Transport and Catabolism of Carbohydrates by \textit{Neisseria meningitidis}

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\textbf{Key Words} \textit{Neisseria meningitidis} · Glucose · Maltose · Gluconate · Virulence

\textbf{Abstract}
We identified the genes encoding the proteins for the transport of glucose and maltose in \textit{Neisseria meningitidis} strain 2C4-3. A mutant deleted for \textit{NMV}\textsubscript{.}1892 (\textit{glcP}) no longer grew on glucose and deletion of \textit{NMV}\textsubscript{.}0424 (\textit{malY}) prevented the utilization of maltose. We also purified and characterized glucokinase and α-phosphoglucomutase, which catalyze early catabolic steps of the two carbohydrates. \textit{N. meningitidis} catabolizes the two carbohydrates either via the Entner-Doudoroff (ED) pathway or the pentose phosphate pathway, thereby forming glyceraldehyde-3-P and either pyruvate or fructose-6-P, respectively. We purified and characterized several key enzymes of the two pathways. The genes required for the transformation of glucose into gluconate-6-P and its further catabolism via the ED pathway are organized in two adjacent operons. \textit{N. meningitidis} also contains genes encoding proteins which exhibit similarity to the gluconate transporter (\textit{NMV}\textsubscript{.}2230) and gluconate kinase (\textit{NMV}\textsubscript{.}2231) of Enterobacteriaceae and Firmicutes. However, gluconate might not be the real substrate of \textit{NMV}\textsubscript{.}2230 because \textit{N. meningitidis} was not able to grow on gluconate as the sole carbon source. Surprisingly, deletion of \textit{NMV}\textsubscript{.}2230 stimulated growth in minimal medium in the presence and absence of glucose and drastically slowed the clearance of \textit{N. meningitidis} cells from transgenic mice after intraperitoneal challenge.

\textbf{Introduction}

The β-proteobacterium \textit{Neisseria meningitidis} is a strictly human pathogen. In about 10% of any population, this commensal microorganism colonizes the nasopharynx [Yazdankhah and Caugant, 2004], where it resides asymptotically [Caugant and Maiden, 2009]. For reasons not yet understood it can occasionally become virulent and cross the epithelial cell layer and enter the blood
In order to explain this dual lifestyle, the hypothesis of the existence of carriage and invasive strains has been put forward [Caugant and Maiden, 2009; Taha et al., 2002]. Only the latter are assumed to cause severe infections leading to sepsis and meningitis, sometimes with a fatal outcome [Zarantonelli et al., 2008]. The current concept of meningococcal pathogenesis is based on the polygenic nature of meningococcal virulence comprising differences in genes involved in diverse aspects of meningococcal biology [Schoen et al., 2008]. However, the genetic and physiological differences between carriage and invasive strains are not clearly understood, but are proposed to be related to 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 [Schoen et al., 2014]. For example, the type of available carbon sources affects meningococcal complement resistance [Exley et al., 2005b].

In contrast to most bacteria, N. meningitidis is known to utilize only a small number of carbon sources, including a few amino acids, such as glutamate [Mallavia and Weiss, 1970], and the two carbohydrates maltose and glucose [Beno et al., 1968]. The tricarboxylic acid cycle intermediate succinate is also taken up and catabolized by meningococci [Weiss, 1970]. In addition, these bacteria can grow on L-lactate as well as pyruvate as the sole carbon source. Interestingly, glucose and lactate are present in millimole per liter amounts in the nasopharynx [Exley et al., 2005a], the usual habitat of N. meningitidis. While the transporter for lactate has been identified [Exley et al., 2005b], the permease involved in glucose uptake is not known. In many bacteria, glucose is efficiently transported via the phosphoenolpyruvate:carbohydrate phosphotransferase system (PTS) [Deutscher et al., 2006]. However, N. meningitidis possesses only an incomplete PTS composed of enzyme I (EI), HPr and two EI1A’s, but lacks known EIIB’s and the membrane components EIIC [Boël et al., 2003]. Nevertheless, it contains the metabolite-controlled HPr kinase, which phosphorylates HPr at Ser–46 [Derkaoui et al., 2016]. In Firmicutes, the resulting P-Ser-HPr is involved in numerous regulatory processes [Deutscher et al., 2006]. Although little is known about the role of the N. meningitidis incomplete PTS, it was suggested to carry out regulatory functions, possibly in meningococcal virulence [Poncet et al., 2009]. Indeed, the meningococcal ptsI and ptsH genes, which encode the general PTS proteins EI and HPr, were reported to be essential for experimental infection in an infant rat model of meningococcal bacteremia [Sun et al., 2000]. 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 [Derkaoui et al., 2016].

