ*ptsH/ptsH*. A pAb that specifically recognizes the serogroup B capsule was used as a negative control. This approach provided results similar to those of the ELISA experiments. Indeed, an about 2-fold lower mean fluorescence intensity (MFI) was measured for the Δ*ptsH* mutant compared to the wild-type strain 2C4-3 (MFI 23.02 for WT and 12.26 for Δ*ptsH*). In contrast, the complemented strain Δ*ptsH/ptsH* provided signals comparable to the wild-type strain (Supplementary Fig. 1). As anticipated, the fluorescence intensity emitted by bacteria labelled with the group B capsule-specific antibody was similar to that of unlabeled bacteria, confirming the labelling specificity (Supplementary Fig. 1). Taken together these results suggest that capsule synthesis is diminished in the Δ*ptsH* mutant.

The effect of the ptsH *deletion on cell adhesion*

It has been postulated that synthesis of high levels of capsule interferes with efficient intimate adhesion of *N. meningitidis* to human target cells (de Vries *et al.*, 1998; Deghmane *et al.*, 2002; Hardy *et al.*, 2000). It was therefore tempting to assume that the lower amount of capsule in the Δ*ptsH* mutant might affect bacteria - host cell interactions, such as cell adhesion. Indeed, loose contact with epithelial cells triggers a signal which leads to reduced capsule production and in turn allows intimate cell adhesion. We therefore studied the effect of the *ptsH* deletion on the adhesion of *N. meningitidis* to human Hec-1-B epithelial cells. After 6 h infection, the Δ*ptsH* mutant showed an almost 3-fold higher level of adhesion to epithelial cells compared to the wild-type strain (Fig. 4). In contrast, complementation of the Δ*ptsH* mutant diminished cell adhesion to a level that was even slightly lower than that observed for wild-type bacteria (Fig. 4).

The effect of ptsH *deletion on induction of apoptosis*

Induction of apoptosis in target cells is an important feature in bacterial pathogenesis and invasiveness. We previously reported that *N. meningitidis* induces apoptosis in response to adhesion to epithelial cells, such as Hec-1-B cells (Deghmane *et al.*, 2009; Zarantonelli *et al.*, 2008). Compared to the wild-type strain, a higher level and an earlier onset of apoptosis were detected with a capsule-deficient *ctrA* mutant. These effects were proposed to be due to unmasking of the bacterial cell surface in the capsule-deficient strain (Deghmane *et al.*, 2009).

In order to test whether the low capsule production by the *ΔptsH* mutant might have a similar effect, we analysed Hec-1-B cells infected either with the wild-type, the *ΔptsH* mutant or the complemented *ΔptsH/ptsH* strain for apoptosis using flow cytometry following Annexin V staining. Necrosis of Hec-1-B epithelial cells was only slightly elevated when they were infected with a meningococcal strain (Fig. 5, regions PI). In contrast, apoptosis of Hec-1-B cells was significantly elevated after incubation with any of the *N. meningitidis* strains (Fig. 5, regions Annexin V). The strongest increase of apoptotic Hec-1-B cells was observed when they were infected with the Δ*ptsH* mutant (about 2-fold higher than Hec-1-B cells infected with the wild-type strain) (Fig. 5A and B). In contrast, infection with the complemented Δ*ptsH/ptsH* strain provided numbers of apoptoic cells similar to those observed with the wild-type strain (Fig. 5, regions Annexin V).

HPr affects binding of CrgA to its own promoter region

The *N. meningitidis* LysR-type transcriptional regulator CrgA is upregulated when bacteria first contact host cells (Deghmane *et al.*, 2000). Indeed, *crgA* appears to be part of a group of genes that are co-ordinately upregulated during initial adhesion (Deghmane *et al.*, 2003; Morelle *et al.*, 2003). Because HPr affects capsule expression and cell adhesion, we tested whether HPr might bind to CrgA and thereby change its affinity for the target site preceding its own gene. In order to test this hypothesis, we carried out electrophoretic mobility shift assays (EMSA) with purified His-tagged CrgA, Strep-tagged HPr and amplified DNA fragments, which contained either the promoter region of *crgA* or a control DNA (see Experimental procedures). We observed that during EMSA the presence of 150 nM CrgA shifted only a small fraction of the 259 bp DNA fragment containing the promoter region of *crgA*. The amount of the shifted DNA increased with the concentration of CrgA (300 and 450 nM) (Fig. 6A, lanes 3 and 4). In contrast, 450 nM CrgA had no effect on the migration behaviour of the control DNA (Fig. 6A, lanes 5 and 6). Interestingly, when HPr (5 μM) was included in the assay mixture containing 150 nM CrgA, a significant increase of the shifted *crgA*-containing DNA fragment was observed (Fig. 6B, lane 3). HPr alone did not alter the migration behaviour of the DNA fragment (Fig. 6B, lane 4). We cut out the shifted DNA band (Fig. 6B, lane 3), digested the associated proteins with trypsine and carried out mass spectrometry. We detected peptides for CrgA (16.7% coverage) and for HPr (20.2% coverage), although both proteins migrate much faster than the shifted DNA band. These

results confirm that HPr increases the affinity of CrgA for its target site located upstream from the *crgA* gene probably by interacting with CrgA.

Interaction of HPr with CrgA

In order to confirm the suggested direct interaction of HPr with CrgA we performed co-immunoprecipitation experiments with protein extracts from various *N. meningitidis* strains (wild-type 2C4-3, Δ*crgA*, Δ*ptsH* and complemented Δ*ptsH/ptsH*). CrgA was detected only in those anti-HPr precipitates which were prepared from strain 2C4-3 or the complemented strain Δ*ptsH/ptsH* (Fig. 7A). Similarly, HPr was detected only in the anti-CrgA precipitates prepared from the same two strains (Fig. 7B). These results confirm that there is a specific interaction of *N. meningitidis* HPr with CrgA.

In vitro *phosphorylation of HPr at Ser-46 by HprK*

HPr is the central phosphocarrier in the PTS phosphorylation cascade and its phosphorylation state might affect its interaction with CrgA. The sequence around Ser-46 in *N. meningitidis* HPr strongly resembles that of HPr in firmicutes (Boël *et al.*, 2003; Hu and Saier Jr, 2002) and we therefore tested whether purified *N. meningitidis* HPr kinase can phosphorylate HPr. Indeed, *N. meningitidis* HprK phosphorylated HPr by using either ATP or PPi as phosphoryl donor and its activity was controlled by inorganic phosphate (Pi) and FBP. Pi has been shown to compete with ATP and PPi for their HprK binding site (Fieulaine *et al.*, 2001) and under the employed reaction conditions Pi higher than 1 mM inhibited both ATP- and PPi-dependent HPr phosphorylation (Fig. 8A). The inhibitory effect of Pi (2.5 mM) was counteracted by FBP at concentrations higher than 2.5 mM (Fig. 8B). The meningococcal enzyme therefore behaves similar to HprK/P of most firmicutes. However, it failed to dephosphorylate [^{32}P]P-Ser-HPr in the presence of Pi (up to 50 mM) (data not shown) and thus differs from HprK/P of most firmicutes, which use a phosphorylase reaction to convert P-Ser-HPr and Pi to HPr and PPi (Mijakovic *et al.*, 2002).

In vitro *reconstitution of the PEP-requring PTS phosphorylation cascade*

In order to reconstiute the *in vitro* meningococcal PTS phosphorylation cascade, we purifed HPr and the two "sugar-specific" PTS-components encoded by the *N. meningitidis* genome, EIIA^{Ntr} and EIIA^{Man}. Unfortunately, we failed to purify *N. meningitidis* EI, which forms inclusion bodies and instead used purified *Bacillus subtilis* EI. The *B. subtilis* protein was

indeed able to phosphorylate HPr from *N. meningitidis* (Fig. 9A, lane 5). We subsequently tested whether P~His-HPr can transfer its phosphoryl group to $EIIA^{Ntr}$ and $EIIA^{Man}$. Phosphorylation of neither $EIIA^{Ntr}$ nor $EIIA^{Man}$ could be detected when these proteins were incubated with [^{32}P]PEP without the general PTS components (Fig. 9A, lanes 3 and 4) or with only EI or only HPr (data not shown). Only in the presence of both, EI and HPr, a strong radioactive band migrating to the position of $EIIA^{Ntr}$ was observed (Fig. 9A, lane 6). Unexpectedly, under the same conditions P~His-HPr could not phosphorylate *N. meningitidis* $EIIA^{Man}$ (Fig. 9A, lane 7).

The incomplete PTS phosphorylation cascade is also active in vivo

After establishing that HPr and $EIIA^{Ntr}$ of *N. meningitidis* can be phosphorylated *in vitro*, we tested whether these proteins are also phosphorylated *in vivo*. To demonstrate that $EIIA^{Ntr}$ is phosphorylated, we made use of the observation that similar to other PTS components, such as $EIIA^{Glc}$ from *E. coli* (Dörschug *et al.*, 1984) and $EIIA^{Ntr}$ from *Pseudomonas putida* (Pflüger and de Lorenzo, 2008), meningococcal P~$EIIA^{Ntr}$ migrates significantly faster than the unphosphorylated protein during electrophoresis on a non-denaturing polyacrylamide gel. We therefore carried out Western blots with rabbit pAb against $EIIA^{Ntr}$ and crude extracts prepared either from the *N. meningitidis* wild-type strain 2C4-3, the Δ*ptsH* mutant derived from it and the complemented strain Δ*ptsH*/*ptsH*. In the wild-type strain and the complemented Δ*ptsH* mutant, two protein bands of similar intensity correrssponding to $EIIA^{Ntr}$ and P~$EIIA^{Ntr}$ were recognized by the antibodies. In contrast, the faster migrating P~$EIIA^{Ntr}$ band was lacking in the Δ*ptsH* mutant (Fig. 9B). This result shows that although incomplete, the meningococcal PTS phosphorylation cascade is functional *in vivo* and that under the employed growth conditions about half of the $EIIA^{Ntr}$ is phosphorylated by EI and HPr inside meningococcal cells.

Discussion

Virulence gene expression and synthesis of virulence-specific proteins are often regulated in response to changes in the host environment and frequently depend on the metabolic state of the bacterial cell with a special emphasis on carbon source availability (Kietzman and Caparon, 2011; Poncet *et al.*, 2009). For example, glycolytic enzymes exhibit additional

('moonlighting') functions and are sometimes involved in virulence regulation, such as the FBP aldolase of *N. meningitidis* that plays a role in meningococcal adhesion to epithelial cells (Tunio *et al.*, 2010). Moreover, components of the carbohydrate-transporting PTS regulate not only many cellular processes, but often also affect the virulence of both Gram-positive and Gram-negative pathogens (Antunes *et al.*, 2011; Choi *et al.*, 2010; Herro *et al.*, 2005; Isaza *et al.*, 2008; Mazé *et al.*, 2014; Mertins *et al.*, 2007; Wang *et al.*, 2015). Most of these effects depend on the phosphorylation state of the PTS proteins.

Sun and coworkers found a correlation between the PTS components EI and HPr and meningococcal virulence in an infant rat infection model (Sun *et al.*, 2000). We therefore decided to study meningococcal virulence in a *ptsH* deletion mutant, which lacks HPr, the central protein of the PTS phosphorylation cascade. In a mouse infection model, the Δ*ptsH* mutant turned out to be less virulent than the wild-type strain. The reduced virulence is probably a consequence of the observed lower amount of capsule produced by the mutant strain. In serogroup C meningococci the capsule is composed of sialic acid polymers and is necessary for survival of *N. meningitidis* in the bloodstream and for intracellular survival in host cells (Spinosa *et al.*, 2007). The observed rapid clearance of the Δ*ptsH* mutant from the blood in mice might therefore be a consequence of the lower capsule level. In addition, changes in capsule synthesis are known to affect other infection-related traits of *N. meningitidis* (Hammerschmidt *et al.*, 1996; Stephens *et al.*, 1993; Virji *et al.*, 1993). For example, we observed that in the Δ*ptsH* mutant cell adhesion and induction of apoptosis were elevated.

CrgA, which belongs to the LysR type transcription regulator (LTTR) (Deghmane *et al.*, 2002) is involved in the switch from initial to intimate adhesion during bacteria/host cell interaction. Interestingly, members of the LTTR family usually require a co-regulator (small effector molecule) in order to be fully active. LTTRs bind their specific co-regulator at the non-conserved C terminal part of the protein, which probably leads to a conformational change, which in turn modulates the binding affinity of LTTRs (for a review, see (Maddocks and Oyston, 2008)). Based on the CrgA crystal structure, an effector binding pocket at the interface between the two C-terminal subdomains RD-I and RD-II has been postulated (Sainsbury *et al.*, 2009). Co-immunoprecipitation experiments revealed a direct interaction between CrgA and HPr and it is therefore likely that the small protein HPr binds to the effector binding pocket. The precise role of the different forms of HPr in CrgA/HPr interaction remains to be analyzed.

The interaction of HPr with CrgA possibly depends on the phosphorylation state of the PTS protein. Although *N. meningitidis* possesses an incomplete PTS unable to transport carbohydrates, the remaining phosphorylation cascade is functional and we could demonstrate that meningococcal HPr becomes phosphorylated by EI and PEP at His-15. The observation that inactivation of both, HPr and EI, similarly affected meningococcal virulence (Sun *et al.*, 2000) suggested that P~His-HPr might phosporylate CrgA and thus regulate its activity. However, when carrying out corresponding experiments we did not observe P~His-HPr-mediated phosphorylation of CrgA (data not shown). HPr is also phosphorylated by HprK, which uses either ATP or PPi as phosphoryl donor. However, meningococcal HprK has no phosphorylase activity and therefore cannot dephosphorylate P-Ser-HPr. *B. melitensis* HprK also lacks a phosphorylase activity (Dozot *et al.*, 2010) and these organisms must therefore use a different P-Ser-HPr dephosphorylation mechanism. For example, *Mycoplasma pneumoniae*, which also contains an HprK with very low phosphorylase activity, possesses a PPM type protein phosphatase that dephosphorylates P-Ser-HPr (Halbedel *et al.*, 2006). Similar as reported for the β-proteobacterium *Ralstonia eutropha* (Kraußе *et al.*, 2009), meningococcal P~His-HPr transfers its phosphoryl group to $EIIA^{Ntr}$, but not to $EIIA^{Man}$. It also phosphorylates *Lactobacillus casei* $EIIA^{Lev}$ (data not shown), another EIIA protein of the mannose PTS family (Mazé *et al.*, 2004). We suspected that replacement of the glycine, which usually follows the phosphorylatable His in EIIA's of the mannose type PTS, with a glutamate in meningococcal $EIIA^{Man}$ might prevent its phosphorylation. However, the purified mutant protein $EIIA^{Man}$(Glu10Gly) was also not phosphorylated by P~His-HPr under the employed assay conditions (data not shown). The reason for the failure of P~His-HPr to phosphorylate $EIIA^{Man}$ *in vitro* therefore remains unknown. It can presently not be excluded that $EIIA^{Man}$ might become phosphorylated under specific *in vivo* conditions.

The ATP- and PPi-dependent HPr phosphorylation at Ser-46 is stimulated by the metabolic intermediate FBP and inhibited by Pi (Fig. 9). The PEP-dependent phosphorylation at His-15 increases when the ratio of intracellular PEP to pyruvate is high (Hogema *et al.*, 1998). These two metabolites are substrate and product of the EI-dependent auto-phosphorylation reaction, respectively. In contrast, formation of P-Ser-HPr slows the phosphoryl group transfer activity of the PTS (Monedero *et al.*, 2001b), because P-Ser-HPr is a poor substrate for P~EI. It will therefore be interesting to study whether the concentration of any of the above metabolites changes when *N. meningitidis* enters into loose contact with host cells, which would alter the phosphorylation state of HPr. Phosphorylation of HPr at His-15 or Ser-46 or on both amino acid residues might affect its interaction with CrgA, which should

lead to alter *crgA* expression and also capsule synthesis, which is required for intimate cell contact.

Experimental procedures

Bacterial strains and growth conditions

E. coli strains NM522 (Stratagene), M15[pREP4] (Qiagen) and BL21-DE3 (Merck Millipore) were used for protein overproduction. Strains harbouring derivatives of the His-tag expression vectors were grown at 37°C under agitation in LB medium supplemented with 100 μg/ml of ampicillin for NM522[pQE30-ptsN] and BL21-DE3[pGP172-ptsH], with 25 μg/ml of kanamycin for BL21-DE3[pET-28b-crgA] and with 50 μg/ml of ampicillin and 12.5 μg/ml of kanamycin for M15[pREP4, pQE30-ptsM] and M15[pREP4, pQE30-hprK].

The meningococcal strain 2C4-3, isolated as clone 12 from the serogroup C, class 1 strain 8013 (Nassif *et al.*, 1993) has the relevant phenotype P^+, Opa^-, Opc^-, $PilC1^+/PilC2^+$. Strain 2C4-3, the Δ*ptsH* mutant derived from it and the complemented strain Δ*ptsH*/*ptsH* were grown in GC medium base (GCB) (Difco) containing the supplements previously described (Kellogg *et al.*, 1963), in Roswell Park Memorial Institute (RPMI) medium and in RPMI medium supplemented with serum. In these media, no significant growth differences could be observed for the three strains (data not shown). We also used a previously constructed meningococcal Δ*crgA* mutant (Deghmane and Taha, 2003). When appropriate, kanamycin or spectinomycin was added at final concentrations of 100 and 75 μg/ml, respectively.

Construction of a N. meningitidis *Δ*ptsH *mutant*

DNA fragments of 500 bp from the upstream (*ptsM*) and downstream (*ptsI*) genes of *ptsH* (Fig. 1) were PCR amplified by using the primer pairs ptsHam-F/ptsHamREcoVR and ptsHavEcoVF/ptsHav-R (Table 1). The primer ptsHav-R contains the *N. meningitidis* DNA uptake sequence TTCAGACGGC, which allows efficient transformation of this bacterium (Table 1). Pfu DNA polymerase (Takara) or GoTaq DNA Polymerase (Promega) were used for all PCRs, and the correct sequence of the PCR products was confirmed by DNA sequencing. The resulting amplicons were restricted with BamHI/EcoRV and EcoRV/KpnI, respectively, and cloned into pBluescript II KS^+ cut with the same enzymes, thus providing plasmid pBluescript-ΔptsH. Finally, a terminator-less kanamycin resistance gene *aphA-3* was

amplified by PCR using plasmid pMGC20 (Nassif *et al.*, 1991) as a template and primers KanaSmaHindF and KanaSmaRev. The resulting DNA fragment was cut with SmaI and cloned into pBluescript-ΔptsH restricted with EcoRV, thus providing pBluescript-ΔptsH/aphA3. The presence of a HindIII site in the primer KanaSmaHindF allowed the identification of plasmids in which the *aphA3* gene was oriented in the same direction as the *ptsI* fragment. Strain 2C4-3 was transformed with one of the correctly oriented pBluescript-ΔptsH/aphA3 plasmids by following a previously described procedure (Taha *et al.*, 1988). Transformants were selected on GCB medium with kanamycin and for one clone the correct deletion was confirmed by PCR amplification with appropriate verification primers (Table 1). In addition, the absence of HPr was confirmed by carrying out a Western blot with antibodies raised against HPr and protein extracts of wild-type and Δ*ptsH* mutant (supplementary Fig. 2). In the isolated Δ*ptsH* mutant, the *ptsI* gene is expressed from the constitutive kanamycin promoter. This was also confirmed by showing that the *in vivo* PTS $EIIA^{Ntr}$ phosphorylation cascade was restored in the complemented Δ*ptsH* mutant. Phosphorylation of $EIIA^{Ntr}$ requires functional EI and HPr.

Complementation of the N. meningitidis *Δ*ptsH *mutant*

For the complementation of the Δ*ptsH* mutant we modified plasmid $pComP_{RBS}$ (Seib *et al.*, 2009), a derivative of pSLComCmr (Ieva *et al.*, 2005). $pComP_{RBS}$ contains the *cat* gene followed by the *tac* promoter and a ribosome binding site. These elements are flanked by two DNA fragments which allow their integration into the *N. meningitidis* large intergenic region between the genes *NMV0958* (*porA*) and *NMV0959* (Fig. 1). In the first step, we replaced the *cat* gene with the spectinomycin resistance gene *aad1*, which was PCR amplified by using plasmid pHP45Ω-spec (Prentki and Krisch, 1984) as a template and primers aad1-KpnI-2 and aad3-XbaI (Table 1). The obtained amplicon was cut with KpnI and XbaI and used to replace the *cat* gene of $pComP_{RBS}$ providing $pComP_{RBS}$-spec. In the second step, the *ptsH* gene was amplified with primers ptsH-F-NdeI and ptsH-R-NsiI. The resulting amplicon was cut with NdeI and NsiI and cloned into $pComP_{RBS}$-spec cut with the same enzymes. In the resulting plasmid $pComP_{RBS}$ptsH the *ptsH* gene is expressed from the *tac* promoter, which in the absence of LacI functions constitutively. This plasmid was subsequently used to transform the Δ*ptsH* mutant. Transformants were selected on GCB medium that contained kanamycin and spectinomycin and insertion at the correct locus was confirmed by sequencing appropriate amplified DNA fragments. In addition, qRT-PCR experiments confirmed the expression of

the *ptsH* gene in the complemented strain (data not shown) and Western blots revealed similar amounts of HPr in wild-type and complemented Δ*ptsH* mutant (Supplementary Fig. 2).

Protein purification

The *ptsH* (*NMV2248*), *hprK* (*NMV1662*), *ptsN* (*NMV1663*), *ptsM* (*NMV2249*), and *crgA* (*NMV2045*) genes were amplified using genomic DNA from strain 2C4-3 and the oligonucleotide pairs ptsHNmStrepForKpnI/ptsHNmStrepRevBam, HprK-Nme5'/HprK-Nme3' SP1/SP2, SP22/SP23, and CrgA2C43BsaIF/CrgA2C43XhoI, respectively (see Table 1). The amplified *ptsN* and *ptsM* genes were cut with BamHI and KpnI and *hprK* was digested with BamHI and HindIII and the three genes were cloned into the His-tag expression vector pQE30 (Qiagen) cut with the corresponding enzymes to provide pQE30-ptsN, pQE30-ptsM, and pQE30-hprK, respectively. The resulting plasmids were used to transform *E. coli* strain NM522 (for pQE30-ptsN) or M15[pREP4] (for pQE30-ptsM and pQE30-hprK). The amplified *crgA* gene was cut with BsaI and XhoI and cloned into the His-tag expression vector pET-28b cut with NcoI and XhoI thus providing plasmid pET28b-crgA. The resulting plasmid was used to transform *E. coli* strain BL21-DE3. Finally, the amplified *ptsH* gene was cut with KpnI and BamHI and cloned into the strep-tag expression vector pGP172 (Merzbacher *et al.*, 2004) cut with the same enzymes to provide pGP172-ptsH. The resulting plasmid was also used to transform *E. coli* strain BL21-DE3.

The *E. coli* M15[pREP4] and NM522 strains harbouring the different pQE30-derived expression vectors and the BL21-DE3 strains containing either plasmid pGP172-ptsH or pET-28b-crgA were grown at 37°C in 500 ml of LB medium supplemented with appropriate antibiotics to an OD_{600} of about 0.5. Expression of the different *N. meningitidis* genes was subsequently induced by growing the cells for 3 h in the presence of 0.1 mM IPTG. For the strains used for the purification of CrgA, $EIIA^{Man}$ and HprK the temperature was shifted to 28°C. Purification of His-tagged HprK, $EIIA^{Ntr}$ and $EIIA^{Man}$ was carried out on Ni-NTA columns under non-denaturing conditions as recommended by the manufacturer. His-tagged CrgA was purified as previously described (Deghmane *et al.*, 2000). Strep-tagged HPr was mainly present in inclusion bodies, which were solubilized with 3 M guanidinium chloride for 2 h at room temperature. The protein solution was dialyzed before it was loaded onto a streptavidin column (IBA, Göttingen). After washing, HPr was eluted with desthiobiotin. The recovered protein seemed to be correctly folded because it could be phosphorylated with *N. meningitidis* HprK (Fig. 8A and B) or *L. casei* HprK/P and ATP (Supplementary Fig. 3) as well as with EI and PEP (Fig. 9A and B). Our attempts to purify active *N. meningitidis* EI

were not successful. We therefore used *B. subtilis* EI purified from strain M15[pREP4, pAG3] (Galinier *et al.*, 1997) for *in vitro* PEP-dependent phosphorylation assays. $EIIA^{Lev}$ from *L. casei* was purified as described in (Mazé *et al.*, 2004).

Protein phosphorylation assays

[^{32}P]PEP was synthesized by using the PEP-pyruvate isotope exchange method in the presence of pyruvate kinase, pyruvate and [γ-^{32}P]ATP (Roossien *et al.*, 1983). Phosphorylation assays with [^{32}P]PEP, EI, HPr and $EIIA^{Ntr}$ or $EIIA^{Man}$ were carried out at 37°C in 40 µl reaction mixtures containing 50 mM Tris-HCl (pH 7.4), 10 µM [^{32}P]PEP (0.1 µCi), 5 mM $MgCl_2$, 5 µg of EI, 5 µg of HPr, and 3 µg of $EIIA^{Ntr}$ or $EIIA^{Man}$. The reactions were stopped after 20 min by the addition of SDS sample buffer. Proteins were separated by electrophoresis on 0.1% SDS-15% polyacrylamide gels, which were dried and exposed over night to a storage phosphor screen. The HPr(Ser) kinase assays were performed in 30 µl reaction mixtures containing 50 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 25 µM [γ-^{32}P]ATP or [^{32}P]PPi (0.35 µCi each), 250 ng of HprK and 3 µg of HPr as well as varying amounts of FBP and Pi. The assay mixtures were incubated for 20 min at 37°C and the reactions were stopped by the addition of SDS sample buffer. Proteins were separated on 0.1% SDS-15% polyacrylamide gels. After electrophoresis, the gels were boiled for 10 min in 0.5 N HCl, dried and exposed to a storage phosphor screen over night.

P-Ser-HPr dephosphorylation assays

In order to carry out P-Ser-HPr phosphorylase assays, *N. meningitidis* P-Ser-HPr was prepared by incubating 2 mg strep-tagged HPr with 0.5 mg His-tagged HprK for 30 min at 37°C in 100 µl 50 mM Tris-HCl (pH 7.4) containing 5 mM $MgCl_2$, 25 µM [γ-^{32}P]ATP (0.35 µCi) and 2.5 mM FBP. Under these conditions, HPr was almost completely phopsphorylated at Ser-46. The assay mixture was then loaded onto a Strep-tag column to remove His-tagged HprK and ATP and P-Ser-HPr was subsequently eluted with biotin and passed over a PD10 column (GE Healthcare) equilibrated with 20 mM ammonium bicarbonate before it was lyophylized. P-Ser-HPr dephosphorylation assays were carried out in 50 µl samples containing 50 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 1 µg P-Ser-HPr, 200 ng HprK and increasing concentrations of potassium phosphate (pH = 7.4). Assay mixtures were incubated for 20 min at 37°C and the reactions were stopped by adding SDS sample buffer. After

electrophoresis, the gel was boiled for 10 min in 0.5 N HCl, dried and exposed to a storage phosphor screen over night.

Detection of in vivo *phosphorylated EIIA^{Ntr} by Western blots*

Rabbit pAbs were raised against purified *N. meningitidis* EIIA^{Ntr} and used to detect EIIA^{Ntr} and P~EIIA^{Ntr} in crude extracts of strain 2C4-3, the isogenic Δ*ptsH* mutant and the complemented strain Δ*ptsH*/*ptsH*. Cells grown on solid GCB medium were resuspended in 10 mM Tris-HCl, pH 8 and crude extracts were prepared. Aliquots of each strain corresponding to 60 µg of total protein were loaded on a non-denaturing 12.5% polyacrylamide gel and separated by electrophoresis. Under these conditions, EIIA^{Ntr} and P~EIIA^{Ntr} migrate to slightly different positions. After electrophoresis, proteins were transferred onto a nitrocellulose membrane, which was treated with anti-EIIA^{Ntr} rabbit pAbs diluted 1:1000-fold. Alkaline phosphatase-conjugate anti-rabbit antibodies at a 1:5000 dilution and the BCIP/NBT color development substrate kit were used for the detection of EIIA^{Ntr} and P~EIIA^{Ntr} bands according to the manufacturer's recommendations (Promega).

Virulence assays in mice

The virulence of meningococcal strains was evaluated by their ability to provoke bacteremia in mice after intraperitoneal challenge. The experimental design was approved by the Institut Pasteur Review Board. Bacterial loads in blood are a good indicator of virulence, as they reflect bacterial survival in blood upon invasion of the bloodstream. Blood bacterial counts in six-week-old female BALB/c mice (Janvier, France) were performed 2, 6 and 24 h after intraperitoneal challenge with standardized inocula of 10^7 colony forming units (cfu) per mouse for each tested strain. Mice were bled and bacterial counts were determined by plating serial dilutions of blood on GCB plates.

Capsule quantification with ELISA and flow cytometry.

Bacteria were cultured on GCB medium with supplements and after 18-20 h of growth bacterial suspensions (2.5 x 10^7 bacteria/ml) were prepared in PBS. Bacteria were inactivated by incubating the suspension for 30 min at 56°C and 100 µl aliquots were subsequently loaded in the wells of maxisorp microtiter plates (NUNC). After overnight incubation at 45°C, the wells were twice washed with PBS containing Tween 80 (0.1%) and subsequently incubated with 5% skimmed milk in PBS/Tween 80 (0.1%) for 30 min at 37°C. Anti-

serogroup C mouse monoclonal antibodies were purchased at the National Institute for Biological Standards and Control (Hertfordshire, UK) were 1000-fold diluted in PBS containing Tween 80 (0.1 %) and skimmed milk (5%) and 100 µl aliquots were subsequently added to the wells. After incubation at 37°C for 1 h the wells were washed as above. The same procedure was repeated with the peroxidase-coupled secondary mouse anti-IgG antibodies, which were diluted 5000-fold. After washing, 100 µl of o-phenylenediamine and hydrogen peroxide dissolved in 0.1 M citrate bufffer, pH 5.5, was added. The plates were kept for 10 to 30 min in the dark before 50 µl of 2 N sulfuric acid was added. The formation of orange colour was subsequently measured with a Multiskan Ascent plate reader (MTX Lab Systems) at OD_{492}.

Alternatively, capsule synthesis was measured using a rabbit pAb specific against meningococcal serogroup C capsule polysaccharides. Briefly, approximately 1×10^7 bacteria (grown on GC plates) were washed twice with PBS. Bacterial pellets were resuspended in 100 µl PBS supplemented with 1% (w/v) bovine serum albumin (BSA). The anti-serogroup C pAb was added at a 1:100 dilution and the reaction was incubated at 37°C for 30 min. After two washes in PBS/1% BSA, bacteria were resuspended in the same buffer in the presence of fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories Inc., Suffolk, UK) at a 1:100 dilution and incubated at 37°C for 30 min. The bacterial pellets were twice washed with PBS/1% BSA, subsequently resuspended in 500 µl PBS and fixed with 1% paraformaldehyde. Rabbit pAb specific to capsular polysaccharide of serogroup B was used as a negative control. For each sample, 10000 events were acquired on a BD FACS Calibur flow cytometer and analyzed using the Cyflogic software. The mean fluorescence intensity (MFI) of the FITC channel was determined for each sample after gating on bacterial cells in the logarithmic FSC versus SSC dot plot.

Host cell adhesion assays

Hec-1-B cells (a human endometrial carcinoma cell line) were maintained in RPMI1640 medium (Gibco BRL) supplemented with 10% heat-inactivated fetal bovine serum. Cells were placed in 24-well plates at $2x10^5$ cells per well and incubated for 24h at 37°C under 5% of CO_2. Meningococcal strains were suspended in the same cell-culture medium at $2x10^6$ cfu/ml (a 10-fold excess over Hec-1-B cells) and 1 ml of the bacterial suspension was used to replace the culture medium in each Hec-1-B cells-containing well. After 6 h infection the medium was removed and the remaining cells were washed before cells and cell-associated

bacteria were lifted off the plates. Adhesion levels were then determined by viable count assays on GCB agar plates as previously described (Deghmane *et al.*, 2000). Adhesiveness is expressed as the ratio of cell-associated cfu and cfu present in the medium multiplied with 100.

Annexin V assay for apoptosis

One of the characteristics of apoptosis is the flipping of phosphatidylserine from the inner to the outer bilayer of the plasma membrane. The localisation change of phosphatidyl-serine in the cell membrane during apoptosis can be detected with annexin V. Co-staining with annexin V and propidium iodide (PI) allows the differentiation between viable cells (annexin V-negative, PI-negative), early apoptotic cells (annexin V-positive, PI-negative) and late apoptotic cells (annexin V-positive, PI-positive). Hec-1-B cells were seeded in 24-well tissue culture plates and infected with bacteria as previously described (Deghmane *et al.*, 2009). After incubation for 24 h, cells were harvested using cold phosphate buffered saline (PBS)/0.02% EDTA and washed twice with PBS. Staining was carried out using the Annexin V-FITC kit (Immunotech, Marseille, France). Briefly, 2 x 10^5 cells were resuspended in binding buffer and incubated with annexin V-FITC and PI for 15 min in a darkroom at ambient temperature. After adequate compensation, annexin V binding was analyzed by using a FACScan cytometer (BD Biosciences) equipped with an FITC signal detector FL1 (excitation = 488 nm, green) and a phycoerythrin emission signal detector FL2 (excitation = 585 nm, red) for PI stained cells. The percentage of apoptotic cells was calculated from a total of 10000 cells using the Cyflogic software (www.cyflogic.com).

Electrophoretic Mobility Shift Assays

DNA fragments (8 nM final concentration) derived from the promoter region of *crgA* or the *ptsH* gene (served as a control) were mixed with various concentrations of the protein CrgA. Only a small fraction of the DNA was shifted when CrgA was present at a concentration of 150 nM. Adding 5 μM HPr significantly increased the shifted band, whereas the HPr storage buffer had no effect. The assay mixture also contained 75 mM NaCl. After mixing all components, samples were immediately loaded on a 7% polyacrylamide gel buffered with 25 mM Tris base/190 mM glycine. DNA fragments were separated by electrophoresis and visualized by UV after incubating the gels in an ethidiumbromide solution (1 μg/ml).

Mass spectrometry

A gel piece containing the shifted DNA band from Fig. 6B, lane 3 was cut out and washed for 15 min with an acetonitrile/100 mM ammonium bicarbonate solution (1:1). Modified trypsin (0.1 µg, Promega, sequencing grade) was added and proteins were digested for 6 h at 37°C in 50 mM ammonium bicarbonate, pH 8.0. The supernatant was removed and peptides remaining in the gel piece were extracted with 5% formic acid in water/acetonitrile (v/v) and centrifuged. The supernatants were pooled, dried, dissolved in 30 µl of 0.1% (v/v) trifluoroacetic acid (TCA) in 2% acetonitrile and separated by HPLC (Ultimate 3000 LC system; Dionex). A 4-µl aliquot was loaded on a pre-column cartridge (flow rate 20 µl/min; stationary phase: C18 PepMap 100 Å, 5 µm; column: 300 µm i.d., length 5 mm; Dionex) and desalted for 4 min with 0.08% TFA in 2% acetonitrile. The precolumn cartridge was subsequently connected to a PepMap C18 column (100 Å, 2 µm; 75 µm i.d., length 50 cm; Dionex). Peptides were eluted with a 0 to 36% gradient of solvent B (0.1% formic acid in 80% acetonitrile; 300 nl/min) within 18 min; solvent A was 0.1% formic acid in 2% acetonitrile. Eluted peptides were analyzed on-line with a LTQ-Orbitrap mass spectrometer (Thermo Electron) using a nanoelectrospray interface as previously described (Misra *et al.*, 2014).

Two databases were used for data processing: the NCBI database for *E. coli* K12 strains from August 2013; 9033 protein entries), we added the sequences of CrgA and HPr from *N. meningitidis* 8013 (accession numbers in UniprotKB: Q9JPU9 and C9X2R2, respectively). This database together with a protein to peptide reverse database (version 2013.02.01.1 created by Database Manager v2.0) and contaminant databases were searched by the algorithm: X!Tandem.

The precursor mass tolerance was 10 ppm and the fragment mass tolerance was 0.5 Daltons. One missed tryptic cleavage was allowed. The carbamidomethylation of cysteine residues was set as a fixed modification and the oxidation of methionine residues as a variable modification. Results were filtered using an in-built X!Tandem parser (X!Tandem version 3.3.1) with peptide E value smaller than 0.05 and protein log E value smaller than -2.8.

In vivo *co-immunoprecipitation*

GCB-grown bacteria were lysed in lysis buffer (25 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 100 mM NaCl, 1% Triton X-100, 0.5% NP-40 and a protease inhibitor cocktail)

for 1 h at 4°C and cell debris was removed by centrifugation at 18000 g for 30 min. Aliquots containing 0.5 mg of soluble proteins were mixed with 5 µg of anti-CrgA or anti-HPr pAbs, and incubated for 2 h at 4°C. The HPr or CrgA protein complexes bound to their antibodies were pulled down with protein A-agarose beads as previously described (Deghmane *et al.*, 2007). The agarose beads were extensively washed and protein complexes were solubilized in Laemmli buffer and subsequently submitted to SDS-PAGE. After electrophoresis, proteins were transferred onto a nitrocellulose membrane and immunoblotted with anti-CrgA or anti-HPr pAbs followed by peroxidase-conjugated anti-IgG antibodies. Co-immunoprecipitated HPr or CrgA were detected with the corresponding antibody using the ECL method (GE Healthcare).

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Table 1. List of primers used in this study.

Primer	Used for	Sequence (5' to 3')
HprK-Nme5'	pQE30-hprK	CTGAAG*GGATCC*ATGCCCAGTTATCTCC[a]
HprK-Nme3'		GCCCG*AAGCTT*ACAGGCCGCTAATC
ptsHNmStrepForKpnI	pGP172-ptsH	GGG*GGTACC*AATGCTCAAACAATCCATCG
ptsHNmStrepReBam		GGC*GGATCC*TTTGCCCGCCGCCACGCCG
SP1	pQE30-ptsN	AAGAG*GGATCC*ATGAGCCCTTATCG
SP2		GCCGGC*GGTACC*GGCCGTCGAAG
SP22	pQE30-ptsM	GGACG*GGATCC*ATGATAGGGCT
SP23		CGGCAGCA*GGTACC*GTTTAAAATG
CrgA2C43-BsaIF	pET28b-crgA	GGG*GGTCTC*CCATGAAAACCAATTCAGAAGAAC
CrgA2C43-XhoI		GGGG*CTCGAG*TCCACAGAGATTGTTTCCC
ptsHam-F	Δ*ptsH* construction	CCGC*GGATCC*GCGTGCAACCGACGGAAGAC
ptsHamEcoVR		TATG*GATATC*GGCAGCAGACGCCGTTTAAAATG
ptsHavEcoVF		CGAC*GATATC*AACGGCTACTTCGGCGAG
ptsHav-R		CCCG*GGTACCC*G**TTCAGACGGC**TTGCAGCATATCCTGC[b]
kanaSmaHindF		ACGT*CCCGGG*AAGCTTCCCAGCGAACCATTTGAGG
kanaSmaRev		TAAA*CCCGGG*TACTAAAACAATTCATCCAG
ptsM1-F	Δ*ptsH* verification	CATCACACACGAAACCATAGG
ptsI4-R		CCACAAGCTCGATGCAGACC
aad3-XbaI	Spectinomycin	ATTCAG*TCTAGA*GCTTAGTGCATCTAACGCTTG
aad1-KpnI-2		ATTCAG*GGATCC*AAGCTCTCGGGTAACATCAAG
ptsH-F-NdeI	Δ*ptsH* complementation	ATCCAG*CATATG*CTCAAACAATCCATCGAAATCATC
ptsH-R-NsiI		ATTCAG*ATGCAT*TTATTCGCCCTCGCCGAAGTAGCCGTTG
COMCFW-NVD	Verify Complementation	CCTCGAGCCGCTGACCGAAGG
COMCREV-NVD		ACCGGCATCGGCAACTACAC
aadA1		TGCCGTCACGCAACTGGTCCA
aadA2		CAACTGATCTGCGCGCGAGGC
CrgAPro98-2F	crgA promoter region	GATGAATGTATTGTGCCGTTTTAC
CrgAPro98-3R		CACCACTTGAACAAATACGGTCAGTTCTTC
ptsHRT-F3	RT-PCR	ATGGGGCTGATGATGCTCGC
ptsHRT-R3		GTAGCCGTTGATTAAGTCGG
ptsIRT-F2		AAGCAGCAGAGCGACAAACT
ptsIRT-R2		GCAAATAGGCATCGTCCA
ptsMRT-F2		ACACGAAACCATAGGCGAAG
ptsMRT-R2		AGTATGCGGACGTTTTCAGG

[a]Restriction sites are underlined and in italics.

[b]The *N. meningitidis* DNA uptake sequence is written in bold letters.

Fig. 1

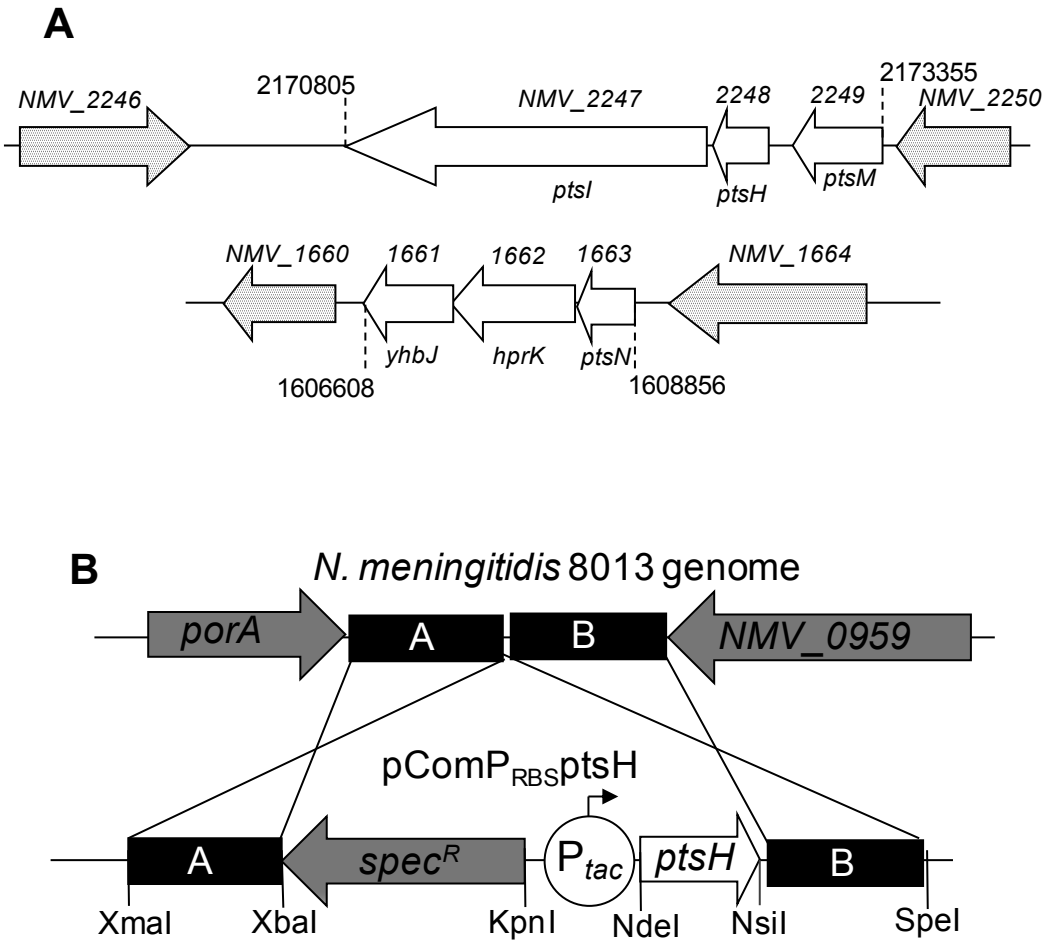


Fig. 1. A. Schematic presentation of the two genomic regions encoding *ptsM-ptsH-ptsI* and *ptsN-hprK-yhbJ*, respectively, in *N. meningitidis* 2C4-3, also known as strain 8013. The numbers indicate the location on the *N. meningitidis* 2C4-3 genome (first base for *ptsM* and *ptsN* and last base of *ptsI* and *yhbJ*). *NMV_2246* encodes a putative ATP bining protein of an ABC transporter; *NMV_2250* a putative hypoxanthine guanine phosphoribosyl transferase; *NMV_1660* a putative GTP binding protein; *NMV_1664* is annotated as *ubiA*, which is involved in ubiquinone biosynthesis.

B. Integration of the Φ(*tacp-ptsH*) transcriptional fusion at the intergenic region upstream from *porA*. The integrative plasmid pComP$_{RBS}$ptsH carries the first part of the intergenic region between the *NMV0959* and *porA* genes, a spectinomycin resistance cassette, the *tac* promoter together with the ribosome binding site, the meningococcal *ptsH* gene, and the second part of the intergenic region. Double crossover allows chromosomal insertion of the spectinomycin resistance cassette and the *tacp*-controlled *ptsH* gene at the intergenic region.

Fig. 2

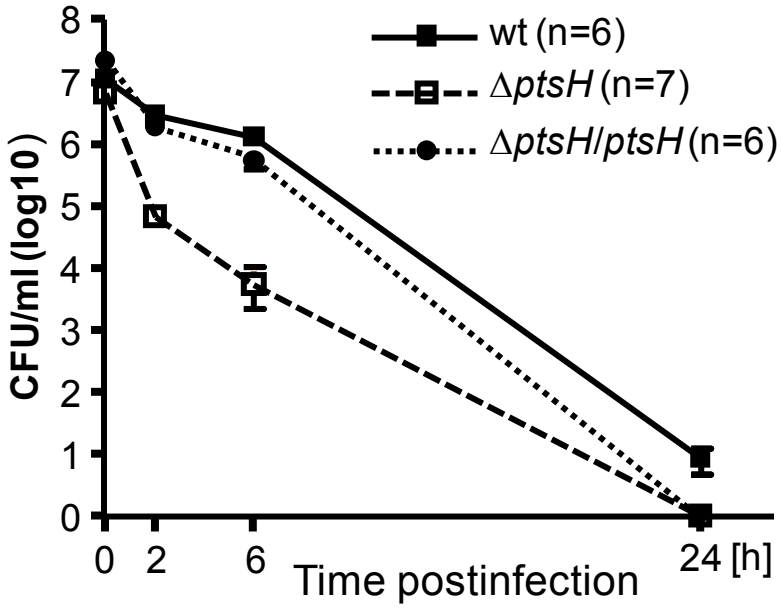


Fig. 2. Virulence of *N. meningitidis* strains in mice.

The virulence of meningococcal strains in BALB/c mice was evaluated after intraperitoneal challenge with standardized inocula of 10^7 bacteria. Each strain was used to infect six or seven mice. Survival of the wild-type 2C4-3 (wt, filled squares), the Δ*ptsH* mutant (open squares) and the Δ*ptsH/ptsH* complemented strain (filled circles) was determined by bacterial counts in the blood of mice 2, 6 and 24 h after intraperitoneal infection. Data were calculated from two independent experiments (mean values with standard errors). ***$P = 0.02$ student *t* test.

Fig. 3

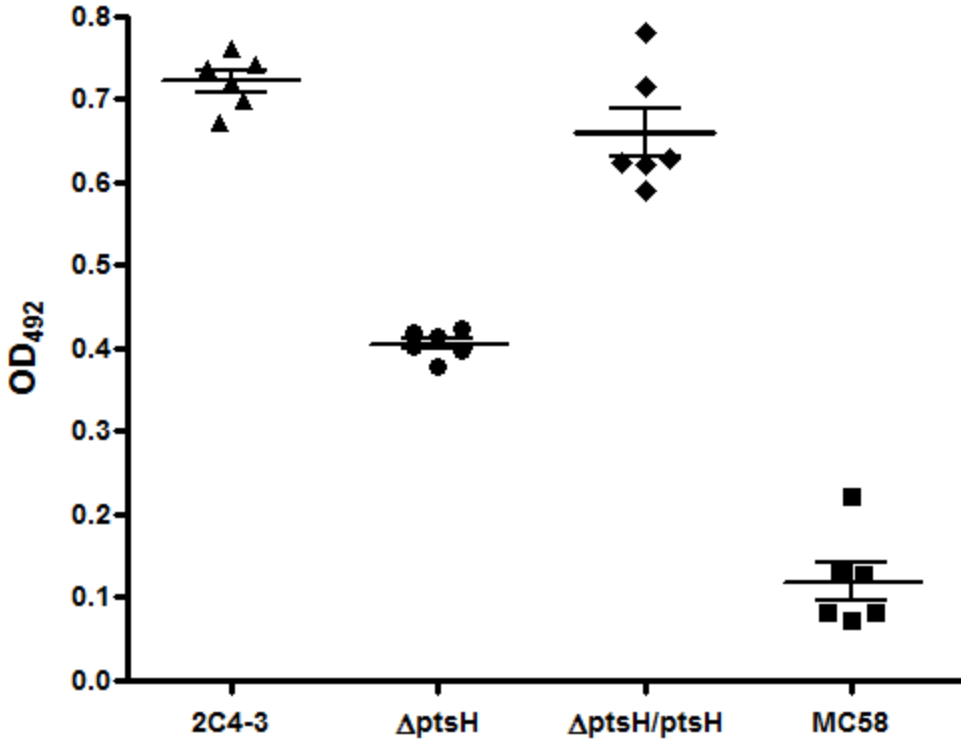


Fig. 3. Quantification of capsule production by ELISA. Bacterial cells of the serotype C wild-type strain 2C4-3, the Δ*ptsH* mutant derived from it and the complemented Δ*ptsH/ptsH* were prepared as described in Experimental procedures. Serotype C capsule-specific monoclonal mouse antibodies were used for the ELISA. The serotype B strain MC58, which produces a different type of capsule, served as a negative control. Cells of each strain were loaded in 6 wells and the measured OD_{492} values are presented. The experiment was repeated 3-times and nearly the same differences in capsule production were always obtained.

Fig. 4

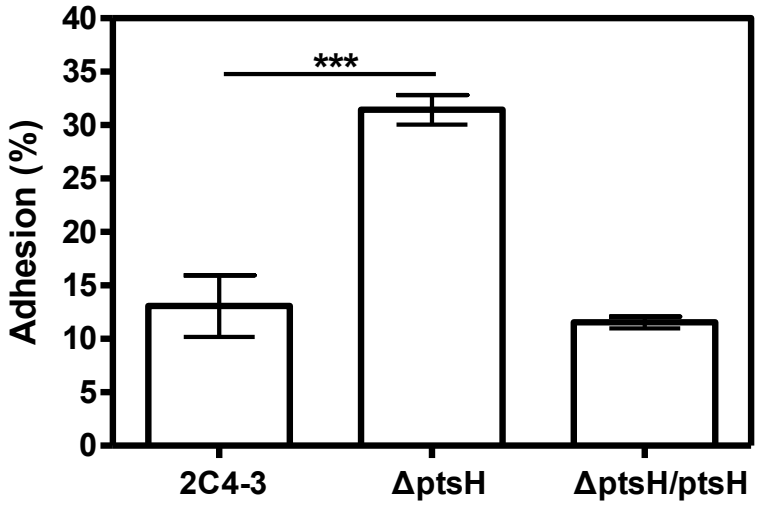


Fig. 4. Comparison of the adhesion efficiency to Hec-1-B cells between the *N. meningitidis* wild-type strain 2C4-3 and the Δ*ptsH* and Δ*ptsH/ptsH* strains. Infection was performed as described in Experimental procedures. At 6 h post-infection, cells and cell-associated bacteria were lifted off the plates and the adhesion level was measured as described in Experimental procedures. Adhesiveness is expressed as the ratio of cell-associated cfu and cfu present in the medium multiplied with 100. Presented are the mean values and standard deviations (vertical bars) calculated from four independent experiments.

Fig. 5

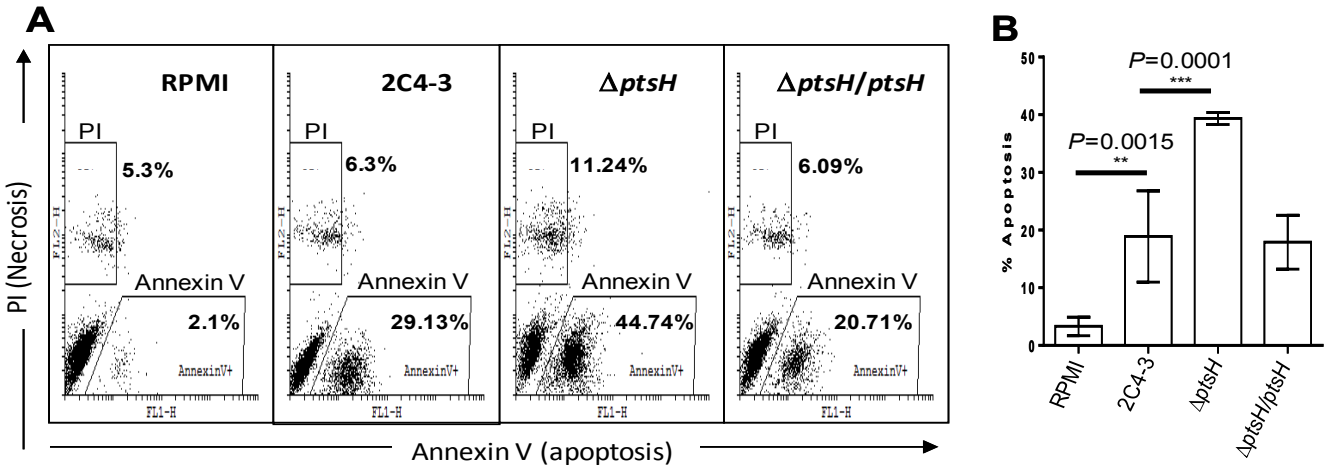


Fig. 5. Cell death of Hec-1-B cells 9 h and 24 h after infection either with *N. meningitidis* strain 2C4-3, the Δ*ptsH* mutant or the complemented Δ*ptsH/ptsH*. Subconfluent monolayers of Hec-1-B epithelial cells were cultured without or with *N. meningitidis* strains 2C4-3, *ΔptsH* or *ΔptsH/ptsH*. Hec-1-B cells were harvested at 24 h after infection and incubated with FITC-conjugated annexin V and propidium iodide (PI).

A. Cell death was measured by flow cytometry and representative dot plots are shown. The annexin V-positive regions (corresponding to apoptotic cells) and PI-positive regions (corresponding to necrotic cells) are indicated. The percentage of each population is listed in the corresponding region.

(B) Presented are the mean values ± standard deviations of the percentage of apoptotic cells determined for the three strains in three independent experiments.

Fig. 6

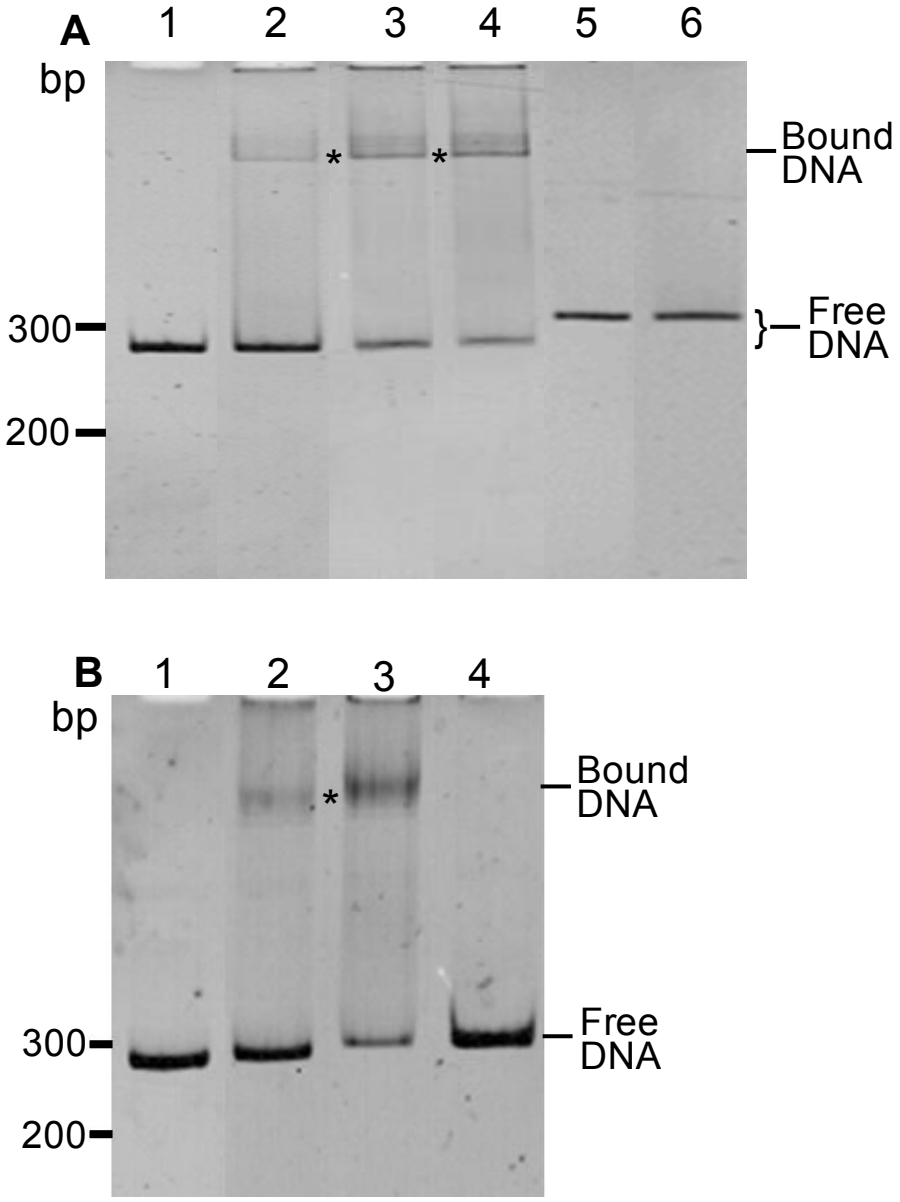


Fig. 6. EMSA experiments on polyacrylamide gels were carried out with CrgA and either the promoter region of the *crgA* gene or a similar sized negative control DNA in the presence or absence of HPr.

A. The samples loaded on lanes 1 to 4 contained 8 nM DNA of the *crgA* promoter and increasing amounts of CrgA (0, 150, 300 and 450 nM). The presence of CrgA leads to the appearance of a slower migrating band indicated with an *. The samples loaded on lanes 5 and 6 contained 8 nM negative control DNA and in lane 6 also 450 nM CrgA. CrgA does not alter the migration behaviour of the contol DNA.

B. All samples loaded on this gel contained 8 nM DNA of the *crgA* promoter. The following proteins were added: Lane 2, 150 nM CrgA; lane 3, 150 nM CrgA and 5 μM HPr; lane 4, 5 μM HPr. HPr does not alter the migration position of the DNA (lane 4), but apparently enhances the affinity of CrgA for its DNA target (lane 3).

Fig. 7

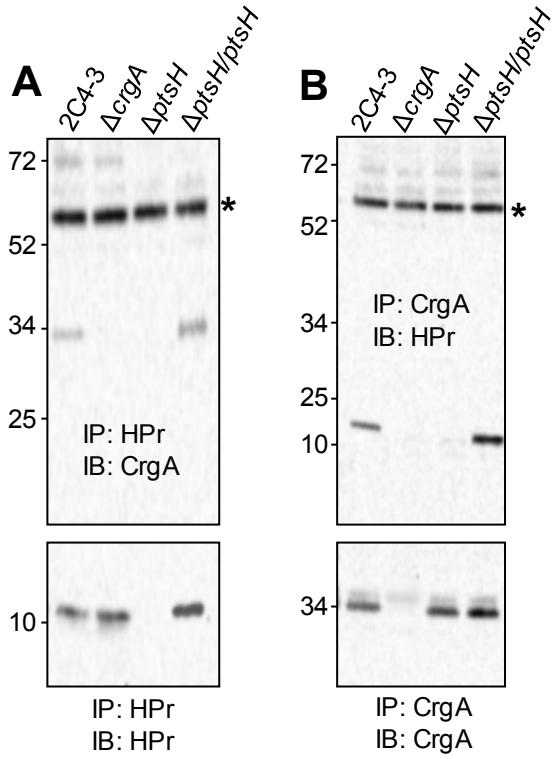


Fig. 7. Co-immunoprecipitation of HPr and CrgA. Immunoprecipitations (IP) were performed with anti-HPr (A) or anti-CrgA antibodies (B) on crude extracts prepared from four *N. meningitidis* strains (wild-type 2C4-3, Δ*crgA*, Δ*ptsH* and Δ*ptsH/ptsH*). Proteins, which were bound to protein A Sepharose, were separated by electrophoresis on SDS polyacrylamide gels. HPr and CrgA were detected by immunoblotting (IB) with the appropriate antibody. Co-immunoprepcipitation was only detected in strains producing both, HPr and CrgA, but was absent in lacking either one of the two proteins. The * indicates the migration position of the heavy chain of the antibodies used for immunoprecipitation.

Fig. 8

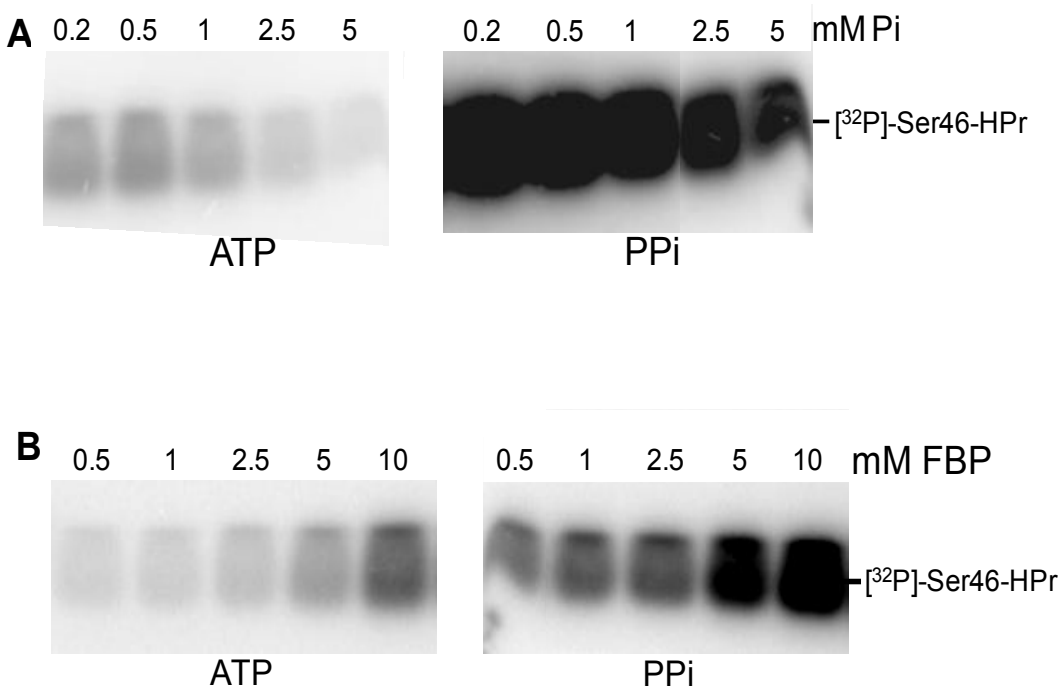


Fig. 8. Autoradiograms of phosphorylation assays with *N. meningitidis* HprK and HPr or [^{32}P]P-Ser-HPr.

A. HPr kinase assays with 250 ng of HprK and 3 μg of HPr in the presence of 10 mM FBP and [γ-^{32}P]ATP or [^{32}P]PPi, and increasing concentrations of potassium phosphate (0.2; 0.5; 1; 2.5 and 5 mM). For all experiments, samples were separated by SDS-PAGE and radioactive bands were detected by exposing the dried gels to a storage phosphor screen over night.

B. HPr kinase assays with 250 ng of HprK and 3 μg of HPr in the presence of 2.5 mM Pi and [γ-^{32}P]ATP or [^{32}P]PPi and increasing concentrations of FBP (0.5; 1; 2; 5 and 10 mM).

Fig. 9

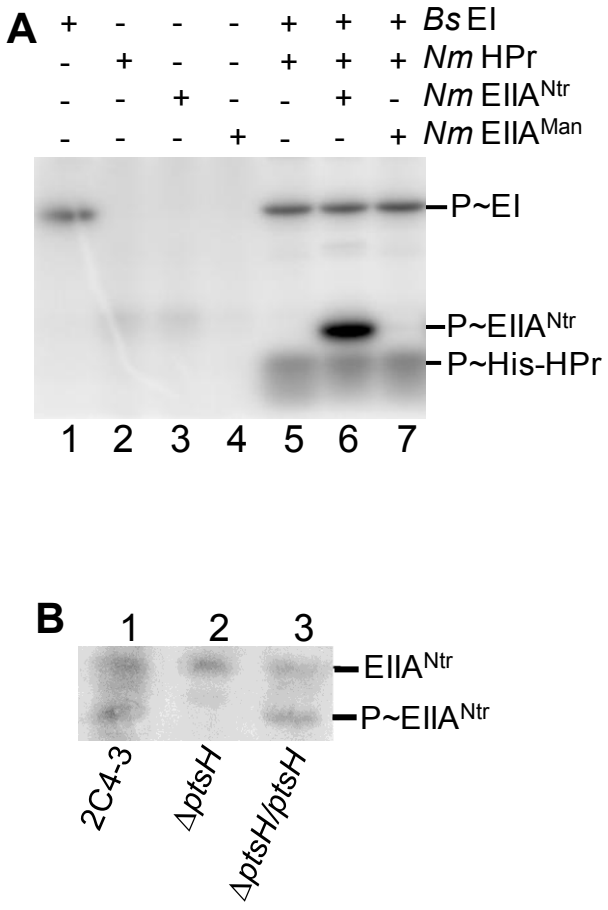
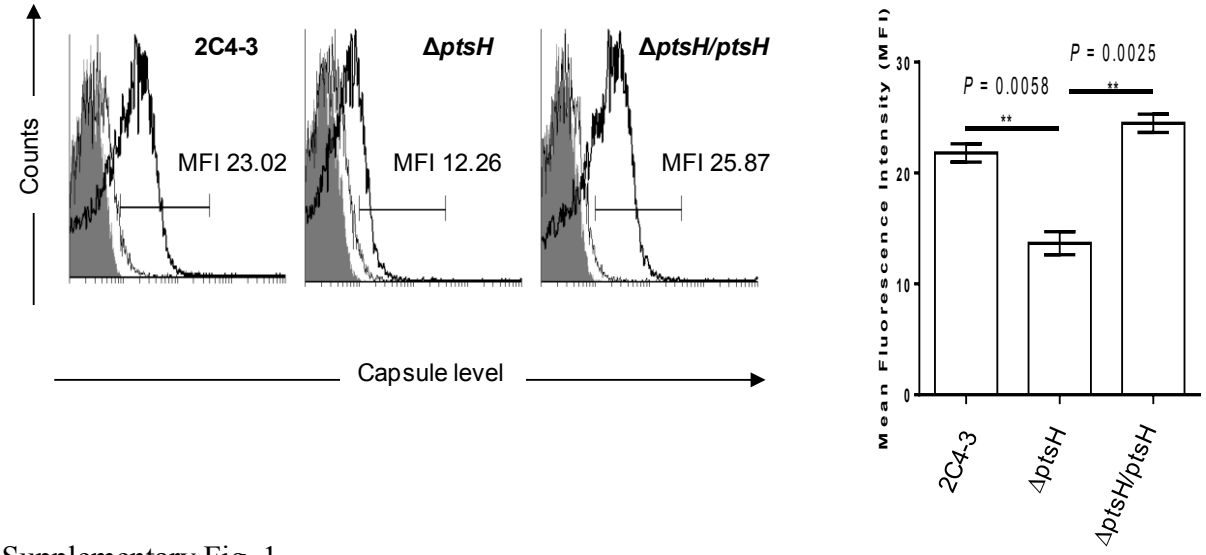


Fig. 9. *In vitro* and *in vivo* PEP-dependent phosphorylation of PTS proteins.

A. Autoradiogram after phosphorylation of PTS proteins with [^{32}P]PEP. Samples containing the indicated purified proteins were separated by SDS-PAGE. Bars indicate the migration positions of phosphorylated *B. subtilis* EI and meningococcal EIIA^{Ntr} and HPr.

B. Western blot with crude extracts prepared from wild-type strain 2C4-3, the Δ*ptsH* mutant and the complemented Δ*ptsH/ptsH* strain after electrophoresis on a non-denaturing 12.5% polyacrylamide gel. The bands corresponding to phosphorylated and unphosphorylated EIIA^{Ntr} were detected with rabbit pAbs against purified His-tagged *N. meningitidis* EIIA^{Ntr} and their migration positions are indicated with bars.

Supplementary Fig. 1

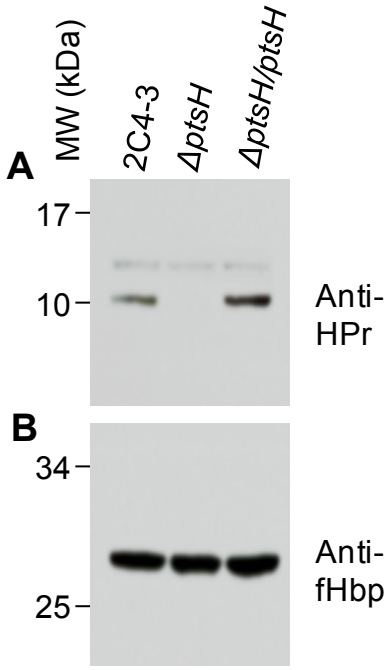


Supplementary Fig. 1.

A. The production of capsular polysaccharides by the different strains was determined by flow cytometry using a specific rabbit anti-serogroup C pAb (bold open histograms). A rabbit anti-serogroup B pAb (regular open histograms) was used as a negative control. Unlabeled bacteria are shown with grey solid histograms. The mean fluorescence intensity is indicated on the top of each marker line.

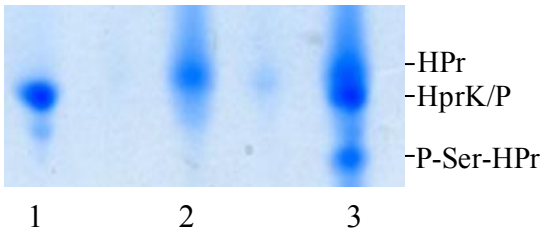
B. Quantification of capsule levels from the above strains expressed as mean fluorescens intensity (means ± SD) from three independent experiments.

Supplementary Fig. 2



Supplementary Fig. 2. A. The presence of HPr was determined in the wild-type strain 2C4-3, the Δ*ptsH* mutant and the complemented strain Δ*ptsH/ptsH* by Western blot analysis. B. Antibodies against the meningococcal factor H binding protein (fHbp) were used as a loading control.

Supplementary Fig. 3



Supplementary Fig. 3. Separation of proteins from an ATP-dependent HPr phosphorylation assay on denaturing polyacrylamide gels containing 6 M urea (Deutscher *et al.*, 1982). All samples contained 5 mM ATP, 10 mM $MgCl_2$, and 20 mM FBP. The following proteins were also added: Lane 1, HprK/P(V267F) from *L. casei* (Monedero *et al.*, 2001b); lane 2, HPr of *N. meningitidis*; lane 3, *L. casei* HprK/P(V267F) and HPr from *N. meningitidis*. In lane 3, a new faster migrating band appeared confirming that more than 50% of the *N. meningitidis* HPr were phosphorylated at Ser-46.

III. Résultats supplémentaires

III. 1. Vérification de la délétion/complémentation du gène *ptsH* par qRT-PCR

Dans le but de vérifier la délétion du gène *ptsH* dans le mutant Δ*ptsH* et la complémentation correcte de ce dernier par le même gène, nous avons réalisé des qRT-PCR sur 80 ng d'ARN total de la souche sauvage, du mutant Δ*ptsH* et du mutant complémenté Δ*ptsH*/*ptsH*. Les résultats ont montré l'expression du gène *ptsH* chez la souche sauvage 2C4-3 et le mutant complémenté Δ*ptsH*/*ptsH* et son absence chez le mutant Δ*ptsH* (Figure 17).

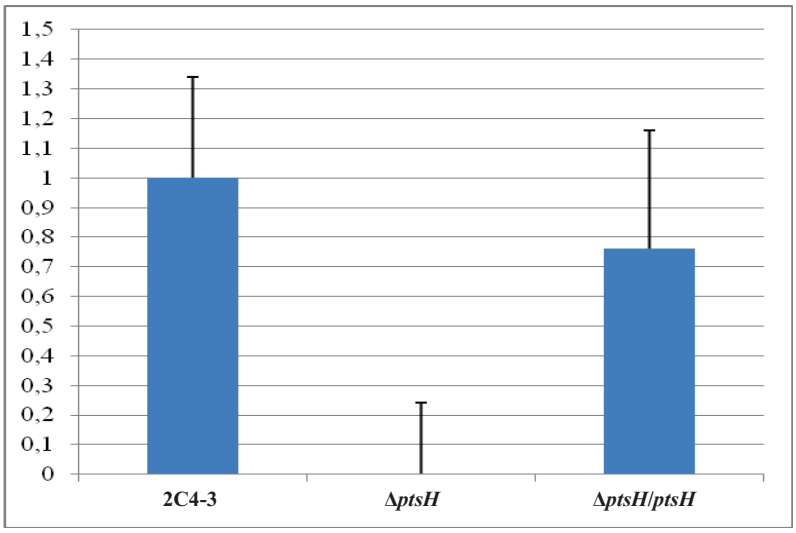


Figure 17 : Résultats de la qRT-PCR pour l'expression du gène *ptsH* chez la souche sauvage 2C4-3, le mutant Δ*ptsH* et le mutant complémenté Δ*ptsH*/*ptsH*.

III. 2. Purification des protéines HPr et CrgA

La protéine CrgA a été purifiée à partir de la souche *E. coli* BL21(DE3) selon le protocole décrit par Deghmane *et al.* (2000). Cette protéine porte une étiquette histidine à son extrémité C-terminale. La protéine CrgA se présente sous forme d'un dimère très stable. Le chauffage à une température de 90°C pendant 10 min ou l'ajout de l'EDTA (10 mM) n'ont pas permis la dissociation de ce dimère. Des analyses de spectrométrie de masse ont été réalisées sur la bande correspondant à une taille de 55 à 70 kDa sur le gel de polyacrylamide (Figure 18) et les résultats ont confirmé que cette bande correspond à la forme dimérique de CrgA.

La protéine HPr a été également purifiée à partir de la souche BL21(DE3) (Figure 18). Les premiers essais de surexpression et de purification de cette protéine ont été infructueux. La protéine HPr est en effet majoritairement accumulée sous forme de corps d'inclusion insolubles chez *E. coli*. Les corps d'inclusion ont été solubilisés avec 3 M de chlorure de guanidinium pendant 2 h à température ambiante. Le chlorure de guanidinium a été ensuite éliminé par dialyse de la solution protéique pendant 24 h. La protéine est ensuite purifiée selon le protocole recommandé par le fournisseur, pour la purification des protéines Strep-tag.

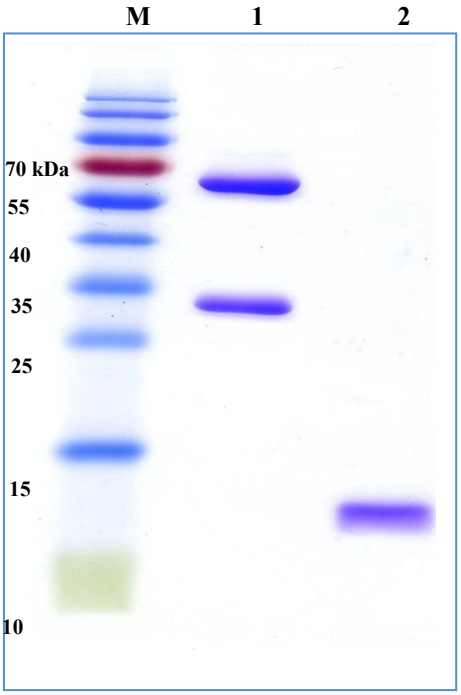


Figure 18 : Electrophorèse en gel de polyacrylamide (Acrylamide 15%, SDS 1%) des protéines CrgA (34 kDa) et HPr (10 kDa) de *N. meningitidis*.

M : Marqueur (*precision plus protéin*), 1 : CrgA His-tag (0.8 µg/µl), 2 : HPr Strep-tag (0.46 µg/µl).

III. 3. Effet de la protéine HPr sur l'affinité de CrgA aux régions promotrices des gènes *pilC1*, *pilE* et *sia*

Les produits d'amplification des régions promotrices des gènes *pilE*, *pilC1* et *sia* (8 nM) codant respectivement la piline, l'adhésine et la capsule, ont été mis en solution avec la protéine CrgA (150 nM) en présence et en absence de HPr à différentes concentrations. Une augmentation de l'affinité de CrgA à la région promotrice de *pilE* a été observée en présence de 5 à 30 µM de HPr (Figure 19 (A), lignes 3 à 8). La région promotrice de *pilC1* a été également mise en solution avec la protéine CrgA en présence et en absence de 25 µM de HPr. Une légère variation de migration de l'ADN a été observée en rajoutant la protéine HPr en plus de CrgA (Figure 19 (B), ligne 3). La présence de l'HPr semble augmenter l'affinité de

CrgA à la région promotrice du gène *pilC1*. En revanche, aucun effet n'a été observé pour l'affinité de CrgA à la région promotrice de *sia* en présence de HPr (5 à 30 µM) (Figure 19 (C), lignes 3 à 8).

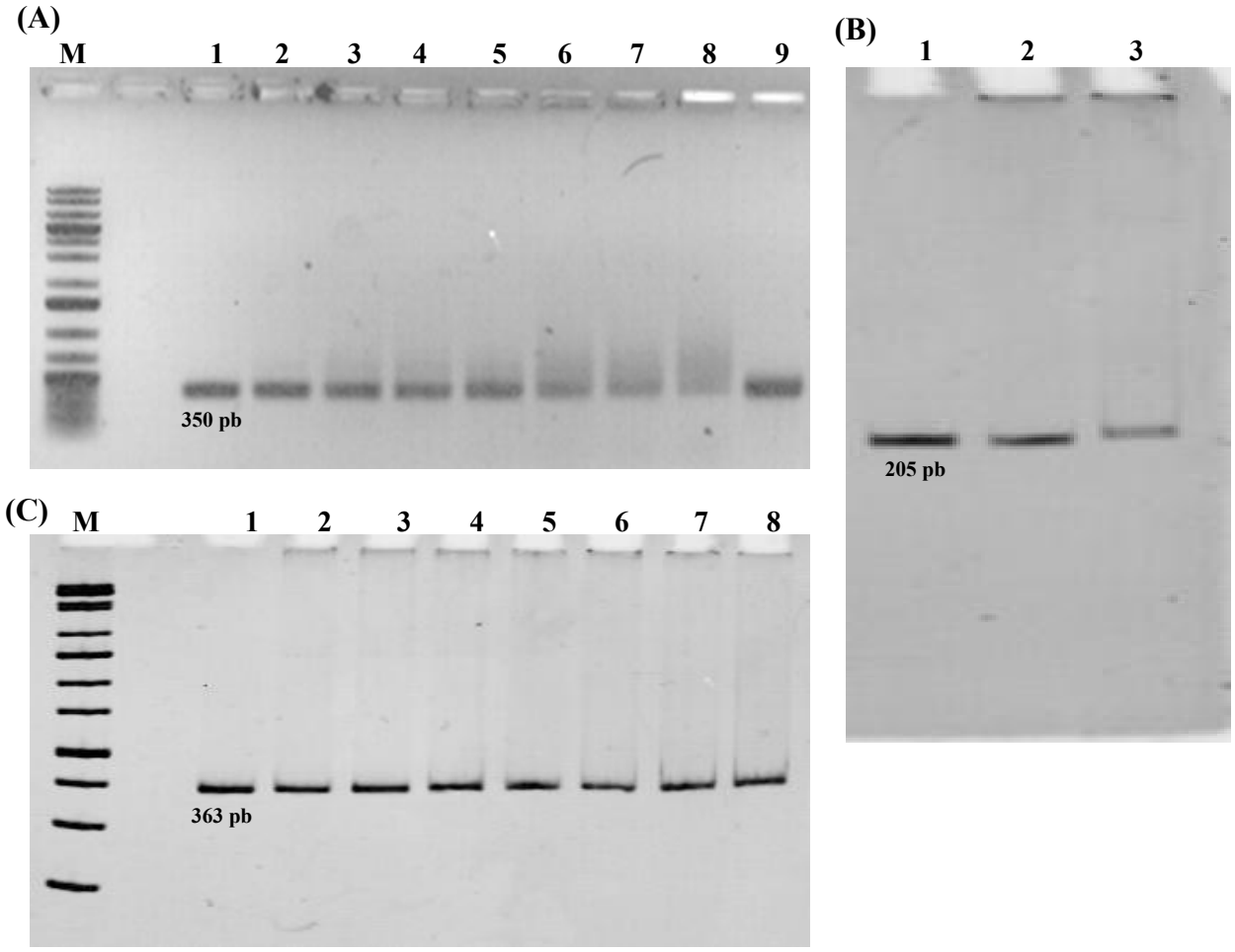


Figure 19 : Affinité de la protéine CrgA aux régions promotrices des gènes *pilC1*, *pilE* et *sia* en absence et en présence de la protéine HPr.

(A) : Région promotrice de *pilE* (RP-*pilE*) : 1 : RP-*pilE* (8 nM), **2 :** RP-*pilE* (8 nM) + CrgA (150 nM). De la ligne 3 à 8, différentes concentrations de HPr ont été rajoutées à RP-*pilE* (8 nM) + CrgA (150 nM) : **3 :** HPr (5 µM), **4 :** HPr (10 µM), **5 :** HPr (15 µM), **6 :** HPr (20 µM), **7 :** HPr (25 µM), **8 :** HPr (30 µM). **9 :** RP-*pilE* (8 nM) + HPr (30 µM).

(B) : Région promotrice de *pilC1* (RP-*pilC1*) : 1 : RP-*pilC1* (8 nM), **2 :** RP-*pilC1* (8 nM) + CrgA (150 nM), **3 :** RP-*pilC1* (8 nM) + CrgA (150 nM) + HPr (25 µM).

(C) : Région promotrice de *sia* (RP-*sia*) : 1 : RP-*sia* (8 nM), **2 :** RP-*sia* (8 nM) + CrgA (150 nM). De la ligne 3 à 8, différentes concentrations de HPr ont été rajoutées à RP-*sia* (8 nM) + CrgA (150 nM) : **3 :** HPr (5 µM), **4 :** HPr (10 µM), **5 :** HPr (15 µM), **6 :** HPr (20 µM), **7 :** HPr (25 µM), **8 :** HPr (30 µM).

III. 4. Co-immunoprécipitation *in vitro* des protéines CrgA, HPr et P-Ser-HPr

Dans l'objectif d'étudier l'effet de la phosphorylation de l'HPr (sur la Ser-46) sur son interaction avec CrgA, nous avons réalisé des tests de co-immunoprécipitation *in vitro* sur les protéines purifiées CrgA His-tag, HPr Strep-tag et sa forme phosphorylée sur la Ser-46 (P-Ser-HPr). La P-Ser-HPr a été produite par phosphorylation de l'HPr sur sa Ser-46 par l'HprK/P(V267F) de *Lactobacillus casei*, en présence de 5 mM ATP, 10 mM $MgCl_2$ et 20 mM FBP (Article 1 : *Supplementary Fig. 3*).

La co-immunoprécipitation a été réalisée, en incubant les mélanges protéiques CrgA+HPr et CrgA+P-Ser-HPr en présence des anticorps anti-CrgA (anti-His-tag de CrgA) ou anti-HPr (anti-Strep-tag de l'HPr). La présence de CrgA, l'HPr ou la P-Ser-HPr dans les précipitats a été vérifiée par Western blot suivant le protocole décrit dans l'article 1 pour la co-immunoprécipitation *in vivo* (partie *Experimental procedures*). Des anticorps IgG de souris qui n'interagissent pas avec CrgA et HPr ont été utilisés comme témoins négatifs de l'immunoprécipitation.

Nous avons observé une co-immunoprécipitation de l'HPr et de la P-Ser-HPr après avoir précipité CrgA avec un anticorps anti-CrgA (Figure 20A). Néanmoins, la quantité de l'HPr détectée dans le Western blot était plus faible que la quantité de la P-Ser-HPr (Figure 20A). Nous avons également observé une co-immunoprécipitation de CrgA après avoir précipité l'HPr ou la P-Ser-HPr avec un anticorps anti-HPr (Figure 20B) . La quantité de CrgA détectée dans le mélange contenant la P-Ser-HPr est plus importante que celle détectée dans le mélange contenant l'HPr (Figure 20B). Ces résultats montrent que CrgA interagit avec l'HPr et également avec la P-Ser-HPr. Cependant, l'affinité de CrgA à la P-Ser-HPr est plus forte que son affinité à l'HPr non phosphorylée. La phosphorylation de l'HPr sur la Ser-46 semble augmenter son interaction avec la protéine CrgA.

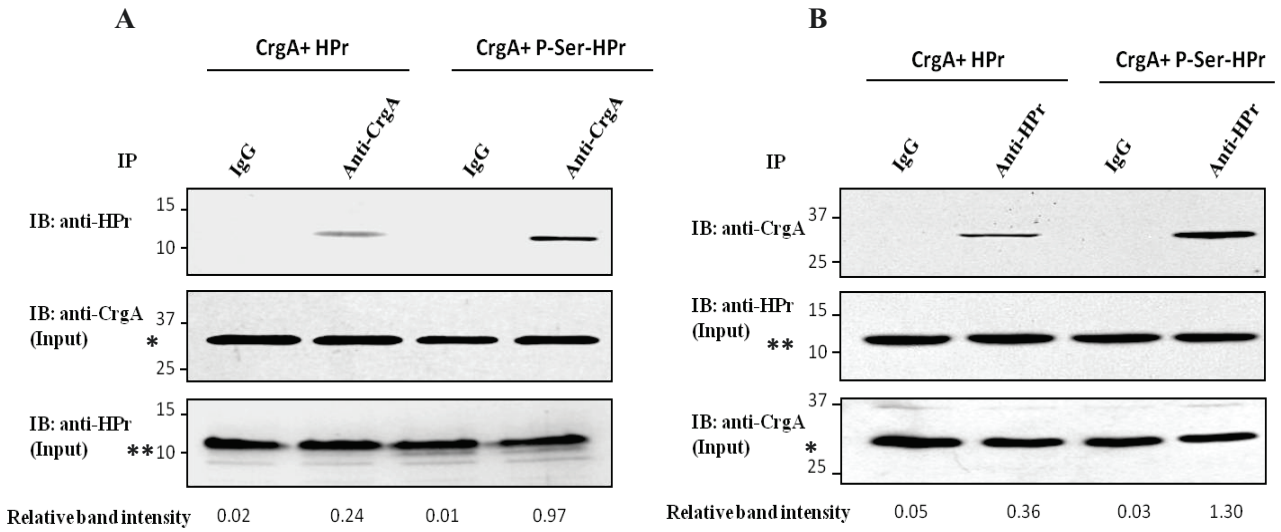


Figure 20 : Test de co-immunoprécipitation *in vitro* des protéines CrgA, HPr et P-Ser-HPr.

(A) : Détection de l'HPr ou la P-Ser-HPr (10 kDa), par Western blot avec un anticorps anti-HPr, dans les co-immunoprécipitats obtenus après précipitation des mélanges CrgA+HPr et CrgA+P-Ser-HPr avec des anticorps anti-CrgA.

(B) : Détection de CrgA (34 kDa), par Western blot avec un anticorp anti-CrgA, dans les co-immunoprécipitats obtenus après précipitation des mélanges CrgA+HPr et CrgA+P-Ser-HPr avec des anticorps anti-HPr.

Les anticorps IgG qui ne reconnaissent ni HPr ni CrgA, ont été utilisés comme témoins négatifs de l'immunoprécipitation. Aucune protéine n'a été détectée en traitant les mélanges CrgA+HPr ou CrgA+P-Ser-HPr avec les anticorps IgG.

* : Quantité de la protéine CrgA présente dans les mélanges protéiques avant précipitation avec les anticorps.

** : Quantité des protéines HPr ou P-Ser-HPr présentes dans les mélanges protéiques avant précipitation avec les anticorps.

IV. Discussion

Chez un grand nombre de bactéries, le transport des hydrates de carbone est assuré par le PTS. Il s'agit d'un système composé d'enzymes générales EI et HPr ainsi que d'une série d'enzymes spécifiques au sucre transporté (EIIA, EIIB, EIIC et parfois EIID) (Postma *et al*., 1993). Chez certaines bactéries, comme *N. meningitidis*, le PTS est déficient des enzymes EIIB et EIIC et ne peut transporter les hydrates de carbone (Boël *et al*., 2003 ; Barabote et Saier, 2005 ; Stonestrom *et al*., 2005). En revanche, il intervient dans plusieurs fonctions régulatrices dans la cellule, y compris la virulence (Mao *et al*., 2007 ; Hondorp et McIver, 2007 ; Noguez *et al*., 2008 ; Bowden *et al*., 2009 ; Pflüger-Grau et Görke, 2010 ; Dozot *et al*., 2010 ; Mazé *et al*., 2014).

L'HPr fait partie des protéines PTS dont le lien avec la virulence a été étudié chez plusieurs bactéries pathogènes telles que *Listeria monocytogenes* (Herro *et al*., 2005), *Salmonella enterica* serovar Typhimurium (Bowden *et al*., 2009) et *Brucella melitensis* (Dozot *et al*., 2010). Chez *N. meningitidis*, les seuls travaux qui décrivaient un lien entre la protéine HPr et la virulence ont été réalisés par Sun *et al*. (2000). Ces derniers ont montré, en analysant une banque de 2850 mutants de *N. meningitidis*, l'implication de plusieurs gènes, dont les gènes *ptsHI* codant l'HPr et l'EI respectivement, dans la virulence du méningocoque. Dans le but d'étudier, de manière plus détaillée, le lien entre la protéine HPr et la virulence de *N. meningitidis*, nous avons construit un mutant *ΔptsH* chez *N. meningitidis* 2C4-3, variant isolé à partir de la souche 8013 du sérogroupe C. Le sérogroupe C est responsable de 30% des infections invasives à méningocoques en France derrière le sérogroupe B (56%) (Nicand, 2015). Le mutant Δ*ptsH* a été complémenté avec le gène *ptsH* dont l'expression est constitutive.

Les tests de survie du méningocoque, réalisés sur des souris après injection par voie intrapéritonéale, de la souche sauvage 2C4-3, du mutant Δ*ptsH* et du mutant complémenté Δ*ptsH*/*ptsH*, ont montré que le mutant Δ*ptsH* est rapidement éliminé de la circulation sanguine par rapport aux deux autres souches. Il semble que le mutant Δ*ptsH* est plus facilement reconnu et éliminé par le système immunitaire des souris.

N. meningitidis est un pathogène à multiplication extracellulaire, capable d'exprimer plusieurs facteurs de virulence qui lui permettent de traverser la barrière rhinopharyngée, survivre et se multiplier dans le sang et éventuellement traverser la barrière hémato-encéphalique (Quagliarello et Scheld, 1992 ; Nassif *et al*., 2002). La capsule constitue le plus important

facteur de virulence qui permet la protection de la bactérie contre la phagocytose et la lyse par les protéines du système du complément (Nassif *et al*., 2002 ; Koedel *et al*., 2002). La production de la capsule chez la souche sauvage 2C4-3, le mutant Δ*ptsH* et le mutant complémenté Δ*ptsH*/*ptsH* a été étudiée par un test ELISA. Le mutant Δ*ptsH* montre une faible production de la capsule par rapport à la souche sauvage et au mutant complémenté Δ*ptsH*/*ptsH*. La faible production de la capsule chez le mutant Δ*ptsH* pourrait expliquer son élimination rapide du sang par le système immunitaire des souris.