Several bacteria transport glucose not only by the PTS, but also at lower efficiency via ion symport permeases, such as GlCP of Bacillus subtilis [Paulsen et al., 1998] or GlcU of Staphylococcus xylosus [Fiegler et al., 1999] and Listeria monocytogenes [Aké et al., 2011]. These permeases usually have a broad substrate specificity for hexoses [Paulsen et al., 1998]. The gene NMV_1892 of N. meningitidis strain 2C4-3 encodes a protein resembling GlcP of Firmicutes. This protein was therefore a likely candidate for the glucose transporter of this pathogen.

Meningococci are not only devoid of PTS-catalyzed transport but also of fructose-6-P 1-kinase [Baart et al., 2010], a key enzyme of the Embden-Meyerhof-Parnas (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 positive effect on the growth of the recombinant strain [Baart et al., 2010]. In contrast, it caused a reduction in biomass production. It was therefore proposed that for preferably oxidative organisms, such as N. meningitidis, catabolism via the EMP pathway would not represent an advantage. Meningococci catabolize glucose mainly via the Entner-Doudoroff (ED) pathway (70%) [Baart et al., 2007], and to a minor extent via the pentose phosphate pathway (PPP) [Schoen et al., 2014]. The transcription regulator HexR has recently been shown to control the expression of several genes presumed to encode enzymes of the PPP and the ED pathway [Antunes et al., 2015]. Part of the pyruvate formed via both pathways or from lactate utilization or taken up from the medium is either further metabolized via the tricarboxylic acid cycle or transformed into acetate, which is mostly secreted into the culture medium [Leighton et al., 2001].

N. meningitidis can also utilize maltose [Beno et al., 1968], which contains two glucose molecules connected via an α-1,4 glycosidic bond. Most bacteria transport the disaccharide either via an ABC transporter [Boos and Shuman, 1998; Monedero et al., 2008] or via a PTS [Mokhtar et al., 2013; Schönert et al., 2006]. As already mentioned, meningococci contain an incomplete PTS unable to transport carbohydrates. In addition, they lack genes encoding the membrane components of a carbohydrate-specific ABC transporter. Nevertheless, we found that N. meningitidis contains a presumed operon encoding three enzymes likely involved in maltose utilization. A fourth
gene encodes a LacY-type permease, which was a possible candidate for the maltose transporter.

In this study, we indeed 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 from Firmicutes and enterobacteria.