La capsule joue également un rôle important dans l'adhérence du méningocoque aux cellules de l'hôte. L'adhérence représente une étape clé dans le cycle infectieux du méningocoque, pendant laquelle plusieurs gènes de virulence sont exprimés (Davidsen et Tonjum, 2006). Le contact initial avec les cellules de l'hôte est assuré par les pili (Naumann *et al*., 1999). Selon Deghmane *et al*. (2000), le méningocoque passe par deux étapes d'adhérence, une adhérence initiale caractérisée par la présence des pili et de la capsule et une adhérence intime où la bactérie rétracte ses pili et résorbe sa capsule. Les tests d'adhérence des souches (2C4-3, Δ*ptsH* et Δ*ptsH*/*ptsH*) aux cellules « Hec-1-B » ont montré que le mutant Δ*ptsH* adhère plus aux cellules épithéliales « Hec-1-B » que la souche sauvage 2C4-3 et le mutant complémenté Δ*ptsH*/*ptsH*. En effet, la capsule, par sa structure juxtaposée, permet de masquer les adhésines de la membrane externe (Virji *et al*., 1992 ; Virji *et al*., 1993 ; Griffiths *et al*., 2007). Dans ce contexte, la faible production de la capsule chez le mutant Δ*ptsH* explique sa meilleure adhérence aux cellules épithéliales, par rapport aux deux autres souches qui produisent plus de capsules.

La faible production de la capsule chez le mutant Δ*ptsH* ainsi que sa meilleure adhérence aux cellules épithéliales « Hec-1-B » suggèrent un lien avec l'induction de l'apoptose. En effet, les travaux réalisés par Deghmane *et al*. (2009) ont montré un niveau élevé de cellules apoptotiques induit par des mutants de *N. meningitidis* ne produisant pas de capsule. Des résultats similaires ont été observés dans notre travail, lors de l'infection de cellules « Hec-1-B » par le mutant Δ*ptsH*. En effet, ce dernier, provoque plus d'apoptose que la souche sauvage 2C4-3 et le mutant complémenté Δ*ptsH*/*ptsH*. Les lipopolysaccharides (LPS) et la protéine PorB représentent d'important facteurs apoptotiques chez *N. meningitidis* (Xaus *et al*., 2000 ; Massari et *al*., 2000). En absence de capsule, ces structures sont démasquées, favorisant ainsi un niveau élevé d'apoptose des cellules-cibles.

Les gènes codant pour la synthèse de la capsule et des pili, composants nécessaires à l'adhérence du méningocoque aux cellules de l'hôte, s'avèrent être contrôlés par CrgA, un

régulateur transcriptionnel de la famille des LTTRs (Zaim et Kierzek, 2003). Les résultats obtenus dans ce travail suggèrent que HPr pourrait contrôler l'activité de CrgA. En effet, les protéines du PTS exercent leurs fonctions régulatrices dans la cellule soit par phosphorylation soit par interaction directe avec d'autres protéines non PTS (Deutscher *et al*., 2014a). Des tests de phosphorylation de CrgA avec l'EI et HPr et avec l'EI, HPr et l'EIIA^{Ntr} n'ont pas mis en évidence une phosphorylation de ce régulateur (Poncet et Mazé, résultats non publiés). En revanche, des tests de co-immunoprécipitation ont montré une interaction entre HPr et CrgA. Cette interaction pourrait avoir un effet sur l'activité de CrgA. En effet, CrgA réprime l'expression des gènes codant des protéines de pili et de la capsule (*pilC1*, *pilE* et *sia*) assurant une meilleure adhérence du méningocoque aux cellules épithéliales (Deghmane *et al*., 2002). Par contre, en absence de cellules épithéliales, CrgA réprime l'expression de son propre gène; l'expression des gènes *pilC1*, *pilE* et *sia* est alors déréprimée (Deghmane *et al*., 2002 ; 2004). Effectivement, les expériences du gel shift ont montré que la protéine HPr augmente l'affinité de CrgA à sa propre région promotrice ainsi que la région promotrice du gène *pilE* et probablement aussi *pilC1*. D'autre part, les études de la structure cristallographique de CrgA ont suggéré la présence d'une région potentielle pour la fixation d'un effecteur putatif (Sainsbury *et al*., 2009). Cet effecteur pourrait être la protéine HPr.

Chez les organismes ayant tous les composants du PTS (EI, HPr, EIIA, EIIB, EIIC et parfois aussi EIID), le transport des sucres se fait par une cascade de phosphorylation qui commence par l'autophosphorylation de l'EI et finit par la phosphorylation du sucre lié à l'EIIC (ou EIIC et EIID) (Postma *et al*., 1993). Dépourvu des enzymes EIIB et EIIC, le PTS de *N. meningitidis* ne permet pas le transport des sucres à l'intérieur de la cellule. Cependant les tests de phosphorylation réalisés *in vitro* sur les protéines PTS de *N. meningitidis* (HPr, EIIA^{Ntr} et EIIA^{Man}) montrent que la cascade de phosphorylation de ces protéines est fonctionnelle. En effet, l'HPr est phosphorylée sur son His-15 par l'EI de *Bacillus subtilis,* avec le PEP comme donneur de phosphate. La P~His-HPr phosphoryle à son tour l'EIIA^{Ntr}. Aucune phosphorylation n'a été observée pour l'EIIA^{Man}. Des résultats similaires ont été obtenus chez *Ralstonia eutropha* H16, une β-protéobactérie comme *N. meningitidis*, pour laquelle la P~His-HPr phosphoryle seulement l'EIIA^{Ntr} (Krausse *et al*., 2009). En revanche, chez *B. melitensis*, une α-protéobactérie, les deux EIIAs (EIIA^{Ntr} et EIIA^{Man}) sont phosphorylées par la P~His-HPr (Dozot *et al*., 2010).

La phosphorylation des protéines du PTS de *N. meningitidis* a été également confirmée *in vivo*. En absence des enzymes EIIB et EIIC, l'EIIA^{Ntr} est l'accepteur final du groupement

phosphate. La présence de la P~EIIA^{Ntr}, détectée dans les extraits cellulaires de la souche sauvage 2C4-3 et du mutant complémenté Δ*ptsH*/*ptsH*, représente un bon indice pour confirmer la cascade de phosphorylation du PTS chez *N. meningitidis*. Par contre, aucune bande correspondant à la forme phosphorylée de l'EIIA^{Ntr} n'a été détectée dans l'extrait cellulaire du mutant Δ*ptsH* dépourvu de l'HPr. Ce qui montre que HPr contribue à la cascade de phosphorylation *in vivo*.

N. meningitidis possède également la protéine HprK/P (HPr kinase/phosphorylase). Chez les firmicutes, l'HprK/P phosphoryle l'HPr sur la Ser-46 en utilisant l'ATP ou le PPi comme donneurs de phosphate. Outre son activité kinase, l'HprK/P déphosphoryle la P-Ser-HPr en utilisant le Pi comme substrat avec la production de l'HPr et du pyrophosphate inorganique (PPi) (Deutscher et Saier, 1983 ; Deutscher *et al*., 1986 ; Kravanja *et al*., 1999 ; Mijakovic *et al*., 2002). Les résultats des tests de phosphorylation ont montré que l'HprK/P de *N. meningitidis* peut également phosphoryler l'HPr sur la Ser-46, avec l'ATP ou le PPi comme donneurs de phosphate. Cette phosphorylation est activée par le FBP et inhibée par de fortes concentrations en phosphate inorganique (Pi) dans le milieu. En effet, le Pi rentre en compétition avec l'ATP pour son site de fixation sur l'HprK/P (Fieulaine *et al*., 2001 ; Mijakovic *et al*., 2002).

La P-Ser-HPr est connue pour son rôle dans la répression catabolique carbonée CcpA-dépendante chez les firmicutes (Poncet *et al*., 2009b) mais elle joue également un rôle important dans la virulence de certaines espèces pathogènes (Herro *et al*., 2005 ; Poncet *et al*., 2009b). Les tests de co-immunoprécipitations réalisés *in vitro* sur les protéines CrgA, HPr et P-Ser-HPr de *N. meningitidis*, ont montré que la phosphorylation de l'HPr sur la Ser-46 augmente son interaction avec la protéine CrgA. Cette phosphorylation pourrait avoir également des effets sur l'affinité de CrgA à sa propre région promotrice ainsi qu'aux régions promotrices des gènes qu'elle contrôle (*pilC1*, *pilE* et *sia*).

La déphosphorylation de la P-Ser-HPr par l'HprK/P de *N. meningitidis* a été également étudiée en présence du Pi. Contrairement à l'activité kinase, l'HprK/P montre une très faible activité phosphorylase. Des résultats similaires ont été observés pour l'HprK/P d'autres espèces, telles que : *Treponema denticola* (Gonzalez *et al*., 2005), *Mycoplasma pneumoniae* (Halbedel *et al*., 2006) et *B. melitensis* (Dozot *et al*., 2010). La faible activité phosphorylase de l'HprK/P de certaines bactéries (α-protéobactéries) est due à l'absence de la région C-terminale nécessaire à la déphosphorylation de l'HPr (Fieulaine *et al*., 2001 ; Monedero *et al*., 2001 ; Poncet *et al*., 2004). Halbedel *et al*. (2006) ont également montré que la

déphosphorylation de la P-Ser-HPr chez *M. pneumoniae* est catalysée par une autre protéine phosphatase nommée PrpC (Halbedel *et al*., 2006).

En plus de son activité kinase/phosphorylase, la protéine HprK/P joue également un rôle important dans la virulence de *N. meningitidis*. En effet, la synthèse de la capsule est fortement augmentée chez un mutant *hprK/P* de *N. meningitidis* (Boël *et al*., 2003) ; la surproduction de la capsule réduit l'adhérence du méningocoque aux cellules de l'hôte.

Article 02

Transport and catabolism of carbohydrates by *Neisseria meningitidis*

I. Présentation générale des résultats

N. meningitidis utilise très peu de sources de carbone dont le glucose et le maltose comme seuls sucres (Beno *et al*., 1968). Cette bactérie utilise également le lactate, le pyruvate et certains acides aminés tels que le glutamate (Mallavia et Weiss, 1970). Le glucose et le lactate sont présents dans plusieurs parties du corps humain, telles que le sérum, le liquide céphalo-rachidien (LCR) et les sécrétions des voies aériennes supérieures (Smith *et al*., 2001). Chez plusieurs bactéries, le transport du glucose et du maltose se fait par le PTS (Deutscher *et al*., 2006 ; Schönert *et al*., 2006). En revanche, chez *N. meningitidis* le PTS est dépourvu des enzymes EIIB et EIIC et ne permet pas le transport des hydrates de carbone chez cette bactérie. Le glucose peut être également transporté par des perméases, comme la perméase à glucose de *B. subtilis* (GlcP) (Paulsen *et al*., 1998).

L'objectif principal de cette deuxième partie du travail a été d'identifier les perméases à glucose et à maltose chez *N. meningitidis* 2C4-3 ainsi que de confirmer l'activité de quelques enzymes clés des différentes voies métaboliques chez cette bactérie. Les gènes *NMV_1892* et *NMV_0424* semblent coder pour des perméases à glucose et à maltose, respectivement. Les résultats du BLAST ont montré un pourcentage de similarité de 46% entre la protéine NMV_1892 de *N. meningitidis* et la perméase à glucose (GlcP) de *B. subtilis*. L'inactivation des gènes *NMV_1892* ou *NMV_0424* chez *N. meningitidis* empêche sa croissance en présence du glucose et du maltose, respectivement, comme seules sources de carbone. De plus, la complémentation de mutants d'*E. coli* incapables d'utiliser le glucose ou le maltose selon le cas, par les gènes *NMV_1892* et *NMV_0424* de *N. meningitidis*, respectivement, a permis de restaurer le phénotype de croissance sur ces sucres par *E. coli*. Ces résultats montrent que les gènes *NMV_1892* et *NMV_0424* codent respectivement, une perméase à glucose, nommée GlcP et une perméase à maltose, nommée MalY chez *N. meningitidis*. Le gène *NMV_0424* codant la perméase MalY de *N. meningitidis*, semble être en opéron avec des gènes codant les enzymes du métabolisme du maltose.

Une perméase putative à gluconate a été également identifiée chez *N. meningitidis*. Cette perméase est codée par le gène *NMV_2230* ; elle représente un pourcentage de similarité de 58% avec la perméase à gluconate (GntP) de *B. subtilis*. Le gène *NMV_2230* est situé en amont d'un autre gène (*NMV_2231)* codant une protéine ayant 56% d'identité avec la gluconate kinase (GntK) d'*E. coli*. Dans le but de vérifier si le gène *NMV_2230* code une perméase à gluconate chez *N. meningitidis* 2C4-3, nous avons construit un mutant par délétion du gène *NMV_2230*, ce mutant a été nommé *ΔgntP*. Des tests de croissance ont été réalisés

avec la souche sauvage 2C4-3 et le mutant *ΔgntP* en présence du gluconate comme seule source de carbone. Cependant, aucune croissance n'a été observée pour les deux souches. Ces résultats suggèrent que le gène *NMV_2230* ne code pas une perméase à gluconate active chez *N. meningitidis* dans les conditions testées. En revanche, une restauration du phénotype de croissance sur gluconate a été observée chez un mutant *ΔgntP* de *B. subtilis*, dépourvu de sa perméase à gluconate, aprés sa complémentation avec le gène *gntP* de *N. meningitidis*. Ceci pourrait indiquer que chez *N. meningitidis*, l'environnement dans la membrane et la composition des lipides ne permettraient pas une insertion active du gluconate à la perméase.

Des tests de croissance sur glucose ont été également réalisés avec la souche sauvage 2C4-3 et le mutant *ΔgntP* de *N. meningitidis*. De façon inattendue, le mutant *ΔgntP* pousse sur glucose mieux que la souche sauvage 2C4-3. Ce résultat nous a incités à réaliser des tests de survie du mutant *ΔgntP* de *N. meningitidis* et de la souche sauvage 2C4-3 chez la souris. Les injections intrapéritonéales des deux suspensions bactériennes ont été suivies de dénombrements dans le sang, après 2 h, 6 h et 24 h d'infections. Les résultats obtenus montrent que le nombre de bactéries qui survivent après 2 h et 6 h, chez les souris infectées avec le mutant Δ*gntP*, est plus élevé que le nombre de bactéries chez les souris infectées avec la souche sauvage 2C4-3. Des essais de complémentation du mutant *ΔgntP* de *N. meningitidis* ont été aussi réalisés. Le gène *gntP* a été cloné dans le vecteur $pComP_{RBS}$ qui permet l'expression constitutive du gène d'intérêt (Ieva *et al.*, 2005 ; Seib *et al.*, 2009). Malgré plusieurs essais, aucun clone ayant une expression constitutive du gène *gntP* n'a été obtenu. Le gène *gntP* a alors été cloné dans un second vecteur « pComPind » (Ieva *et al*., 2005 ; Seib *et al*., 2009) permettant une expression inductible du gène d'intérêt par l'IPTG. Nous avons réussi à obtenir des mutants complémentés en utilisant ce vecteur en absence de l'IPTG. Les effets liés à la présence de l'IPTG n'ont pas été testés.

Des études antérieurs, réalisées sur l'utilisation du glucose par *N. meningitidis*, ainsi que des tests d'activité enzymatique en utilisant des extraits protéiques bruts, ont confirmé la présence des enzymes de la voie d'Embden-Meyerhof-Parnas (EMP ou glycolyse), de la voie d'Entner-Douderoff (ED) et de la voie des pentoses phosphates (PP) (Jyssum *et al*., 1961; Jyssum, 1962a, b, c, Holten, 1974, Holten 1975). Cependant, le glucose est entièrement métabolisé via la voie d'ED et la voie des PP (Baart *et al*., 2010 ; Schoen *et al*., 2014). La glycolyse est partiellement fonctionnelle chez *N. meningitidis*. En effet, cette dernière ne possède pas le gène codant la fructose-6-P 1-kinase (Pfk), enzyme catalysant la phosphorylation irréversible du fructose-6-phosphate en fructose-1,6-bisphosphate (FBP)

(Baart *et al*., 2010). Le glycéraldéhyde-3-P formé par la voie des PP et la voie d'ED est métabolisé via la partie basse de la glycolyse (Figure 21). Néanmoins, nous avons testé l'activité enzymatique d'une protéine (NMV_1007), de fonction inconnue chez *N. meningitidis*, afin de savoir s'il s'agit bien de la Pfk. De manière intéressante, le gène codant la protéine NMV_1007 est situé en aval du gène *pgi1* (*NMV_1006*) codant une glucose-6-phosphate isomérase (Pgi1) (ou 6-phosphohexose isomérase). La Pgi1 catalyse la conversion du glucose-6-phosphate en fructose-6-phosphate, substrat de la Pfk (Figure 21).

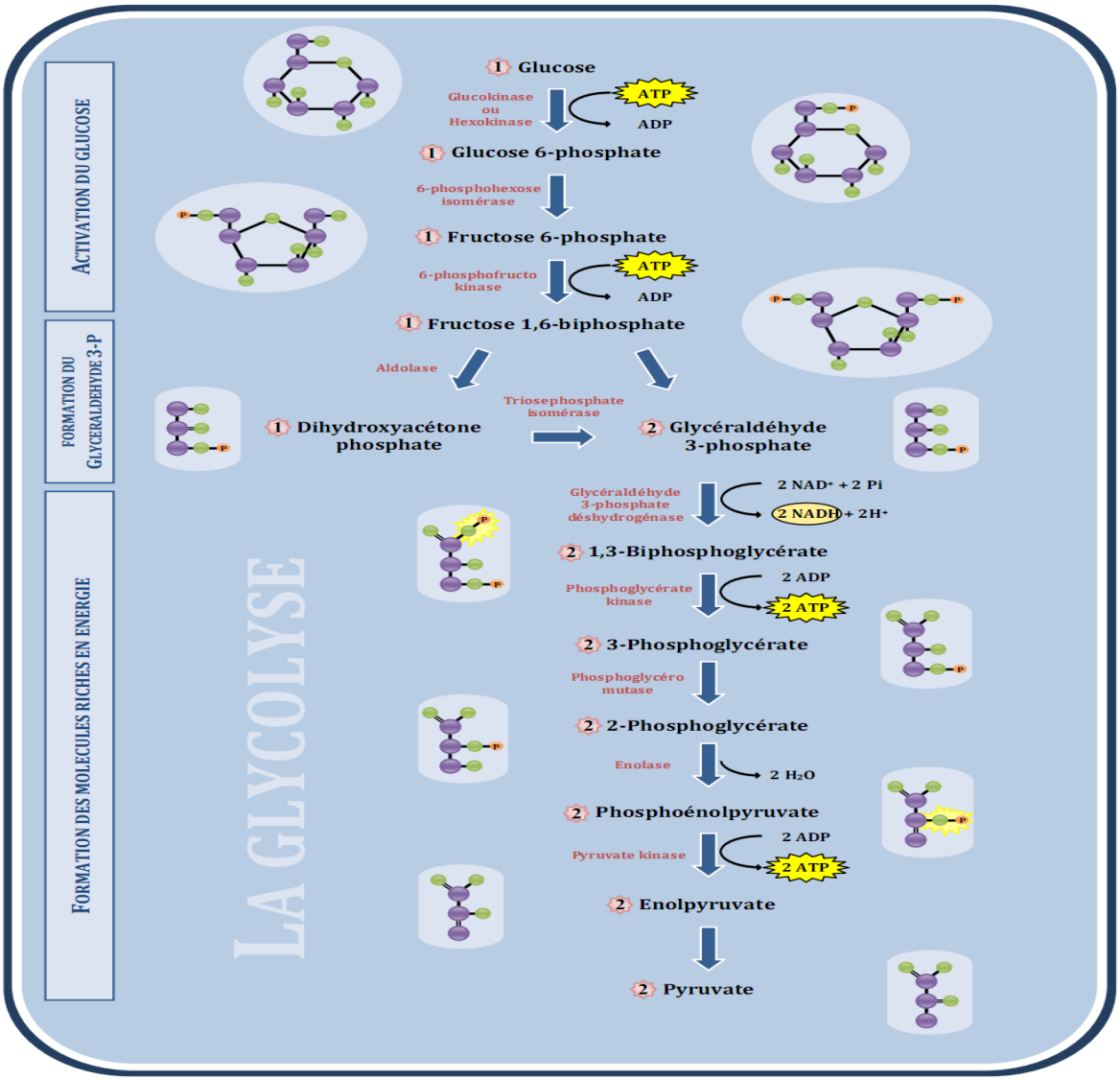


Figure 21 : Représentation schématique de la glycolyse chez les bactéries.

Les différentes réactions de la glycolyse sont regroupées en trois grandes étapes :

1. Activation des hexoses par phosphorylations successives aboutissant à la formation du fructose-1,6-bisphosphate (FBP).

2. Une aldolase clive le FBP en glycéraldéhyde-3-P (GAP) et dihydroxyacétone-P (Dha-P).

3. Le GAP rentre dans plusieurs réactions aboutissant à la formation de l'ATP et du pyruvate.

N. meningitidis possède toutes les enzymes de la glycolyse à l'exception de la fructose-6-P 1-kinase (Pfk).

Le gène *NMV_1007* a été cloné dans le vecteur pQE30 afin de purifier une protéine NMV_1007 His-tag. L'activité de NMV_1007 His-tag (14 µg) a été testée par spectrophotométrie (340 nm), dans une solution de 50 mM Tris-HCl pH 7.4, en présence de 1 mM fructose-6-P, 1.4 U fructose-1,6-bisphosphate aldolase, 2.8 U triosephosphate isomérase+Glycérol-3-P déshydrogénase, 1 mM NADH, 10 mM $MgCl_2$ et en utilisant 1 mM d'ATP ou de PPi, comme donneurs de phosphate. Dans les conditions testées, aucune activité n'a été détectée pour la protéine NMV_1007. Ce résultat signifie que la protéine NMV_1007 ne code pas pour une Pfk, mais pour une protéine dont la fonction reste inconnue.

Au cours de ce travail, nous avons cherché à étudier plus en détails le métabolisme carboné de *N. meningitidis*. Pour cela, nous avons purifié les principales enzymes des voies métaboliques du glucose et du maltose. Au total 8 enzymes ont été purifiées, dont la glucokinase (NMV_1004, Glk) et l'α-phosphoglucomutase (NMV_0427, PgcM), qui catalysent des étapes précoces et spécifiques pour l'utilisation du glucose et du maltose, respectivement. Nous avons également confirmé l'activité de trois enzymes de la voie des PP incluant la 6-phosphogluconate déshydrogénase (NMV_0016, Gnd), la D-ribose-5-P isomérase A (NMV_0882, RpiA) et la D-ribulose-5-P 3-épimérase (NMV_1185, Rpe), ainsi qu'une enzyme de la voie d'ED, la gluconate-6-P déhydratase (NMV_1001, Edd). Finalement, nous avons testé la fructose-1,6-bisphosphate aldolase (NMV_2059, FbaA) et la gluconate kinase (NMV_2231, GntK) (Figure 22). Les gènes codant ces enzymes ont été clonés dans le vecteur pQE30 qui permet la surexpression de protéines recombinantes fusionnées dans leurs extrémités N-terminales à une étiquette His-tag. Les protéines recombinantes ont été surproduites chez *E. coli* NM522 [pREP4-GroES/EL] et purifiées par chromatographie sur une résine Ni-NTA agarose (Figure 23). L'activité des enzymes purifiées a été confirmée par des tests spectrophotométriques à 340 nm, cette longueur d'onde permet de détecter la production ou l'utilisation du NADH ou NADPH au cours de la réaction enzymatique. Les milieux réactionnels utilisés pour chacune des enzymes sont indiqués dans le Tableau 4 (à voir dans la partie « Matériel et Méthodes »).

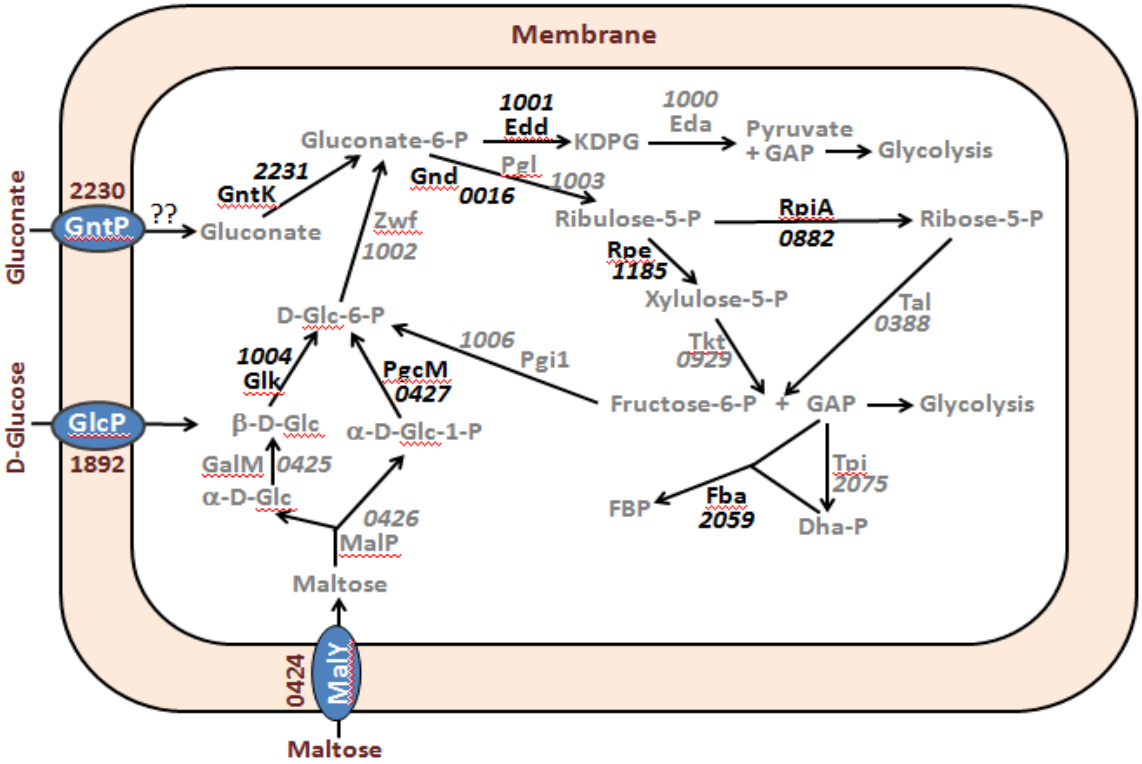


Figure 22 : Représentation schématique des différentes voies du catabolisme du glucose et du maltose chez *N. meningitidis*.

Les enzymes purifiées et caractérisées au cours de cette étude sont notées en noir. Les chiffres indiquent le nom du gène codant l'enzyme ; ces chiffres sont précédés de la mention « *NMV_* » non indiquée sur ce schéma. Les perméases à glucose, à maltose et à gluconate sont indiquées par des cercles bleus. Les points d'interrogation pour la perméase à gluconate désignent sa fonction inconnue.

Le glucose et le maltose rentrent dans la cellule à travers les perméases GlcP et MalY, respectivement. Le D-glucose-6-P (D-Glc-6-P), produit lors de la conversion du α-D-glucose-1-P (α-D-Glc-1-P) par l'α-phosphoglucomutase (PgcM) et de la phosphorylation du β-D-glucose par la glucokinase (Glk), est transformé en gluconate-6-P par une glucose-6-phosphate 1-déshydrogénase (Zwf). Le gluconate-6-P est alors métabolisé via la voie d'Entner-Douderoff (ED) ou la voie des pentoses phosphates (PP). Le glycéraldéhyde-3-P (GAP) produit par ces deux voies est métabolisé via la partie fonctionnelle de la glycolyse. Une partie du GAP est aussi isomérisée en dihydroxyacétone-P (Dha-P) par la triose-phosphate isomérase (TpiA). Le GAP et la Dha-P sont transformés en fructose-1,6-bisphosphate par la fructose-1,6-bisphosphate aldolase (FbaA). Le fructose-6-P produit par la voie des PP est isomérisé en glucose-6-P par la phosphoglucose isomérase (Pgi1). Le glucose-6-P rentre ensuite dans la voie d'ED et la voie des PP.

Glc : glucose, **MalP :** maltose phosphorylase, **GalM :** aldose 1-epimerase, **Glk :** glucokinase, **PgcM :** α-phosphoglucomutase, **Zwf :** glucose-6-phosphate 1-déshydrogénase, **GntK :** gluconate kinase, **Edd:** gluconate-6-P déhydratase, **KDPG :** 2-keto-3-deoxy-6-P gluconate, **Eda :** 2-keto-3-deoxy-6-P gluconate aldolase, **GAP :** glycéraldéhyde-3-P, **Gnd :** 6-phosphogluconate déshydrogénase, **RpiA :** D-ribose-5-P isomérase A, **Rpe :** D-ribulose-5-P 3-épimérase, **Tkt :** transketolase, **Tal** : transaldolase, **Pgi1 :** glucose-6-phosphate isomérase, **Tpi :** triose-phosphate isomérase, **Dha-P** : dihydroxyacétone-P, **FbaA :** fructose-1,6-bisphosphate aldolase, **FBP :** fructose-1,6-bisphosphate.

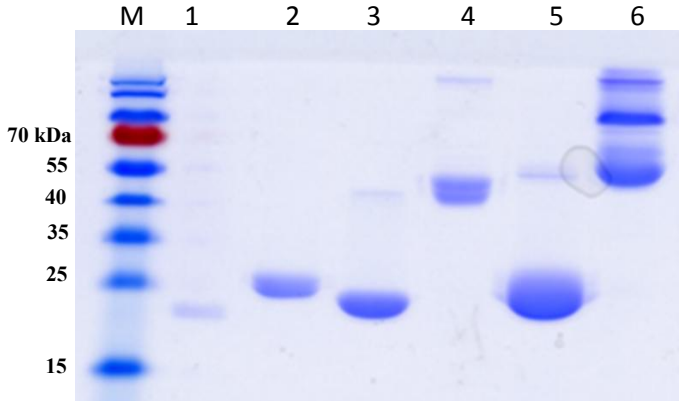


Figure 23 : Electrophorèse en gel de polyacrylamide (acrylamide 12,5%, SDS 1%) des protéines purifiées pour l'étude du métabolisme carboné de *N. meningitidis*.

M : Marqueur (*precision plus protein*), 1: GntK (18 kDa), 2: RpE (26 kDa), 3: PgcM (24 kDa), 4: Gnd (53 kDa), 5: RpiA (24 kDa), 6: Edd (67 kDa).

Contribution aux travaux de l'article

- Construction du vecteur de délétion du gène *gntP* de *N. meningitidis* 2C4-3.

- Construction du mutant Δ*gntP* chez *N. meningitidis* 2C4-3.

- Construction des vecteurs de complémentation du mutant Δ*gntP*, à partir des plasmides $pComP_{RBS}$ et $pComP_{ind}$.

- Tests de croissance de *N. meningitidis* 2C4-3 et du mutant Δ*gntP* sur RPMI et RPMI+glucose (0.5%) et RPMI+gluconate (0.5%).

- Purification et caractérisation de l'activité des enzymes suivantes: glucokinase (Glk), α-phosphoglucomutase (PgcM), 6-phosphogluconate déshydrogénase (Gnd), D-ribose-5-P isomérase A (RpiA), D-ribulose-5-P 3-épimérase (Rpe), gluconate-6-P déhydratase (Edd), fructose-1,6-bisphosphate aldolase (FbaA), gluconate kinase (GntK) et NMV_1007.

Transport and catabolism of carbohydrates by *Neisseria meningitidis*

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Short title: Carbohydrate utilization by *Neisseria meningitidis*

ABSTRACT

The human pathogen *Neisseria meningitidis* has the capacity to use glucose and maltose as carbon sources. We identified the genes encoding the proteins for the transport of these two carbohydrates in strain 2C4-3. A mutant deleted for *NMV_1892* (*glcP*) no longer grew on glucose and deletion of *NMV_0424* (*malY*) prevented the utilization of maltose. We also purified and characterized the enzymes catalyzing the early catabolic steps of the two carbon sources (glucokinase, α-phosphoglucomutase). *N. meningitidis* lacks fructose-6-P 1-kinase of the Embden-Meyerhof-Parnass pathway and the two carbohydrates are therefore catabolized either via the Entner-Doudoroff or the pentose phosphate pathway. Both catabolic routes lead to the formation of glyceraldehyde-3-P and to either pyruvate or fructose-6-P, respectively. We also purified and characterized several key enzymes of the two pathways. The genes encoding the enzymes, which catalyze the transformation of glucose into D-ribulose-5-P, are organized in an operon. Interestingly, *N. meningitidis* also contains the genes for a presumed gluconate transporter (*NMV_2230*, *gntP*) and a gluconate kinase (*NMV_2231*, *gntK*). The two proteins exhibit significant similarity to the corresponding enzymes of enterobacteriaceae and firmicutes. Indeed, the meningococcal *gntP* gene restored gluconate utilization in a *Bacillus subtilis* Δ*gntP* mutant. We also confirmed that purified NMV_2231 has gluconate kinase activity. However, although the *gntP* and *gntK* genes encode functional enzymes, *N. meningitidis* was not able to grow on gluconate as the sole carbon source. Surprisingly, deletion of *gntP* stimulated growth in glucose-containing medium and drastically slowed the clearance of *N. meningitidis* cells from transgenic mice after intraperitoneal challenge.

INTRODUCTION

The β-proteobacterium *Neisseria meningitidis* is a strictly human pathogen. In about 10% of a population, this commensal microorganism colonizes the nasopharynx (1), where it resides asymptomatically (2). For reasons not yet understood it can occasionally become virulent and cross the epithelial cell layer and enter the blood stream (3). In order to explain this dual lifestyle, the hypothesis of the existence of carriage and invasive strains has been put forward (2, 4). Only the latter are assumed to cause severe infections leading to septicemia and meningitis with sometimes fatal outcome (5). The current concept of meningococcal pathogenesis is based on the polygenic nature of meningococcal virulence comprising differences in genes involved in divers aspects of meningococcal biology (6). However, the genetic and physiological differences between these two presumed types of strains are not clearly understood, but were proposed to include amino acid metabolism and oxidative stress response. In addition, differences in carbon catabolism are also thought to be decisive whether a strain is invasive or not (7). Available carbon sources also affect meningococcal resistance against complement (8).

Compared to most bacteria, *N. meningitidis* can utilize only a small number of carbon sources, including not only carbohydrates, but also amino acids, such as glutamate (9). The tricarboxylic acid cycle intermediate succinate is also taken up and catabolized by meningococci (10). In addition, these bacteria can grow on L-lactate as well as pyruvate as the sole carbon source. Only two carbohydrates are so far known to be utilized by *N. meningitidis*, with glucose being one of them (11). Interestingly, glucose and lactate have been reported to be present in mM amounts in the nasopharynx (12), the usual habitat of *N. meningitidis*. While the transporter for lactate has been identified (8), the meningococcal enzymes involved in glucose uptake are not known. In many bacteria, glucose is efficiently transported via the phosphoenolpyruvate (PEP):carbohydrate phosphotransferase system (PTS) (13). However, *N. meningitidis* possesses only an incomplete PTS lacking any known membrane component (14). Nevertheless, it contains the metabolite-controlled HPr kinase, which phosphorylates the PTS protein HPr at Ser-46 (15). In firmicutes, the resulting P-Ser-HPr is involved in numerous regulatory processes (13). Although little is known about the role of the *N. meningitdis* incomplete PTS, it was suggested to carry out regulatory functions, possibly in meningococcal virulence (16). Indeed, the meningococcal PTS genes *ptsH* and *ptsI* were reported to be essential for experimental infection in an infant rat model of

meningococcal bacteremia (17). In addition, the PTS component HPr was found to interact with the transcription regulator CrgA and its deletion affects virulence and capsule synthesis of *N. meningitidis* (15). In several bacteria, glucose was reported to be transported not only by the PTS, but also slowly via ion symport permeases, such as GlcP of *Bacillus subtilis* (18) or GlcU of *Staphylococcus xylosus* (19) and *Listeria monocytogenes* (20). These permeases usually have a broad substrate specificity for hexoses (18). A gene (*NMV_1892*) encoding a protein resembling GlcP of firmicutes is present in *N. meningitidis* strain 2C4-3. This protein was therefore a likely candidate for the glucose transporter in *N. meningitidis*.

Meningococci are not only devoid of PTS-catalyzed transport but also of fructose-6-P 1-kinase (21), a key enzyme of the Embden-Meyerhof-Parnass (EMP) pathway, which uses ATP to transform fructose-6-P into fructose-1,6-bisphosphate (FBP). Heterologous expression of a *pfk* gene in *N. meningitidis*, which allowed the metabolism of glucose via the EMP pathway, had no detectable positive effect on growth of the recombinant strain (21). In contrast, it caused a reduction in biomass production. For preferably oxidative organisms, such as *N. meningitidis*, catabolism via the EMP pathway does not seem to represent an advantage. Meningococci therefore catabolize glucose mainly via the Entner Doudoroff (ED) pathway (70%) (22) and to a minor extent via the pentose phosphate pathway (PPP) (7). Pyruvate formed via both pathways or from lactate utilization or taken up from the medium is further metabolized via the tricarboxylic acid cycle (23). In addition, cells, which grow on glucose, lactate or pyruvate, secret significant amounts of acetate into the culture medium (23). The genes, which encode the enzymes presumed to catalyze the transformation of glucose into glyceraldehyde-3-P and pyruvate via the ED pathway are clustered within a specific region of the meningococcal genome (Fig. 1A). This region also contains the genes for 6-P-gluconolactonase (*pgl*), the entrance point into the PPP, and phosphoglucose isomerase (*pgi1*), which recycles fructose-6-P formed via the PPP by converting it into glucose-6-P. The genes encoding other enzymes of the PPP are randomly distributed over the meningococcal chromosome.

N. meningitidis can also utilize maltose (11), which is formed by two glucose molecules connected via an α-1,4 glycosidic bond. The disaccharide is taken up by most bacteria either via an ABC transporter (24, 25) or via a PTS (26, 27). As already mentioned, meningococci contain an incomplete PTS unable to transport carbohydrates. In addition, no genes encoding the membrane components of a carbohydrate-specific ABC transporter could be detected. Nevertheless, we found that *N. meningitidis* contains a presumed operon

encoding three enzymes involved in maltose utilization. A fourth gene encodes a permease, which was a likely candidate for the maltose transporter.

In this study, we identified the two permeases which catalyze the uptake of the carbohydrates glucose and maltose. We also characterized the enzymes, which catalyze their first catabolic steps as well as several key enzymes of the two carbon catabolic pathways used by *N. meningitidis*. Finally, we searched in the meningococcal genome for permeases transporting additional carbon sources and discovered a transport protein resembling gluconate permeases.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The meningococcal strain 2C4-3, isolated as clone 12 from the serogroup C, class 1 strain 8013 (28) has the relevant phenotype P^+, Opa^-, Opc^-, $PilC1^+/PilC2^+$. This strain as well as the *glcP*, *malU*, and *gntP* deletion mutans derived from it were grown either in GC medium base (GCB) (Difco) containing the supplements previously described (29), in Roswell Park Memorial Institute (RPMI) medium, and in RPMI medium supplemented with serum. When appropriate, kanamycin or spectinomycin was added at a final concentration of 100 or 75 μg/ml, respectively.

Escherichia coli strains NM522 (Stratagene) or NM522 transformed with plasmid pREP4-GroES/EL (30) were used for protein overproduction. Strains harbouring derivatives of the His-tag expression vector pQE30 (Qiagen) were grown at 37°C under agitation in LB medium supplemented with 100 μg/ml ampicillin for NM522 and with 50 μg/ml ampicillin and 12.5 μg/ml kanamycin for NM522[pREP4-GroES/EL].

Growth of the *Bacillus subtilis* BSB168-derived *gntP* mutant and its transformant containing pIC634-gntP-2C4-3 was measured in C minimal medium containing 25 mM gluconate in the presence or absence of 1 mM IPTG.

Plasmids used in this study. The plasmid pSU18c confers resistance to chloramphenicol and is a pACYC184-derived vector containing the multiple cloning site and the *lacZ*α reporter gene of pUC18 (31). Plasmid pQE30 (Qiagen) was used for the overproduction of His-tagged poteins. Plasmid pIC634 is a derivative of plasmid pDG1664 (32), which contains the *E. coli lacI* gene and the *spac* promoter inserted at the BamHI/HindIII/EcoRI restriction sites (gift of Dominique Le Coq, Jouy en Josas, France). It

also contains two *B. subtilis* DNA fragments allowing its integration by dual recombination in the *thrC* locus of this organism.

Purification of His-tagged proteins. The genes encoding the proteins glucokinase (Glk), α-phosphoglucomutase (PgcM), gluconate kinase (GntK), gluconate-6-P dehydrogenase (Gnd), gluconate-6-P dehydratase (Edd), D-ribulose-5-P 3-epimerase (Rpe), D-ribose-5-P isomerase A (RpiA), and fructose-1,6-bisphosphate aldolase (Fba) were PCR amplified by using *N. meningitidis* 2C4-3 genomic DNA as template and the corresponding primer pairs listed in Table 1. The amplicons were cut with the appropriate restriction enzymes and cloned into the His-tag expression vector pQE30 (Qiagen) cut with the corresponding enzymes. The correct sequence of each insert was confirmed by DNA sequencing. The resulting plasmids were used to transform the *E. coli* strain NM522[pREP4-GroES/EL] (30). Expression of the various genes by induction with IPTG and purification of the His-tagged proteins on Ni-NTA columns (Qiagen) was carried out as previously described (33). To purify the ED pathway enzyme 2-dehydro-3-deoxyphosphogluconate aldolase from *E. coli* the corresponding gene *eda* was PCR amplified by using genomic DNA of *E. coli* strain NM522 as matrix and primers edaFcoliBam and edaRcoliSal (Table 1). The resulting amplicon was cut with BamHI and SalI, inserted into pQE30 cut with the same enzymes and subsequently cloned into *E. coli* strain NM522. For one clone, the correct sequence of the insert was confirmed by DNA sequencing. Subsequent purification of Eda was carried out as previously described (34). Maltose phosphorylase of *Enterococcus faecalis* and ribitol-5-P 2-dehydrogenase from *Lactobacillus casei* were purified as described in (27) and (35), respectively.

Spectrophotometric enzyme assays. To measure the activity of the purified enzymes we carried out appropriate spectrophotometric assays. The tests were performed in a total volume of 500 µl and the change in OD_{340} was continuously followed with a Kontron Bio-Tek spectrophotometer using the 'Autorate' programme. All mixtures contained 50 mM Tris/HCl, pH 7.4, and 10 mM $MgCl_2$. To measure the various enzyme activities we added for glucokinase: 10 mM glucose, 1 mM NADP, 1 mM ATP, 10 µg of glucose-6-phosphate 1-dehydrogenase (Sigma), and 8 µg of glucokinase; for α-phosphoglucomutase: either 10 mM maltose and 4 µg of maltose phosphorylase from *E. faecalis* (27) or 10 mM β-D-glucose-1-P, for both tested substrates we added 1 mM NADP, 10 µg of glucose-6-phosphate 1-dehydrogenase, and 20 µg of α-phosphoglucomutase; for gluconate kinase: 1 mM gluconate, 1 mM NADP, 1 mM ATP, 25 µg of 6-phosphogluconate dehydrogenase (Sigma), and 2 µg of

gluconate kinase; for gluconate-6-P dehydrogenase: 1 mM gluconate-6-P, 1 mM NADP, and 10 μg gluconate-6-P dehydrogenase; for gluconate-6-P dehydratase: 1 mM gluconate-6-P, 1 mM NADH, 30 μg of 2-dehydro-3-deoxyphosphogluconate aldolase (*E. coli*), 20 μg of triosephosphate isomerase (Sigma), 24 μg of glycerol-3-P dehydrogenase (Sigma), and 40 μg of gluconate-6-P dehydratase; for D-ribulose-5-P 3-epimerase: 0.5 mM D-xylulose-5-P, 1 mM NADH, and 18 μg of *L. casei* D-ribitol-5-P 2-dehydrogenase (35), and 10 μg of D-ribulose-5-P 3-epimerase; for D-ribose-5-P isomerase: 1 mM ribose-5-P, 1 mM NADH, 18 μg of *L. casei* ribitol-5-P 2-dehydrogenase, and 30 μg of D-ribose-5-P isomerase; for fructose-1,6-bisphosphate aldolase: 5 mM FBP, 1 mM NADH, 20 μg of triosephosphate isomerase, 24 μg of glycerol-3-P dehydrogenase and 5 μg of fructose-1,6-bisphosphate aldolase. After preincubation of the assay mixture for 5 to 10 min at ambient temperature, the reaction was started by adding the enzyme to be tested.

Construction of *N. meningitidis* Δ*glcP*, Δ*malY* and Δ*gntP* mutants. The three deletion mutants were constructed by first amplifying upstream and downstream regions of the targeted gene by using the primers listed in Table 1. The two upstream and downstream DNA fragments of the *glcP* and the *malY* genes were cloned into plasmid pQE30 cut with BamHI/HindIII for *glcP* and BamHI/KpnI for *malY*. The *glcP*-related fragments were cut with BamHI/EcoRV and EcoRV/HindIII and the *malY* fragments with BamHI/EcoRV and EcoRV/KpnI. In each case, the simultaneous insertion of the two fragments created an EcoRV site in the middle. In the case of *glcP*, a spectinomycin cassette was inserted in the EcoRV site and a kanamycin cassette in the EcoRV site of the *malY* fragments. The two antibiotic resistance cassettes were amplified with primers, which created SmaI sites at their ends and thus allowed blunt end ligation. For *gntP* deletion, a BamHI/EcoRI upstream fragment was first cloned into pBluescript KS+, followed by a kanamycin resistance cassette amplified with EcoRI and HindIII sites. Finally, the downstream *gntP* fragment amplified with HindIII and SalI sites was added. The three deletion plasmids also contained the uptake sequence for *N. meningitidis* and they were used to transform *N. meningitidis* strain 2C4-3. Transformants with the correct antibiotic resistance were selected and the correct deletion of each gene (*glcP, gntP*, and *malY*) was verified by PCR with appropriate primers and subsequent DNA sequencing of the amplicon.

Glucose and maltose utilization assays. The *N. meningitidis* wild-type strain 2C4-3 and the *glcP* mutant derived from it were grown in cystine trypticase agar (CTA) medium (Difco) containing 1% glucose. Similarly, the wild-type strain and the *malY* mutant were

grown in CTA medium containing 1% maltose. The medium also contains 17 mg/l phenol red as pH indicator, which changes its color from red to yellow during acidification of the medium owing to the utilization of the added sugar (36).

Complementation of an *E. coli* strain unable to utilize glucose with the *NMV_1892* gene. Heterologous complementation assays were carried out by following a previously described method (37). The *N. meningitidis glcP* gene *NMV_1892* together with its ribosome binding site was amplified by PCR with *N. meningitidis* genomic DNA as template and the oligonucleotides SP219 and SP220, which introduce BamHI and HindIII restriction sites (Table 1). The resulting PCR fragment was digested with the indicated restriction enzymes and ligated into vector pSU18c in the same orientation as the 5'-part of *lacZ* encoding the α-fragment of β-galactosidase (38), thus putting the *NMV_1892* gene under control of the promoter preceding *lacZ*. In order to avoid fusion of *NMV_1892* to the N-terminus of the α-fragment the 5' oligonucleotides were chosen in such a way that they created a stop codon located upstream from the ribosome binding site. The stop codon was in frame with the *NMV_1892* start codon. The correct sequence of the inserts was confirmed by DNA sequencing. The resulting plasmid was introduced into *E. coli* strain LJ140, which carries a *ptsHIcrr* deletion and therefore exhibits very poor fermentation of glucose (39). Transformants were plated on MacConkey agar, which contained 1 mM IPTG and 1% glucose in order to test for heterologous complementation of the glucose-negative phenotype. Empty pSU18c was used to transform *E. coli* strains NM522 and LJ140, thus providing positive and negative controls, respectively, for glucose fermentation.

Complementation of an *E. coli malE* mutant with the *NMV_0424* gene. In order to complement the *E. coli* strain GSJ100 (40), which lacks the periplasmic maltose binding protein MalE and therefore cannot utilize maltose, we used the same approach as described above for the complementation of the glucose-negative *E. coli* mutant. The *N. meningitidis malY* gene *NMV_0424* together with its ribosome binding site was amplified by PCR with the oligonucleotides SP236 and SP237. The amplicon was cloned into vector pSU18c cut with BamHI and HindIII. The resulting plasmid was used to transform the *E. coli malE* mutant GSJ100 (40). Fermentation assays were carried out on MacConkey agar, which contained 1 mM IPTG and 1% maltose. Empty pSU18c was used to transform *E. coli* strains NM522 and GSJ100, thus providing positive and negative controls, respectively, for maltose fermentation.

Complementation of a *B. subtilis gntP* mutant with the *NMV_2230* gene. Because NMV_2230 of *N. meningitidis* resembles strongly GntP of firmicutes, we decided to test whether this protein could complement a *B. subtilis gntP* mutant. In the first step we deleted

the *gntP* gene in the *B. subtilis* strain BSB168. For that purpose, the upstream region of *gntP* and the *aphA3* kanamycin resistance gene were amplified by using *B. subtilis* genomic DNA and plasmid pGEM-T(aphA3) (33), respectively, as template and appropriate primer pairs (Table 1). The forward primer for *aphA3* overlapped the reverse primer for the *gntP* upstream region, which allowed the fusion of the two DNA fragments by PCR amplification by using the two amplicons as template and the two external oligonucleotides as primers. The fused DNA was cut with ApaI and SpeI and inserted into plasmid pGEM-T Easy (Promega) cut with the same enzymes. The downstream region of *B. subtilis gntP* was subsequently amplified by using genomic *B. subtilis* DNA as template and appropriate primers (Table 1). The resulting amplicon was cut with SpeI and SalI and cloned into pGEM-T Easy containing *aphA3* and the upstream region of *gntP*. The resulting plasmid pGEM-T-ΔgntP-Kan was used to transform the *B. subtilis* strain BSB168 and a double recombination allowed the replacement of *gntP* with the kanamycin resistance gene *aphA3*. The correct deletion of *gntP* was confirmed by PCR amplification of the corresponding region and DNA sequencing of the amplicon.

In the second step, the *gntP*-like gene of *N. meningitidis* was PCR amplified by using genomic DNA as template and the primers D-GntP-HindIII and R-GntP-SphI (Table 1). The resulting DNA fragment was cut with HindIII and SphI and inserted into plasmid pIC634, which is a derivative of pDG1664 (32) containing the *E. coli lacI* gene and the *spac* promoter inserted at the BamHI/HindIII/EcoRI restriction sites. The correct sequence of the *gntP*-like gene, which is under control of the *spac* promoter, was verified by DNA sequencing. Plasmid pIC634 also contains the upstream and downstream regions of the *B. subtilis thrC* gene and thus allowed the simultaneous integration of *lacI*, *erm*, and the P*spac*-controlled *gntP* gene at the *thrC* locus of the *B. subtilis* wild-type strain BSB168.

Virulence assays in mice. The virulence of meningococcal strains was evaluated by their ability to provoke bacteremia in mice after intraperitoneal challenge. The experimental design was approved by the Institut Pasteur Review Board. Bacterial loads in infected six-week-old female BALB/c mice (Janvier, France) were determined from blood samples taken at 2, 6 and 24 h after intraperitoneal challenge with standardized inocula of 10^7 cfu per mouse for each tested strain. Bacterial counts were determined by plating serial dilutions of blood on GCB plates.

RESULTS AND DISCUSSION

Identification of the meningococcal glucose permease. *N. meningitidis* is known to utilize glucose. However, the enzymes catalyzing the uptake of glucose and the first catabolic steps have not been identified. Based on sequence similarity to bacterial glucose permeases, NMB0535 of *N. meningitidis* strain MC58 had been suggested to function as glucose transporter (41). This protein is nearly identical to NMV_1892 of strain 8013, which exhibits 46% sequence similarity to *B. subtilis* GlcP, which has been shown to take up glucose and mannose (18). In order to test whether NMV_1892 catalyzes glucose transport in *N. meningitidis*, we deleted the *NMV_1892* gene by replacing it with a spectinomycin resistance cassette. The resulting mutant had indeed lost the capacity to grow in RPMI medium containing glucose as the sole carbon source (Fig. 2) and to acidify CTA medium (Supplementary Fig. 1). To further confirm the glucose transport function of NMV_1892, we cloned its gene together with the ribosome binding site into plasmid pSU18c (38), where it is transcribed from the *lac* promoter (see Materials and Methods). The resulting plasmid was subsequently used to transform the *E. coli* Δ*ptsHIcrr* mutant LJ140, which is not able to utilize glucose (20, 37). In contrast, the resulting transformant was able to ferment this sugar when grown on MacConkey agar plates similar to the *E. coli* wild-type strain NM522 transformed with empty pSU18c (Fig. 3A). LJ140 transformants carrying the empty plasmid pSU18c were not able to ferment glucose.

Characterization of enzymes involved in glucose transport and catabolism via the ED pathway. In most bacteria, glucose taken up via an ion symporter is first phosphorylated to glucose-6-P by an enzyme called glucokinase (Glk) or hexokinase. Glk activity has previously been detected in crude extracts of *N. meningitidis*, but the enzyme has not been purified and characterized (42). In order to identify the *N. meningitidis* Glk we carried out a BLAST search with Glk from *E. coli*. This search revealed that the *N. meningitidis NMV_1004* gene encodes a protein exhibiting 62% sequence similarity to *E. coli* Glk. We purified His-tagged NMV_1004 as described in Materials and Methods and tested whether it can phosphorylate glucose. Indeed, NMV_1004 used ATP to transform D-glucose into D-glucose-6-P and we therefore called it Glk (Table 2).

Interestingly, the genes located upstream and downstream from *NMV_1004* seemed to encode several other enzymes involved in glucose metabolism (Fig. 1A). These enzymes include a potential P-glucose isomerase (NMV_1006) (Fig. 4), which interconverts glucose-6-

P and fructose-6-P; a glucose-6-P dehydrogenase (NMV_1002), which oxidizes glucose-6-P to gluconate-6-P; a 6-phosphogluconolactonase (NMV_1003), which transforms gluconate-6-P into 6-P-glucono-δ-lactone; a gluconate-6-P dehydratase (NMV_1001), which converts gluconate-6-P into 2-keto-3-deoxy-6-P gluconate (branching point to the ED pathway); a 2-keto-3-deoxy-6-P gluconate aldolase (NMV_1000), which cleaves 2-keto-3-deoxy-6-P gluconate into glyceraldehyde-3-P and pyruvate; and finally a RpiR-like repressor (NMV_1005). The genes *NMV_1002* to *NMV_1006* are oriented in the same direction and probably form an operon, while the ED pathway genes *NMV_1000* and *NMV_1001* are oriented in the opposite direction (Fig. 1A). *N. meningitidis* catabolizes glucose mainly via the ED pathway (70%) (22) and we therefore wanted to confirm the presumed gluconate-6-P dehydratase activity of NMV_1001. We purified this enzyme with a His-tag and carried out a coupled spectrophotometric assay as described in Materials and Methods. NMV_1001 exhibited indeed gluconate-6-P dehydratase activity (Table 2).

Characterization of enzymes involved in glucose catabolism via the PPP. Catabolism via the PPP is more complex and requires five additional enzymes. We carried out BLAST searches with the corresponding *E. coli* and *B. subtilis* proteins and could identify candidates for the five enzyme activities: gluconate-6-P dehydrogenase (NMV_0016); D-ribulose-5-P 3-epimerase (NMV_1185); D-ribose-5-P isomerase (NMV_0882); transketolase (NMV_0929); and transaldolase (NMV_0388) (Fig. 4). The genes for the presumed PPP enzymes are not organized in an operon, but are randomly distributed over the meningococcal chromosome. In order to confirm that this pathway is indeed active, we purified three of the five enzymes as described in Materials and Methods. By carrying out appropriate spectrophotometric assays we could demonstrate that NMV_0016 converts gluconate-6-P into ribulose-5-P (branching point to the PPP). This activity has previously been measured in *N. meningitidis* crude extracts, but the enzyme has not been purified and characterized (43). We found in addition that NMV_1185 transforms D-ribulose-5-P into D-xylulose-5-P, whereas NMV_0882 isomerizes D-ribulose-5-P to D-ribose-5-P. During the metabolism of glucose via the PPP, three molecules of glucose are transformed into 2 molecules of fructose-6-P, one molecule of glyceraldehydes-3-P and 3 CO_2. While glyceraldehyde-3-P is metabolized via the lower part of glycolysis, fructose-6-P is recycled to glucose-6-P by one of the presumed phosphoglucose isomerases NMV_1006 (Pgi1) or NMV_0369 (Pgi2). *N. meningitidis* lacks fructose-6-P 1-kinase activity, which would allow the phosphorylation of fructose-6-P to FBP (Fig. 4). Nevertheless, *N. meningitidis* can probably form FBP from glyceraldehydes-3-P, which is partly converted into dihydroxyacetone phosphate by a presumed triosephosphate

isomerase (NMV_2075). Indeed, we could demonstrate that purified NMV_2059 functions as a fructose-1,6-bisphosphate aldolase, which uses the two triosephosphates to form FBP (Fig. 4). In numerous organisms, FBP is an important allosteric regulator of metabolic enzymes, such as pyruvate kinase (44), and of signal transduction proteins, such as HPr kinase (45), which probably explains why this glycolytic intermediate is synthesized in *N. meningitidis*, although it cannot be further metabolized.

Identification of enzymes catalyzing maltose uptake and catabolism. *N. meningitidis* is also known to efficiently utilize the disaccharide maltose, but the enzymes, which catalyze its uptake and catabolism have not been identified. Meningococci do not possess the components of an ABC transporter for maltose. Nevertheless, maltose phosphorylase activity had been detected in meningococcal crude extracts precipitated with ammonium sulfate (46). Usually, maltose phosphorylase catalyzes the first step of maltose catabolism, i.e. its phosphorolysis into D-glucose and D-glucose-1-P, in bacteria transporting the disaccharide via an ABC transporter. We therefore carried out a BLAST search with the sequence of the *L. casei* maltose phosphorylase MapA (LCABL_11440) (25). We indeed identified a gene in the *N. meningitidis* strain 2C4-3, *NMV_0426*, which encodes a protein that exhibits strong sequence identity (59%) to *L. casei* maltose phosphorylase. Interestingly, the genes upstream and downstream from *NMV_0426* also encode enzymes potentially involved in maltose catabolism (Fig. 1B): *NMV_0425* encodes a presumed aldose 1-epimerase, which transforms α-D-glucose into β-D-glucose and NMV_0427 encodes a presumed phosphoglucomutase, which interconverts D-glucose-1-P and D-glucose-6-P (Fig. 4). A meningococcal phosphoglucomutase (Pgm) has previously been identified (47). Pgm is encoded by the gene NMV_1606 and was reported to be important for the synthesis of lipooligosaccharides, because the size of these surface compounds was largely reduced in a mutant carrying a transposon insertion in the *pgm* gene. NMV_1606 interconverts β-D-glucose-6-P and β-D-glucose-1-P (47). NMV_0427 shares no sequence similarity with the much longer meningococcal Pgm, but is 46% identical to PgcM from the *B. subtilis* maltodextrin operon (26), which converts both α- and β-D-glucose-1-P into glucose-6-P (48). In addition, NMV_0427 lacks the phosphorylation site signature sequence [T(P/A)SHNP] of bacterial phosphoglucomutases (49). In order to test whether it nevertheless functions as phosphoglucomutase we purified His-tagged NMV_0427 as described in Materials and Methods. Purified NMV_0427 was able to convert α-D-glucose-1-P into D-glucose-6-P (Table 2), but did not react with β-D-glucose-1-P. The activity of NMV_0427 therefore

resembles α-phosphoglucomutases, such as PgmH of *Lactococcus lactis* (50). However, NMV_0427 exhibits no sequence similarity to PgmH and we therefore preferred to call it PgcM according to the highly similar *B. subtilis* enzyme, which reacts with α- and β-D-glucose-1-phosphate (48).

The three genes encoding the presumed enzymes for maltose catabolism probably form an operon together with a fourth gene, *NMV_0424* (Fig. 1B), which is annotated as a sucrose/H^+ cotransporter-like transmembrane protein of the major facilitator superfamily. We therefore suspected that NMV_0424 might catalyze maltose uptake in *N. meningitidis*. Indeed, NMV_0424 exhibits strong sequence similarity (61%) to the protein MalY of the α–proteobacterium *Caulobacter crescentus*, which had been shown to function as maltose transporter in this organism (51). In order to test whether NMV_0424 of *N. meningitidis* strain 2C4-3 is indeed able to transport maltose, we deleted the *NMV_0424* gene by replacing it with a kanamycin resistance cassette. The resulting mutant grew normally on glucose (data not shown), but had lost the capacity to utilize maltose (Supplementary Fig. 1). To further confirm that NMV_0424 functions as maltose transporter, we cloned its gene together with the ribosome binding site into plasmid pSU18c (31), where it is transcribed from the *lac* promoter (see Materials and Methods). The resulting plasmid was subsequently used to transform the *E. coli malE* mutant GSJ100 (40), which lacks the periplasmic maltose binding protein MalE and therefore is not able to utilize maltose. In contrast, a resulting transformant, which contained pSU18c carrying *NMV_0424* was able to ferment this disaccharide when grown on maltose-containing MacConkey agar plates similar to the *E. coli* wild-type strain NM522 transformed with empty pSU18c (Fig. 3B). In contrast, GSJ100 transformants carrying the empty plasmid pSU18c were not able to ferment maltose.

We found that similar maltose-like transporters are present in lactobacilli that lack a maltose-specific ABC transporter, such as *Lactobacillus salivarius* and *Lactobacillus reuteri*. NMV_0424 exhibits up to 82% sequence identity to some of these *Lactobacillus* transporters. This unusually high sequence identity suggests that *N. meningitidis* probably acquired the maltose transporter gene by horizontal gene transfer from one of these lactobacilli. The donor could well be *L. salivarius*, which not only possesses a *malY* gene (*LSL_1282*), but contains a maltose operon with the same gene order as *N. meningitidis* (*malY*, *galM*, *mapA*, *pgcM*) (Fig. 1B). Even more important, these two bacteria share a common habitat, the nasopharynx. *Neisseria gonorrhoeae*, which colonizes a different habitat, lacks the enzymes for maltose utilization.

In conclusion, *N. meningitidis* strain 2C4-3 takes up maltose via the presumed H^+ symport permease NMV_0424, which we dubbed MalY in agreement with the name given to the *C. crescentus* maltose transporter (51). Intracellular maltose is phosphorolyzed into α-D-glucose and α-D-glucose-1-P by the enzyme maltose phosphorylase, MalP (NMV_0426) (Fig. 4). The two products of this reaction, α-D-glucose and α-D-glucose-1-P, are subsequently converted into D-glucose-6-P either by the enzymes aldose 1-epimerase (GalM, NMV_0425) and glucokinase (Glk, NMV_1004) or by the α-phosphoglucomutase (PgcM, NMV_0427), respectively. Similar to glucose, maltose is therefore probably mainly metabolized via the ED pathway and to a minor extent via the PPP.

***N. meningitidis* 2C4-3 contains a presumed gluconate permease and gluconate kinase.** In order to identify additional carbohydrate transporters in *N. meningitidis*, we carried out BLAST searches with various carbohydrate permeases and catabolic enzymes from *E. coli* and *B. subtilis*. Interestingly, when we used the gluconate permease from *B. subtilis* as query, we detected a protein (NMV_2230) which exhibits 58% sequence similarity to the *B. subtilis* transporter. In addition, the protein encoded by the downstream gene *NMV_2231* (Fig. 1C) showed 56% sequence similarity to gluconate kinase (GntK) from *E. coli*. We therefore constructed an NMV_2230 deletion mutant. Surprisingly, when we carried out growth studies with wild-type and mutant strain, both were unable to utilize gluconate as the sole carbon source. The failure to utilize gluconate was also observed with a *N. meningitidis* serotype B strain (data not shown). It was possible that the *NMV_2230* and *NMV_2231* genes were not expressed or that one of them contains a mutation leading to the inactivation of the encoded enzyme. To test the second possibility, we purified His-tagged NMV_2231 and carried out gluconate kinase assays. Indeed, the purified enzyme exhibited very strong gluconate kinase activity (Table 2). We subsequently tested whether GntP can transport gluconate when produced in another organism. To that end, we constructed in a first step a *gntP* deletion mutant of *B. subtilis* strain BSB168, which in contrast to the wild-type strain no longer grew on gluconate as the sole carbon source (Fig. 5), but grew normally on minimal C medium containing 25 mM glucose (data not shown). In the second step, we amplified the NMV_2230 gene and cloned it into the shuttle vector pIC634, where it is under control of the IPTG-inducible *spac* promoter. This plasmid also contains two *B. subtilis* DNA fragments allowing its integration into the *thrC* site of *B. subtilis*. The resulting plasmid pIC634-gntP was used to transform the *B. subtilis gntP* mutant and for three transformants their correct integration into the *thrC* site was confirmed by PCR and subsequent sequencing of the amplified DNA

fragments. Only when 1 mM IPTG was present in the growth medium, the complemented strains were able to utilize gluconate as the sole carbon source. Nevertheless, they grew at a slower rate than the *B. subtilis* wild-type strain BSB168, as shown for one of the integrants (Fig. 5). This result was surprising because in *N. meningitidis* 2C4-3 the *gntP*-like gene NMV_2230 does not support growth on gluconate.

Deletion of the *gntP-like* gene enhances *N. meningitidis* virulence. We tested whether any other phenotypes might be associated with the deletion of the *gntP*-like gene *NMV_2230*. During its catabolism in *N. meningitidis*, glucose is converted to gluconate-6-P, which is also the product of the GntK-catalyzed reaction. We therefore tested whether deletion of the *NMV_2230* gene might affect growth on glucose. Surprisingly, the *NMV_2230* mutant strain reproducibly grew significantly faster and to a higher cell density than the wild-type strain (Fig. 2). Glucose transport and/or catabolism seem to be more efficient in the absence of GntP thus allowing faster growth.

In addition, we tested whether deletion of the *gntP*-like gene might affect the virulence of this pathogen. For that purpose we measured the clearance of *N. meningitidis* cells at 2, 6 and 24 h after intraperitoneal challenge with standardized inocula of 10^7 cfu per mouse for the wild-type strain and the *NMV_2230* mutant. Interestingly, clearance of the *NMV_2230* mutant from the bloodstream of mice was much slower than for the wild-type strain (Fig. 6). The enhanced resistance against clearance from the blood stream might partly be due to the observed faster growth of the *gntP*-like mutant in glucose-containing environment (Fig. 2).

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Table 1. List of primers used in this study.

Name	Used for	Sequence (5' to 3')
glcPBamHI	N.m. Δ*glcP*	CAC**GGATCC**GCGGAAGTGAAGGCGGAAA[a]
glcPEcoRV1		GGG**GATATC**AAGACGACCAATGGGGACG
glcPEcoRV2		CCC**GATATC***TTCAGACGGC*CCGGTGTACTGTGTACCGCG[b]
glcPHindIII		ATT**AAGCTT**CAACGCGCCTATCATGCCC
specSmaIFor		ATA**CCCGGG**GATCCGGTGATTGATTGAGC
specSmaIRev		CGGC**CCCGGG**TTGAACGAATTGTTAGAC
malYBamHI	N.m. Δ*malY*	GGT**GGATCC**GCCCGCGCCGTTCAAACCG
malYEcoRV1		ATC**GATATC**GTGCTTTTT GCCAGCGCGG
malYEcoRV2		ATC**GATATC**GCTTGGGCGGGCATTATC
malYKpnI		ACG**GGTACC**GGCCAGCCCGTGCGAACCGC
KanSmaI1		GGG**CCCGGG**CCCAGCGAACCATTTGAGG
KanSmaI2		AAA**CCCGGG***TTCAGACGGC*TACTAAAACAATTCATCCAG
AMgntP2C-BamF	N.m. Δ*gntP*	GCAC**GGATCC**TGCCGCTGCCTGAAATACCGTCC
AMgntP2C-ECORIR		AAGC**GAATTC**CTGTAATAAAAGTACAAAATTGTCAGG
KangntP2C-ECORIF		GGGGG**GAATTC**CCCAGCGAACCATTTGAGG
KangntP2C-HindR		CTAAA**AAGCTT***TTCAGACGGC*TACTAAAACAATTCATCCAG
AvgntP2C-HindF		CATC**AAGCTT**CAGACGGAAAGGATAGTAAATG
AvgntP2C-SalIR		GGG**GTCGAC**GGCTTCAGACGGCATATTGCTTC
Deltagntp-F	Δ*gntP* verificat.	GCCCGCCACCAACGTGGGCATC
DeltagntP-F1		CTTGCACAAACCCCTG
Deltagntp-R		GGTGCTTCAGCACCTTAGAGAATC
KanF1		GATGTGGAACGGGAAAAGGACATGATG
KanR1		TCTTCATACTCTTCCGAGCAAAGGACGCC
amont-gntP-D-ApaI	BSB168 Δ*gntP*	CGT**GGGCCC**CAAATGGGAATCGATCCGGAAACGCC
amont-gntP-R-SacII		ACGT**CCGCGG**CAAGTGCAACGATGATTAACGGCATGG
aval-gntP-D-SpeI		ACGT**ACTAGT**AAGTTTAGTTGTATGAAATTAAGAAGG
aval-gntP-R-SalI		ACGT**GTCGAC**AAGAGCCTGTCTGACGTATTCAATCC
amont-D-Kana	*aphA3*	CGTTGCACTTG**CCGCGG**ACGTGTTCTTGAAAACGGCGGAGAAGC
aval-R-Kana		CAACTAAACTT**ACTAGT**ACGTCCCATGTAAAGGAATTCTGTACGG
GlkNmBaFor	pQE30-glcK	GTGA**GGATCC**ATGTCTTCTACGCCGAAT
GlkNmHiRev		ATAT**AAGCTT**CGATAATGCAGCCCCGCTG
GntKNmBaHiFor	pQE30-gntK	AGGA**GGATCC**ATGACTACGCATTTTGTCG
GntKNmBaHiRev		TGTC**AAGCTT**CAGACGGCATATTGCTTCAA
Gnd8013KpnIF	pQE30-gnd	GCTT**GGTACC**ATGAAAGGCGATATTGGTG
Gnd8013HindR		CGGC**AAGCTT**ATCAAATATCGTAGG
rpiA8013bamF	pQE30-rpiA	GGAC**GGATCC**ATGACGACACAAGACGAACTCAAGCG
rpiA8013HindR		GACGG**AAGCTT**AACGTCAGGATTACCCTTGGCAGGG
Rpe8013BamF	pQE30-rpe	GGA**GGATCC**ATGACCGCCTACCGTATCGCACCCAG
Rpe8013KpnIR		CTGAA**GGTACC**CTAATTTTCAGACGGCATTTCTATTTC
Edd8013BamF	pQE30-edd	GAGA**GGATCC**GTGAACACTACTCCTATCCACTCC

Edd8013HindR		TGAA**AAGCTT**GTCTGAAACGCGCATCAG
FbaNmBaHiFor	pQE30-fba	GGAG**GGATCC**ATGGCACTCGTATCCATGCG
FbaNmBaHiRev		AAGC**AAGCTT**TTATTTGACGATTTGGTTC
pgmB8013BamF	pQE30-pgmB	GGG**GGATCC**ATGACGTTTACCGCAGTGCTCTTCG
pgmB8013HindR		CTTA**AAGCTT**TATCTGACGCGTTTTACCTGCCC
edaFcoliBam	pQE30-edaEc	AAA**GGATCC**ATGAAAAACTGGAAAACAAGTG
edaRcoliSal		ATC**GTCGAC**CGGGCATTTTGACTTTTACAG
SP219	pSU18c-glcP	CGC**GGATCC**TAGATAGTAATATTCCGACACC
SP220		CTCAAGA**AAGCTT**TTATTTGTCCGCCC
SP236	pSU18c-malY	CGC**GGATCC**TAGATACGAGGCAACGCCGTACTGG
SP237		TTCCC**AAGCTT**TCAAACCCCGCCGTGTGTTTG
D-GntP-HindIII	pIC634-gntP	ACGT**AAGCTT**ACATAAGGAGGAACTACTATGGACGGCTGGACACAGACGC
R-GntP-SphI		ACGT**GCATGC**TCAGACGATGGCGAACAGCAGTGC

[a]Restriction sites are underlined and in bold letters.

[b]The *N. meningitidis* DNA uptake sequence is written in italics.

TABLE 2 : Enzyme activities of purified proteins.

Gene	Enzyme activity	Specific activity (µmol/min and mg protein)
glK (*NMV_1004*)	Glucokinase	15
gnd (*NMV_0016*)	6-phosphogluconate dehydrogenase	2
rpiA (*NMV_0882*)	D-Ribose-5-P isomerase A	3.66
rpe (*NMV_1185*)	D-Ribulose-5-P 3-epimerase	2
pgcM (*NMV_0427*)	α-Phosphoglucomutase	12.5
edd (*NMV_1001*)	Gluconate-6-P dehydratase	2.5
fbaA (*NMV_2059*)	Fructose-1,6-bisphosphate aldolase	56
gntK (*NMV_2231*)	Gluconate kinase	150

Fig. 1

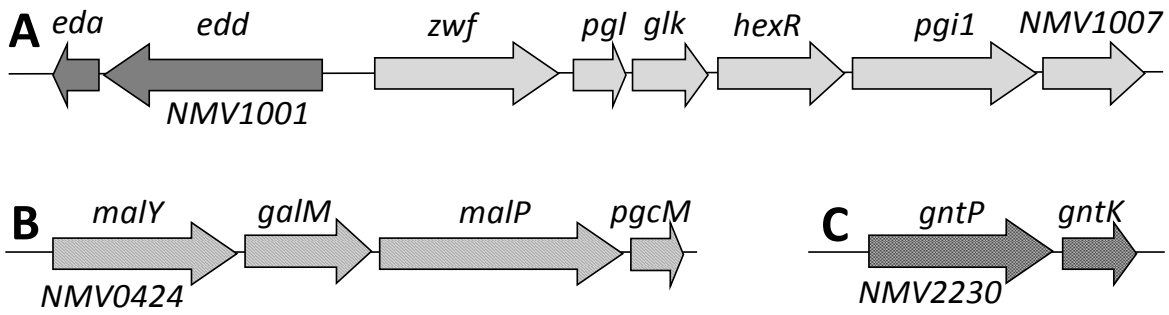


FIG 1 Schematic representation of the *N. meningitidis* genomic regions containing the genes encoding the enzymes necessary for the metabolism of (A) glucose and (B) maltose via the ED pathway. Although gluconate is not utilized by *N. meningitidis*, the genes *gntP* and *gntK* are present in 2C4-3 (C) and other meningococcal strains. The encoded GntK is functional in *in vitro* tests and GntP can complement a *B. subtilis gntP* mutant. In (A), the genes encoding the ED enzymes Eda and Edd are in dark grey. The genes encoding the maltose permease MalY (B) and gluconate permease GntP (C) are associated with the corresponding catabolic genes, whereas the gene encoding the glucose permease GlcP (NMV_1892) is located elsewhere on the genome. The genes encoding the PPP enzymes are randomly distributed over the whole chromosome.

Fig. 2

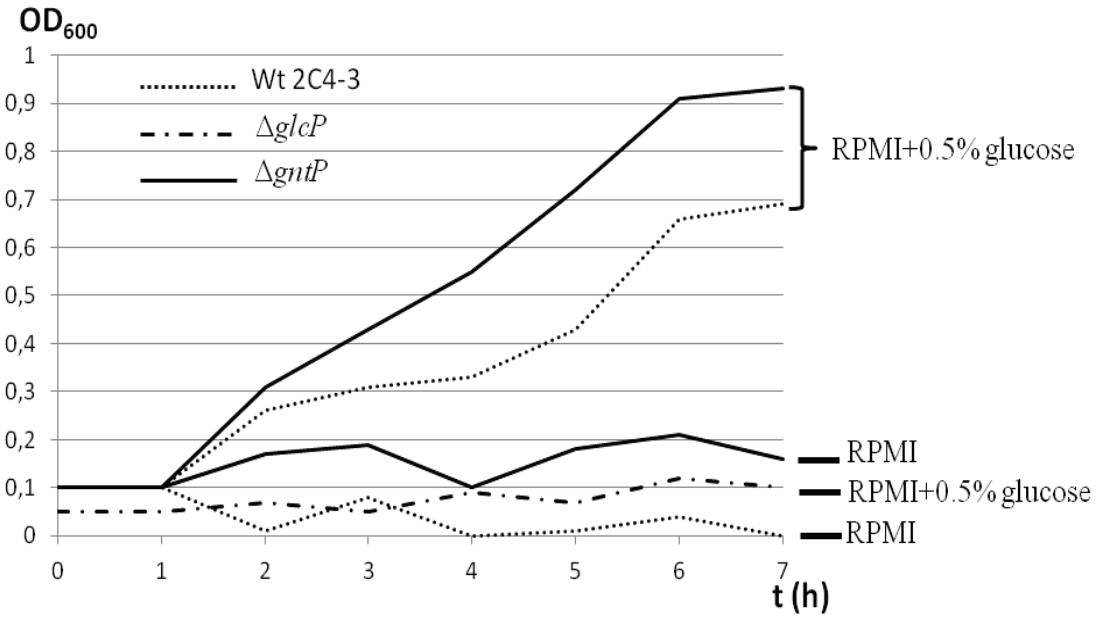


FIG 2 Growth of the *N. meningitidis* wild-type strain 2C4-3 (dotted lines) and the Δ*glcP* mutant derived from it (broken line) on RPMI medium in the absence and presence of 0.5% glucose. We also tested growth of the Δ*gntP* mutant in the presence and absence of 0.5% glucose (solid lines). In three independent experiments, the Δ*gntP* mutant grew significantly faster on glucose than the wild-type strain. For all strains, no significant growth occurred in the absence of glucose.

Fig. 3

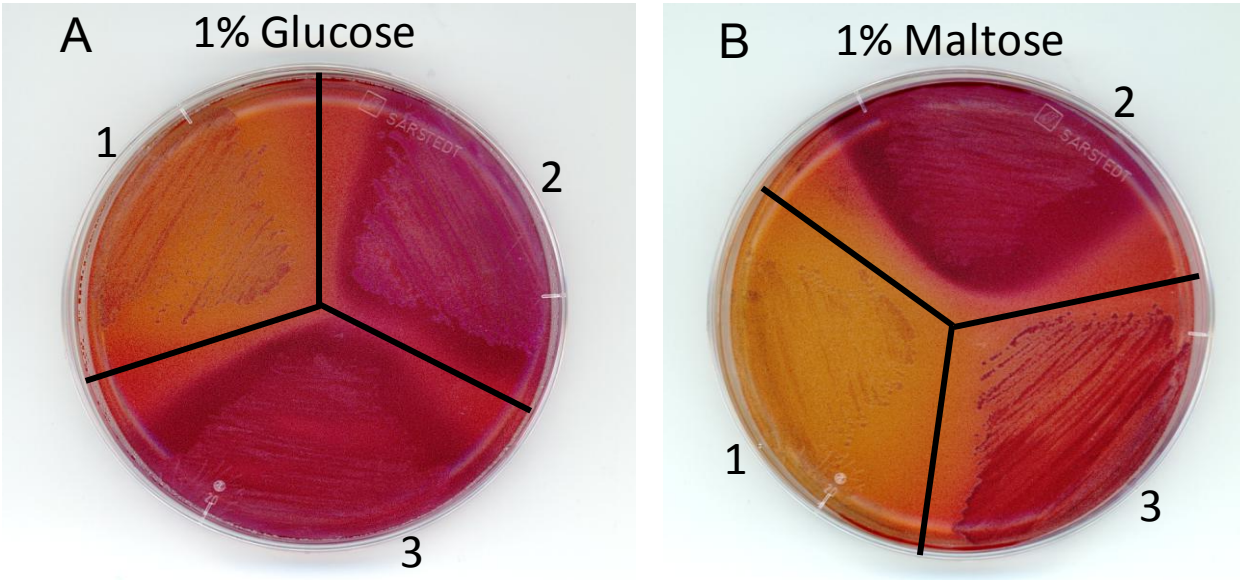


FIG 3 The meningococcal glucose permease GlcP and maltose permease MalY were able to complement the *E. coli* strains LJ140 and GSJ100 unable to utilize glucose and maltose, respectively. Fermentation assays were carried out on MacConkey agar plates containing 0.5 mM IPTG and either (A) 1% glucose or (B) 1% maltose. In (A) we tested strain LJ140 transformed with either empty plasmid pSU18c (sector 1) or plasmid pSU18c-glcP, in which the *N. meningitidis glcP* gene is expressed from the *spac* promoter (sector 3). The strong red coloration in sector 3 compared to sector 1 indicates that expression of the *N. meningitidis glcP* gene restores glucose utilization in strain LJ140. The *E. coli* strain NM522 was used as a positive control (sector 2). Similarly, in (B) we tested fermentation of maltose by strain GSJ100 transformed either with empty pSU18c (sector 1) or with pSU18c-malY (sector 3). Again, the red coloration in sector 3 indicates that expression of the *N. meningitidis malY* gene restores maltose utilization in strain GSJ100. Strain NM522 was used as a positive control (sector 2).

Fig. 4

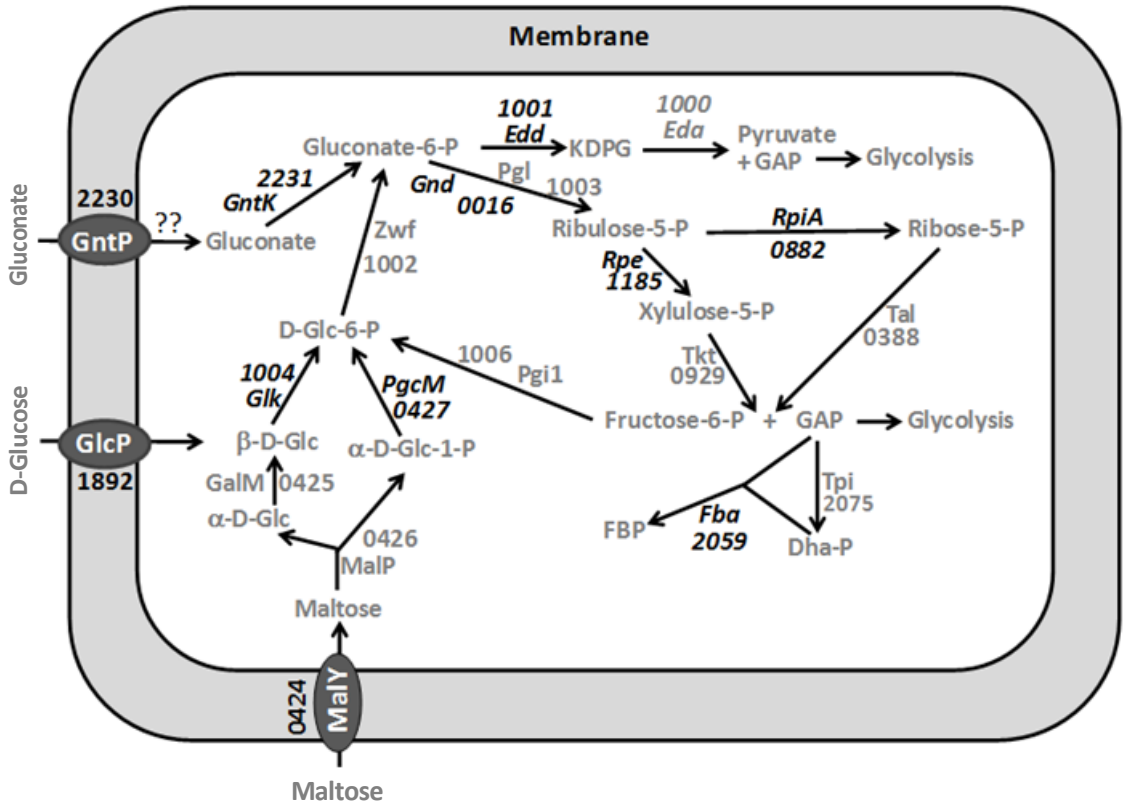


FIG 4 Proposed model of glucose and maltose catabolism via the ED pathway and PPP in *N. meningitidis*. The eight enzymes written in black and italics were purified and their activity has been dermined by carrying out appropriate spectrophotometric assays. The protein numbers of *N. meningitidis* strain 2C4-3 are normally preceded by "NMV_"; for better clarity we wrote the protein numbers without the prefix. The glucose and maltose transport activities of GlcP and MalY, respectively, were confirmed by growth studies with the corresponding deletion mutants and by complementation of appropriate *E. coli* mutants with the meningococcal *glcP* or *malY* gene. Surprisingly, although purified GntK was able to phosphorylate gluconate and expression of *N. meningitidis gntP* in a *B. subtilis* Δ*gntP* mutant restored growth on gluconate, *N. meningitidis* was not able to grow in minimal medium containing gluconate as the sole carbon source. The physiological function of GntP and GntK remains therefore unclear, as is indicated by the question marks.

Fig. 5

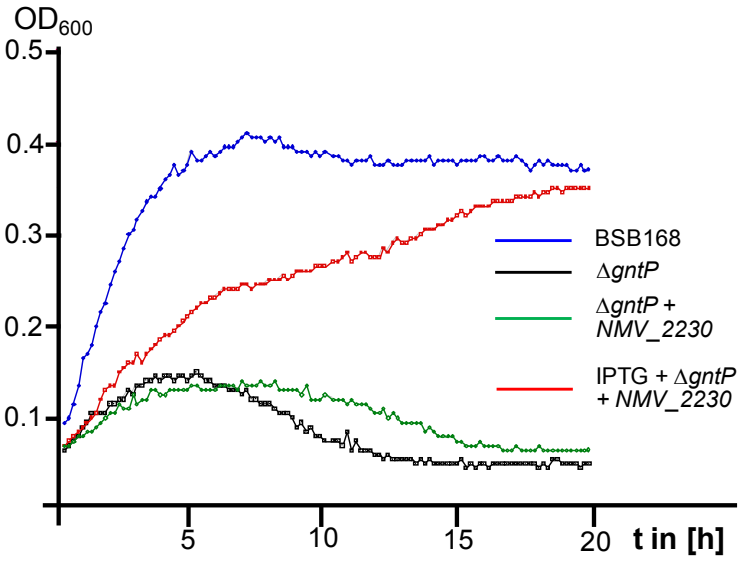


FIG 5 Growth of a *B. subtilis* Δ*gntP* mutant and a transformant complemented with the *N. meningitidis gntP* gene in minimal medium containing 0.5% gluconate or 0.5% gluconate plus 1 mM IPTG. The Δ*gntP* mutant as well as a transformant containing pIC634-gntP were not able to grow on gluconate as the sole carbon source. The transformant grew only when 1 mM IPTG was added. Under these conditions, the complemented strain grew about half as fast as the *B. subtilis* wild-type strain BSB168, but both strains reached a similar final OD_{600}.

Fig. 6

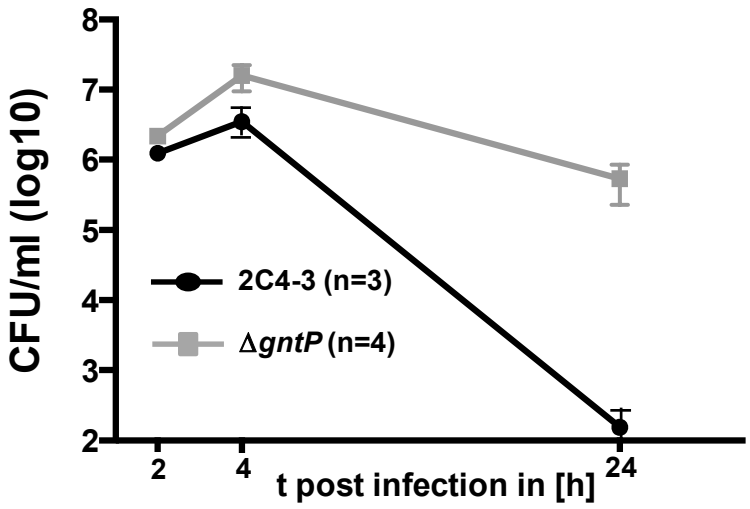
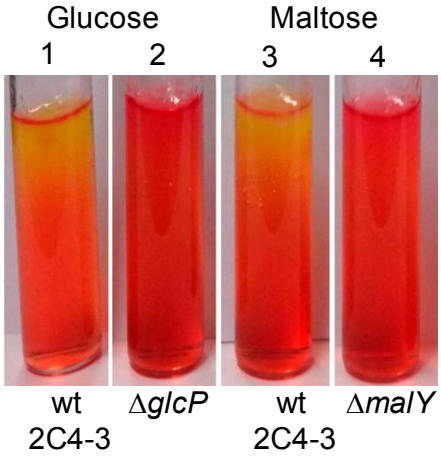


FIG 6 Enhanced virulence of the *N. meningitidis* Δ*gntP* mutant in mice. The virulence was evaluated by the levels of bacterial load in blood samples from BALB/c mice after intraperitoneal challenge with standardized inocula of 10^7 cfu. The Δ*gntP* mutant (filled grey squares) showed significantly higher virulence compared to the *N. meningitidis* wild-type strain 2C4-3 (filled black circles). Three mice were infected with the wild-type strain and four with the Δ*gntP* mutant. Survival of the wild-type strain 2C4-3 and the Δ*gntP* mutant was determined by bacterial counts from blood samples of infected mice 2, 6 and 24 h after intraperitoneal infection. Data were calculated from two independent experiments and mean values together with standard deviations (errors bars) are presented.

Supplementary Figure 1



Acidification assays with the *N. meningitidis* wild-type strain 2C4-3 and the Δ*glcP* mutant grown in CTA medium containing 0.5% glucose (tubes 1 and 2) or with the wild-type strain and Δ*malY* mutant grown in CTA medium containing 0.5% maltose. The yellow color formed in the upper part of tubes 1 and 3 indicates that the wild-type strain 2C4-3 is able to ferment both sugars, whereas the Δ*glcP* and Δ*malY* mutant no longer ferment glucose and maltose, respectively.

III. Discussion

N. meningitidis utilise très peu de sources de carbone, dont le glucose et le maltose. Ces sucres ne peuvent être transportés par le PTS vue l'absence de l'EIIB et l'EIIC. En revanche nous avons identifié une perméase à glucose (GlcP) et une perméase à maltose (MalY) qui assurent le cotransport de ces sucres avec des ions chez *N. meningitidis* 2C4-3. Chez la plupart des bactéries, le maltose est transporté soit par le PTS soit par un transporteur ABC (Mokhtari *et al*., 2013). Des homologues de MalY sont également présents chez *Caulobacter crescentus* (Lohmiller *et al*., 2008) et quelques lactobacilles tels que *Lactobacillus salivarius* (Mokhtari *et al*., 2013). Cette dernière, tout comme *N. meningitidis*, colonise le rhinopharynx. Il est possible que *N. meningitidis* ait acquis, par transfert horizontal, les gènes pour l'utilisation du maltose de *L. salivarius*. *N. gonorrhoeae*, l'espèce proche du meningocoque, ne possède pas *malY*.

Le métabolisme du glucose et du maltose chez *N. meningitidis* est entièrement assuré par la voie d'Entner-Douderoff (ED) et la voie des pentoses phosphates (PP) (Baart *et al*., 2010 ; Schoen *et al*., 2014). *N. meningitidis* est dépourvue de la fructose-6-P 1-kinase (Pfk). Par conséquent, elle ne peut utiliser que la partie basse de la voie d'Embden-Meyerhof-Parnas (EMP ou glycolyse), à partir du glycéraldéhyde-3-P, pour métaboliser les sucres. Cet intermédiaire glycolytique est produit quand des sources de carbone sont métabolisées via la voie des PP ou la voie d'ED. La Pfk est absente chez plusieurs α- et β-protéobactéries, les chlamydiées et les archées (Baart *et al*., 2010). À l'exception de la Pfk, des homologues de toutes les enzymes de la voie d'ED, des PP et de la glycolyse sont présents chez *N. meningitidis* (Jyssum *et al*., 1961 ; Jyssum, 1962a, b, c, Holten, 1974, Holten 1975). La plupart de ces enzymes ont été purifiées et leurs activités ont été confirmées par des tests d'activité enzymatique *in vitro*.