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 was suggested to function as a glucose transporter [Echenique-Rivera et al., 2011]. This protein is nearly identical to NMV_1892 of strain 8013 and exhibits 46% sequence similarity to B. subtilis GlcP, which has been shown to take up glucose and mannose [Paulsen et al., 1998]. In order to test whether NMV_1892, which is encoded by a presumably monocistronic gene, catalyzes glucose transport in N. meningitidis, we deleted the NMV_1892 gene by replacing it with a spectinomycin resistance cassette. The resulting mutant lost the capacity to grow in glutamine-free Roswell Park Memorial Institute (RPMI) medium containing glucose as the sole carbon source (fig. 1) and failed to acidify glucose-containing cystine trypticase agar (CTA) medium (Difco) (online suppl. fig. 1; for all online suppl. material, see www.karger.com/doi/10.1159/000447093). To further confirm the glucose transport function of NMV_1892, we cloned its gene together with the ribosome binding site into plasmid pSU18c [Chandler, 1991], where it is transcribed from the lac promoter (see Materials and Methods). The resulting plasmid was subsequently used to transform the Escherichia coli ΔptsHIcrr mutant LJ140, which cannot utilize glucose [Aké et al., 2011; Pimentel-Schmitt et al., 2009]. However, when transformed with pSU18c containing the meningococcal NMV_1892 gene, it was able to ferment this sugar when grown on MacConkey agar plates (fig. 2a) similar to the Escherichia coli wild-type strain NMS22 transformed with empty pSU18c (fig. 2a). LJ140 transformants carrying empty pSU18c were not able to ferment glucose (fig. 2a).

Characterization of Enzymes Involved in Glucose Catabolism via the ED Pathway

In most bacteria, glucose taken up via a non-PTS transporter is first phosphorylated to glucose-6-P by an enzyme called glucokinase (Glk) or hexokinase [Wagner et al., 1995]. Glk activity has previously been detected in crude extracts of N. meningitidis [Holten, 1974b], but the enzyme has not been purified and characterized. In order to identify the N. meningitidis Glk, we carried out a BLAST search with Glk from E. coli. The 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 1).

NMV_1004 is part of an operon containing several other genes that, based on sequence similarities, probably encode enzymes involved in glucose metabolism (fig. 3a),
such as: a glucose-6-P dehydrogenase (NMV_1002), which oxidizes glucose-6-P to 6-P-glucono-δ-lactone and a 6-P-gluconolactonase (NMV_1003), which transforms 6-P-glucono-δ-lactone into gluconate-6-P. Together with kinase (Glk), these enzymes transform glucose into gluconate-6-P, the branching point for the PPP and ED pathways. The operon also contains the gene for a P-glucose isomerase (NMV_1006), which interconverts glucose-6-P and fructose-6-P. A protein of unknown function is encoded by NMV_1007. The expression of the operon has recently been shown to be controlled by the RpiR-like repressor HexR, the gene of which, NMV_1005, is also part of this operon [Antunes et al., 2015].

HexR was also reported to control the expression of the adjacent genes NMV_1000 and NMV_1001, which are divergently oriented to the NMV_1002 to NMV_1007

Table 1. Specific enzyme activities of purified proteins

<table>
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<th>Gene</th>
<th>Enzyme activity</th>
<th>Specific activity, μmol/min and mg protein</th>
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<tr>
<td>glk (NMV_1004)</td>
<td>glucokinase</td>
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<td>gnd (NMV_0016)</td>
<td>6-phosphogluconate dehydrogenase</td>
<td>2</td>
</tr>
<tr>
<td>rpiA (NMV_0882)</td>
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<td>3.66</td>
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<tr>
<td>rpe (NMV_1185)</td>
<td>D-ribulose-5-P 3-epimerase</td>
<td>2</td>
</tr>
<tr>
<td>pgcM (NMV_0427)</td>
<td>α-phosphoglucomutase</td>
<td>12.5</td>
</tr>
<tr>
<td>edd (NMV_1001)</td>
<td>gluconate-6-P dehydratase</td>
<td>2.5</td>
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<tr>
<td>fbaA (NMV_2059)</td>
<td>fructose-1,6-bisphosphate aldolase</td>
<td>56</td>
</tr>
<tr>
<td>gntK (NMV_2231)</td>
<td>gluconate kinase</td>
<td>150</td>
</tr>
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</table>