En faisant des BLASTS avec des séquences de transporteurs et des enzymes cataboliques, nous avons également identifié une perméase putative à gluconate (GntP) et une gluconate kinase (GntK) chez *N. meningitidis* 2C4-3. Les gènes codant ces deux protéines forment probablement un opéron. La perméase représente un pourcentage d'identité de 58% avec la perméase à gluconate de *B. subtilis*. En revanche, aucune croissance n'a été observée pour la souche sauvage 2C4-3 en présence du gluconate comme seule source de carbone. Ces résultats remettent en question l'implication de cette perméase dans le transport du gluconate. Cependant, la perméase GntP de *N. meningitidis* permet le transport du gluconate chez un mutant de *B. subtilis* dépourvu de sa perméase à gluconate. De plus, une très forte activité

gluconate kinase a été observée chez la protéine purifiée GntK de *N. meningitidis*. Les résultats obtenus suggèrent que la présence de la perméase GntP pourrait nuire au méningocoque et que ce dernier l'a inactivé pour une raison inconnue. Effectivement, la délétion du gène *gntP* codant cette perméase a permis une meilleure croissance du mutant Δ*gntP* sur glucose et une meilleure survie dans le sang des souris infectées par ce mutant, par comparaison à la souche sauvage. La perméase GntP serait alors impliquée dans la virulence de *N. meningitidis*. De plus, les nombreux essais de complémentation du mutant Δ*gntP* avec le gène *gntP*, sous le contrôle d'un promoteur permettant son expression constitutive, ont été infructueux. Néanmoins, en absence d'IPTG nous avons obtenu des mutants Δ*gntP* complémentés avec le gène *gntP*. La fonction réelle de la perméase GntP ainsi que les mécanismes par lesquels cette dernière contribue à la virulence de *N. meningitidis* restent inconnus et méritent d'être étudiés de manière plus développée.

CONCLUSION

ET

PERSPECTIVES

Le lien entre les protéines du PTS et la virulence des bactéries pathogènes a fait l'objet de plusieurs études. Dans ce travail, nous avons confirmé l'implication de la protéine HPr, un composant général du PTS, dans la virulence de *N. meningitidis*, principal agent de la méningite bactérienne. Nous avons montré que l'HPr interagit avec le régulateur transcriptionnel CrgA. CrgA régule l'expression des gènes codant des protéines de pili et de la capsule au cours de l'adhérence du méningocoque aux cellules épithéliales.

Le PTS de *N. meningitidis* est incomplet et ne permet pas le transport des sucres chez cette bactérie. Néanmoins, nous avons montré que la cascade de phosphorylation des protéines PTS est fonctionnelle et que la protéine HPr est phosphorylée sur son histidine 15 par l'EI et sur sa sérine 46 par l'HprK/P. Sous ses deux formes phosphorylées, la protéine HPr intervient dans plusieurs fonctions régulatrices dans la cellule. Il serait donc important d'étudier l'impact de ces phosphorylations sur la virulence de *N. meningitidis*. Pour cela, on envisage de complémenter le mutant Δ*ptsH* avec différents allèles de *ptsH* codant des protéines qui miment : la P-Ser-HPr (sérine 46 mutée en aspartate), la P~His-HPr (histidine 15 mutée en aspartate) et les formes non phosphorylées de l'HPr (histidine 15 et sérine 46 mutées en alanine). Les mutants complémentés feront l'objet de plusieurs éxpériences *in vivo* et *in vitro* afin d'étudier les effets de l'état de phosphorylation de l'HPr sur la survie du méningocoque chez la souris, la synthèse de la capsule, l'apoptose et l'adhérence aux cellules épithéliales.

La phosphorylation de l'HPr sur la sérine 46 augmente son interaction avec CrgA. Les conséquences de cette phosphorylation sur l'affinité de CrgA à sa propre région promotrice ainsi qu'aux régions promotrices des gènes *pilC1*, *pilE* et *sia* seront également étudiées. L'effet de la phosphorylation de l'histidine 15 sur l'interaction HPr/CrgA sera plus difficile à étudier à cause de l'hydrolyse rapide de la P~His-HPr. Cependant, on essayera de faire des tests de titration calorimetrique isotherme pour remédier à ce problème.

Au cours de ce travail, nous avons également identifié deux perméases chez *N. meningitidis*, une perméase pour le transport du glucose (GlcP) et une perméase pour le transport du maltose (MalY). Etant dépourvue de la fructose-6-P 1-kinase, *N. meningitidis* ne métabolise pas ces sucres via la voie d'Embden-Meyerhof-Parnas (EMP). Ces deux sucres sont métabolisés via la voie d'Entner-Doudoroff (ED) et la voie des pentoses phosphates (PP). Les principales enzymes de ces voies métaboliques ont été purifiées et leurs activitées ont été confirmées par des tests enzymatiques *in vitro*.

Une perméase putative à gluconate ainsi qu'une gluconate kinase qui catalyse la première étape catabolique du gluconate, ont été également identifiées au cours de cette étude.

L'implication de cette perméase dans le transport du gluconate chez *N. meningitidis* n'a pas pu être démontrée dans les conditions expérimentales testées. Néanmoins, le rôle de cette perméase dans le transport du gluconate a été confirmé chez un mutant de *B. subtilis* dépourvu de sa perméase à gluconate. L'activité de la gluconate kinase a été également confirmée par des tests enzymatiques *in vitro*.

La perméase à gluconate de *N. meningitidis* semble aussi avoir un lien avec la virulence de cette bactérie. La délétion du gène *gntP* codant cette perméase induit une meilleure croissance sur glucose et une bonne survie du mutant chez la souris, par rapport à la souche sauvage. La complémentation du mutant Δ*gntP* de *N. meningitidis* a été également réalisée, avec un gène *gntP* sous le contrôle transcriptionnel d'un promoteur permettant son expression inductible par l'IPTG. Aucun mutant complémenté permettant l'expression constitutive du gène *gntP* n'a été obtenu. Il est donc intéressant d'étudier l'effet de l'IPTG sur la survie des mutants complémentés. Les conséquences de la délétion du gène *gntP* sur la production de la capsule, l'apoptose et l'adhérence aux cellules épithéliales font également partie des perspectives pour ce travail.

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Annexe

Dans cette partie, quelques articles dont je suis co-auteur sont présentés :

- **Joyet P, Derkaoui M, Bouraoui H, Deutscher J. 2015.** PTS-Mediated Regulation of the Transcription Activator MtlR from Different Species: Surprising Differences despite Strong Sequence Conservation. J. Mol. Microbiol. Biotechnol. 25 : 94-105.

- **Deutscher J, Aké FM, Derkaoui M, Zébré AC, Cao TN, Bouraoui H, Kentache T, Mokhtari A, Milohanic E, Joyet P. 2014.** The bacterial phosphoenolpyruvate:carbohydrate phosphotransferase system: regulation by protein phosphorylation and phosphorylation-dependent protein-protein interactions. Microbiol. Mol. Biol. Rev. 78: 231-256.

Research Article

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PTS-Mediated Regulation of the Transcription Activator MtlR from Different Species: Surprising Differences despite Strong Sequence Conservation

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Key Words

Mannitol · Phosphotransferase system · Phosphotransferase system regulation domain · Transcription activator · Protein phosphorylation

Abstract

The hexitol D-mannitol is transported by many bacteria via a phosphoenolpyruvate (PEP):carbohydrate phosphotransferase system (PTS). In most Firmicutes, the transcription activator MtlR controls the expression of the genes encoding the D-mannitol-specific PTS components and D-mannitol-1-P dehydrogenase. MtlR contains an N-terminal helix-turn-helix motif followed by an Mga-like domain, two PTS regulation domains (PRDs), an EIIB^{Gat}- and an EIIA^{Mtl}-like domain. The four regulatory domains are the target of phosphorylation by PTS components. Despite strong sequence conservation, the mechanisms controlling the activity of MtlR from *Lactobacillus casei*, *Bacillus subtilis* and *Geobacillus stearothermophilus* are quite different. Owing to the presence of a tyrosine in place of the second conserved histidine (His) in PRD2, *L. casei* MtlR is not phosphorylated by Enzyme I (EI) and HPr. When the corresponding His in PRD2 of MtlR from *B. subtilis* and *G. stearothermophilus* was replaced with alanine, the transcription regulator was no longer phosphorylated and remained inactive. Surprisingly, *L. casei* MtlR functions without phosphorylation in PRD2 because in a *ptsI* (EI) mutant MtlR is constitutively active. EI inactivation prevents not only phosphorylation of HPr, but also of the PTS^{Mtl} components, which inactivate MtlR by phosphorylating its EIIB^{Gat}- or EIIA^{Mtl}-like domain. This explains the constitutive phenotype of the *ptsI* mutant. The absence of EIIB^{Mtl}-mediated phosphorylation leads to induction of the *L. casei mtl* operon. This mechanism resembles *mtlARFD* induction in *G. stearothermophilus*, but differs from EIIA^{Mtl}-mediated induction in *B. subtilis*. In contrast to *B. subtilis* MtlR, *L. casei* MtlR activation does not require sequestration to the membrane via the unphosphorylated EIIB^{Mtl} domain.

Introduction

D-Mannitol is the most abundant hexitol in nature and is produced by numerous plants, yeasts, algae, lichen and fungi. It is therefore not surprising that many bacteria utilize it as a carbon and energy source. Bacteria take up D-mannitol by at least three different mechanisms: ion-driven cotransport (MtlT), ATP-binding cassette transport (MtlEFGK), or transport via the phosphoenolpyru-

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vate (PEP):glycose phosphotransferase system (PTS^{Mtl}) [Deutscher et al., 2013]. The latter D-mannitol transport system is used by a large number of bacteria, including Firmicutes [Behrens et al., 2001; Fischer et al., 1991; Honeyman and Curtiss III, 2000; Reiche et al., 1988; Watanabe et al., 2003] and Enterobacteriaceae [Davis et al., 1988; Kumar et al., 2011; Otte and Lengeler, 2001]. Several Actinobacteria, such as *Leifsonia xyli* and *Arthrobacter aurescens*, also contain genes encoding a presumed mannitol PTS and D-mannitol-1-P dehydrogenase [Deutscher et al., 2013]. The PTS is usually composed of five proteins or protein domains, four of which form a phosphorylation cascade [Deutscher et al., 2006]. Enzyme I (EI) autophosphorylates at the expense of PEP [Alpert et al., 1985] and subsequently transfers the phosphoryl group to histidine (His)-15 in HPr. EI and HPr are unique in most organisms and are therefore called the general PTS components. In contrast, P~His-HPr phosphorylates one of usually several carbohydrate-specific EIIA components present in a bacterium, which subsequently transfer the phosphoryl group to their corresponding EIIB protein. The PTS proteins are phosphorylated at His (EI, HPr, EIIA and some EIIB) or Cys residues (most EIIB). In the last step, the P~EIIBs phosphorylate the carbohydrate bound to the cognate EIIC (in some cases to EIIC and EIID components). The crystal structure of the N,N′-diacetylchitobiose-specific *Escherichia coli* EIIC component [Cao et al., 2011] suggests that phosphorylation of the carbohydrate bound to the transmembrane-spanning EIIC (or EIIC and EIID) lowers its affinity for the PTS permease, thus favoring its release into the cytoplasm. PTS substrates are usually phosphorylated at the C-6 position. However, with D-mannitol being a symmetric molecule with a 2-fold symmetry axis, the 1 and 6 positions are indistinguishable and the hexitol phosphorylated during its transport by the PTS is normally called D-mannitol-1-P and not D-mannitol-6-P.

The D-mannitol-specific PTS transporter MtlA of *E. coli* has been extensively studied [Jacobson et al., 1979; Pas et al., 1988]. It was the first PTS permease for which the gene was identified and sequenced [Lee and Saier, 1983]. The operon encoding the PTS component(s) usually also contains a gene encoding a D-mannitol-1-P dehydrogenase [Deutscher et al., 1994; Fischer et al., 1991; Otte and Lengeler, 2001], which converts D-mannitol-1-P into the glycolytic intermediate fructose-6-P. MtlA of Enterobacteriaceae is usually composed of the three D-mannitol-specific components $EIIC^{Mtl}$, $EIIB^{Mtl}$ and $EIIA^{Mtl}$, fused to each other in the indicated order. In contrast, in Firmicutes $EIIA^{Mtl}$ (previously also called Enzyme III^{Mtl}) is a distinct protein encoded by the *mtlF* gene [Fischer et al., 1991; Reiche et al., 1988; Reizer et al., 1992] and the *mtl* operon usually has the gene order *mtlARFD* [Deutscher et al., 2013] with *mtlR* encoding a regulator (fig. 1a). Interestingly, in the actinobacterium *L. xyli*, the mannitol-specific genes *mtlAFD* (without the regulator gene *mtlR*) are preceded by the genes *ptsHI* for the general PTS proteins HPr and EI [Deutscher et al., 2013]. This gene organization might indicate that D-mannitol is an important carbon source for this plant pathogen.

Expression of the *mtl* operon in Firmicutes and many Actinobacteria requires the transcription activator MtlR, the gene of which is usually part of the *mtl* operon. *Bacillus subtilis* and some Actinobacteria, such as *L. xyli* and *Clavibacter michiganensis*, are an exception to this rule. In the two Actinobacteria, *mtlR* is located just upstream from the *ptsHI*,*mtlAFD* operon or separated by a couple of genes and oriented in the opposite direction. In *B. subtilis*, *mtlR* is located even 14 kb downstream from the *mtl* operon [Watanabe et al., 2003] (fig. 1a). MtlR binds to several small regions within an approximate 50-bp region located upstream from the *mtlA* promoter [Henstra et al., 1999; Heravi et al., 2011]. In *B. subtilis*, and presumably also Actinobacteria, MtlR controls its own expression [Heravi et al., 2011; Watanabe et al., 2003]. MtlR is composed of an N-terminal DNA-binding domain followed by a domain resembling the N-terminal part of Mga and four regulatory domains (fig. 1b) [Joyet et al., 2013]. The first two regulatory domains are PTS regulation domains (PRDs), each usually containing two conserved histidines, which are potential phosphorylation sites for PTS components [Deutscher et al., 2006]. The two C-terminal regulatory domains resemble the PTS components $EIIB^{Gat}$ and $EIIA^{Mtl}$. MtlR proteins can therefore contain up to six potential PTS phosphorylation sites. PRD-containing transcription regulators are usually controlled by two PTS-mediated phosphorylations exerting antagonistic effects on their activity. MtlR of *Geobacillus stearothermophilus* (previously called *Bacillus stearothermophilus*) and *B. subtilis* need to be activated by phosphorylation by PEP, EI and HPr in their PRD2 (fig. 1b), which is prevented when either one of the two conserved His of this domain is replaced with a nonphosphorylatable alanine [Henstra et al., 2000; Joyet et al., 2010]. The absence of P~His-HPr-mediated phosphorylation serves as a carbon catabolite repression mechanism. When an efficiently metabolizabled PTS substrate, such as glucose, is present in the growth medium, HPr is barely phosphorylated at His-15 [Monedero et al., 2001; Singh et al., 2008]. As the phosphoryl group transfer between PTS components

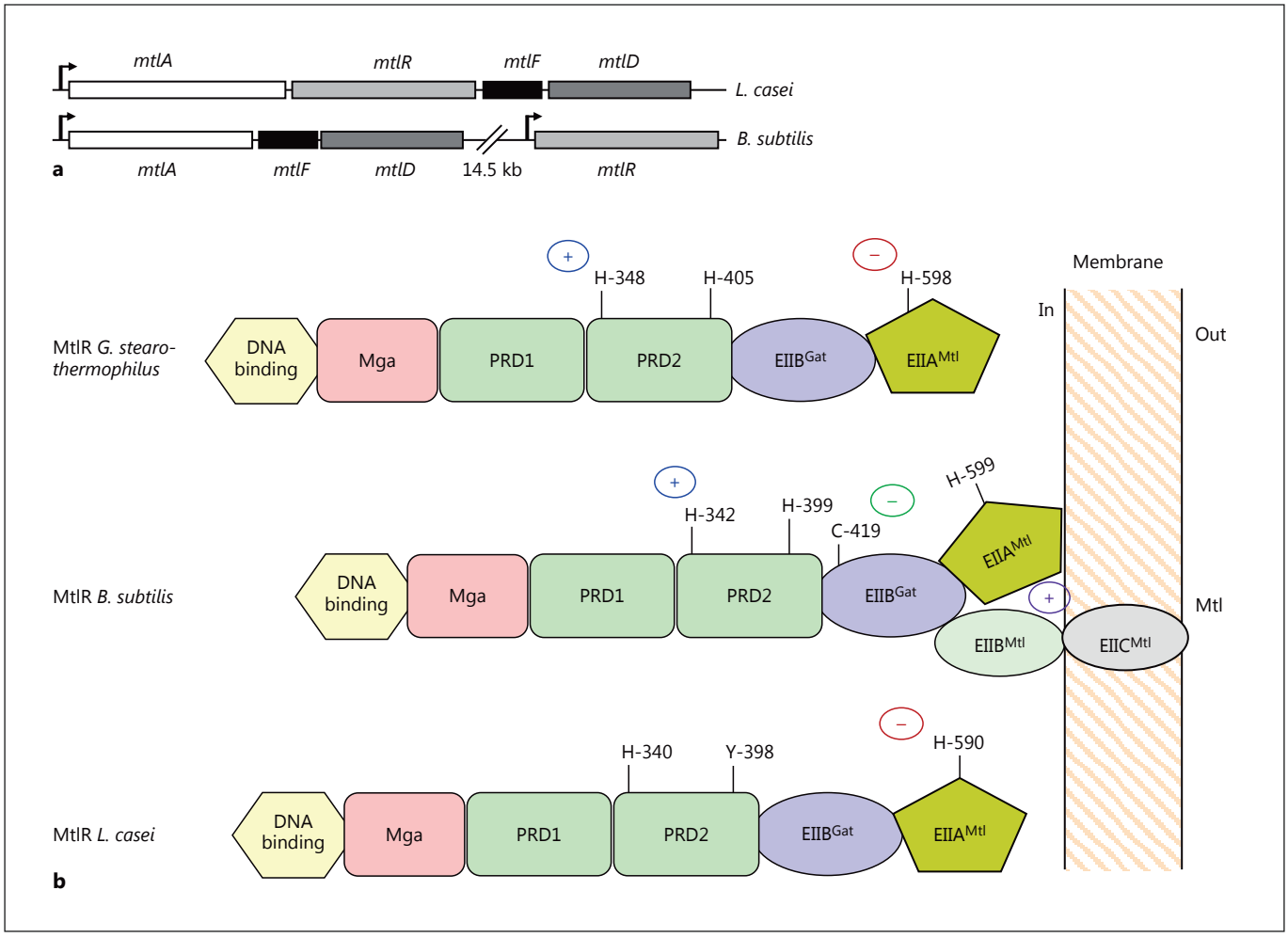

Fig. 1. a Presentation of the organization of the *mtl* operon in *L. casei* and *B. subtilis*. In the latter organism, the *mtlR* gene is not part of the *mtl* operon, but is located 14.5 kb downstream from *mtlD*. *G. stearothermophilus* has the same operon organization as *L. casei*, with the only difference that the $EIIB^{Mtl}$ domain at the C-terminus of MtlA is not duplicated. There is also only one $EIIB^{Mtl}$ domain fused to MtlA of *B. subtilis*. **b** Comparison of the domain organization and the known regulatory sites in MtlR from *G. stearothermophilus* and *B. subtilis* to those in *L. casei*. Phosphorylation of MtlR from *G. stearothermophilus* and *B. subtilis* at the first conserved His in PRD2 (blue-colored '+' at histidines 348 and 342, respectively) by PEP, EI and HPr stimulates their activity. In contrast, phosphorylation of *G. stearothermophilus* MtlR in the $EIIA^{Mtl}$-like domain by $P\sim EIIB^{Mtl}$ (red-colored '–' at His 598) and of *B. subtilis* MtlR in the $EIIB^{Gat}$-like domain by $P\sim EIIA^{Mtl}$ (green-colored '–' at cysteine 419) inhibits their activity. In addition, *B. subtilis* MtlR needs to interact with the unphosphorylated $EIIB^{Mtl}$ domain of MtlA in order to be active (the violet-colored '+'). In contrast to MtlR from *G. stearothermophilus* and *B. subtilis*, *L. casei* MtlR is not phosphorylated by PEP, EI or HPr, but is active without being phosphorylated in PRD2 (EI and HPr independent). Similar to *G. stearothermophilus* MtlR, phosphorylation in the $EIIA^{Mtl}$-like domain by $P\sim EIIB^{Mtl}$ inhibits the activity of MtlR from *L. casei* (red-colored '–' at His 590).

and their target proteins is reversible [Deutscher and Sauerwald, 1986], the presence of glucose also leads to the dephosphorylation of PRD2 in MtlR, and hence to its inactivation [Joyet et al., 2010] (fig. 1b). Phosphorylation of the $EIIA^{Mtl}$-like domain of *G. stearothermophilus* MtlR is catalyzed by EI, HPr, $EIIA^{Mtl}$ and the $EIIB^{Mtl}$ domain of MtlA [Henstra et al., 2000] (fig. 1b). It leads to the inhibition of MtlR activity and dephosphorylation of the $EIIA^{Mtl}$-like domain is used as an induction mechanism of the *mtl* operon. In the presence of D-mannitol, the carbohydrate-specific PTS components, and consequently also the $EIIA^{Mtl}$-like domain of MtlR, are mostly dephosphorylated and MtlR is active. Replacement of the phosphorylatable His in the $EIIA^{Mtl}$-like domain of MtlR

therefore leads to constitutive activity of the transcription activator. Surprisingly, although MtlR from *G. stearothermophilus* and *B. subtilis* exhibit significant sequence similarity, MtlR of the latter organism was found to require only EI, HPr and EIIA^{Mtl} for the inhibiting phosphorylation, which occurs at the conserved Cys in the EIIB^{Gat}-like domain [Joyet et al., 2010] (fig. 1b). In addition, MtlR from *B. subtilis* but not *G. stearothermophilus* needs to be sequestered to the membrane via the unphosphorylated EIIB^{Mtl} domain of MtlA in order to be active [Bouraoui et al., 2013; Joyet et al., 2013]. The significant differences in regulation of MtlR in two bacteria of the family Bacillaceae prompted us to carry out a BLAST search in order to detect MtlRs in which important regulatory sites might not be conserved. Indeed, the lactic acid bacterium *Lactobacillus casei* was found to possess an MtlR in which four of the six potentially phosphorylatable amino acids were not conserved. For example, while the first His in PRD2 of *L. casei* MtlR is present, the second is replaced with a tyrosine (fig. 1b). This substitution was expected to affect MtlR function, because replacement of either one of the two histidines in PRD2 of *G. stearothermophilus* and *B. subtilis* MtlR caused a loss of activity. We therefore decided to study the regulation of the *L. casei* MtlR protein in detail.

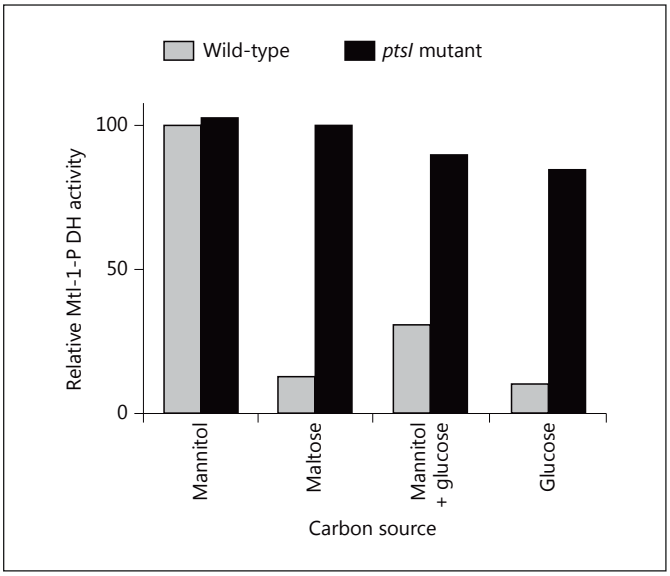

Fig. 2. Mannitol-1-P dehydrogenase (DH) activity measured in crude extracts of the *L. casei* BL23 wild-type strain and a Δ*ptsI* mutant grown on various carbon sources (mannitol, maltose, mannitol plus glucose, glucose). The mannitol-1-P dehydrogenase activity measured for the wild-type strain grown on mannitol was set to 100. The results from three independent experiments are presented. The standard deviations were always within ±12%.

Results

Activity of L. casei MtlR Does Not Depend on a Functional PTS

When carrying out a BLAST search with the MtlR sequences of *B. subtilis* and *G. stearothermophilus* at UniProt (http://www.uniprot.org/blast/uniprot/) we observed that in the four regulatory domains of MtlR of various *L. casei* strains only two of the six phosphorylatable amino acids are conserved. First, the two phosphorylatable His in PRD1 are replaced with Leu and Glu, respectively (fig. 1b). These amino acid exchanges are expected to have no drastic effects on the activity of the regulator, because replacement of these two His with Ala or Asp in MtlR of *B. subtilis* [Joyet et al., 2010] or *G. stearothermophilus* [Henstra et al., 1999] had no or only little effect on their transcription activator function. Secondly, the phosphorylatable Cys in the EIIB^{Gat}-like domain, which in *B. subtilis* becomes phosphorylated by P~EIIA^{Mtl}, leading to inactivation of MtlR [Bouraoui et al., 2013], is replaced with a Thr in *L. casei* MtlR. Third and most importantly, while the first phosphorylatable His in PRD2 is conserved in MtlR from *L. casei*, the second is replaced with a Tyr (fig. 1b). In MtlR from *B. subtilis* [Joyet et al., 2010] and *G. stearothermophilus* [Henstra et al., 2000] replacement of either one of the two conserved His in PRD2 with Ala prevented the phosphorylation by PEP, EI and HPr, and therefore strongly inhibited the function of the transcription activator. MtlR inhibition was also observed when the second His in PRD2 (His-399) of *B. subtilis* MtlR was replaced with Asp. We therefore decided to test how deletion of *ptsI*, which prevents both phosphorylation by P~His-HPr and by either P~EIIA^{Mtl} or P~EIIB^{Mtl}, would affect the activity of MtlR in *L. casei* strain BL23.

The *mtl* operon of *L. casei* BL23 has the gene order *mtlARFD*, which is typical of Firmicutes and identical to that in *G. stearothermophilus*, but different from that in *B. subtilis*, where the *mtlR* gene is located about 14 kb downstream from *mtlAFD* (fig. 1a). The last gene encodes mannitol-1-P dehydrogenase (MtlD) and we therefore determined the activity of this enzyme in crude extracts prepared from either the *L. casei* wild-type strain BL23 or the *ptsI* mutant derived from it [Viana et al., 2000]. In the wild-type strain, mannitol-1-P dehydrogenase activity was induced during growth on mannitol when compared to growth on the non-PTS sugar maltose [Monedero et al., 2008] and repressed about 3-fold in the presence of

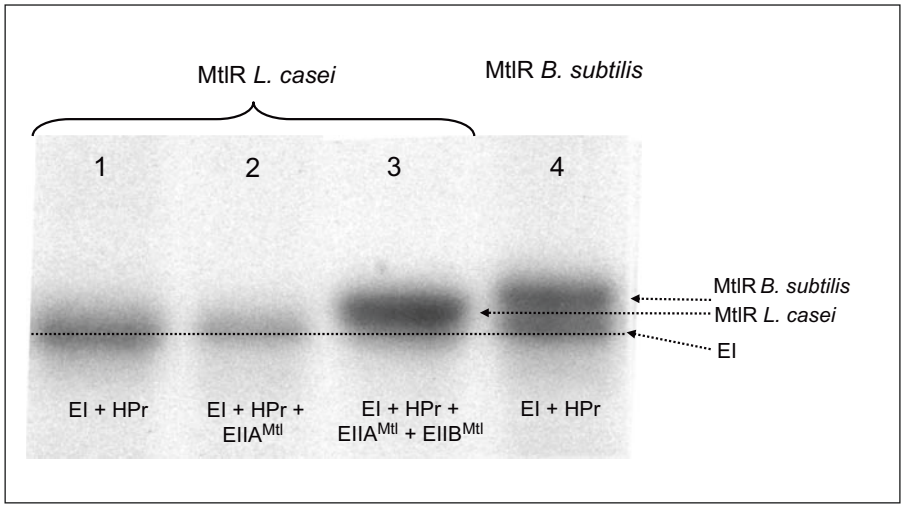

Fig. 3. Autoradiogram of a 1% SDS/10% polyacrylamide gel on which MtlR from *L. casei* and *B. subtilis* had been separated after phosphorylation with [^{32}P]PEP and various PTS proteins. Only the upper part of the gel is shown. The migration positions of EI and MtlR from *L. casei* and *B. subtilis* are indicated by arrows. For lanes 1–3, MtlR from *L. casei* was incubated with: EI and HPr (lane 1); EI, HPr and EIIA^{Mtl} (lane 2), and EI, HPr, EIIA^{Mtl} and the two EIIB^{Mtl} domains from the C-terminus of MtlA (lane 3). For lane 4, MtlR from *B. subtilis* was incubated with EI and HPr. For all experiments, EI from *B. subtilis* was used, whereas HPr, EIIA^{Mtl} and EIIB^{Mtl} were from *L. casei*, except for lane 4, where HPr from *B. subtilis* was used.

mannitol and glucose (fig. 2). The lowest activity was observed when the cells were grown in the presence of only glucose. Surprisingly, while deletion of *ptsI* in *B. subtilis* caused a loss of MtlR activity, MtlR in the *L. casei ptsI* mutant was constitutively active, because mannitol-1-P dehydrogenase activity was similar in mannitol- and maltose-grown *ptsI* mutant cells and glucose had almost completely lost its repressing effect (fig. 2). This result indicates that *L. casei* MtlR does not need to be activated by P~His-HPr-mediated phosphorylation. In addition, the observed constitutive expression of the *mtl* operon suggested that the activity of MtlR is inhibited via phosphorylation by the mannitol-specific PTS components. This phosphorylation might be catalyzed either by P~EIIA^{Mtl} in the EIIB^{Gat}-like domain or by P~EIIB^{Mtl} in the EIIA^{Mtl}-like domain. Both mannitol-specific PTS components need EI and HPr for their own phosphorylation.

MtlR of L. casei Is Not Phosphorylated by P~His-HPr

In MtlR from *G. stearothermophilus* [Henstra et al., 2000] and *B. subtilis* [Joyet et al., 2010] replacement of the second conserved His in PRD2 with an Ala prevented the phosphorylation of the first His in this domain. For this reason, we tested whether the 'natural' replacement of the equivalent His (His-398) in *L. casei* MtlR with a Tyr (fig. 1b) would have a similar effect. Indeed, despite the presence of the first conserved His in PRD2 (His-340), no phosphorylation of *L. casei* wild-type MtlR was detected when it was incubated together with [^{32}P]PEP, EI and *L. casei* HPr (fig. 3, lane 1). Under the same conditions, wild-type MtlR from *B. subtilis* becomes phosphorylated with *B. subtilis* EI and HPr (fig. 3, lane 4).

The P~His-HPr-Independent MtlR Activity Is Not due to the 'Natural' His288Glu or His398Tyr Substitutions

We suspected that the P~His-HPr-independent activity of *L. casei* MtlR might be caused by mutations affecting one of the usually conserved histidines. This included the potentially phosphomimetic natural replacement of His-288 in PRD1 with Glu and the replacement of His-398 with Tyr. In order to test whether this assumption was correct, MtlR alleles were constructed in which Glu-288 and Tyr-398 were replaced with His, Asp and Ala, and the effect of these replacements on the expression from the *L. casei mtlA* promoter (P*mtlA*) fused to the *E. coli lacZ* gene was measured. To carry out the necessary assays, the *L. casei* P*mtlA-lacZ* fusion was integrated into the *amyE* gene of either the *B. subtilis* wild-type strain, the Δ*ptsI* or the Δ*mtlAFD* mutant. In all three strains, the P*mtlA-lacZ* fusion was not expressed, independently of whether the strains were grown in the presence or absence of mannitol. This result indicates that *B. subtilis* MtlR does not recognize the operator of the *L. casei mtlARFD* operon. Various *L. casei mtlR* alleles preceded by an IPTG-inducible promoter and their natural Shine Dalgarno box were integrated into the *thrC* site of the three *B. subtilis* strains (see Materials and Methods). Integration of the wild-type *mtlR* allele in either one of the three *B. subtilis* mutants caused constitutive expression of the *L. casei* P*mtl-lacZ* fusion (fig. 4, results not shown for the *ΔmtlAFD* mutant). Constitutive MtlR activity was expected for the Δ*ptsI* and Δ*mtlAFD* mutants, because, as will be shown in the following chapter, MtlR needs EI and HPr as well as EIIA^{Mtl} and EIIB^{Mtl} to become phosphorylated at its in-

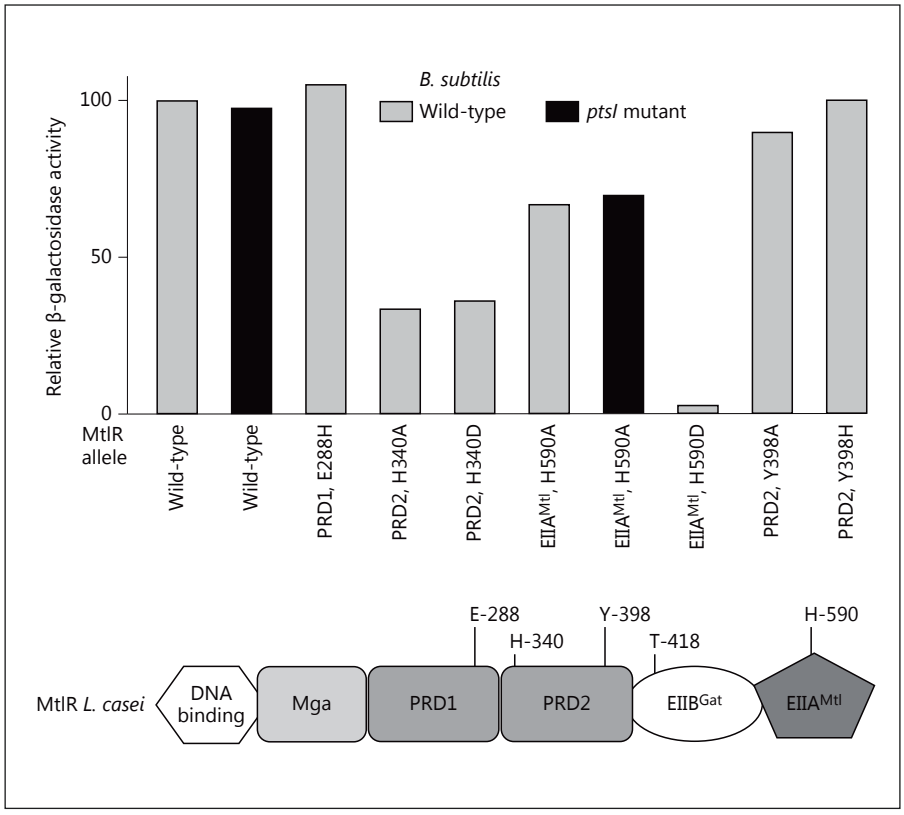

Fig. 4. β-Galactosidase activity measured in crude extracts of a *B. subtilis* wild-type strain and a Δ*ptsI* mutant, which all contained the *lacZ* reporter gene fused to the *L. casei* mannitol promoter (inserted at *amyE*) as well as one of the various *L. casei mtlR* alleles expressed from the *spac* promoter (inserted at *thrC*). The β-galactosidase activity measured for the wild-type strain, which produces *L. casei* wild-type MtlR, was set to 100. The results from three independent experiments are presented. The standard deviations were always within ±14%.

activating site. The deletion of either one of these PTS components therefore leads to constitutive MtlR activity. However, constitutive expression of the *L. casei* P*mtl-lacZ* fusion was not expected for the wild-type strain. A possible explanation is that *L. casei* MtlR is not or only poorly phosphorylated by the P~EIIA^{Mtl} and P~EIIB^{Mtl} components of *B. subtilis*. Constitutive expression of the *lacZ* fusion in the three genetic backgrounds was also observed for the *mtlR* Glu288His and Tyr398His alleles, suggesting that neither of the two 'natural' His replacements are responsible for the constitutive *L. casei* MtlR activity (fig. 4). Their replacements with Ala or Asp had similar effects on the expression of the *L. casei* P*mtl-lacZ* fusion (data not shown). We therefore tested whether mutation of His-340 would have an effect on MtlR activity. Indeed, replacement of His-340 in PRD2 with Ala or Asp led to a more than 2-fold reduction of MtlR activity, indicating that this His, although not phosphorylated by PEP, EI and HPr in *L. casei* MtlR, is important for MtlR activation. It should nevertheless be noted that in MtlR from *G. stearothermophilus* and *B. subtilis* the corresponding Ala replacement caused a complete loss of MtlR activity, whereas the Asp replacement induced fully constitutive MtlR function [Henstra et al., 2000; Joyet et al., 2010].

MtlR of L. casei Is Phosphorylated by the P~EIIB^{Mtl} Domains

In MtlR from *L. casei*, the phosphorylatable Cys in the EIIB^{Gat}-like domain is replaced with a Thr, whereas the phosphorylatable His in the C-terminal EIIA^{Mtl}-like domain is conserved (fig. 1b). It was therefore likely that the induction mechanism of the *mtl* operon of *L. casei* would follow the one described for *G. stearothermophilus* (P~EIIB^{Mtl}-mediated phosphorylation of the EIIA^{Mtl}-like domain of MtlR) [Henstra et al., 2000] and not the one reported for *B. subtilis* (P~EIIA^{Mtl}-mediated phosphorylation of the EIIB^{Gat}-like domain of MtlR) [Joyet et al., 2010]. To test whether this assumption was correct, we carried out MtlR phosphorylation experiments in the presence of the EII components of *L. casei*. It should be noted that *L. casei* MtlA contains two EIIB^{Mtl} domains fused in tandem to its C-terminus [Mazé et al., 2010]. We therefore cloned a DNA fragment coding for both EIIB^{Mtl} domains into the His-tag expression vector and purified the corresponding pro-

tein composed of the two *L. casei* EIIB^{Mtl} domains. No phosphorylation of *L. casei* MtlR could be observed in the presence of [^{32}P]PEP, *B. subtilis* EI, *L. casei* HPr and EIIA^{Mtl} (fig. 3, lane 2). However, when in addition the duplicated EIIB^{Mtl} protein was included in the phosphorylation assay, a radioactive band appeared right above the migration position of EI and just below MtlR from *B. subtilis* (fig. 3, lane 3). These results suggest that, like in *G. stearothermophilus*, the conserved His in the EIIA^{Mtl}-like domain of MtlR is phosphorylated. The presence of mannitol in the growth medium leads to induction of the *L. casei mtl* operon by causing the dephosphorylation of the EIIB^{Mtl} domain(s) of MtlA, and presumably also of His-590 in the EIIA^{Mtl}-like domain of MtlR.

Replacement of His-590 with Asp Strongly Inhibits L. casei MtlR Activity

To further support the presumed induction mechanism, we studied the effect of the replacement of Thr-418 and His-590 on the expression from the *L. casei mtlA* promoter (P*mtlA*) fused to the *E. coli lacZ* gene. Cys419 in the EIIB^{Gat}-like domain of MtlR from *B. subtilis*, which is the equivalent of Thr418, was found to be phosphorylated by P~EIIA^{Mtl}. We therefore inserted the following *mtlR* alleles into the *B. subtilis* wild-type strain, the Δ*ptsI* and the Δ*mtlAFD* mutants carrying the *L. casei* P*mtl-lacZ* fusion: Thr418Ala and Thr418Asp as well as His590Ala and His590Asp. Integration of the *mtlR* alleles encoding a transcription activator with Thr418Ala or Thr418Asp replacements in the EIIB^{Gat}-like domain in either one of the three *B. subtilis* strains lowered the expression of the *L. casei* P*mtl-lacZ* fusion almost 2-fold compared to wild-type MtlR (data not shown). A similar inhibition was observed when His590 was replaced with alanine (fig. 4). These results suggest that the potential phosphorylation sites are important, but not essential for MtlR activity. In contrast, a nearly total loss of expression of the *L. casei* P*mtl-lacZ* fusion occurred when the allele encoding His590Asp mutant MtlR was integrated in the *thrC* site of one of the three *B. subtilis* strains (fig. 4). The finding that the phosphomimetic His590Asp replacement had a very strong repressive effect on MtlR supports the concept that induction of the *L. casei mtl* operon requires dephosphorylation of His-590 by the unphosphorylated EIIB^{Mtl} domain(s) of MtlA. In addition, the observation that wild-type and His590Ala mutant MtlR are also constitutively active in the *ptsI* (fig. 4) and *mtlAFD* deletion strains (data not shown) confirms that *L. casei* MtlR does not require activation via phosphorylation by PEP, EI and HPr, or by membrane sequestration mediated by one of the two EIIB^{Mtl} domains of MtlA.

Discussion

A detailed comparison of the regulatory mechanisms controlling the transcription activator MtlR from three different Firmicutes revealed drastic differences despite significant overall amino acid sequence conservation. While MtlR from *G. stearothermophilus* and *L. casei* share an identical induction mechanism (P~EIIB^{Mtl}-mediated phosphorylation in the C-terminal EIIA^{Mtl}-like domain), MtlR from *B. subtilis* has developed two new modes of mannitol-mediated transcription activation. In the absence of mannitol, *B. subtilis* EIIA^{Mtl} phosphorylates the conserved Cys-419 in the EIIB^{Gat}-like domain of MtlR, which inhibits the transcription activator (fig. 1b). When mannitol is present, the mannitol-specific EIIA^{Mtl} becomes dephosphorylated and consequently also the EIIB^{Gat}-like domain of MtlR. However, dephosphorylation of MtlR at Cys-419 is not sufficient for its activation. *B. subtilis* MtlR also needs to interact with the unphosphorylated EIIB^{Mtl} domain of MtlA and to be sequestered to the membrane in order to be functional (fig. 1b). In contrast, P~EIIB^{Mtl}, which prevails in *B. subtilis* cells when mannitol is absent from the growth medium, does not bind to the C-terminal domain of MtlR and stimulate its activity [Joyet et al., 2013]. The interaction of EIIB^{Mtl} with MtlR therefore depends on the phosphorylation state of the mannitol-specific EII component and probably serves as a second induction mechanism.

MtlR from *G. stearothermophilus* and *B. subtilis* share the same CcpA-independent carbon catabolite repression mechanism, which involves P~His-HPr-mediated phosphorylation in PRD2 (fig. 1b). This phosphorylation is essential for MtlR activity. If in addition to mannitol a repressing carbohydrate, such as glucose, is present in the growth medium, mostly HPr and P-Ser-HPr but little P~His-HPr are found in the cells [Monedero et al., 2001; Singh et al., 2008]. As a consequence, MtlR is barely phosphorylated in PRD2 and remains inactive. The absence of this stimulating phosphorylation is used as a CcpA-independent carbon catabolite repression mechanism not only for MtlR of *G. stearothermophilus* and *B. subtilis*, but numerous other PRD-containing transcription regulators [Deutscher et al., 2014; Krüger et al., 1996]. The conservation of two histidines in PRD2 of MtlR is required for the P~His-HPr-mediated phos-

phorylation and activation. In MtlR from *L. casei*, the second His in PRD2 (His398) is replaced with a Tyr. Probably due to this amino acid exchange, MtlR from *L. casei* was not phosphorylated by P~His-HPr. However, MtlR of *L. casei* was found to be active without being phosphorylated by P~His-HPr. The 'PTS-independent transcription activation' phenotype does not seem to be caused by the His398Tyr substitution because, when we replaced Tyr398 with a His or Ala, *L. casei* MtlR remained PTS-independent and constitutively active. Similarly, the substitution of the usually conserved His-288 in PRD1 with glutamic acid probably did not cause the PTS-independent phenotype. The mutation leading to PTS independence therefore remains unknown. Two successive events were probably necessary to create the present regulatory characteristics of *L. casei* MtlR. The first and still unknown mutation caused PTS independence, thus making phosphorylation at His398 unnecessary and allowing its replacement with tyrosine without loss of activity.

MtlR from *L. casei* is the first PRD-containing transcription activator reported to be functional without being phosphorylated by PEP and the common PTS components EI and HPr. This seems to be a characteristic for certain lactobacilli because in MtlR of *L. paracasei*, *L. rhamnosus* and *L. mali* the second conserved His in PRD2 is also replaced with a Tyr, suggesting that these MtlRs are also PTS-independent. Nevertheless, PTS independence seems to occur more frequently in antiterminators, which are composed of an N-terminal RNA-binding domain and two PRDs, but lack EIIA- and EIIB-like domains. For example, *B. subtilis* contains two antiterminators, SacY and GlcT, which control the expression of sucrose- and glucose-specific PTS components, respectively, but which do not need to be activated via phosphorylation by PEP, EI and HPr [Crutz et al., 1990; Stülke et al., 1997]. In antiterminators, the activating P~His-HPr-mediated phosphorylation usually also occurs in PRD2 [Lindner et al., 1999]. In contrast to *L. casei* MtlR, the two PTS-independent antiterminators SacY and GlcT were still phosphorylated by PEP, EI and HPr in their PRD2. Interestingly, replacing PRD2 of the PTS-independent *B. subtilis* antiterminator SacY with PRD2 from the PTS-dependent LicT rendered the hybrid protein PTS-dependent and vice versa [Tortosa et al., 2001]. In addition, a sophisticated mutagenesis screen allowed the isolation of a series of PTS-independent *licT* (LicT^{Pia}) alleles [Lindner et al., 2002]. Interestingly, most of the *licT*^{Pia} mutations were located in the vicinity of a salt bridge, which is formed by Glu-121 from PRD1 and Lys-209 from PRD2 and which has been proposed to be the major route of signal transduction from PRD2 to PRD1.

An obvious question is why these three Firmicutes developed such significantly different mechanisms of MtlR regulation? It is likely that these differences result from adaptation to the different environmental conditions encountered by the three organisms. For example, mannitol might be an important carbon source for *L. casei*, which grows on decaying plants, and relief of the mannitol operon from strong glucose repression might therefore be an advantage. The differences in MtlR regulation might also partly reflect the different metabolic and catabolic processes operative in these organisms. The absence of a functional tricarboxylic acid cycle in *L. casei* certainly has a strong impact on the mode and efficiency of the metabolism of carbohydrates, and might also be partly responsible for the observed poor glucose repression of the *mtlARFD* operon. Unfortunately, our present knowledge of bacterial physiology is not sufficiently advanced to allow the understanding of the consequences imposed by such subtle alterations in regulatory processes.

Materials and Methods

Bacterial Strains and Growth Conditions

L. casei strain BL23 [Mazé et al., 2010] and a previously constructed *ptsI* deletion mutant derived from it [Viana et al., 2000] were grown in carbohydrate-free MRS fermentation medium (Scharlau, Barcelona, Spain) containing 1% mannitol, 1% maltose, and 1% mannitol plus 1% glucose or 1% glucose. This medium contains chlorophenol red, which acts as indicator of carbohydrate utilization. The *B. subtilis* wild-type strain BSB168 and the *ptsI* and *mtlAFD* mutants derived from it as well as various integrants, which all contain an *L. casei* P*mtlA-lacZ* fusion and different *L. casei mtlR* alleles, were grown in Luria-Bertani (LB) medium (Difco) containing 1% mannitol, 1% glucose or 1% mannitol plus 1% glucose, 1 mM IPTG and the appropriate antibiotics.

Introduction of a L. casei PmtlA-lacZ Fusion into B. subtilis Wild-Type and ptsI and ΔmtlFAD Mutants

The 420-bp promoter region upstream from *mtlA* of *L. casei* was amplified by PCR by using *L. casei* genomic DNA as a template and the oligonucleotides D-PromtlA and R-PromtlA as primers (table 1). The PCR-introduced *Eco*RI and *Bam*HI restriction sites allowed insertion of the amplicon into plasmid pDG1661 [Guérout-Fleury et al., 1996] and consequently *L. casei* P*mtlA*-controlled expression of the promoterless *lacZ* gene present in this plasmid. Plasmid pDG1661 also carries DNA fragments allowing its integration into the *amyE* site of *B. subtilis*. The pDG1661-derived plasmid containing *L. casei* P*mtlA* was therefore used to transform the *B. subtilis* wild-type strain as well as Δ*ptsI* [Joyet et al., 2010] and Δ*mtlAFD* mutants [Bouraoui et al., 2013] derived from it. Chloramphenicol-resistant and spectinomycin-sensitive colonies had the pDG1661-derived plasmids integrated at the

Table 1. Primers used for the amplification of the genes encoding MtlR, EIIA^{Mtl}, the EIIB^{Mtl} domain and the 420-bp *mtlA* promoter/operator region

Name	Sequence	Amplified gene	Restriction site
D-PromtlA	CCGGAATTCTCATTGATATCCCCCTATCT	5′ *mtlA*	*Eco*RI
R-PromtlA	CGCGGATCCGGCAAACACACTCCTTTCCC	5′ *mtlA*	*Bam*HI
R-mtlR	CGCGGATCCATGTTACTAACGAATCGTGAAC	*mtlR*	*Bam*HI
D-mtlR	CCCAAGCTTTCACCTTCGATCAATTTTTTTGATTTCATTC	*mtlR*	*Hin*dIII
D-EIIA	AAATGCGCATGCATGATGAAAGGACTAGACG	*mtlF*	*Sph*I
R-EIIA	AAGCATAAGCTTTTATTCAACTTCCTTCAGTAAATTG	*mtlF*	*Hin*dIII
R-EIIB	AAAGCTCTGCAGTTATTTCATCTTCTCAATAAGC	3′ part *mtlA*	*Pst*I
D-EIIB	AACGCGGATCCGCAGCTAAAGATGTGATGTCAG	3′ part *mtlA*	*Bam*HI

Restriction sites are underlined.

Table 2. Primers used for the construction of *mtlR* mutants

Name	Sequence	Restriction sites
D-mtlRpIC	ACGTAAGCTTACATAAGGAGGAACTACTATGTTACTAACGAATCGTG	*Hin*dIII
R-mtlRpIC	ACGTGCATGCTCACCTTCGATCAATTTTTTTGATTTCATTC	*Sph*I
D-E288D	CAGTTTCTTGCCGTTGACCTCAGTACCATTCG	
R-E288D	CGAATGGTACTGAGGTCAACGGCAAGAAACTG	
D-E288H	CAGTTTCTTGCCGTTCATCTCAGTACCATTCG	
R-E288H	CGAATGGTACTGAGATGAACGGCAAGAAACTG	
D-Y398A	CGGGTATCTGGTGTTGGGCTTTGCTTCAGTCTTGG	
R-Y398A	GCCCATAGACCACAACCCGAAACGAAGTCAGAACC	
D-Y398H	CGGGTATCTGGTGTTGCACTTTGCTTCAGTCTTGG	
R-Y398H	CCAAGACTGAAGCAAAGTGCAACACCAGATACCCG	
D-Y398D	CGGGTATCTGGTGTTGGACTTTGCTTCAGTCTTGG	
R-Y398D	CCAAGACTGAAGCAAAGTCCAACACCAGATACCCG	
D-H340A	CTGTTGGCTGCTGTCCAACGAACCGCTGGCGGT	
R-H340A	TCGTTGGACAGCAGCCAACAGCGATGAGTAAAG	
D-H340D	CTGTTGGCTGATGTCCAACGAACCGCTGGCGGT	
R-H340D	TCGTTGGACATCAGCCAACAGCGATGAGTAAAG	
D-T418A	ctTGCTGATTGCGAGCGATGGCCCGGGGACCGGC	
R-T418A	GCCATCGCTCGCAATCAGCAAGACAGCTGCTG	
D-T418D	ctTGCTGATTGATAGCGATGGCCCGGGGACCGGC	
R-T418D	GCCATCGCTATCAATCAGCAAGACAGCTGCTG	
D-H590A	GCTATGATCGCTACCAGTAGTCAGGGTGTGACA	
R-H590A	ACTACTGGTAGCGATCATAGCGAGTCCGGTATC	
D-H590D	GCTATGATCGATACCAGTAGTCAGGGTGTGACAG	
R-H590D	ACTACTGGTATCGATCATAGCGAGTCCGGTATCG	

Restriction sites and mutated nucleotides are underlined.

amyE locus. *B. subtilis* MtlR is inactive in the Δ*ptsI* [Joyet et al., 2010] and the Δ*mtlAFD* mutant [Bouraoui et al., 2013]. In addition, in the wild-type strain expression of the P*mtlA-lacZ* fusion was not induced by growth on mannitol, suggesting that *B. subtilis* MtlR does not bind to the *L. casei* operator site of the *mtlAFD* operon. The three strains can therefore be used to determine the activity of *L. casei* wild-type and mutant MtlRs by measuring β-galactosidase activity.

Purification of His-Tagged B. subtilis and L. casei Proteins

His-tagged *B. subtilis* EI, HPr and EIIA^{Mtl} as well as the soluble EIIB^{Mtl} domain from the C-terminus of MtlA were overproduced and purified as previously described [Galinier et al., 1997; Joyet et al., 2010]. Similarly, we used a previously constructed plasmid for the overproduction and purification of His-tagged HPr from *L. casei* [Mazé et al., 2004].

The *L. casei* wild-type *mtlR* gene was amplified by PCR using oligos R-mtlR and D-mtlR as primers (table 1) and genomic DNA

as template. The resulting amplicon was cloned into the His-tag expression vector pQE30 cut with BamHI and HindIII providing plasmid pQE30MtlR. The *L. casei* genes for $EIIA^{Mtl}$ *(mtlF)* and the two soluble $EIIB^{Mtl}$ domains from the C-terminus of MtlA [Mazé et al., 2010] were also amplified by PCR, and the two amplicons were cloned into the His-tag expression vector pQE30 cut with SphI and HindIII for *mtlF* and PstI and HindIII for the DNA fragment encoding the $EIIB^{Mtl}$ domains *(mtlA-3′)*, thus providing plasmids pQE30mtlF and pQE30mtlA-3′, respectively. The ligation mixtures were used to transform either *E. coli* strain XL1-Blue (Stratagene; for *mtlF* and *mtlA*-3′) or NM522[pREP4-GroESL] (for *mtlR*) [Amrein et al., 1995]. The correct sequence of each gene was verified by DNA sequencing. For the overproduction and purification of His-tagged $EIIA^{Mtl}$ and the two fused $EIIB^{Mtl}$ domains we followed the usual protocol [Galinier et al., 1997]. In contrast, MtlR easily formed inclusion bodies and we therefore used only 0.05 mM IPTG for the 3-hour induction during growth at 28°C. The presence of the chaperone GroES/GroEL and addition of 200 mM of NaCl to the buffer during the preparation of cell extracts and dialysis of the purified protein also improved its solubility.

Introduction of Various L. casei mtlR Alleles into B. subtilis Strains Carrying the L. casei Pmtl-lacZ Fusion

In order to allow the three *B. subtilis* strains carrying the *L. casei* P*mtlA-lacZ* fusion to produce *L. casei* wild-type and mutant MtlRs we used plasmid pIC634, which is a derivative of plasmid pDG1664 [Guérout-Fleury et al., 1996] containing the *E. coli lacI* gene and the *spac* promoter inserted at the *Bam*HI/*Hin*dIII/*Eco*RI restriction sites (a gift from Dominique Le Coq, Jouy en Josas, France). The *L. casei* wild-type *mtlR* allele as well as the Glu288Asp, Glu288His, Tyr398Ala, Tyr398His, Tyr398Asp, His340Ala, His340Asp, Thr418Ala, Thr418Asp, His590Ala and His590Asp mutant alleles were amplified using D-MtlRpIC and R-MtlRpIC as distal primers combined with appropriate mutagenic primers (table 2) and genomic *L. casei* DNA as template. The primer D-MtlRpIC was designed in such a way that the resulting amplicon also contained the Shine Dalgarno box of *mtlR*. The PCR products corresponding to the various *mtlR* alleles were subsequently digested with the restriction enzymes *Sph*I and *Hin*dIII and inserted into pIC634 cut with the same enzymes, thus allowing *mtlR* expression from the IPTG-inducible *spac* promoter. The correct sequence of all *mtlR* alleles was verified by DNA sequencing. The pIC634-derived plasmids also contain the upstream and downstream regions of *B. subtilis thrC* and thus allowed the simultaneous integration of the *lacI*, *mtlR* and *erm* genes, as well as the *spac* promoter at the *thrC* site of the *B. subtilis* wild-type strain and the *ptsI* and *mtlAFD* mutants.

Synthesis of [^{32}P]PEP

[^{32}P]PEP was synthesized from [γ-^{32}P]ATP by using the pyruvate kinase isotope exchange reaction method at equilibrium described by Roossien et al. [1983].

[^{32}P]PEP-Requiring Phosphorylation of Wild-Type L. casei and B. subtilis MtlR and Several Mutant Proteins

Phosphorylation of *L. casei* and *B. subtilis* MtlR was carried out with purified PTS proteins of the corresponding organism with the exception of *B. subtilis* EI, which was used for the phosphorylation of MtlR from both organisms. Assay mixtures of 30 µl contained 50 mM of Tris-HCl (pH 7.4), 5 mM of $MgCl_2$ and 0.01 mM of [^{32}P]PEP (0.5 µCi; specific radioactivity 3 Ci mmol^{-1}). The samples contained various mixtures of the following proteins: 2 µg of wild-type or one of the different mutant MtlRs, 3 µg of EI, 1.6 µg of HPr, 1 µg of $EIIA^{Mtl}$ and 1 µg of the two $EIIB^{Mtl}$ domains of the mannitol-specific PTS permease MtlA. The assay mixtures were incubated for 30 min at 37°C before the reactions were stopped by adding SDS sample buffer. The proteins were subsequently separated by electrophoresis on denaturing 1% SDS/15% polyacrylamide gels. After drying, the gels were exposed to a storage phosphor screen (STORM).

Spectrophotometric Assay of Mannitol-1-P Dehydrogenase Activity

Mannitol-1-P dehydrogenase activity in crude extracts of *L. casei* wild-type and *ptsI* mutant was measured by following the reduction of NAD^+ to NADH + H^+, which accompanies the oxidation of mannitol-1-P to fructose-6-P. We followed the procedure described for measuring the mannitol-1-P dehydrogenase activity in *B. subtilis* strains [Deutscher et al., 1994].

β-Galactosidase Activity Assays

β-Galactosidase activity in toluenized *B. subtilis* BSB168 wild-type containing the *L. casei* P*mtlA* promoter fused to the *lacZ* gene integrated at *amyE*, various *mtlR* alleles integrated at *thrC*, and various mutant strains derived from it, was measured as previously described [Miller, 1972]. β-Galactosidase activity was also measured in BSB168-derived *ptsI* and *mtlAFD* mutants carrying the same integrations in *amyE* and *thrC*. In short, 3 ml of LB medium containing erythromycin and no carbohydrate or either 1% mannitol, 1% glucose or 1% mannitol plus 1% glucose were inoculated with 200 µl of an overnight culture (LB containing the appropriate antibiotics) and grown for 4 h at 37°C before an aliquot of 1 ml was withdrawn. Cells were harvested by centrifugation, resuspended in 500 µl of Z buffer and permeabilized with toluene. β-Galactosidase assays were performed with o-nitrophenyl-β-D-galactopyranoside as substrate and the change in OD_{420} was continuously followed with a Kontron Bio-Tek spectrophotometer using the 'AutoRate' program. The specific activity is expressed as ΔOD_{420} per minute and microgram of protein. Protein concentrations were determined by using the protein-dye binding method with Coomassie brilliant blue [Bradford, 1976].

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The Bacterial Phosphoenolpyruvate:Carbohydrate Phosphotransferase System: Regulation by Protein Phosphorylation and Phosphorylation-Dependent Protein-Protein Interactions

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The Bacterial Phosphoenolpyruvate:Carbohydrate Phosphotransferase System: Regulation by Protein Phosphorylation and Phosphorylation-Dependent Protein-Protein Interactions

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SUMMARY

The bacterial phosphoenolpyruvate (PEP):carbohydrate phosphotransferase system (PTS) carries out both catalytic and regulatory functions. It catalyzes the transport and phosphorylation of a variety of sugars and sugar derivatives but also carries out numerous regulatory functions related to carbon, nitrogen, and phosphate metabolism, to chemotaxis, to potassium transport, and to the virulence of certain pathogens. For these different regulatory processes, the signal is provided by the phosphorylation state of the PTS components, which varies according to the availability of PTS substrates and the metabolic state of the cell. PEP acts as phosphoryl donor for enzyme I (EI), which, together with HPr and one of several EIIA and EIIB pairs, forms a phosphorylation cascade which allows phosphorylation of the cognate carbohydrate bound to the membrane-spanning EIIC. HPr of firmicutes and numerous proteobacteria is also phosphorylated in an ATP-dependent reaction catalyzed by the bifunctional HPr kinase/phosphorylase. PTS-mediated regulatory mechanisms are based either on direct phosphorylation of the target protein or on phosphorylation-dependent interactions. For regulation by PTS-

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mediated phosphorylation, the target proteins either acquired a PTS domain by fusing it to their N or C termini or integrated a specific, conserved PTS regulation domain (PRD) or, alternatively, developed their own specific sites for PTS-mediated phosphorylation. Protein-protein interactions can occur with either phosphorylated or unphosphorylated PTS components and can either stimulate or inhibit the function of the target proteins. This large variety of signal transduction mechanisms allows the PTS to regulate numerous proteins and to form a vast regulatory network responding to the phosphorylation state of various PTS components.

INTRODUCTION

PTS-Mediated Sugar Transport and Phosphorylation

Many bacteria (1, 2) as well as some archaea (3) take up sugars and sugar derivatives, such as sugar alcohols, amino sugars, glycuronic acids, disaccharides, and numerous other carbon sources, via the phosphoenolpyruvate (PEP):carbohydrate phosphotransferase system (PTS). The PTS is usually composed of one membrane-spanning protein and four soluble proteins. Enzyme I (EI) and HPr are the general cytoplasmic PTS components, which in most organisms are involved in the uptake of all PTS carbohydrates; their genes are usually organized in the *ptsHI* operon, with *ptsH* coding for HPr and *ptsI* for EI. In contrast, the EIIA, EIIB, and EIIC (and in mannose-type PTSs also EIID) proteins are usually specific for one substrate or, in a few cases, for a small group of closely related carbohydrates (1, 2, 4). To indicate their substrate specificity, a three letter code is used, which is added as superscript to the corresponding protein name (5). For example, $EIIA^{Glc}$ stands for the glucose-specific EIIA component, $EIIB^{Fru}$ for the fructose-specific EIIB, $EIID^{Man}$ for the mannose-specific EIID, etc. The genes encoding the PTS components specific for a certain sugar are normally organized in an operon, which frequently also contains the genes required for the catabolism of the transported substrate.

With the exception of several actinobacteria, such as *Agromyces italicus*, *Cellulomonas flavigena*, or *Actinomyces turicensis*, which possess a regular HPr in addition to a DhaM protein composed of an HPr-like domain and an $EIIA^{Man}$-like domain, Gram-positive bacteria usually contain only one EI and one HPr. In contrast, *Enterobacteriaceae* usually produce several EI and HPr homologues or paralogues, such as nitrogen-related EI^{Ntr} and NPr and the fructose-specific FPr. Both types of bacteria normally contain several operons encoding the sugar-specific PTS components. According to the sequence and composition of the membrane components, seven families of PTS can be distinguished: the glucose, fructose, lactose, glucitol, galactitol, mannose, and ascorbate families (6). Interestingly, numerous alpha-, beta-, gamma-, and deltaproteobacteria also contain an incomplete PTS lacking any known EIIB and EIIC component. These PTSs are therefore probably not involved in carbohydrate transport but carry out only regulatory functions. Finally, in certain bacteria the phosphorylation of intracellular dihydroxyacetone requires EI, HPr, and an EIIA of the mannose family (7).

PTS proteins are often fused to each other, thus forming multifunctional polypeptides made up of two or more domains. Frequently, one or two of the soluble PTS components are fused to the N or C terminus of the membrane-spanning transport protein and are therefore located at the cytosolic side of the membrane. For example, the oligo-β-glucoside-specific PTS of *Bacillus subtilis* is composed of three distinct proteins, whereas in the mannitol-specific PTS permease of the same organism, the EIIB component is fused to the C terminus of the membrane-spanning EIIC and only $EIIA^{Mtl}$ is a distinct protein (Fig. 1). The mannose-type PTSs are an exception because they possess two transmembrane proteins, as is the case for most mannose/glucose-specific PTSs of firmicutes as well as the low-efficiency fructose-specific levan PTS of *B. subtilis* (Fig. 1).

The PTS uses PEP as an energy source for the uptake of its substrates and as a phosphoryl donor for their phosphorylation. In order to phosphorylate the carbohydrates during their transport, the soluble PTS components form a phosphorylation cascade beginning with EI, which autophosphorylates at the N-ε3 position of a conserved histidyl residue at the expense of PEP (Fig. 1) (8). Phosphorylated EI (P∼His-EI) transfers the phosphoryl group to the N-δ1 position of His-15 in HPr (9), and histidyl-phosphorylated HPr (P∼His-HPr) passes the phosphoryl group on to one of several sugar-specific EIIAs usually present in a bacterium. (The symbol "∼" indicates an energy-rich phosphoryl bond, in contrast to, for example, seryl-phosphorylated proteins, which are written as P-Ser throughout this article.) EIIAs are also phosphorylated at the N-ε3 position of a histidyl residue (10, 11). P∼EIIAs, however, phosphorylate their cognate EIIB at a cysteyl residue (12), except the EIIBs of the mannose PTS family, which are phosphorylated at the N-δ1 position of a conserved histidine (13). In the last step, P∼EIIB transfers its phosphoryl group to a carbohydrate molecule bound to the cognate EIIC. There is so far only one carbohydrate, fucosyl-α-1,3-*N*-acetylglucosamine, which is transported by a PTS (that of *Lactobacillus casei*) without being phosphorylated (14). All other carbohydrates are phosphorylated during their transport and subsequently converted into phosphorylated intermediates of either the Embden-Meyerhof-Parnas, pentose phosphate, or Entner-Doudoroff pathway. Only maltose taken up via a PTS by *Enterococcus faecalis* cells was reported to be first dephosphorylated to maltose by the maltose-6′-phosphate (maltose-6′-P)-specific phosphatase MapP (15). Intracellular maltose is subsequently cleaved by the enzyme maltose phosphorylase into glucose-1-P and glucose, which are converted into glycolytic intermediates.

The crystal structure of the *N*,*N*′-diacetylchitobiose-specific EIIC component of *Bacillus cereus* with the disaccharide bound to the active site has been determined and indeed confirmed that the phosphorylatable hydroxyl group at the C-6′ position of the disaccharide is located close to the cytoplasm and can therefore be accessed by the P∼EIIB component (16). Phosphorylation of the carbohydrate is thought to lower its affinity for EIIC, and the phosphorylated carbohydrate is therefore released into the cytosol.

Among the about 230 archaea for which the genome sequence has been determined, more than 50 contain the general PTS components EI and HPr; most of these belong to the genus *Haloferax*. It seems that these organisms use the PTS mainly to transport and phosphorylate fructose (3, 17) or to phosphorylate dihydroxyacetone (18). A previous deduction that archaea are devoid of any PTS is therefore no longer valid (6). *Methanopyrus kandleri* AV19 possesses a protein (MK1512) corresponding to the short form of HprK/P present in alphaproteobacteria (19). As will be explained below, HprK/P phosphorylates HPr at Ser-46 (Fig. 1). The N-terminal part of MK1512 exhibits 55% sequence identity to a ca. 85-amino-acid-long region of HprK/P from firmicutes compris-

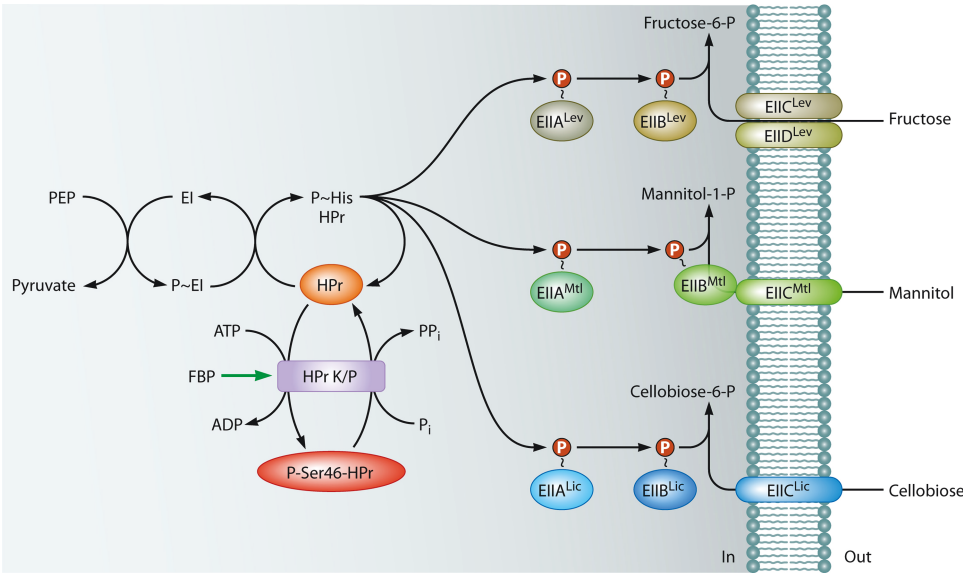

FIG 1 Schematic presentation of the phosphorylation cascade formed by the *B. subtilis* PTS components necessary for the uptake of fructose (PTS^{Lev}), mannitol (PTS^{Mtl}), and cellobiose (PTS^{Lic}). The two general PTS components EI and HPr phosphorylate the EIIAs, which are specific for a certain carbohydrate. *B. subtilis* contains nine entire PTSs, six PTSs lacking an EIIA component, and one PTS lacking EIIA and EIIB components (49). For the seven incomplete PTSs, EIIA and EIIB components of other PTS, most likely from the same family, probably complement the transport and phosphorylation functions. Nevertheless, the PTSs usually exhibit different sugar specificities. The P~EIIAs transfer the phosphoryl group to their cognate EIIB, which finally phosphorylates the carbohydrate bound to the corresponding membrane-integral EIIC or, for the fructose-specific levan PTS, to EIIC and EIID. The phosphorylated carbohydrate is subsequently released into the cytoplasm. While the PTS phosphorylation cascades for cellobiose and fructose are formed by EI, HPr, and two distinct EIIA and EIIB proteins, the EIIB component of PTS^{Mtl} is fused to the C terminus of the EIIC domain and is therefore attached to the cytoplasmic side of the membrane. Shown are also the fructose-1,6-bisphosphate (FBP)-stimulated and ATP-requiring phosphorylation of HPr at Ser-46 as well as the dephosphorylation of P-Ser-HPr, which follows a phosphorolysis reaction with P-Ser-HPr and P_i being the substrates and HPr and pyrophosphate (PP_i) the products (22). The ATP-dependent phosphorylation of HPr occurs in firmicutes but also in many proteobacteria containing HprK/P and the HPr paralogue NPr.

ing the active site with the P loop. This organism also contains an HPr-like domain present in a protein of unknown function (MK0716). Interestingly, in the HPr-like domain the phosphorylatable His is replaced with a Glu, whereas the ATP-dependent phosphorylation site around Ser-46 is conserved. It is therefore likely that in some archaea PTS proteins also carry out regulatory functions, which are associated with phosphorylation of this serine residue.

Regulatory Functions of the PTS

Soon after the discovery of the PTS in *Escherichia coli* (20), it was realized that it not only transports and phosphorylates carbohydrates but also carries out regulatory functions. In *Enterobacteriaceae*, $EIIA^{Glc}$ (also called Crr, for catabolite repression resistance) was found to be the central regulator of carbon metabolism. In firmicutes, this function is carried out by HPr, which in these organisms becomes phosphorylated not only at His-15 by PEP and EI but also at Ser-46 by ATP and the metabolite-controlled bifunctional kinase/phosphorylase HprK/P (Fig. 1) (21, 22). The latter HPr modification plays no role in sugar uptake and phosphorylation but serves exclusively regulatory functions. In addition to $EIIA^{Glc}$ and HPr, many other PTS proteins also play regulatory roles. Intriguingly, numerous proteobacteria possess EI, HPr, and one or two EIIAs but lack any known EIIB and EIIC proteins. Although *E. coli* and other *Enterobacteriaceae* are devoid of HprK/P and the region around Ser-46, the ATP-dependent phosphorylation site in HPr, is not conserved, HPr proteins of proteobacteria with an incomplete PTS usually possess an HprK/P, and the region around Ser-46 strongly resembles that in firmicutes. These observations suggest that in these proteobacteria, the incomplete PTS is not involved in carbohydrate transport but carries out only regulatory functions by interacting with or by phosphorylating non-PTS target proteins.

Indeed, in most cases phosphorylation of non-PTS proteins by PTS components serves regulatory functions. There are nevertheless at least two reported cases where PTS proteins phosphorylate a non-PTS protein or its bound cofactor for enzymatic purposes. First, the phosphoryl group of P~EI from *Salmonella enterica* serovar Typhimurium can be transferred to the intermediately phosphorylated active-site residue of acetate kinase (23). Second, P~EIIA of the dihydroxyacetone phosphorylation system of numerous bacteria and several archaea (24) transfers its phosphoryl group to an ADP molecule tightly bound to the active site of the dihydroxyacetone kinase subunit DhaL, which subsequently phosphorylates dihydroxyacetone bound to DhaK (18). Some enterobacteria possess a more complex dihydroxyacetone-specific multidomain protein composed of an EIIA-like domain and an HPr-like domain followed by a truncated EI-like domain containing the conserved phosphorylatable histidine. This protein is phosphorylated by PEP, EI, and HPr at the truncated EI-like domain, and after primarily intramolecular transfer of the phosphoryl group to the HPr- and EIIA-like domains, it phosphorylates the ADP bound to DhaL (7).

In most cases, however, phosphorylation of non-PTS proteins by PTS components serves regulatory purposes. To allow the PTS to carry out its regulatory functions, several different mechanisms evolved. They include direct phosphorylation of the target pro-

teins by PTS components. Phosphorylation can occur at PTS domains fused to the target proteins or at specific phosphorylation sites or domains that evolved in or were fused to the target protein. Alternatively, PTS components, in either phosphorylated or unphosphorylated form, interact with their target proteins. PTS-catalyzed phosphorylation of a target protein can stimulate or inhibit its activity, and this also holds for the interaction of phosphorylated and unphosphorylated PTS components with their target proteins. We describe these different scenarios in detail below.

PTS-MEDIATED REGULATION BY PHOSPHORYLATION

Since all cytoplasmic PTS proteins or membrane-associated hydrophilic PTS domains undergo transient phosphorylation, it is not surprising that numerous regulatory functions of the PTS are mediated by phosphoryl group transfer to the target proteins. Identical to the case for PTS proteins, all presently known PTS-regulated target proteins also become phosphorylated at histidine (phosphoamidate) or cysteine (thiophosphate) residues. This renders all phosphorylation steps reversible, because the standard free energies of the various high-energy phosphate bonds (P∼Nδ-1 or P∼Nε-3 in P∼histidines and P∼S in cysteines) are very similar (25–27). Consequently, if the PTS proteins are present primarily in phosphorylated form, the PTS-regulated target proteins are also mainly phosphorylated.

Two major factors were so far reported to affect the phosphorylation state of the PTS proteins in *Enterobacteriaceae* and probably most other bacteria. First, the uptake of an efficiently metabolized PTS sugar, such as glucose, and its concomitant phosphorylation lead to dephosphorylation of the PTS components. The phosphoryl group transfer from most P∼EIIBs to their cognate substrates seems to be more rapid than rephosphorylation via the PTS cascade. Second, changes in the ratio of PEP to pyruvate, the two compounds which are the substrate and product of the autophosphorylation of EI (Fig. 1), also affect the phosphorylation state of the PTS proteins (28, 29). The PEP-to-pyruvate ratio changes in response to the metabolic state of the cell. Starving cells have a high PEP-to-pyruvate ratio, and under resting conditions the PTS proteins are therefore mainly phosphorylated (28, 30) and the cells are primed to take up any PTS substrate they might encounter. In metabolically active cells, the PEP-to-pyruvate ratio is low and the PTS proteins are barely phosphorylated at His and Cys residues (30). Very low phosphorylation of the PTS proteins is therefore usually observed in cells efficiently metabolizing PTS sugars, such as glucose (28, 31). However, growth on non-PTS sugars can also lower the PEP-to-pyruvate ratio and therefore the phosphorylation state of the PTS proteins (28). Finally, α-ketoglutarate, which accumulates in *E. coli* cells exposed to nitrogen-limiting conditions, inhibits the autophosphorylation activity of EI. Nitrogen limitation therefore inhibits glucose uptake. In contrast, sudden nitrogen availability almost immediately increases glucose uptake and consumption without significantly altering the concentration of glycolytic intermediates (32).

In firmicutes, the metabolite-controlled HprK/P-mediated phosphorylation of HPr at Ser-46 (33) during the rapid metabolism of a carbohydrate slows the phosphoryl group transfer within the PTS phosphorylation cascade (34). The kinase function of HprK/P is enhanced during growth on efficiently utilized carbon sources (30, 35), and the resulting seryl-phosphorylated HPr (P-Ser-HPr) is a poor substrate for the PEP-dependent phosphorylation by P∼EI (36, 37). This mechanism further lowers the poor PEP-requiring phosphorylation of HPr and the EIIA and EIIB components in firmicutes growing on an efficiently metabolizable carbon source. The alterations of the phosphorylation state of the general and sugar-specific PTS components in response to the metabolic state of the cell and/or to the presence of a distinct carbohydrate are used to control numerous cellular functions.

Proteins Containing Their Own PTS-Recognized Phosphorylation Sites

One might expect that the development of a phosphorylation site recognized by PTS components would be the most straightforward and simplest way to render a protein "PTS controlled." However, there is so far only one well-established example in which a protein developed a specific regulatory site (as opposed to the enzymatic sites described above) for phosphorylation by a PTS protein. This is glycerol kinase (GlpK) from firmicutes (38). GlpKs from firmicutes possess a surface-exposed flexible loop close to the dimer interface, which contains a histidyl residue surrounded by three aromatic amino acids, Aro-His-Aro-Aro (with Aro being Tyr or Phe in arbitrary order, depending on the organism) (39). This sequence does not resemble any known phosphorylation site in PTS proteins. The histidine residue in the above tetrapeptide (His-230 in GlpK from *B. subtilis*) (40) becomes phosphorylated at the N-ε3 position by PEP, EI, and HPr (38, 41). Despite a usually 60% amino acid sequence identity between GlpKs from firmicutes and other bacterial phyla, the phosphorylatable His is present only in GlpKs of firmicutes. Accordingly, GlpKs of no other phylum were so far found to be phosphorylated by PTS proteins, suggesting that this regulatory mechanism developed relatively late in evolution. In fact, GlpKs from *Enterobacteriaceae* possess at the same position a similar but histidine-less flexible loop for the binding of fructose-1,6,-bisphosphate (FBP), which leads to the formation of inactive tetramers (42). Proteins strongly resembling GlpKs from firmicutes but phosphorylating other carbohydrates, such as the presumed fuculose kinase Lmo1034 of *Listeria monocytogenes* (43), also lack the phosphorylation loop with the conserved histidine. The phosphorylation of GlpKs from firmicutes is reversible, and in contrast to the inhibitory effect of FBP binding on GlpKs of *Enterobacteriaceae*, it stimulates the GlpK-catalyzed ATP-dependent phosphorylation of glycerol 10- to 15-fold (44). Addition of an excess of HPr to purified phosphorylated GlpK leads to rapid dephosphorylation of the kinase and consequently lowers its activity (38). The uptake of efficiently metabolized PTS sugars, which leads to the dephosphorylation of PTS components and therefore also of GlpK, inhibits the activity of the kinase. Indeed, firmicutes in which EI or HPr have been inactivated not only are unable to utilize PTS carbon sources but also are unable to grow on glycerol as the sole carbon source (40, 45). The absence of GlpK phosphorylation leads to inducer exclusion, one of the regulatory mechanisms contributing to carbon catabolite repression, which will be discussed in detail in the section "Inducer exclusion in *Enterobacteriaceae*." The regulatory His of GlpK is located about 20 Å from the active site. PTS-mediated GlpK stimulation therefore follows a long-range activation mechanism. Studies with an *E. faecalis* GlpK mutant protein in which the regulatory His-232 had been replaced with an arginine, which leads to a fully active enzyme without phosphorylation (46), suggested that phosphorylation induces structural rearrangements along the dimer interface that allow an

TABLE 1 Proteins fused to PTS domains with proven or presumed regulatory functions

Organism(s)	Protein function	Fused PTS domain	Location of PTS domain	Conserved phosphorylation site	Effect of phosphorylation
Clostridium acetobutylicum	NtrC-type response regulator HprR CA_C3088	HPr	N terminus	His-15	?
Eubacterium limosum	BkdR-like transcription regulator	HPr	N terminus	His-16	?
Tepidanaerobacter acetatoxydans Re1	BkdR-like transcription regulator	HPr	N terminus	His-15	?
Streptococcus thermophilus	Lactose/galactose antiporter LacS	$EIIA^{Glc}$	C terminus	His-552	+
Streptococcus salivarius	Lactose/galactose antiporter LacS	$EIIA^{Glc}$	C terminus	His-552	?
Several *Borrelia* species	Na^+/H^+ symporter	$EIIA^{Ntr}$	C terminus	His-622	?
Pasteurella multocida	Triosephosphate isomerase	$EIIB^{Glc}$	C terminus	Cys-304	?
Actinobacillus succinogenes	Triosephosphate isomerase	$EIIB^{Glc}$	C terminus	Cys-297	?
Several *Vibrio* species	Cyclic diguanylate phosphodiesterase-like	$EIIC^{Lac}$	C terminus	171–432[a]	?
Firmicutes, actinobacteria, and a few proteobacteria	LevR-type transcription activators	$EIIA^{Man}$	Fourth domain	His-585[b]	+
		$EIIB^{Gat}$	Penultimate domain	Cys-718	−
Numerous *Clostridiales*, *Clostridium beijerinckii* NCIMB 8052	LevR-type transcription activator, YP_001309609.1, YP_001307365.1, YP_001307684.1	$EIIA^{Man}$	Fourth domain	His-629[c]	?
		$EIIB^{Gat}$	Penultimate domain	Cys-760	?
		$EIIA^{Mtl}$	C terminus	His-919	?
Numerous *Clostridiales*, *Thermoanaerobacter ethanolicus* JW200	LevR-type transcription activator, EGD53072.1	$EIIA^{Man}$	Fourth domain	His-606	?
		$EIIB^{Gat}$	Penultimate domain	Cys-740	?
		$EIIA^{Mtl}$	C terminus	His-896	?
Several *Selenomonadales* and other firmicutes, *Pelosinus fermentans* B3	LevR-type transcription activator, EIW31722.1	$EIIA^{Man}$	Fourth domain	His-604	?
		$EIIB^{Gat}$	Penultimate domain	Cys-744	?
		$EIIA^{Mtl}$	C terminus	His-898	?
Firmicutes, actinobacteria, and a few proteobacteria	MtlR/LicR-type transcription activators	$EIIB^{Gat}$	Penultimate domain	Cys-419[d]	−
		$EIIA^{Mtl}$	C terminus	His-599	−

[a] The number refers to the region corresponding to the $EIIC^{Lac}$ domain.
[b] The numbers refer to the *B. subtilis* LevR sequence.
[c] The numbers refer to the *C. beijerinckii* LevR-like protein with the ID YP_001309609.1.
[d] The numbers refer to the *B. subtilis* MtlR sequence.

optimal positioning of a crucial arginine residue (Arg-18) in the active site (47).

Proteins Containing a PTS Component Fused to the N or C Terminus

Another mechanism of PTS-mediated regulation that developed during bacterial evolution was the fusion of a PTS component to a target protein. In most cases the phosphorylatable His or Cys is conserved in the regulatory PTS domain and can therefore become phosphorylated by the proteins of the PTS phosphorylation cascade. For some proteins it was not an entire PTS domain that was fused to the target polypeptide but only a relatively small fragment containing the phosphorylatable histidine or cysteine. Phosphorylation or dephosphorylation of the PTS domain induces structural changes in the target protein, which lower or stimulate its activity. Proteins possessing a regulatory PTS domain include non-PTS transporters (48), various types of transcription activators (49–51), and two-component system response regulators (52). Several proteins and protein families containing a clearly identifiable PTS domain are listed in Table 1. There are also numerous proteins of unknown function that contain a PTS domain (6). The PTS-mediated regulation of the lactose transporters from *Streptococcus thermophilus* (53) and *Streptococcus salivarius* (54) and of several transcription activators from firmicutes (1) and some *Enterobacteriaceae*, such as FrzR of *E. coli* (55), has been studied in detail.

Transcriptional activators. The PTS domain-containing transcription activators are mainly found in firmicutes and in actinobacteria but are less frequent in proteobacteria. Some of these bacteria possess transcription activators that can contain up to three EIIA and EIIB domains (Table 1). In fact, two types of EII domain-containing transcription activators can be distinguished. The LevR-type activators possess an N-terminal DNA binding helix-turn-helix motif followed by a homologue of the central ATP binding cassette-containing domain of NtrC-type regulators and four regulatory domains; two or, in a few cases, three of the regulatory domains are EIIA and EIIB domains of the mannose/glucose, galactitol, and mannitol/fructose PTS classes (1). These transcription activators contain other regulatory domains (Fig. 2), which are discussed below (see "Proteins Containing a Specific PTS-Recognized Phosphorylation Domain, the PRD"). The NtrC-type regulators containing three EII domains belong primarily to the *Clostridiales*, including *Clostridia*, *Thermoanaerobacter*, *Carboxydibrachia*, and a few other firmicutes (Table 1). The members of the second type of EII domain-containing transcription activators are composed of a DeoR-like helix-turn-helix motif followed by a winged helix-turn-helix domain resembling that in the *Streptococcus pyogenes* virulence gene regulator Mga and

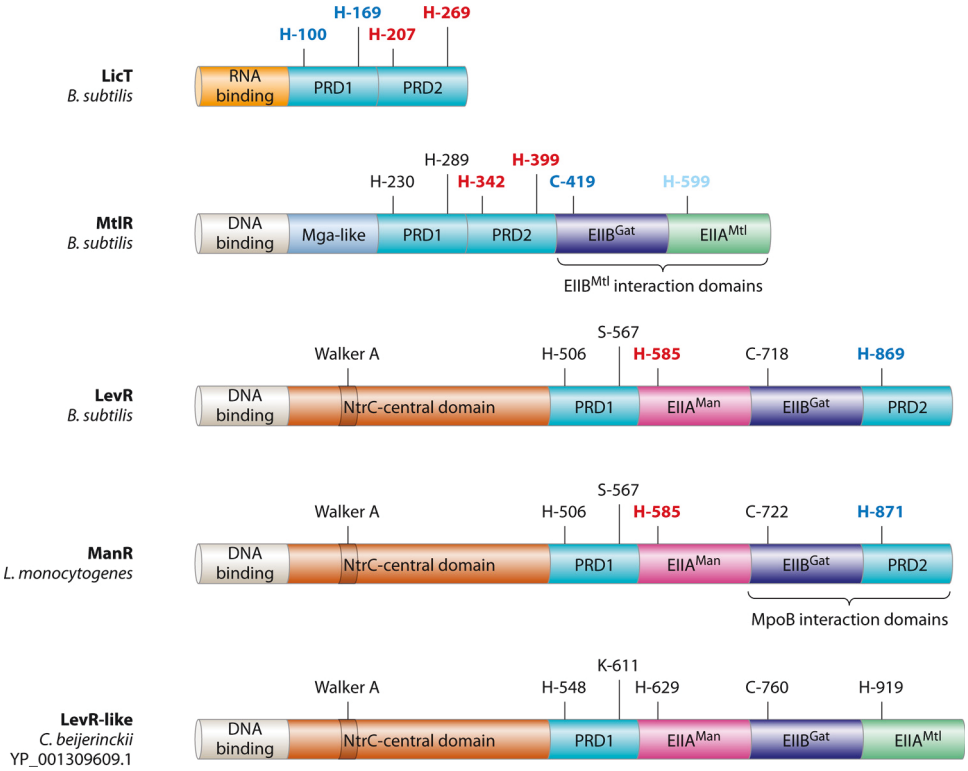

FIG 2 Schematic presentation of the different regulatory domains in PRD-containing proteins. Shown are the *B. subtilis* antiterminator LicT (which binds to RNA) and several transcription activators (which bind to DNA), including the *B. subtilis* regulators MtlR and LevR. Antiterminators are composed of two PRDs fused to the RNA binding domain. The two PRDs usually contain four potential sites of PTS-mediated phosphorylation (73). Similarly, MtlR-like transcription activators contain two PRDs fused to the DNA binding and Mga-like domains (71). However, in MtlR-like transcription activators, the PRDs are followed by an $EIIB^{Gat}$-like domain and an $EIIA^{Mtl}$-like domain. *B. subtilis* MtlR needs to be activated both by phosphorylation at His-342 and by the interaction of its C-terminal $EIIB^{Gat}$- and $EIIA^{Mtl}$-like domains (marked with a bracket) with the $EIIB^{Mtl}$ domain of the mannitol-specific PTS permease MtlA (146) (Fig. 1). The domain order in the *B. subtilis* transcription activator LevR is different from that in MtlR: the DNA binding and NtrC-like domains are followed by PRD1, $EIIA^{Man}$- and $EIIB^{Gat}$-like domains, and finally a truncated PRD2 (which contains only one conserved His). In all of the presented proteins the known stimulating phosphorylation sites (by P∼His-HPr) are indicated by red numbers, and inhibitory phosphorylation sites (by P∼EIIA or P∼EIIB) are written in blue (pale blue in MtlR indicates slight phosphorylation). Also presented in this figure is the LevR-like transcription activator ManR from *L. monocytogenes*, which, similar to the case for MtlR from *B. subtilis*, needs to be activated by both phosphorylation by P∼His-HPr at His-585 in the $EIIA^{Man}$-like domain (A. Zébré, E. Milohanic, and J. Deutscher, unpublished results) and interaction with the EIIB component MpoB (78). It should be noted that the phosphorylation sites are not always conserved. For example, ManR from *L. innocua*, which is almost identical to *L. monocytogenes* ManR, was reported to become phosphorylated by P∼His-HPr at His-506 in the PRD1 domain (77). Finally, in some LevR-like regulators of the order *Clostridiales*, the truncated PRD2 can be replaced with an $EIIA^{Mtl}$-like domain, as is the case for the *C. beijerinckii* protein with ID number YP_001309609.1.

four regulatory domains, one of which is an EIIB of the galactitol-type PTS and one an EIIA of the mannitol/fructose class PTS (Fig. 2). According to the first members described for this family of transcription activators, they are called MtlR/LicR-type regulators (51, 56).