Fig. 2. Fermentation assays on MacConkey agar plates containing 0.5 mM IPTG and either 1% glucose (a) or 1% maltose (b). The meningococcal glucose permease GlcP restored glucose utilization in E. coli strain LJ140 and the maltose permease MalY maltose utilization in GSJ100. In a we tested strain LJ140 transformed with either empty plasmid pSU18c or plasmid pSU18c-glcP, in which the N. meningitidis glcP gene is expressed from the lac promoter. The strong red color (appears as dark gray in the print version) formed by LJ140[pSU18c-glcP] indicates that the synthesis of N. meningitidis GlcP restores glucose utilization in strain LJ140. E. coli strain NM522[pSU18c] was used as a positive control. Similarly, in b we tested fermentation of maltose by strain GSJ100 transformed either with empty pSU18c or with pSU18c-malY. Again, the red color (dark gray) formed by GSJ100[pSU18c-malY] indicates that expression of the N. meningitidis malY gene restores maltose utilization in strain GSJ100. Strain NM522[pSU18c] was used as a positive control.
Based on sequence similarities, they were likely to encode the ED pathway enzymes gluconate-6-P dehydratase (Edd; NMV_1001), which converts gluconate-6-P into 2-keto-3-deoxy-6-P gluconate (KDPG) and KDPG aldolase (NMV_1000), which cleaves KDPG into glyceraldehyde-3-P and pyruvate. N. meningitidis catabolizes glucose mainly via the ED pathway (70%) [Baart et al., 2007]. We therefore confirmed the presumed Edd activity of NMV_1001 by carrying out a spectrophotometric assay as described in Materials and Methods. Purified NMV_1001 indeed converts gluconate-6-P into KDPG (table 1).

Interestingly, in two γ-proteobacteria, KDPG controls HexR by dissociating and thus inactivating the oligomeric regulator [Daddaoua et al., 2009; Leyn et al., 2011]. However, no such effect of KDPG was observed for N. meningitidis HexR [Antunes et al., 2015].

**Characterization of Enzymes Involved in Glucose Catabolism via the PPP**

Catabolism via the PPP 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 (Gnd; NMV_0016); D-ribulose-5-P 3-epimerase (Rpe; NMV_1185); D-ribose-5-P isomerase A (RpiA; 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 meningooccal chromosome. We purified three of the five enzymes and confirmed their activity. We found that NMV_0016 is a decarboxylating Gnd, which converts gluconate-6-P into ribulose-5-P (branching point to the PPP; fig. 4). This activity has previously been measured in N. meningitidis crude extracts, but the enzyme has not been purified and characterized [Holten, 1974a]. We confirmed that NMV_1185 transforms D-ribulose-5-P into D-xylulose-5-P, and that NMV_0882 isomerizes D-ribose-5-P to D-ribose-5-P. During the metabolism of glucose via the PPP, three molecules of glucose are transformed into two molecules of fructose-6-P, one molecule of glyceraldehyde-3-P and three of CO₂. 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 phosphoglucone isomerasers, 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 glyceraldehyde-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 an FBP aldolase (Fba). It probably also catalyzes the reverse reaction by using the two triosephosphates to form FBP (fig. 4). In numerous organisms, FBP is an important allosteric regulator of metabolic enzymes, such as pyruvate kinase [Waygood and Sanwal, 1974], and of signal transduction proteins, such as HPr kinase [Derkaoui et al., 2016; Deutscher and Engelmann, 1984], which might explain 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 that 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 [Fitting and Doudoroff, 1952]. Bacteria transporting the disaccharide via an ABC transporter usually use maltose phosphorylase to catalyze the first step of maltose catabolism, i.e. its phosphorolysis into D-glucose and D-glucose-1-P. We therefore carried out a BLAST search with the sequence of *Lactobacillus casei* maltose phosphorylase MalP (LCABL_11440) [Monedero et al., 2008]. 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. In addition, the genes upstream and downstream from *NMV_0426* encode enzymes also potentially involved in maltose catabolism (fig. 3b): *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). *NMV_0427* is 46% identical to α-phosphoglucomutase (PgcM) from the *B. subtilis* maltodextrin operon [Schönert et al., 2006], which converts both α- and β-D-glucose-1-P into glucose-6-P [Mesak and Dahl, 2000]. However, glucose and maltose transport activities of GlcP and MalY, respectively, were confirmed by appropriate assays, the substrate of the GntP-like *NMV_2230* remains obscure. Although *NMV_2231* efficiently phosphorylates gluconate, *N. meningitidis* was not able to grow in MM containing gluconate as the sole carbon source. The substrate of *NMV_2230* might therefore be one of the other 15 hexonates, which after its uptake is converted to gluconate.