Depending on the phosphoryl donor, phosphorylation of the EII domains in these transcription activators either stimulates their activity (phosphorylation by P∼His-HPr) or inhibits it (phosphorylation by P∼EIIA or P∼EIIB). For example, P∼His-HPr-mediated phosphorylation of the $EIIA^{Man}$-like domain of LevR from *B. subtilis* (57) and *L. casei* (50), the first intensively studied members of the EII and NtrC domain-containing regulators, activates the expression of the *lev* operon, which encodes the proteins for the extracellular degradation of the polysaccharide levan to fructose monomers and the components for a low-efficiency PTS specific for fructose and mannose. The absence of P∼His-HPr-mediated phosphorylation during the uptake of an efficiently metabolized carbon source represents one of the carbon catabolite repression (CCR) mechanisms operative in firmicutes. Nevertheless, in many firmicutes synthesis of the PTS transport components for the major repressing carbohydrates, glucose and mannose (ManLMN), is also regulated by a LevR-like protein (ManR), which in *L. monocytogenes* needs to be activated by P∼His-HPr-mediated phosphorylation at the $EIIA^{Man}$ domain (A. Zébré, E. Milohanic, and J. Deutscher, unpublished results). An autoregulation mechanism for glucose transport therefore seems to be operative. In contrast, phosphorylation by the cognate P∼EIIB component usually inhibits the activity of transcription activators, and its absence is used for induction of the corresponding PTS operon. Such a regulatory mechanism has been reported, for example, for *B. subtilis* LicR, which controls the expression of an operon encoding the components of a cellobiose-specific PTS. The uptake of cellobiose by the PTS^{Cel} is assumed to lead to dephosphorylation of the corresponding EIIB component and therefore to prevent the $EIIB^{Cel}$-mediated phosphorylation of the C-terminal $EIIA^{Mtl}$-like domain of the transcription activator LicR, thereby allowing the expression of the *cel* (or *lic*) operon. A similar regulation mechanism is probably operative for those

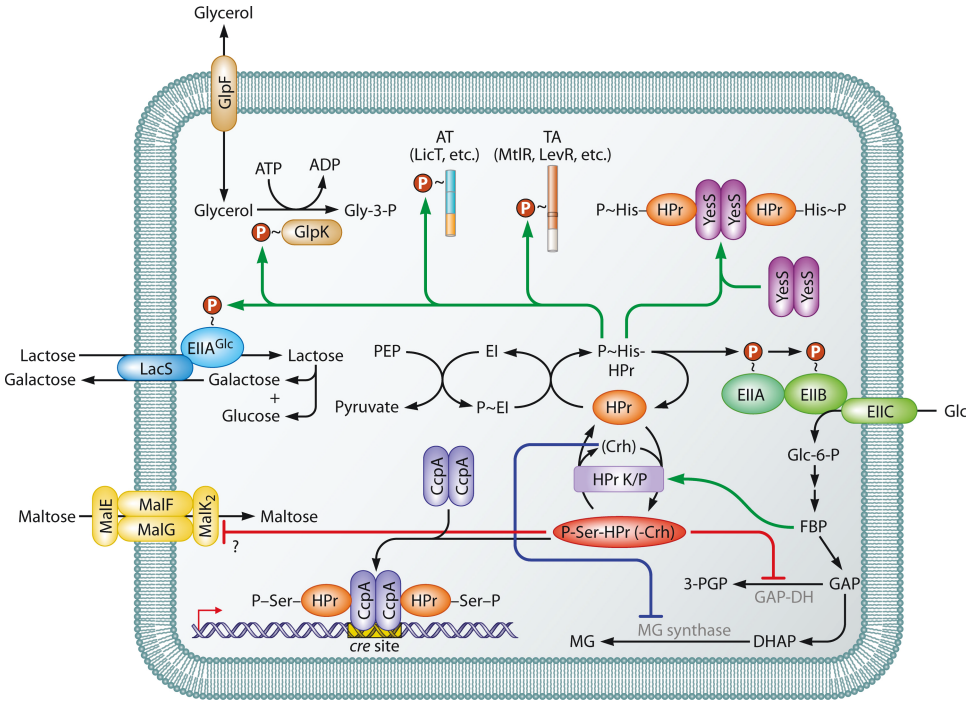

FIG 3 PTS-catalyzed glucose uptake and the HPr/Crh "regulon" in firmicutes. HPr, the central regulator of carbon metabolism in firmicutes, exists in four different forms: unphosphorylated, phosphorylated at His-15, phosphorylated at Ser-46, and doubly phosphorylated. *B. subtilis* contains in addition the HPr paralogue Crh, which lacks His-15 and therefore can be phosphorylated only at Ser-46. Histidyl-phosphorylated HPr prevails when a less favorable carbon source is utilized, whereas P-Ser-HPr and P-Ser-Crh are formed when glucose or other preferred sugars are metabolized. The utilization of glucose leads to an increase of the concentration of the glycolytic intermediate FBP, which stimulates the kinase function of HprK/P. Either the different forms of HPr (and *B. subtilis* Crh) interact with their target proteins (YesS, MG synthase, CcpA, and MalK) or P∼His-HPr phosphorylates them (glycerol kinase, PRD-containing transcription activators and antiterminators, and LacS). The following regulatory functions of HPr and Crh and their various phosphorylated forms are presented. (i) Unphosphorylated Crh of *B. subtilis* interacts with methylglyoxal synthase (MG synthase) and inhibits its activity. Methylglyoxal synthase is an enzyme at the entry point of the methylglyoxal bypass of glycolysis that catalyzes the transformation of dihydroxyacetone-P into methylglyoxal. (ii) P-Ser-HPr as well as P-Ser-Crh interacts with the *B. subtilis* glycolytic enzyme glyceraldehyde-3-P DH (GAP DH) and inhibits its activity. (iii) P-Ser-HPr and in *B. subtilis* also P-Ser-Crh interact with CcpA and stimulate its repressor function for CCR by binding to the *cre* operator sites of numerous catabolic genes. (iv) P-Ser-HPr of lactobacilli and lactococci inhibits maltose uptake by an inducer exclusion mechanism by probably directly interacting with a component of the ABC transporter. (v) P∼His-HPr interacts with and stimulates the *B. subtilis* transcription activator YesS, which controls the expression of the pectin/rhamnogalacturonan genes. (vi) P∼His-HPr phosphorylates and activates several PRD-containing antiterminators (AT) and transcription activators (TA), and the absence of their phosphorylation during glucose metabolism is used as a CcpA-independent CCR mechanism. (vii) P∼His-HPr also phosphorylates and activates glycerol kinase (GlpK). The absence of GlpK phosphorylation leads to inducer exclusion. (viii) Finally, in streptococci, P∼His-HPr phosphorylates the $EIIA^{Glc}$-like domain of LacS and stimulates the lactose/galactose exchange reaction catalyzed by this protein.

LevR-like transcription activators that contain a C-terminal $EIIA^{Mtl}$-like domain (Fig. 2). In contrast, induction of the mannitol operon of *B. subtilis*, which is controlled by the transcription activator MtlR, occurs only when the P∼$EIIA^{Mtl}$-mediated phosphorylation of MtlR at the conserved cysteyl residue in the penultimate $EIIB^{Gat}$-like domain is prevented by the presence of mannitol in the growth medium (58). So far, *B. subtilis* MtlR is the only example of P∼EIIA-mediated phosphorylation of a transcription activator at the conserved Cys in the $EIIB^{Gat}$-like domain (Fig. 2).

The lactose-specific transporter LacS. LacS from *S. thermophilus* contains an $EIIA^{Glc}$-like domain fused to its C terminus (Table 1 and Fig. 3). This natural hybrid protein catalyzes slow H^+/lactose symport as well as fast lactose/galactose counterflow (59). Intracellular lactose is cleaved into glucose and galactose. The latter hexose cannot be metabolized by *S. thermophilus* and is therefore secreted. The export of galactose is coupled to the uptake of lactose via a counterflow mechanism. Phosphorylation of the $EIIA^{Glc}$-like domain of LacS was demonstrated for *S. thermophilus* (60) and *S. salivarius* (61). It increases the affinity of LacS for its substrate and the speed of the counterflow reaction (62). Efficient uptake of lactose therefore requires a functional β-galactosidase and phosphorylation of LacS by PEP, EI, and HPr (Fig. 3). By carrying out *in vitro* and *in vivo* complementation studies with truncated LacS lacking the $EIIA^{Glc}$-like domain, it could be established that the unphosphorylated $EIIA^{Glc}$-like domain does not bind to truncated LacS. In contrast, the phosphorylated $EIIA^{Glc}$-like domain interacted with truncated LacS and stimulated its lactose/galactose counterflow activity (63).

HPr domain-containing proteins. Several proteins were found to contain an HPr-like domain fused either to their N or C termini (Table 1). The presence of an HPr-like domain at the N terminus of an NtrC-type regulator with a C-terminal DNA binding domain (but without EII domains) in *Clostridium acetobutylicum* ATCC 824 suggested a possible cross talk between this transcription regulator (called HprR) and the PTS (52), with the HPr-like domain functioning as the receiver module (Fig. 4). His-15, the PEP-dependent phosphorylation site for P∼EI, is nicely conserved in the HPr-like domain, whereas the surrounding of Ser-46

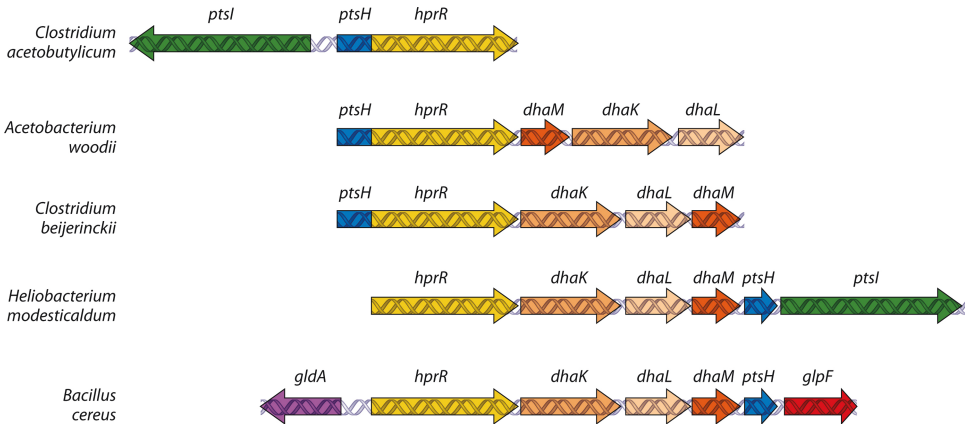

FIG 4 Gene arrangements around the *hprR* gene, encoding a σ^{54}-dependent regulator, in different organisms. The *hprR* gene is represented by the yellow arrows; when it is fused to *ptsH*, we call the gene *ptsH-hprR* and the protein accordingly PtsH-HprR. The *hprR* gene fused to *ptsH* was discovered in *C. acetobutylicum*, where a *ptsI* gene oriented in opposite direction is located upstream from *ptsH-hprR*. The *ptsI* gene is expressed from a σ^{54}-dependent promoter and therefore probably controlled by PtsH-HprR (CA_C3088). In other organisms, *ptsH-hprR* is part of the *dhaMKL* (*A. woodii*, Awo_c08990) or *dhaKLM* (*C. beijerinckii*, Cbei_2147) operon. The latter order of the *dha* genes is more common and is also found in *H. modesticaldum* and *B. cereus*. However, HprR of these two organisms (HM1_0841 and II5_05639, respectively) does not contain an HPr domain fused to its N terminus. Instead, a separate *ptsH* gene is located downstream from the *dhaKLM* genes, which is followed by a *ptsI* gene in *H. modesticaldum* and by a *glpF*-like gene in *B. cereus*. In the latter organism, a gene encoding a glycerol dehydrogenase-like protein (*gldA*) is located upstream from *hprR* and oriented in the opposite direction.

differs from that usually present in HPr of firmicutes. Indeed, a gene encoding a paralogue of EI (with 54% sequence identity to *B. subtilis* EI) is located just upstream from *hprR* and oriented in the opposite direction (Fig. 4). This is the only *ptsI* gene present in *C. acetobutylicum*, although a second *ptsH* gene is located elsewhere in the genome (unpublished observation). HPr encoded by the second *ptsH* also contains a well-conserved Ser-46. It is likely that the EI protein not only phosphorylates the second HPr but also transfers the phosphoryl group to His-15 in the HPr domain of HprR. Phosphorylation probably leads to structural changes affecting the affinity of HprR for its DNA target site. HprR does not contain the receiver module with the Asp phosphorylation site normally present in NtrC-like regulators. Activation of HprR by inter- or intramolecular transfer of the phosphoryl group from the HPr-like domain to a second phosphorylation site in the NtrC-like regulator is therefore unlikely. Similar to the case for other NtrC-type regulators, HprR probably controls the expression of RpoN (σ^{54})-dependent transcription units. Interestingly, the gene encoding the EI paralogue is preceded by a typical RpoN-dependent promoter, and its expression was therefore proposed to be controlled by HprR (52); HprR might nevertheless control the expression of other genes.

Genome sequencing revealed that HprR is present not only in *C. acetobutylicum* but also in many other bacteria of the order *Clostridiales*. In addition, an identical organization of the genes encoding HprR and the EI paralogue is found in several of them. In other *Clostridiales* the *hprR* gene is followed by the genes encoding the proteins for dihydroxyacetone phosphorylation, in the order *dhaMKL* or *dhaKLM* (Fig. 4). Interestingly, in a few species, such as *Halobacteroides halobius* and *Heliobacterium modesticaldum*, HPr is not fused to HprR, but *ptsH* and *ptsI* genes are located downstream from *hprR*, with the *dhaKLM* genes inserted between *hprR* and *ptsHI* (Fig. 4). Because in certain bacteria HprR exists without an HPr domain, we suggest that the form without the PTS protein be called HprR and that it be named PtsH-HprR when the PTS domain is fused to it. Accordingly, the gene names should be *hprR* and *ptsH-hprR* (Fig. 4). *H. halobius* and *H. modesticaldum* contain only the *ptsH* and *ptsI* genes located next to *hprR*. The gene arrangement in these organisms suggests that HprR might regulate the expression of the *dha* operon in response to dihydroxyacetone or glycerol availability. This hypothesis is further supported by our observation that in *Bacillus thuringiensis* IBL 200 and *Bacillus cereus* MSX-A1, the *hprR* gene is preceded by a gene encoding a glycerol dehydrogenase and followed by *dhaK*, *dhaL*, *dhaM*, *ptsH*, and *glpF*, with the last gene encoding a glycerol facilitator-like protein (Fig. 4). A *ptsHI* operon in these two organisms is located elsewhere on the genomes of the two bacilli. Glycerol metabolism via the enzymes encoded by the *dha* operon and the adjacent genes in the two bacilli thus resembles the glycerol utilization systems present in *L. monocytogenes* (64) and *E. faecalis* (65), which first oxidize intracellular glycerol to dihydroxyacetone, which is subsequently phosphorylated by the DhaKLM system.

HprR and PtsH-HprR probably control the expression of the glycerol/dihydroxyacetone system, depending on the phosphorylation state of the HPr-like domain or protein encoded by the associated or fused *ptsH* gene. In fact, in organisms possessing only the *ptsH* gene fused to *hprR*, such as *Clostridium beijerinckii*, the HPr-like domain of HprR probably participates in dihydroxyacetone phosphorylation by transferring the phosphoryl group received from P~EI to DhaM ($EIIA^{Dha}$), which in turn passes it on to the ADP molecule bound to DhaL. As already mentioned, the resulting ATP is used by DhaL to phosphorylate dihydroxyacetone bound to DhaK. It is therefore likely that the presence of dihydroxyacetone will lead to dephosphorylation of the HPr-like domain of HprR. Therefore, either the unphosphorylated general PTS component stimulates the transcription activator function of HprR or the phosphorylated HPr domain inhibits it. A similar activation or inhibition of HprR by HPr or P~His-HPr, respectively, probably occurs in bacteria where the phosphocarrier protein is encoded by a distinct protein. Finally, it should be noted that *C. acetobutylicum* does not possess a *dha* operon in its ge-

nome. The *ptsI* gene located upstream from *hprR* (Fig. 4) therefore remains the only presumed target for the σ^{54}-dependent regulator in this organism.

Interestingly, a protein belonging to the Fis family of transcriptional regulators from the two firmicutes *Eubacterium limosum* and *Tepidanaerobacter acetatoxydans* Re1 also contains an HPr-like domain (unpublished observation). These proteins exhibit significant sequence identity to BkdR of *B. subtilis*; however, *B. subtilis* BkdR, which controls the expression of the genes necessary for the utilization of the branched-chain amino acids valine and isoleucine (66), lacks an HPr-like domain. Unfortunately, for none of the HPr domain-containing proteins listed in Table 1 has the presumed regulatory role of the PTS domain been studied in detail.

Pyruvate kinase of firmicutes is a well-known example of proteins which do not contain an entire PTS domain fused to them but only a relatively small fragment, including the phosphorylation site with the conserved histidine or cysteine. In the case of pyruvate kinase, a peptide composed of about 50 amino acids exhibiting strong similarity to the surrounding of the EI autophosphorylation site is fused to its C terminus. The phosphorylatable His is located at position 539 in the sequence of *B. subtilis* pyruvate kinase, and the surrounding sequence (GGLTS**H**AAV) is nicely conserved compared to the phosphorylation site of *B. subtilis* EI (GGRTS**H**SAI). The role of the EI fragment is not known. Pyruvate kinase binds PEP to its active center but does not autophosphorylate at the conserved histidine in the EI fragment. The *B. subtilis* enzyme is also not phosphorylated by P∼EI or P∼His-HPr (G. Boël and J. Deutscher, unpublished results). It is therefore tempting to assume that HPr in phosphorylated or unphosphorylated form might bind to the EI-like fragment and thus affect the activity of pyruvate kinase.

Proteins fused to other PTS domains. In *Pasteurella multocida* Pm70 (6) and several other *P. multocida* strains as well as in *Actinobacillus succinogenes* 130Z, an entire $EIIB^{Glc}$-like domain is fused to another glycolytic enzyme, namely, triosephosphate isomerase. The phosphorylatable Cys is conserved in all these proteins, suggesting that their activity might be controlled by PTS-mediated phosphorylation in response to the presence or absence of glucose, which in most *Pasteuriaceae* is taken up via a PTS. The Na^+/H^+ symporter of *Borrelia burgdorferi*, the causative agent of borreliosis, and of other *Borrelia* strains carries an $EIIA^{Ntr}$-like domain fused to its C terminus (6). The phosphorylatable His residue is well conserved in the $EIIA^{Ntr}$-like domain. $EIIA^{Ntr}$ is a PTS protein assumed to be involved in the regulation of nitrogen metabolism (for a recent review, see reference 67). Finally, several *Vibrio* species possess an EAL domain characteristic of cyclic diguanylate phosphodiesterases (68), which contains fused to its C terminus an $EIIC^{Lac}$-like domain lacking about 170 amino acids at the N terminus. An $EIIB^{Lac}$-like domain usually fused to $EIIC^{Lac}$ is also absent. This protein therefore cannot be regulated by phosphorylation, because the EIIC domain does not contain a PTS phosphorylation site, but rather might respond to the binding of an extracellular substrate or interact with an $EIIB^{Lac}$ protein or domain of another PTS.

Another example of a protein containing a fragment of a PTS component is the C4-dicarboxylate transporter DcuA of *Enterobacteriaceae*, which contains at its C terminus a region of about 45 amino acids strongly resembling (45% sequence identity) the central part of $EIIA^{Glc}$, including the phosphorylation site (11). Although the surrounding of the phosphorylatable His of $EIIA^{Glc}$ is nicely conserved in DcuA (GVELFV**H**FG in $EIIA^{Glc}$ and GVALA V**C**FG in DcuA), the His itself is replaced with a Cys. However, Cys residues are also sometimes used as phosphorylation sites in PTS proteins (EIIB domain) and also in at least one PTS-controlled transcription activator (58). It is therefore tempting to assume that the DcuA transport activity might be controlled via either phosphorylation by or interaction with HPr or the $EIIB^{Glc}$ domain of the glucose-specific PTS permease. However, it should be noted that according to topology predictions, the C-terminal part of DcuA is quite hydrophobic and is thought to be embedded in the membrane.

There are numerous other proteins which contain clearly identifiable regulatory PTS domains, but their physiological role remains obscure. A list of some of these PTS domain-containing proteins of unknown function was presented by Barabote and Saier (6). Several of them contain a presumed helix-turn-helix motif fused to $EIIA^{Ntr}$; others were found to contain an $EIIA^{Fru}$-like domain at the N terminus and an FPr-like domain (fructose-specific HPr) at the C terminus. It will be interesting to unravel the role of some of these presumed regulatory PTS domains.

Proteins Containing a Specific PTS-Recognized Phosphorylation Domain, the PRD

Structures of PRD and PRD-containing proteins. In the course of evolution, bacteria must have produced a peptide composed of about 85 amino acids forming principally two slightly twisted antiparallel α-helices connected via an unstructured loop. The second α-helix contains a histidine, which is arranged in such a way that it can be phosphorylated by at least two different PTS proteins. The DNA region encoding this small peptide was probably duplicated in order to encode a protein fragment of about 170 amino acids. This polypeptide forms dimers (69, 70), thus finally providing what is known as the PTS regulation domain (PRD). Owing to duplication and dimerization, a PRD usually contains four conserved potential PTS phosphorylation sites. PRDs are frequently found to be fused to transcription regulators in firmicutes and actinobacteria but are less abundant in gammaproteobacteria (mostly in the family *Enterobacteriaceae*) and seem to be absent from alpha-, beta-, delta-, and epsilonproteobacteria. It is therefore likely that the PRDs evolved before the separation of firmicutes and actinobacteria and that some gammaproteobacteria acquired them later by horizontal gene transfer. Horizontal gene transfer probably also explains the presence of PRD-containing regulators in a few spirochaetes (treponemae and brachyspirae). Interestingly, in several pathogenic *E. coli* strains, genes encoding PRD-containing regulators are sometimes located in pathogenicity islands (55), which also supports the concept of horizontal gene transfer.

PRDs have so far been found in antiterminators and two types of transcription activators. These transcription activators were discussed in the preceding section, because they also contain EIIA and EIIB domains. Two PRDs are usually fused to the RNA binding (coantiterminator) domains of transcription antiterminators (called BglG/SacY-type antiterminators according to the first discovered members). The schematic presentation of LicT (a well-studied BglG/SacY family representative) in Fig. 2 shows only the LicT monomer. However, the crystal structure revealed that LicT forms dimers in which the two LicT subunits are aligned in paral-

lel. PRD1 and PRD2 of one subunit interact with PRD1 and PRD2, respectively, of the second subunit (70).

Two PRDs are also fused to the DNA binding domain and the Mga-like domain of MtlR/LicR type transcription activators (Fig. 2) (1). Again, only a monomer of the *B. subtilis* MtlR is presented in Fig. 2. However, the transcription activator is likely to form dimers similar to the model proposed in reference 71, with PRD1 and PRD2 of each subunit interacting with PRD1 and PRD2, respectively, of the second subunit. Finally, two PRDs are usually also present in LevR-type transcription activators, which contain a helix-turn-helix motif followed by an NtrC-like central domain and the first PRD (72) (Fig. 2). In these transcription activators, an $EIIA^{Man}$-like domain and an $EIIB^{Gat}$-like domain separate PRD1 from the usually truncated PRD2 containing only one phosphorylatable His. As already mentioned, in LevR-like proteins of several *Clostridiales* and a few other firmicutes, the second PRD is sometimes replaced with an $EIIA^{Mtl}$-like domain (Table 1) (Fig. 2).

PRD-containing antiterminators. The well-studied antiterminator LicT of *B. subtilis* controls the expression of the *bglPH* and *bglS* genes, which encode the PTS permease and catabolic enzymes for the uptake and metabolism of β-glucosides. The second PRD of LicT drastically alters its structure in response to specific mutations (His-Asp replacements) rendering the regulator constitutively active (69, 70). Similar structural changes are assumed to occur when PRD2 is phosphorylated by P-His-HPr or when other activating mutations are introduced into PRD2 (73, 74). These changes are probably transmitted to the RNA binding domain via PRD1 and finally lead to enhanced affinity of LicT for its target site, the ribonucleotidic antitermination target (RAT), on the corresponding nascent mRNA (1).

Unfortunately, LicT is so far the only PRD-containing protein for which the crystal structures of the two regulatory PRDs in active and inactive forms have been solved, but it is likely that similar phosphorylation-induced structural changes occur in PRDs of other antiterminators and probably also of the transcription activators. PRDs become phosphorylated by P∼His-HPr or P∼EIIBs. In most antiterminators, P∼His-HPr phosphorylates one or two histidine residues in PRD2 (73) and P∼EIIB phosphorylates one or two histidine residues in PRD1 (75).

PRD-containing transcription activators. The phosphorylation of PRDs in transcription activators is more variable. In some transcription activators, such as MtlR, only one PRD becomes phosphorylated by EI and HPr (58, 76), whereas in others, such as LicR, all four histidines of the two PRDs are phosphorylated by the general PTS components and mutation of either one of these histidines leads to a loss of function (56). In most LevR proteins (50, 57) and LevR-like transcription activators (77, 78), the C-terminal PRD2 is phosphorylated by EI, HPr, and the cognate EIIA and EIIB components. Surprisingly, the LevR-like ManR protein of *Listeria innocua* was reported to become phosphorylated by EI and HPr at PRD1 (77), whereas the almost identical ManR of *L. monocytogenes* was found to be phosphorylated by EI and HPr in the $EIIA^{Man}$-like domain (Fig. 2) (A. Zébré, F. Aké, E. Milohanic, and J. Deutscher, unpublished results). The general rule seems to be that phosphorylation of a PRD by P∼His-HPr stimulates the activity of the regulator, whereas phosphorylation by P∼EIIB inhibits it. There is at least one antiterminator, BglG from *E. coli*, which might not follow this rule. BglG was reported to be stimulated by the general PTS components EI and HPr in a phosphorylation-independent manner (79). However, recently BglG from *E. coli* was reported to become phosphorylated also by PEP, EI, and HPr as well as by PEP, EI, and FruB, a hybrid protein composed of the fructose-specific FPr and $EIIA^{Fru}$ (80). There is so far no explanation for these conflicting results.

In transcription activators, such as MtlR from *B. subtilis*, the stimulating signal has to be transmitted from amino acid His-342 in PRD2 across PRD1 and the Mga-like domain to the N-terminal DNA binding domain (71). The inactivating signal for LevR-type transcription activators is possibly transmitted even over a longer distance: from His-869 either to the active site for ATP hydrolysis in the NtrC-like central domain around amino acid 150 or all the way down to the N-terminal DNA binding domain (Fig. 2). As described above for the phosphorylation of EII domains in transcription activators, the absence of phosphorylation by P∼His-HPr owing to the uptake of an efficiently utilized carbon source is used as a CCR mechanism. In contrast, the absence of phosphorylation by the cognate P∼EIIB owing to the presence of the corresponding PTS substrate in the environment leads to the induction of the respective operon (1). A few antiterminators, such as *B. subtilis* GlcT, which controls the expression of the gene encoding the glucose-specific PTS permease and of *ptsHI*, do not require P∼His-HPr-mediated activation. In addition, P∼His-HPr-independent mutants affected in PRD1 or PRD2 could be obtained for antiterminators, such as LicT. These mutants allowed a conclusive confirmation that P∼His-HPr-mediated phosphorylation of these transcription regulators plays a role in CCR (74). The residual CCR observed for certain operons in firmicutes, in which the gene encoding the LacI/GalR-like catabolite control protein A (CcpA), the major player in CCR, had been deleted, disappeared when the corresponding regulator was mutated to a **P**TS-**i**ndependent **a**ntiterminator (Pia) or when the formation of P-Ser-HPr was prevented. In the latter case, the absence of P-Ser-HPr allows an efficient phosphorylation of the antiterminator by P∼His-HPr, even when glucose or other repressing sugars are present (74). It should be noted that in certain firmicutes, other pleiotropic CCR mechanisms exist, such as glucose kinase-mediated CCR in *Staphylococcus xylosus* (81). Even in a *ccpA* background, *pia* mutants might therefore not always cause a complete relief from CCR.

The Mga regulator of *S. pyogenes*. Mga, one of the regulators of *Streptococcus pyogenes* virulence genes, was also reported to be phosphorylated by EI and HPr (82). Phosphorylation was found to occur at a specific histidine in the central part of Mga, which was claimed to resemble PRDs. However, there does not seem to be significant sequence similarity between Mga and PRDs, and the alignment presented by Hondorp et al. (82) is not convincing. Significant sequence similarity between Mga and MtlR/LicR-type transcription activators was observed for only a small fragment of about 70 amino acids from the N-terminal part, which precedes PRD1 in the transcription activators (71). Hondorp et al. (82) also mention structural similarities between an *E. faecalis* Mga-like protein, for which the crystal structure has been solved, and the PRDs of LicT (69, 70). However, the tertiary structure of the dimers formed by these two proteins is completely different. The two subunits of unphosphorylated wild-type LicT are aligned in parallel, allowing the four histidines in the PRD1 and PRD2 dimers to face each other. In contrast, the two subunits of the *E. faecalis* Mga-like protein are oriented in an antiparallel fashion with mainly the C-terminal domain making contact to the other

subunit. The regions supposed to represent PRDs and containing the presumed phosphorylatable His are therefore located on opposite ends of the dimer. Again, only about 70 amino acids from the N-terminal part of the *E. faecalis* Mga-like protein exhibit structural similarity to the region preceding PRD1 in PRD-containing transcription activators (71), which might contribute to DNA binding. It is therefore likely that what the authors report in reference 82 might be more important than just another PRD. Similar to the case for GlpK, Mga and related transcription regulators, such as AtxA (83), might have developed their own PTS-specific phosphorylation sites.

Independently from the question whether Mga contains PRDs or not, Hondorp et al. unequivocally show that PEP-dependent phosphorylation of *S. pyogenes* Mga requires the general PTS components EI and HPr (82). PEP-dependent phosphorylation of Mga strongly inhibited *in vitro* expression of *emm*, one of the Mga-regulated virulence genes. However, there remain some uncertainties concerning the site of phosphorylation in Mga (82). Replacement of His-270, one of the histidines proposed to be located in the presumed PRD1, did not significantly diminish Mga phosphorylation. Surprisingly, Hondorp et al. did not change the two other histidines proposed to resemble the phosphorylatable His in PRDs based on their sequence alignment (His-175 and His-301) but instead replaced His-204 with an alanine. This mutation also had no significant effect on Mga phosphorylation compared to the wild-type protein. Accordingly, doubly mutated Mga(His-204-Ala,His-270-Ala) was also still phosphorylated. Finally, triply mutated Mga(His-204-Ala,His-270-Ala,His-324-Ala), with His-324 also not being one of the presumed conserved PRD-related histidines, was only slightly phosphorylated. However, several radioactive bands of lower molecular weight appeared, suggesting that the triply mutated protein might be unstable and be degraded. It remains to be determined whether replacement of the presumed phosphorylatable His-175 and His-301 has an effect on Mga phosphorylation. Nevertheless, the results presented in reference 82 suggest that PTS-mediated phosphorylation does not occur in the C-terminal part of Mga, because truncated Mga lacking the last 139 amino acids was as strongly phosphorylated by PEP, EI, and HPr as the full-length protein.

The utilization of glucose and other rapidly metabolizable carbon sources by *S. pyogenes* stimulates the expression of its virulence genes (84). Two regulatory mechanisms are probably responsible for this effect. First, expression of *mga* was found to be stimulated by carbon catabolite activation. The elevated amounts of glucose-6-P and FBP present in cells utilizing glucose or other efficiently metabolized carbon sources probably allow binding of CcpA to a specific operator site preceding *mga* (84). Second, in the absence of an efficiently transported PTS sugar, the PTS components are predominantly phosphorylated at their conserved His and Cys residues. The results reported previously (82) suggest that under these conditions Mga is also phosphorylated, leading to its inhibition and therefore to poor expression of virulence genes. In contrast, in the presence of glucose, firmicutes convert most of their HPr into P-Ser-HPr, and little P~His-HPr is therefore present. Mga is probably also barely phosphorylated and remains active, thus leading to strong virulence gene expression. Mutants producing Mga proteins with the above-described His-Ala replacement, but also additional mutants with presumed phosphomimetic His-Asp replacements, were tested as to whether they exhibit altered expression of Mga-regulated genes (82). The Ala replacements had only slight effects on the expression of the Mga-controlled *arp* and *sof* genes. In contrast, the presumed phosphomimetic mutants producing Mga(His-204-Asp,His-270-Asp) and Mga(His-204-Asp,His-270-Asp,His-324-Asp) exhibited very low Mga activity similar to that of an *mga* deletion mutant. However, owing to the uncertainty about the PTS phosphorylation site(s), it remains to be confirmed that there is a correlation between PTS phosphorylation and Mga activity.

REGULATION BY PROTEIN-PROTEIN INTERACTION

PTS components control the activity of their target proteins not only via phosphorylation but also via direct interaction. Interaction-mediated regulation seems to be even more frequent than regulation by phosphorylation. As described above for PTS-catalyzed phosphorylation of proteins at His or Cys residues, interactions with target proteins have been reported for HPr, EIIA, and EIIB components and in one case also for EI. Interactions occur with the unphosphorylated or phosphorylated forms of these proteins. The target proteins carry out various cellular functions, with the majority acting either as transport proteins or as transcription regulators.

Interaction with Unphosphorylated PTS Proteins

The interaction with a PTS protein and the accompanying activation or inhibition of the non-PTS target protein usually depend on the phosphorylation state of the PTS component. In fact, the phosphorylatable amino acids of PTS proteins and their surrounding always seem to be part of the interface of the protein-protein complex (85). If phosphorylation of a PTS component prevents the interaction with its target protein, the phosphorylatable amino acid is usually facing a negatively charged region. The phosphorylatable His, Cys, or Ser (in seryl-phosphorylated HPr) frequently adds to the stability of the complex by forming hydrogen bonds with amino acids of the target protein, which, of course, is prevented when the PTS protein is phosphorylated.

Inducer exclusion in *Enterobacteriaceae*. In most established PTS-mediated regulation mechanisms, PTS components interact in their unphosphorylated forms with non-PTS proteins. The first PTS components shown to interact with a non-PTS protein were the Crr proteins from *E. coli* and *S. enterica* serovar Typhimurium, which were later identified as glucose-specific EIIAs of the PTS ($EIIA^{Glc}$) (86). *E. coli* mutants defective in EI and/or HPr had lost the capacity to synthesize the proteins necessary for the uptake and metabolism of several non-PTS carbon sources, including glycerol, lactose, and maltose (87). The repressive effect of the *ptsHI* mutation could be overcome by a second mutation located in a gene close to the *ptsHI* operon. This gene was called *crr* for **c**atabolite **r**epression **r**esistant, because *crr* mutations also prevented the repressive effect of efficiently metabolizable carbohydrates on the synthesis of the enzymes necessary for the transport and metabolism of less favorable carbon sources.

Inactivation of *ptsHI* prevents the phosphorylation of $EIIA^{Glc}$, and it was therefore thought that under conditions where $EIIA^{Glc}$ would be present mainly in unphosphorylated form, it would inhibit the synthesis of the catabolic enzymes required for the metabolism of non-PTS carbon sources. Unphosphorylated $EIIA^{Glc}$ indeed prevails when an efficiently metabolized carbon source, such as glucose, is taken up (28). This concept was also in agreement with the observation that inactivation of the *crr* gene could overcome the repressive effect of the *ptsHI* mutation. However,

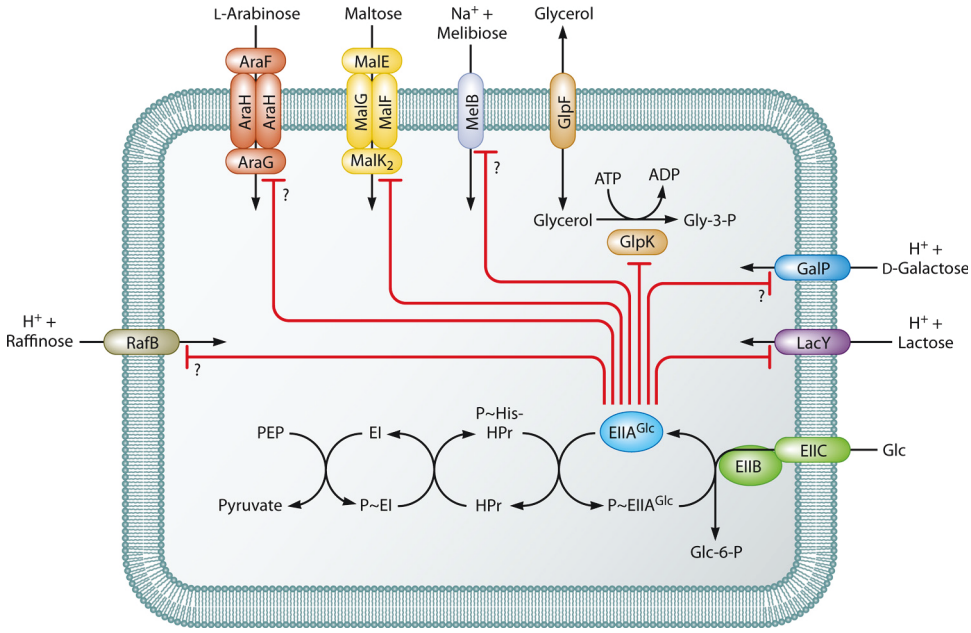

FIG 5 PTS-catalyzed glucose uptake and the EIIA^{Glc} inducer exclusion "regulon" of *E. coli*. Unphosphorylated EIIA^{Glc} but not phosphorylated EIIA^{Glc} interacts with several transport proteins or catabolic enzymes and inhibits their activity. Direct interactions have been shown for the catabolic enzyme glycerol kinase GlpK (88), the ATP binding subunit MalK of the maltose/maltodextrin-specific ABC transport system (89, 90), and the lactose permease LacY (91, 92). The crystal structures of the complexes formed between EIIA^{Glc} and the ATP-hydrolyzing MalK protein (106) as well as EIIA^{Glc} and GlpK, which catalyzes the ATP-dependent phosphorylation of glycerol to glycerol-3-P, have been solved (85). The uptake of the other four carbohydrates, L-arabinose, D-galactose, melibiose, and raffinose, is also subject to inducer exclusion, which is prevented when the EIIA^{Glc}-encoding *crr* gene is deleted. For the transporters labeled with a question mark, a direct interaction with EIIA^{Glc} is suggested by genetic data but has so far not been established by using biochemical or immunological methods.

mutants in which the repression caused by the *ptsHI* mutation was relieved only for one specific carbon source and not the others could be isolated. These mutations mapped not within *crr* but within the genes encoding the corresponding transporter, which therefore suggested that EIIA^{Glc} might interact with and inhibit proteins necessary for the uptake of the various carbon sources affected by the *ptsHI* mutation. Indeed, it was subsequently demonstrated that in *E. coli* and *S. enterica* serovar Typhimurium EIIA^{Glc} interacts with glycerol kinase (GlpK) (88) and the ATP binding protein of the maltose/maltodextrin ABC transport system (MalK) (89, 90) and that in *E. coli* EIIA^{Glc} interacts also with the lactose permease (LacY) (91, 92). In all cases, the interaction with unphosphorylated EIIA^{Glc} leads to inhibition of the non-PTS proteins (93, 94), which are usually transporters or transporter-associated components. Their inhibition prevents the uptake of the corresponding carbon source and consequently the formation of the necessary inducer; this regulation mechanism was therefore called inducer exclusion. In subsequent studies it was found that in *Enterobacteriaceae*, melibiose (95, 96), raffinose (97), galactose (98), and arabinose (99) also belong to the set of non-PTS sugars subject to inducer exclusion. The different carbohydrate utilization systems controlled by EIIA^{Glc} in *E. coli* are summarized in Fig. 5. EIIA^{Glc}-mediated inducer exclusion seems to be restricted to the family *Enterobacteriaceae*, indicating that this regulatory mechanism evolved relatively recently. It nevertheless represents the major CCR mechanism operative in *Enterobacteriaceae* (100). The term inducer exclusion is also used for EIIA^{Glc}-mediated GlpK inhibition, although GlpK is not a transport protein. Nevertheless, GlpK catalyzes the ATP-dependent phosphorylation of glycerol to glycerol-3-P (Fig. 5), the inducer of the glycerol operon in proteobacteria and most other bacteria. Because glycerol enters bacteria by facilitated diffusion, GlpK activity is important for efficient glycerol uptake. The transport-related activity of GlpK also explains why GlpK inhibition in firmicutes, which is not mediated via interaction with EIIA^{Glc} but occurs when the conserved phosphorylatable histidine in GlpK is not efficiently phosphorylated by P~His-HPr (see "Proteins Containing a Specific PTS-Recognized Phosphorylation Domain, the PRD," above), is also considered inducer exclusion (40). It is interesting to note that regarding GlpK regulation, two completely different inducer exclusion mechanisms evolved in firmicutes and *Enterobacteriaceae*, both of which nevertheless are mediated by PTS proteins: activation by P~His-HPr-mediated phosphorylation in firmicutes and inhibition by interaction with unphosphorylated EIIA^{Glc} in *Enterobacteriaceae*.

Other EIIA^{Glc} interaction partners. A regulatory role of EIIA^{Glc} completely different from that in inducer exclusion was reported for this PTS component in *Vibrio vulnificus*. EIIA^{Glc} as well as P~EIIA^{Glc} was found to interact with a bacterial 100-kDa protein which exhibits strong similarity to mammalian insulysins (101). Insulysins are peptidases that degrade insulin, and the *V. vulnificus* protein was therefore called vIDE for *Vibrio* insulin-degrading enzyme. Although both unphosphorylated and phosphorylated EIIA^{Glc} interact with vIDE, only the unphosphorylated PTS protein stimulated its insulin-degrading activity. Deletion of the vIDE-encoding *ideV* gene significantly lowered the survival and virulence of *V. vulnificus* in mice. It was therefore concluded that the vIDE/EIIA^{Glc} complex plays an important role in the survival of the bacterium in the host cell by sensing glucose (101).

In a more general study, it was attempted to detect interaction

partners of wild-type and unphosphorylatable (with replacement of the phosphorylatable His-90 with Ala) EIIA^{Glc} (11) of the pathogen *Vibrio cholerae* in planktonic and biofilm cells by carrying out a tandem affinity analysis. Several EIIA^{Glc} interaction partners were identified, some of which had already previously been shown to bind to the PTS protein in *Enterobacteriaceae*, such as glycerol kinase (see above) and adenylate cyclase (see "Interaction with phosphorylated EIIA components" below). However, several other proteins were also found to interact with EIIA^{Glc}. Interaction partners in planktonic cells include the glycolytic enzyme 6-phosphofructokinase, the purine repressor PurR, and a protein containing the GGDEF motif characteristic of diguanylate cyclases (VC0900) (102). In biofilm cells, wild-type and His-91-Ala mutant EIIA^{Glc} interacted with the gluconeogenic enzyme PEP carboxykinase. The two forms of EIIA^{Glc} also interacted with MshH, a homologue of the carbon storage regulator CsrD (103), a Mg^{2+} transporter, and a protein of unknown function (VC1291). Because for these three proteins the number of peptides found to interact with wild-type EIIA^{Glc} was significantly higher than the number of peptides binding to His-91-Ala mutant EIIA^{Glc}, it is likely that they interact primarily with P∼EIIA^{Glc}.

Among the proteins so far identified as EIIA^{Glc} interaction partners, the *E. coli* fermentation/respiration switch protein FrsA seems to have the highest affinity for unphosphorylated EIIA^{Glc}. A dissociation constant as low as 2×10^{-7} M was measured for the FrsA (previously called YafA) complex with EIIA^{Glc} (104). This protein is present in most facultative anaerobic gammaproteobacteria and is also found in a few alpha- and betaproteobacteria, such as *Thauera selenatis* and *Hirschia maritima*. Its deletion in *E. coli* leads to increased cellular respiration, whereas its overexpression causes elevated fermentation. FrsA was therefore proposed to regulate the partitioning of the flux through either the respiration or fermentation pathways in response to the phosphorylation state of EIIA^{Glc} (104).

The EIIA^{Glc} interface in protein-protein interactions. The surface of EIIA^{Glc} interacting with the various target proteins is almost always the same. For the complex formed between *E. coli* EIIA^{Glc} and GlpK, the crystal structure has been solved (85). The phosphorylatable His-90 of EIIA^{Glc} (11) is part of the interface and is exposed to a negatively charged surface of GlpK, including Glu-478. This explains why phosphorylation of EIIA^{Glc} strongly lowers its affinity for GlpK. In contrast, the affinity of EIIA^{Glc} for GlpK was strongly enhanced by the presence of a Zn^{2+} ion in the complex, which was found to be liganded by amino acid residues from both proteins: His-90 of EIIA^{Glc} and Glu-478 of GlpK (105). Recently, the structure of the maltose-specific ABC transporter with bound EIIA^{Glc} has also been determined (106). Each of the two EIIA^{Glc} molecules bound to the ABC transporter interacts with both MalK subunits. The surface of EIIA^{Glc} containing the phosphorylatable His-90 (11) interacts with the nucleotide binding domain of MalK, and a second region centered around Lys-130 interacts with the dimerization domain of the other subunit. Binding of EIIA^{Glc} prevents MalK closure by stabilizing a resting state of the ABC transporter. MalK closure is required for the interaction of the maltose-loaded periplasmic maltose binding protein with the transmembrane proteins MalF and MalG and for the binding of ATP to MalK (Fig. 5). These two events induce the outward-facing conformation of the membrane proteins, which is essential for transferring maltose from the maltose binding protein to the binding site in the transmembrane proteins (106). Interestingly, out of the 17 residues of EIIA^{Glc} making contacts with the nucleotide binding domain of MalK, 11 are also involved in the interaction with GlpK (85), HPr (107), and EIIB^{Glc} (108), thus confirming that EIIA^{Glc} uses nearly identical surfaces for the interaction with its partner proteins in order to exert its catalytic and regulatory functions.

PTS^{Ntr}. The other EIIA component that has been shown to interact with numerous non-PTS proteins in an unphosphorylated form is EIIA^{Ntr}, an EIIA of the fructose/mannitol PTS family, which exhibits neither sequence nor structure similarity to EIIA^{Glc} (109). In addition, the phosphorylation sites of the two proteins are quite different (11, 19, 110). In alpha-, beta-, and gammaproteobacteria, the *ptsN* gene, which encodes EIIA^{Ntr}, is frequently located downstream from *rpoN* (111). The RpoN protein, also known as σ^{54}, is required for the expression of several genes encoding enzymes for the utilization of nitrogen sources, but RpoN also regulates the expression of many other genes and operons. Inactivation of the *ptsN* gene from *Klebsiella pneumoniae* lowered the expression of several RpoN-controlled transcription units (112), suggesting that EIIA^{Ntr} might directly or indirectly affect RpoN activity. Also sometimes associated with the *rpoN* region is the *npr* gene (also called *ptsO*), which encodes NPr, a paralogue of HPr containing a phosphorylatable His-15 and, with the exception of *Enterobacteriaceae*, also a phosphorylatable Ser-46. In *Enterobacteriaceae*, a paralogue of EI is encoded by the *ptsP* gene (usually located distant from *rpoN*, *ptsO*, and *ptsN*). Together the three proteins EI^{Ntr} (PtsP), NPr (PtsO), and EIIA^{Ntr} (PtsN) form what is referred to as the nitrogen PTS (PTS^{Ntr}) (113). In *Enterobacteriaceae*, phosphorylation of NPr at His-15 is catalyzed mainly by EI^{Ntr}. Nevertheless, in bacteria also possessing EI, HPr, and sugar-specific EIIB and EIIC components, such as most *Enterobacteriaceae*, a cross talk between the two types of PTS exists (114, 115). The activity of EI^{Ntr} was recently shown to be antagonistically regulated by the metabolic intermediates situated at the intersection between carbon and nitrogen metabolism, namely, α-ketoglutarate and glutamine (116). Components of the PTS^{Ntr} are found not only in *Enterobacteriaceae* but also in many proteobacteria that are devoid of any known EIIB and EIIC components. In these organisms, the proteins of the PTS^{Ntr} are sometimes called EI, HPr, and EIIA^{Fru}. An HPr kinase able to phosphorylate NPr at Ser-46 and an EIIA of the mannose class PTS are usually also found in organisms containing the incomplete PTS^{Ntr} (111), except in *Enterobacteriaceae*, which are devoid of HprK/P. The genes encoding these five proteins are usually organized in two operons, as was shown for *Brucella melitensis* (19) and *Ralstonia eutropha* (110). In most alphaproteobacteria, such as *B. melitensis* and *Agrobacterium tumefaciens*, the *hprK* and *ptsO* genes are located downstream from the genes encoding a two-component system called *chvI*/*chvG* in *A. tumefaciens* (for chromosomal virulence) (111). In most betaproteobacteria, such as *R. eutropha* and *Neisseria meningitidis*, the EIIA^{Man}-encoding gene as well as *ptsO* and *ptsP* seem to form an operon, whereas *ptsN* and *hprK* are located in a second operon with *hprK* being followed by the *rapZ* (former *yhbJ*) gene (110). In *E. coli*, RapZ acts as adaptor protein allowing RNase E to degrade the small RNA GlmZ, which is required for the translation of the glucosamine-6-P synthase-encoding RNA (117).

Surprisingly, only EIIA^{Ntr} and not EIIA^{Man} of *R. eutropha* could be phosphorylated with PEP and purified EI^{Ntr} and NPr (110), although the phosphorylatable His residue is conserved in

$EIIA^{Man}$. Owing to the absence of an entire PTS able to transport carbohydrates, it is not clear what exactly controls the phosphorylation states of the usually four soluble PTS components in these organisms. It is likely that the PEP/pyruvate ratio (28) and also the α-ketoglutarate/glutamine ratio (116) affect the phosphorylation states of these PTS proteins. In *E. coli*, the two metabolites α-ketoglutarate and glutamine bind to EI^{Ntr} and exert antagonistic effects on its activity. In contrast, in *Sinorhizobium meliloti* only glutamine was found to bind to the N-terminal GAF domain of EI^{Ntr} and to stimulate its autophosphorylation activity (118). In addition, it seems that the ATP-dependent phosphorylation of NPr affects the PTS phosphorylation cascade, because deletion of the *hprK* gene in *B. melitensis* led to significantly increased phosphorylation of $EIIA^{Ntr}$ compared to that in the wild-type strain (19). In *R. eutropha*, an *hprK* mutant could be isolated only in an *npr* (*ptsO*) background, suggesting that elevated phosphorylation of HPr at His-15 owing to the absence of phosphorylation at Ser-46 negatively affects growth of this organism (110). Indeed, the *npr* mutant could easily be complemented with wild-type *npr* and the *npr*(His15Ala) allele, whereas no transformants could be obtained with the *npr*(Ser46Ala) allele.

Regulation of K^+ transport. The components of the PTS^{Ntr} are involved not only in the regulation of nitrogen-related genes and proteins (113). $EIIA^{Ntr}$ also seems to play an important role in the control of low- and high-affinity potassium (K^+) transport systems. Indeed, the PTS component was shown to interact with TrkA (119), a soluble protein required for K^+ uptake via the transporter TrkH (120). TrkA forms a tetrameric ring which needs to bind ATP in order to exert its positive effect on the single-channel activity of TrkH (121). It is therefore tempting to assume that the interaction of $EIIA^{Ntr}$ with TrkA prevents its stimulating effect on TrkH and therefore on the uptake of K^+. Indeed, a *ptsN* mutant accumulates high concentrations of K^+ inside the cells. K^+ differentially affects the binding of σ^{70} and σ^S to apo RNA polymerase and thus influences σ factor selectivity (122). In contrast to $EIIA^{Ntr}$, phosphorylated $EIIA^{Ntr}$ does not interact with TrkA.

In addition to the low-affinity Trk system, *E. coli* also possesses a high-affinity transporter for K^+ composed of the KdpFABC components, which form a P-type ATPase (123). The expression of the genes encoding these proteins is regulated by the two-component system KdpD/KdpE, with KdpD being a kinase sensing extracellular K^+ probably via aspartyl residues located in its periplasmic loops (124) and KdpE being the response regulator. The two regulator genes *kdpD* and *kdpE* are located downstream from *kdpFABC*. When the K^+ concentration in the medium is low, KdpD autophosphorylates and subsequently passes the phosphoryl group on to KdpE. The phosphorylated response regulator promotes transcription initiation of the *kdpFABCDE* genes (125). Unphosphorylated $EIIA^{Ntr}$ was shown to bind to KdpD and to stimulate its kinase activity, thereby increasing the amount of phosphorylated KdpE and hence the expression of the *kdpFABCDE* genes (126). In agreement with this result, inactivation of the *ptsN* gene caused strongly reduced expression of the *kdpFABCDE* genes. As observed for other $EIIA^{Ntr}$ interaction partners, phosphorylation of the PTS component prevents its interaction with KdpD. Owing to the already-mentioned cross talk in *E. coli* between the components of the regular PTS and the PTS^{Ntr}, growth on glucose also leads to dephosphorylation of $EIIA^{Ntr}$ and consequently to elevated expression of the *kdpFABCDE* genes (126). The opposing regulation of the two K^+ transport systems by $EIIA^{Ntr}$ allows *E. coli* to shut down the low-affinity transporter TrkA when the bacterium is exposed to low K^+ concentrations and to shut down KdpFABC when facing high K^+ concentrations.

Interestingly, mutants of *Rhizobium leguminosarum* lacking both $EIIA^{Ntr}$ proteins present in this organism were unable to grow at low K^+ concentrations (127). Unphosphorylated $EIIA^{Ntr}$ was assumed to interact with KdpD/E and to stimulate the expression of the genes encoding the high-affinity K^+ transporter. Bacterial two-hybrid experiments indeed revealed an interaction of $EIIA^{Ntr}$ with the transcription regulator proteins KdpD/E. In agreement with this concept, a *ptsP* mutant containing only unphosphorylated $EIIA^{Ntr}$ due to the absence of EI^{Ntr} grew normally at low K^+ concentrations. Interestingly, $EIIA^{Ntr}$ was found to regulate several other ABC transporters in *R. leguminosarum* (127).

Other $EIIA^{Ntr}$ interaction partners. Another *E. coli* sensor kinase that was reported to be regulated by $EIIA^{Ntr}$ is PhoR (128). Together with the response regulator PhoB, PhoR controls the expression of the *pho* regulon, which includes more than 30 genes organized in nine transcription units. When *E. coli* cells are exposed to low phosphate concentrations, the kinase activity of PhoR increases, which results in an elevated amount of P∼PhoB. The phosphorylated response regulator binds to specific sites on the DNA, the so-called Pho boxes, and stimulates the expression of the genes of the *pho* regulon. Mutants devoid of $EIIA^{Ntr}$ exhibit reduced expression of the *pho* regulon. Deletion of *ptsN* lowers the expression of the *pho* regulon because in order to be fully active, the sensor kinase PhoR needs to interact with $EIIA^{Ntr}$. This activation seems to occur with unphosphorylated $EIIA^{Ntr}$, because mutants unable to phosphorylate the EIIA component also exhibited increased expression of the *pho* regulon (128).

Unphosphorylated $EIIA^{Ntr}$ was also found to interact with the E1 subunit of *Pseudomonas putida* pyruvate dehydrogenase and to inhibit its activity (129). This enzyme produces acetyl coenzyme A (acetyl-CoA) from pyruvate, and the presence of $EIIA^{Ntr}$ therefore inhibits the metabolism through the tricarboxylic acid cycle. Interestingly, through yeast two-hybrid experiments the $EIIA^{Man}$ component of the incomplete *B. melitensis* PTS was demonstrated to interact with the E1 subunit (SucA) of 2-oxoglutarate dehydrogenase, which has a subunit composition similar to that of pyruvate dehydrogenase (19). In addition, a fusion protein composed of SucA and DivIVA sequestered $EIIA^{Man}$ to the two cell poles, thus confirming the interaction of $EIIA^{Man}$ with SucA. 2-Oxoglutarate dehydrogenase is part of the tricarboxylic acid cycle and catalyzes the transformation of 2-oxoglutarate into succinyl-CoA. The effect of phosphorylation of $EIIA^{Man}$ on this protein-protein interaction and its physiological role have not been studied.

The virulence of the pathogen *S. enterica* serovar Typhimurium is also controlled by PTS proteins. First, it has been reported that inactivation of the general PTS components EI and HPr prevents the replication of this bacterium in macrophages (130). Second, deletion of $EIIA^{Ntr}$ was found to indirectly affect the virulence of this pathogen. $EIIA^{Ntr}$ was reported to interact with the response regulator SsrB, which is required for the expression of numerous genes located within pathogenicity island 2 of this bacterium (131). The interaction with $EIIA^{Ntr}$ prevents SsrB from stimulating the expression of the genes in pathogenicity island 2.

Unphosphorylated $EIIA^{Ntr}$ was also reported to be necessary for the derepression of the *E. coli ilvBN* genes, which encode acetohydroxy acid synthase I, the first common enzyme for the synthesis of branched-chain amino acids (132). Mutants with *ptsN*

deletions were extremely sensitive to leucine-containing peptides, whereas *ptsP* and *npr* (*ptsO*) mutants were more resistant than the wild-type strain. However, the interaction partner of $EIIA^{Ntr}$ for the regulation of the *ilvBN* genes has not yet been identified.

In the betaproteobacterium *R. eutropha*, $EIIA^{Ntr}$ was found to interact with the bifunctional ppGpp synthase/hydrolase (SpoT1), an enzyme involved in the stringent response. Bacterial two-hybrid assays indicated that this interaction occurs only with the unphosphorylated form of $EIIA^{Ntr}$ (133).

Interaction with EIIB components. There are also several examples where EIIB components (as distinct proteins or as domains) interact with transcription regulators. When the EIIB component is fused to the cognate transmembrane permease, the target proteins are sequestered to the membrane. The first such example was the *E. coli* repressor Mlc, which was reported at nearly the same time by three different groups (134–136) to interact with the $EIIB^{Glc}$ domain of PtsG, the glucose-specific PTS permease. Mlc is a repressor of the ROK (**r**epressors, **o**pen reading frames, and **k**inases) family, and it was discovered because transformation of an *E. coli* wild-type strain with a plasmid into which the *mlc* gene had been inserted led to the formation of colonies with increased size when the transformants were grown on glucose-containing solid medium (137). The name *mlc* (**m**aking **l**arge **c**olonies) was deduced from this phenotype. The *mlc* gene turned out to be allelic with the previously described *dgsA* (2-**d**eoxy-D-**g**lucose **s**ensitive) locus (138).

E. coli Mlc is a regulator which represses the expression of several genes, including *mlc*, *ptsG* (*ptsG* encodes the $EIIB^{Glc}$ and $EIIC^{Glc}$ domains of the glucose-specific PTS permease), *ptsHI-crr*, *manXY*, and *malT* (1, 2, 4). The interaction of Mlc with the unphosphorylated $EIIB^{Glc}$ domain inhibits its repressor function (for reviews, see references 1, 2, and 4). Deletion of the *ptsG* gene therefore prevented the expression of all Mlc-controlled genes (139, 140).

An interesting aspect of this regulation mechanism is that Mlc needs to be sequestered to the membrane by the $EIIB^{Glc}$ domain in order to become inactive. When $EIIB^{Glc}$ was produced as a distinct cytoplasmic protein instead of being fused to the membrane-integral $EIIC^{Glc}$, it was still able to bind Mlc but no longer inhibited its activity (141). Possibly, cytoplasmic $EIIB^{Glc}$ has a lower affinity for Mlc and might therefore have lost its inhibiting effect. However, fusing the $EIIB^{Glc}$ domain to another membrane protein prevented Mlc activity, suggesting that the interaction with the membrane environment is important for Mlc regulation (141, 142). The crystal structure of the complex formed by the $EIIB^{Glc}$ domain and Mlc revealed that Mlc forms tetramers with each subunit binding an $EIIB^{Glc}$ domain. In fact, $EIIB^{Glc}$ binds to Mlc in the central part of the protein between the N-terminal DNA binding motif and the C-terminal dimerization domain. This interaction lowers the affinity of the repressor for its target sites (142). However, interaction with the $EIIB^{Glc}$ domain alone does not seem to be sufficient for Mlc inactivation. Interaction with the hydrophobic membrane environment also seems to be required (143). $EIIB^{Glc}$-mediated membrane sequestration was proposed to cause structural restrictions leading to reduced flexibility and therefore to lower affinity for its DNA targets (142). Arg-424 located close to the phosphorylatable Cys-421 in PtsG makes polar contacts with the carboxylate of the C-terminal glycine of Mlc (136, 144). Unphosphorylated $EIIB^{Glc}$ prevails when the cells take up glucose. As a consequence, the uptake of glucose leads to $EIIB^{Glc}$-mediated sequestration of Mlc to the membrane and therefore to increased expression of Mlc-repressed genes (140). It should be noted that Mlc activity is also inactivated by the interaction with the cytoplasmic protein MtfA (Mlc titration factor A) when this protein is overproduced (145).

Other proteins regulated by interaction with unphosphorylated EIIB domains or distinct EIIB proteins include PRD-containing transcription activators. The first example was MtlR from *B. subtilis*, the DeoR-type transcription activator of the mannitol operon, which is also regulated via phosphorylation by P∼His-HPr and $P{\sim}EIIA^{Mtl}$ (see "Proteins Containing a PTS Component Fused to the N or C Terminus" and "Proteins Containing a Specific PTS-Recognized Phosphorylation Domain, the PRD" above). In addition, the $EIIB^{Mtl}$ domain of the mannitol-specific PTS permease interacts with the two C-terminal regulatory domains of MtlR and thereby stimulates its transcription activation function (146). $EIIB^{Mtl}$ fused to the tyrosine kinase modulator YwqC (147), a transmembrane protein not related to mannitol transport, also interacted with MtlR and stimulated its activity. However, similar to what was reported for Mlc, interaction with the $EIIB^{Mtl}$ domain is not sufficient for MtlR activation, because overproduction of $EIIB^{Mtl}$ as a distinct cytoplasmic protein in the *B. subtilis* wild-type strain 168 prevented induction of the *mtl* operon. In this strain, soluble and membrane-associated $EIIB^{Mtl}$ probably compete for binding MtlR, and owing to the excess of soluble $EIIB^{Mtl}$, little MtlR is sequestered to the membrane and activated. The interaction with the membrane environment is therefore also required for MtlR stimulation. This interaction seems to occur only with unphosphorylated $EIIB^{Mtl}$, because first, the Cys-Asp replacement in $EIIB^{Mtl}$ prevented the interaction with MtlR in yeast-two hybrid experiments (146) and second, replacement of the phosphorylatable Cys with a Ser in soluble $EIIB^{Mtl}$ prevented the inhibitory effect on P*mtlA* expression observed with wild-type $EIIB^{Mtl}$ (71). In contrast to cysteyl-phosphorylated $EIIB^{Mtl}$, seryl-phosphorylated $EIIB^{Mtl}$ cannot transfer its phosphoryl group to mannitol (148) and, owing to the low-energy phosphate bond, probably cannot pass it back to $EIIB^{Mtl}$. As a consequence, P-Ser-$EIIB^{Mtl}$ accumulates in the cytoplasm, and the phosphorylated mutant $EIIB^{Mtl}$ apparently no longer competes with the $EIIB^{Mtl}$ domain of MtlA for binding MtlR and rendering it inactive (71).

Several results contradictory to the above model have been published by the group of Altenbuchner. For example, it was reported that the simultaneous deletion of *mtlA* and *mtlF* causes strong constitutive expression from the P*mtlA* promoter (149), whereas according to the results reported in reference 58, deletion of both *mtlA* and *mtlF* led to MtlR inactivation and only the single deletion of *mtlF* caused constitutive MtlR activity. In a more recent study from the Altenbuchner laboratory, deletion of the entire *mtl* operon, including *mtlD*, was found to inhibit expression from P*mtlA* (76). Although MtlR regulation in other bacterial species, such as *Geobacillus stearothermophilus* and *L. casei* (P. Joyet and J. Deutscher, unpublished results), significantly differs from that observed in *B. subtilis*, it is unlikely that the use of different *B. subtilis* strains (3NA and 168) might account for the observed differences in MtlR regulation. A more likely explanation might be the use of a plasmid-borne reporter gene fusion with an optimized P*mtlA* promoter in one study and a chromosome-integrated reporter gene fusion with wild-type P*mtlA* in the other. Heravi and Altenbuchner also recently reported that a *B. subtilis*

mutant MtlR in which His-342 was replaced with an Asp and Cys-419 with an Ala was active even when produced in a mutant lacking MtlA (EIICB^{Mtl}) (76). These results were also thought to be contradictory to the above model of EIIB^{Mtl}-mediated MtlR activation. A possible explanation for this EIIB^{Mtl}-independent MtlR activity could be that the *mtlR*(His-342-Ala,Cys-419-Ala) double mutation not only prevents the inactivating phosphorylation at Cys-419 but also induces structural changes in the transcription activator resembling those caused by EIIB^{Mtl}-mediated membrane sequestration.

The second PRD-containing transcription activator regulated by the interaction with an EIIB domain is the LevR-like ManR from *L. monocytogenes*. This protein controls the expression of the *manLMNO* operon, which encodes the major glucose transporter of this human pathogen (78, 150). However, ManR activity is regulated not by the components of the mannose-type PTS ManLMN but by the proteins of another mannose-type PTS, MpoABCD, a low-affinity glucose/mannose-specific PTS that functions mainly as glucose sensor (78). An interaction between EIIB^{Mpo} (MpoB) and ManR was suggested by results from genetic experiments. While deletion of EIIA^{Mpo} led to constitutive expression of the *man* operon, deletion of EIIB^{Mpo} or of both EIIA^{Mpo} and EIIB^{Mpo} completely inhibited it, suggesting that EIIB^{Mpo} is required for ManR activity (78). An interaction of EIIB^{Mpo} with ManR could indeed be established by carrying out yeast two-hybrid experiments. Similar to the interaction of EIIB^{Mtl} with MtlR, EIIB^{Mpo} also interacts with two of the four regulatory domains of ManR (A. C. Zébré, M. Ventroux, M.-F. Noirot-Gros, J. Deutscher and E. Milohanic, unpublished results). However, in contrast to EIIB^{Mtl}, EIIB^{Mpo} is not fused to one of the two membrane components MpoC or MpoD but is a distinct cytoplasmic protein. As a consequence, it is unlikely that membrane sequestration plays a role in listerial ManR regulation.

PTS components also play a role in biofilm formation, as has been shown for EI and HPr of *V. cholerae* (151). In a recent study, the utilization of mannitol by this organism was reported to activate biofilm formation and the expression of the *vps* genes, which encode the proteins required for the synthesis of the exopolysaccharides forming the biofilm matrix (152). Even when grown on other carbon sources, the mannitol-specific PTS permease MtlA, an EIICBA protein, was found to be necessary for biofilm formation. In fact, it turned out that the EIIB^{Mtl} domain was sufficient for the stimulating effect. Synthesis of this domain from an IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible allele in cells growing in the absence of mannitol also caused elevated biofilm formation. Phosphorylation of the EIIB^{Mtl} domain is not required for this effect, because a mutant overproducing EIIB^{Mtl}, in which the phosphorylatable His of the EIIA domain was replaced with an alanine, also exhibited elevated biofilm formation (152).

Finally, an interesting presumably phosphorylation-independent interaction occurs between the peptide SgrT and the EIIC-EIIB domains of the glucose/*N*-acetylglucosamine family of PTS proteins. SgrT is encoded by the 5′ end of the small RNA SgrS, whereas its 3′ part is complementary to the 5′ end of the *ptsG* mRNA and thus allows the formation of an RNA-RNA hybrid, which inhibits translation of *ptsG* and is a target for RNase E (153). The small peptide SgrT inhibits the transport function of the PTS transporter probably by binding to the linker region connecting the EIIC^{Glc} and EIIB^{Glc} domains. Indeed, replacement of a proline (Pro-384) present in a conserved region (-L-K-T-**P**-G-R-E-D-) of the linker in *E. coli* PtsG with an arginine prevented the repressive effect of SgrT on glucose utilization (154). Surprisingly, the conserved linker motif is also found in the PTS permeases PtsG, GamP, and NagP of *B. subtilis*, although there is no evidence for the presence of an SgrT homologue in firmicutes. The conserved linker motif might therefore carry out additional functions.

Interaction with HPr and its paralogues Crh and NPr. A highly interesting interaction of HPr from enterococci with the response regulator of the CroRS two-component system was recently detected by carrying out a protein fragment complementation assay (155). CroR is required for intrinsic cephalosporin resistance in *E. faecalis*. It was therefore not surprising that deletion of HPr causes a cephalosporin hyperresistance phenotype, confirming that the interaction of HPr with CroR diminishes the intrinsic cephalosporin resistance of *E. faecalis*. Hyperresistance toward cephalosporins was also observed when either one of the two phosphorylatable amino acids of HPr, His-15 or Ser-46, was replaced with alanine. This result suggests that phosphorylation of HPr by either PEP and EI or ATP and HprK/P prevents the interaction with CroR. In agreement with these results, cephalosporin resistance of *E. faecalis* was also influenced by the utilization of carbon sources (155). While growth on the non-PTS carbon sources pyruvate and citrate, which leads to high levels of P∼His-HPr, caused enhanced cephalosporin resistance, growth on the PTS substrates glucose and *N*-acetylglucosamine, which leads to low levels of P∼His-HPr, had no such effect. The nutrient effect disappeared in a *croR* mutant, suggesting that it depends on the interaction between HPr and CroR.

Unphosphorylated HPr from *E. coli* and the two unphosphorylated HPr paralogues Crh from *B. subtilis* and NPr from *E. coli* were also found to interact with non-PTS target proteins. HPr from *E. coli* interacts with a regulatory protein and an enzyme involved in carbon storage. In the latter case, unphosphorylated as well as phosphorylated *E. coli* HPr was reported to bind with high affinity to glycogen phosphorylase (156). However, only the unphosphorylated PTS protein has a significant stimulating effect on glycogen phosphorylase activity (157). Neither NPr nor FPr (an *E. coli* HPr homologue specific for the transport of fructose) binds to glycogen phosphorylase. Interaction with HPr increases the V_{max} and lowers the K_m of glycogen phosphorylase for its substrate. It was proposed that under conditions where the PEP level drops and the PTS proteins are barely phosphorylated, the interaction with HPr will favor the breakdown of glycogen. Recently, HPr from *E. coli* was also reported to interact with the anti-σ^{70} factor Rsd (31). Only unphosphorylated HPr formed a tight complex with Rsd and thus prevented the inhibitory effect of Rsd on σ^{70}-dependent transcription, as was shown in *in vivo* and *in vitro* experiments.

B. subtilis Crh contains a well-conserved Ser-46 phosphorylation site, but lacks His-15, which is replaced with a glutamine. Crh is therefore not phosphorylated by PEP and EI. However, similar to the case for *B. subtilis* HPr, this protein is phosphorylated by ATP and HprK/P. Unphosphorylated Crh was reported to interact with the methylglyoxal synthase MgsA, an enzyme of the methylglyoxal bypass of glycolysis, and to inhibit its activity (158). Phosphorylation of Crh at Ser-46 by ATP and HprK/P prevents this interaction. As a consequence, flux through the methylglyoxal bypass is probably low when no efficiently metabolized carbohydrate is taken up via the PTS. Under these conditions, *B. subtilis* cells contain high levels of unphosphorylated Crh (35), which inhibits

MgsA. Interestingly, regulation of MgsA is a Crh-specific phenomenon because no interaction occurs between MgsA and HPr (158). It is therefore likely that Gln-15 of Crh, which distinguishes it from the His-15-containing HPr, plays an important role in this interaction.

Unphosphorylated NPr from *E. coli* was reported to regulate the production of lipid A, a component of the lipopolysaccharide layer. For that purpose, NPr interacts with the enzyme LpxD (159), an acetyltransferase essential for the synthesis of lipid A (160). This interaction seems to inhibit the activity of LpxD, because an *npr* mutant showed increased lipopolysaccharide synthesis. Phosphorylated NPr as well as unphosphorylated HPr did not interact with the acetyltransferase. The physiological role of PTS-mediated regulation of the production of the bacterial endotoxin lipid A is not yet understood. By carrying out yeast two-hybrid experiments, it was found that NPr from *B. melitensis* interacts with a pyrophosphate phosphatase (Ppa) (19). No further studies were carried out to demonstrate the potential physiological relevance of this protein-protein interaction.

Interaction with EI and EI^{Ntr}. The PTS is also involved in carbohydrate-mediated chemotaxis, a regulatory phenomenon in which the frequency of the change of the direction of flagellar rotation (change between tumbling and swimming) determines the speed at which a bacterium approaches an attracting carbon source or moves away from a repellent (161). A central protein in the chemotactic signaling pathway is the autophosphorylating sensor kinase CheA, which passes its phosphoryl group on to the CheY protein. P∼CheY interacts with the lower part of the switch complex, which is composed of the FliG, FliM, and FliN proteins and associated with the flagella rotor, thus inducing clockwise rotation and therefore tumbling (162). *In vitro* studies with purified enzymes showed that *E. coli* EI, but not P∼EI, inhibits the autophosphorylation activity of CheA (163). Similar experiments with the *B. subtilis* proteins revealed an opposite mechanism, i.e., inhibition of CheA activity by P∼EI and not EI (164). These contradictory results are not surprising since in *B. subtilis* CheY exerts an effect on flagellar rotation antagonistic to that in *E. coli*. However, for neither the *E. coli* nor the *B. subtilis* system has a direct interaction between CheA and EI or P∼EI, respectively, been established by using biochemical or immunological methods, although the inhibiting effect of EI on CheA activity in *in vitro* experiments suggests a direct interaction of the two proteins (163).

EI^{Ntr} and aspartokinase from *Bradyrhizobium japonicum* were also reported to interact with each other (165). This interaction, which was confirmed by *in vitro* pulldown experiments, inhibits the autophosphorylation activity of EI^{Ntr}. Aspartokinase is encoded by the *lysC* gene, and its deletion prevented the uptake of δ-aminolevulinic acid and the utilization of prolyl-glycyl-glycine as a proline source. This phenotype could be compensated for by expressing *ptsP* in *trans* from a multicopy plasmid. The result suggests that aspartokinase exerts an effect on EI^{Ntr} that can be mimicked by the overproduction of the PTS protein.

Interaction with Phosphorylated PTS Proteins

Interactions with phosphorylated PTS components seem to be less frequent than interactions with the unphosphorylated proteins. One reason might be that they are more difficult to detect. Nevertheless, several interactions with phosphorylated HPr and HPr paralogues as well as phosphorylated EIIA and EIIB components have been reported. HPr of firmicutes as well as its paralogue Crh (catabolite repression HPr) present in bacilli is also phosphorylated at Ser-46 by ATP and HprK/P. This modification also occurs with the HPr paralogue NPr of HprK/P-containing proteobacteria (19, 110). The seryl-phosphorylated PTS components also carry out important regulatory functions by interacting with several specific target proteins. The phosphorylation sites of the PTS components are usually part of the interface, and the phosphoryl group faces positively charged amino acids in the target proteins, mostly arginine, which increases the stability of the protein-protein complex (166).

Interaction with phosphorylated EIIA components. Genetic data had suggested that in *Enterobacteriaceae* phosphorylated EIIA^{Glc} stimulates the activity of adenylate cyclase (167). This enzyme converts ATP into cyclic AMP (cAMP), which is an important second messenger in prokaryotes and eukaryotes. Low intracellular levels of cAMP are observed in *E. coli* and *S. enterica* serovar Typhimurium cells grown on an efficiently metabolized carbon source or in mutants preventing the formation of P∼EIIA^{Glc}. In *Enterobacteriaceae*, cAMP binds to the cAMP receptor protein (Crp), and the resulting complex functions as transcription activator for numerous catabolic genes and operons (for a review, see reference 1). These results therefore strongly suggested that P∼EIIA^{Glc} interacts with adenylate cyclase and stimulates its catalytic activity. More specifically, cAMP formation seems to affect the lag phase characteristic for diauxie, which is observed when cells grow on a mixture of a preferred and a less favorable carbon source. Under these conditions, bacteria utilize the less favorable carbon source only when the preferred carbon source is exhausted (168), usually leading to two growth phases (hence the name diauxie) separated by a lag phase, during which the enzymes required for the transport and metabolism of the less-favorable carbon source are synthesized. Addition of extracellular cAMP either shortens or entirely eliminates the lag phase (169). However, addition of extracellular cAMP cannot prevent CCR, i.e., the preferred use of, for example, glucose over a less favorable carbon source, such as lactose (170). The interaction of EIIA^{Glc} with adenylate cyclase could indeed be confirmed by fusing adenylate cyclase to the *E. coli* integral membrane protein Tsr. Unphosphorylated EIIA^{Glc} as well as P∼EIIA^{Glc} was found to bind to the C-terminal regulatory domain of membrane-anchored adenylate cyclase. However, when adenylate cyclase activity assays were carried out in the presence of *E. coli* crude extracts, only P∼EIIA^{Glc} was able to stimulate the enzyme activity (171). An interaction of unphosphorylated EIIA^{Glc} with adenylate cyclase could also be demonstrated by *in vivo* experiments with the His-91-Ala EIIA^{Glc} mutant from *V. cholerae* by using the tandem affinity purification method (102).

Interaction with phosphorylated EIIB components. While some PRD-containing transcription activators are regulated via interaction with unphosphorylated EIIB domains or proteins, antiterminators seem to be regulated rather by the interaction with phosphorylated EIIB. A well-established example of the interaction of an antiterminator with a phosphorylated EIIB component is the *E. coli* antiterminator BglG. Similar to the case for other PRD-containing antiterminators, BglG activity is inhibited by phosphorylation catalyzed by the P∼EIIB^{Bgl} domain of BglF (see "Proteins Containing a Specific PTS-Recognized Phosphorylation Domain, the PRD" above). In addition, BglG binds to the P∼EIIB^{Bgl} domain of the β-glucoside-specific PTS permease BglF

(172). In the absence of a BglF-recognized substrate (salicin or arbutin), the EIIB^{Bgl} domain of BglF is mainly phosphorylated and interacts with the antiterminator BglG. While EIIB^{Mtl}-mediated membrane sequestration leads to activation of *B. subtilis* MtlR, EIIB^{Bgl}-mediated membrane sequestration inhibits the antiterminator activity of BglG. As soon as a substrate for the PTS^{Bgl} (salicin or arbutin) becomes available, the EIIB^{Bgl} domain of BglF will be dephosphorylated, which weakens its affinity for BglG. The antiterminator is therefore released into the cytoplasm, thereby regaining its activity and allowing efficient expression of the *bgl* operon.

In a recent study, the *B. subtilis* antiterminator LicT was also found to change its cellular localization in response to substrate availability. In the absence of a substrate, LicT is phosphorylated in its PRD1 by the P∼EIIB^{Bgl} domain, and the antiterminator is equally distributed in the cell. In the presence of a substrate, LicT is no longer phosphorylated by the P∼EIIB^{Bgl} domain and is located in subpolar regions (173). Although there is no evidence that the presence or absence of EIIB^{Bgl} or P∼EIIB^{Bgl} affects the activity of LicT by direct interaction, phosphorylation of the PRD1 of LicT by P∼EIIB^{Bgl} seems to play an important role in the cellular localization of the antiterminator.

Interaction with histidyl-phosphorylated HPr. Presently, there is only one reported example where P∼His-HPr interacts with and controls a target protein. This is the *B. subtilis* transcription activator YesS, which belongs to the AraC/XylS family of regulators (174). YesS contains a C-terminal DNA binding domain, and it controls the expression of the pectin/rhamnogalacturonan utilization genes of *B. subtilis* (175). A *ptsH* deletion mutant exhibited significantly reduced YesS activity. Yeast two-hybrid and "three-hybrid" experiments revealed that YesS interacts with HPr and P∼His-HPr but not with P-Ser-HPr. In three-hybrid experiments, the additional synthesis of HprK/P prevented the interaction of HPr with YesS, whereas the synthesis of EI had no effect (175). The interaction with HPr and P∼His-HPr occurs within a central regulatory domain of YesS (amino acids 406 to 510), whose sequence seems to be unique to YesS. Although HPr and P∼His-HPr bind to YesS, only phosphorylated HPr stimulates its activity (Fig. 3). This was inferred from experiments with a *ptsI* mutant and a *ptsH*(H15A) mutant, neither of which allowed formation of P∼His-HPr and which exhibited significantly reduced YesS activity compared to that of the wild-type strain. In contrast, inactivation of the HprK/P-encoding *hprK* gene or of *crh*, which encodes the HPr paralogue Crh, did not lower YesS activity (175).

Interactions with P-Ser-HPr and P-Ser-Crh. As mentioned above, HPr of firmicutes becomes phosphorylated not only at His-15 by PEP and EI but also at Ser-46 by ATP and HprK/P (21). This is also true for the HPr paralogue Crh present in bacilli, in which His-15 is replaced with a Gln, whereas Ser-46 and its surrounding region are well conserved compared to those in HPr of firmicutes (41). Homologues of Crh are found in bacilli, geobacilli, and oceanobacilli (1). HprK/P is a bifunctional enzyme also capable of dephosphorylating P-Ser-HPr and P-Ser-Crh by catalyzing a phosphorolysis reaction producing unphosphorylated protein and pyrophosphate (22). Numerous proteobacteria contain the HPr paralogue NPr (PtsO), in which Ser-46 and its surrounding region are well conserved. They also contain an HprK/P homologue, and *in vitro* phosphorylation of NPr has been established in the two alphaproteobacteria *B. melitensis* (19) and *Gluconobacter oxydans* (176) and the betaproteobacterium *R. eutropha* (110). A physiological function of P-Ser-NPr has so far not been established, but this posttranslational modification seems to be essential for growth of *R. eutropha* (110). In contrast, the role of P-Ser-HPr as a corepressor in CCR in firmicutes is well established and has been extensively reviewed (1, 177, 178). We therefore provide here only a brief description of this regulation mechanism.

The uptake of an efficiently metabolized carbon source by firmicutes leads to an increase of the FBP and a decrease of the phosphate concentration. These conditions favor the kinase activity of the bifunctional HprK/P (Fig. 1) and therefore cause an increase of the amount of P-Ser-HPr in the cells. P-Ser-HPr interacts with the catabolite control protein A (CcpA) (179) and allows this LacI-type repressor to bind to the catabolite response elements (*cre*), its target sites on the DNA (Fig. 3) (180). The *cre* site either can overlap the promoter region, in which case CcpA/P-Ser-HPr prevents binding of the RNA polymerase holoenzyme, or can be located downstream from the transcription initiation site with CcpA/P-Ser-HPr functioning as a roadblock. Mutants in which *ccpA* or *hprK* is deleted or Ser-46 of HPr is replaced with an alanine (181) are relieved from CcpA-mediated carbon catabolite repression. As already mentioned, carbon catabolite repression in firmicutes can be mediated by other mechanisms based on PRD-containing regulators, on inducer exclusion (1), or, in some bacteria, on glucose kinase (81). For certain catabolic genes or operons, the CcpA/P-Ser-HPr complex binds upstream from the promoter, which leads to catabolite activation instead of catabolite repression (182). In bacilli and a few other firmicutes, P-Ser-Crh can also bind to CcpA and function as a corepressor or coactivator for certain catabolic genes and operons during the utilization of an efficient carbon source (Fig. 3) (183, 184).

P-Ser-HPr of *B. subtilis* as well as its paralogue P-Ser-Crh has also been reported to interact with the glycolytic enzyme glyceraldehyde-3-P dehydrogenase (GapA) (185). In fact, both proteins interact with GapA in their unphosphorylated and seryl-phosphorylated forms, but only the seryl-phosphorylated forms of the two proteins were able to inhibit GapA (Fig. 3). P-Ser-HPr probably also plays an indirect role in *S. pyogenes* (84, 186) and *Bacillus anthracis* (187) virulence regulation, because in these pathogens CcpA controls the expression of several virulence genes, one being the streptolysin S-encoding gene of *S. pyogenes* (186).

There is strong evidence that in certain lactic acid bacteria P-Ser-HPr is also involved in inducer exclusion. Similar to what has been reported for *Enterobacteriaceae*, the uptake of the non-PTS sugar maltose by *L. casei* or *Lactococcus lactis* is immediately arrested when glucose or another efficiently metabolized carbon source is added (188–190). A similar observation was also made for the transport of ribose by *L. lactis* (190). These non-PTS sugars are taken up by ABC transport systems (191). Mutations in the *hprK* or *ptsH* gene preventing the formation of P-Ser-HPr also prevented the inhibitory effect of glucose on the uptake of the non-PTS sugars (188, 190). In contrast, mutations which caused an increase of the intracellular amount of P-Ser-HPr permanently inhibited the uptake of the non-PTS sugar (192). These results strongly suggest that similar to inducer exclusion in *Enterobacteriaceae*, where EIIA^{Glc} interacts with several non-PTS permeases, inducer exclusion in firmicutes is mediated by the interaction of P-Ser-HPr with a component of the ABC transporters (Fig. 3); unfortunately, this protein has not yet been identified.

Phosphorylation of HPr was discovered in connection with another regulatory phenomenon called inducer expulsion, which occurs in several firmicutes, including streptococci, lactococci, and lactobacilli (21, 193). Most of these bacteria transport the nonmetabolizable carbohydrate derivatives 2-deoxy-D-glucose (2-DG) and thiomethyl-β-D-galactopyranoside (TMG) via a PTS and therefore accumulate them as P derivatives in the cell. Addition of glucose or another efficiently metabolizable carbon source to cells preloaded with P-2DG or P-TMG led to rapid expulsion of the nonmetabolizable carbon source (194, 195). In fact, inducer expulsion was found to be a two-step process (193). In the first step, accumulated P-2DG or P-TMG is dephosphorylated by a P-sugar phosphatase, and in the second step, 2-DG or TMG is expelled. The conditions triggering inducer expulsion (metabolism of an efficiently utilized carbon source) also lead to the formation of P-Ser-HPr, and it was therefore suspected that P-Ser-HPr might be involved in inducer expulsion. Indeed, several P-sugar phosphatases of streptococci, lactococci, and enterococci were reported to be regulated by P-Ser-HPr (196–198). However, *L. casei* and *L. lactis* mutants unable to form P-Ser-HPr (because of *ptsH1* mutation or *hprK* deletion) still exhibited inducer expulsion, establishing that P-Ser-HPr is not essential for this regulatory process. Interestingly, a second type of inducer expulsion was reported for some heterofermentative lactobacilli, such as *Lactobacillus brevis*. P-Ser-HPr was reported to be involved in this second type of inducer expulsion, which affects only non-PTS carbohydrates (199). The galactose/H^+ symporter of *L. brevis* catalyzes not only galactose transport but also the uptake of the nonmetabolizable TMG, which in this organism is accumulated in the unphosphorylated form. The presence of glucose prevents the accumulation of TMG and causes the efflux (expulsion) of already-accumulated thio-sugar. Binding of P-Ser-HPr to the non-PTS permease was assumed to transform the symporter into a uniporter (facilitator) by uncoupling sugar transport from proton symport, thereby allowing the efflux of accumulated substrates. This concept was supported by the reconstitution of the HPr regulatory system (HPr, EI, and HprK/P) of *L. brevis* in a *B. subtilis* mutant devoid of HprK/P but still containing HPr. The strain also produced the *L. brevis* galactose permease GalP (200). When this strain was transformed with a plasmid encoding wild-type *L. brevis* HPr, it was not able to accumulate TMG when glucose was present in the medium. In contrast, glucose could not prevent TMG accumulation by a strain producing Ser-46-Ala mutant HPr. A strain transformed with a plasmid encoding *L. brevis* Ser-46-Asp mutant HPr was not able to accumulate TMG even when glucose was absent.

CONCLUSIONS AND PERSPECTIVES

The PTS was discovered 50 years ago in the laboratory of Saul Roseman at the University of Michigan, Ann Arbor, with the first article describing a PTS component, the HPr from *E. coli*, and its role in hexose phosphorylation being published in 1964 (20). Three years later, evidence for the presence of a PTS in a firmicute was obtained in the laboratory of Melvine Laurance Morse at the University of Colorado, Denver. They identified a PTS catalyzing the transport and phosphorylation of lactose in *Staphylococcus aureus* (201). In the following years, the proteins forming the glucose- and mannose-specific PTSs in *E. coli* (202, 203) and the lactose-specific PTS in *S. aureus* (204) were identified, and their role in transport and phosphorylation of the two hexoses and the disaccharide was established. Since then, a huge number of PTSs transporting a large variety of substrates, including hexoses, 6-deoxy-hexoses (14), amino sugars, *N*-acetyl-amino sugars, gluconic acids (205), pentitols (206, 207), ascorbate (208), and disaccharides, have been identified. We recently obtained evidence that the *E. faecalis* maltose-specific EIICBA^{Mal} (MalT) (15) also transports and phosphorylates the trisaccharide maltotriose and the tetrasaccharide maltotetraose (J. Deutscher, A. Hartke, J. Thompson, C. Magni, C. Henry, V. Blancato, G. Repizo, N. Sauvageot, A. Pikis, T. Kentache, and A. Mokhtari, unpublished results). A previous report had already suggested that the homologous maltose transporter MalT from *Streptococcus mutans* might take up maltotriose and maltotetraose (209).

PTS proteins are also involved in the phosphorylation of intracellular dihydroxyacetone (7), which enters the cells by facilitated diffusion or is formed from glycerol in a reaction catalyzed by glycerol dehydrogenase (64). Soon after its discovery, it was realized that the PTS not only transports and phosphorylates carbohydrates but also carries out regulatory functions related to carbon metabolism and sugar transport, such as catabolite repression and inducer exclusion. As described in the preceding sections, in the last 25 years PTS proteins were found also to regulate numerous cellular functions not or only indirectly related to carbon metabolism and transport, and this number increases steadily. PTS components control nitrogen and phosphate metabolism as well as potassium transport, antibiotic resistance, biofilm formation, and endotoxin production and also regulate the virulence of several pathogens. Given the high physiological impact of its regulatory functions, the PTS can no longer be considered merely a sugar transport and phosphorylation system that also plays some regulatory roles. The two functions rather seem to be of equal importance. In addition, in view of the tight connection between carbohydrate transport and metabolism and the regulatory functions of the PTS, it seems inappropriate to ask which of the two activities of the PTS is more important. Nevertheless, from an evolutionary point of view it would be interesting to understand how the multicomponent or multidomain PTS developed its different activities. Was the PTS originally a transport system that acquired regulatory functions, or was it an early regulatory system responding to environmental signals that later developed carbohydrate transport and phosphorylation activities, or, finally, did the transport and regulation systems evolve separately and became connected at a later stage in evolution in order to allow a correlation of transport and phosphorylation of carbohydrates with the different regulatory functions? The occurrence of an "incomplete PTS" composed of EI, HPr, and two EIIAs in numerous proteobacteria seems to favor the last hypothesis. In addition, many regulatory functions of the PTS seem to have developed late in evolution.

Concerning the PEP-requiring dihydroxyacetone phosphorylation system, we might be witnessing the creation of a novel PTS. At the present stage, transport and phosphorylation of dihydroxyacetone are two distinct steps, with the second being catalyzed by EI, HPr, EIIA, and DhaL and the first by facilitated diffusion. To make the system resemble a classical PTS and thus provide it with high transport efficiency, the dihydroxyacetone facilitator might integrate the dihydroxyacetone binding domain of DhaK. DhaL then needs to transform its ADP binding site into an EIIA-recognized Cys or His phosphorylation site in order to make it an EIIB-like protein. Given the slow pace of evolution, we will certainly not

witness whether one day indeed a classical dihydroxyacetone-specific PTS with the proposed characteristics will be formed.

Whatever the course of evolution of the PTS was, regulatory functions probably played an important role in early stages of the PTS development. The fact that all phosphoryl group transfer steps, with the exception of the last one, i.e., the phosphorylation of the carbohydrate substrate, are reversible makes the PTS an efficient sensor and a fast signal transduction system. In addition, the PTS responds to the most significant signal for bacterial cell growth and proliferation, i.e., the availability of carbon sources in the environment and their efficient metabolism. Together these characteristics explain why the PTS controls so many cellular functions and developed so many different regulatory mechanisms, each probably optimally adapted to the needs of different bacterial species. By developing an additional signal entry via the metabolite-controlled HprK/P in firmicutes and numerous proteobacteria, the complexity of PTS-mediated regulation grew even larger. HprK/P probably evolved from an ancestral kinase and retained the capacity to utilize pyrophosphate as a phosphoryl donor for its kinase activity. Oligophosphates were probably already abundant when life developed on our planet. By reversing the phosphorylation reaction, HprK/P uses P_i for the dephosphorylation of P-Ser-HPr, thereby producing pyrophosphate (22) (Fig. 1). HprK/P probably gained the ability also to utilize ATP when this nucleoside triphosphate became available.

Despite extensive efforts in numerous laboratories, we understand only a few PTS-mediated regulation systems. Little is known about the role of HprK/P in proteobacteria, and the different functions of the PTS^{Ntr} in *Enterobacteriaceae* are barely understood (113). In addition, what is the role of the N-terminal domain of HprK/P, which is absent in HprK/Ps of alphaproteobacteria and which can be deleted from HprK/Ps from firmicutes without affecting their known activities (210)? In the crystal structure, this domain binds P_i to conserved arginine residues, but P_i does not seem to be a physiological effector. PTS domains are found in numerous proteins, and the list of Table 1 is far from being complete; there are numerous other proteins containing a domain of a PTS protein or a small fragment of it composed of about 50 amino acids. Only in a few cases has the regulatory function of these PTS domains been determined.

Phosphoproteome analyses revealed that numerous PTS components become phosphorylated at Ser or Thr residues, including the general PTS proteins EI and HPr as well as many sugar-specific PTS components (211–217). In firmicutes, HPr was found to be phosphorylated not only at Ser-46 but also at position 12, which can be Ser or Thr (212, 213). Other HPr phosphorylation sites were also found (213). Other frequent targets of phosphorylation are the EII components of the mannose PTS (211, 213, 215–217). With the exception of P-Ser46-HPr and P-Ser46-Crh, nothing is known about the enzymes catalyzing these protein phosphorylations and the physiological role of Ser/Thr-phosphorylated PTS components.

Highly interesting is the role of the PTS in certain pathogens, such as *S. enterica* serovar Typhimurium, *V. cholerae*, *V. vulnificus*, *S. pyogenes*, or *L. monocytogenes*. In *L. monocytogenes*, the utilization of an efficient carbon source, such as cellobiose, glucose, fructose, mannose, etc., inhibits the expression of the virulence genes, which are controlled by PrfA, a Crp-like transcription activator (218, 219). The detailed mechanism is not yet understood, but it is likely that a PTS component is involved (220, 221). Many other PTS-related regulatory systems exist for which the mechanism and/or function are not understood, and new systems are constantly being discovered, clearly showing that 50 years after the discovery of the PTS, its regulatory functions are far from being fully understood.

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