**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 (blue in the online version) were purified and their activity was determined 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. While the glucose and maltose transport activities of GlcP and MalY, respectively, were confirmed by appropriate assays, the substrate of the GntP-like *NMV_2230* remains obscure. Although *NMV_2231* efficiently phosphorylates gluconate, *N. meningitidis* was not able to grow in MM containing gluconate as the sole carbon source. The substrate of *NMV_2230* might therefore be one of the other 15 hexonates, which after its uptake is converted to gluconate.
While the wild-type strain grew to a high cell density in the presence (solid line) or absence (dashed line) of 0.5% maltose.

We noticed that MalY-like transporters are present in lactobacilli that lack a maltose-specific ABC transporter, such as L. salivarius (LSL_1282) and L. 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 contains a maltose operon with the same gene order as N. meningitidis (malY, galM, malP, pgcM; fig. 3b). Even more important, these two bacteria share a common habitat — the nasopharynx. N. gonorrhoeae, which colonizes a different habitat, lacks the enzymes for maltose utilization.

In summary, N. meningitidis 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 [Lohmiller et al., 2008]. Intracellular maltose is phosphorylated to α-D-glucose and α-D-glucose-1-P by the enzyme maltose phosphorlase, 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 Glk (NMV_1004), or by the 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 GntP-Like 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. We found that NMV_2230 exhibits significant similarity (54–59%) to hexonate permeases from B. subtilis and E. coli (GntP, GntT, GntU, and IdnT). In addition, the protein encoded by the downstream gene NMV_2231 (fig. 3c) showed 56% sequence similarity to gluconate kinase (GntK) from E. coli. However, the wild-type strain 2C4-3 was not able to grow on Na+–gluconate as the sole carbon source. Failure to utilize gluconate was also observed for the N. meningitidis wild-type strain. Both strains reached a similar final OD600 after the addition of IPTG. The ΔgntP mutant and the complemented strain were not able to grow on gluconate as the sole carbon source. However, when 1 mM of ΔgntP was added the complemented strain grew half as fast as the B. subtilis wild-type strain. Both strains reached a similar final OD600.

Deletion of the gntP-Like NMV_2230 Enhances N. meningitidis Virulence

We also tested the impact of the deletion of the gntP-like gene on meningococcal invasiveness into the blood after intraperitoneal challenge of BALB/c mice. We determined the colony-forming units per milliliter of N. meningitidis in the blood at 2, 6 and 24 h after infection with standardized inocula of 10^7 CFU per mouse. Interestingly, compared to the wild-type strain, the NMV_2230 mutant exhibited a stronger increase of bacterial counts and subsequently persisted at higher levels (fig. 7). After 24 h the difference between the wild-type and the NMV_2230 mutant was more than three orders of magnitude. The enhanced resistance against clearance from the blood might partly be due to the observed faster growth of the gntP-like mutant in a glucose-containing environment (fig. 1).
maltose via the PPP or the ED pathway have also been characterized. Finally, we also identified a novel transport-er resembling gluconate permeases. However, N. me-nigitidis is not able to utilize gluconate and the substrate of this permease might therefore be another hexonate. Surprisingly, deletion of the presumed hexonate transporter increased the survival rate of N. meningitidis in a mouse infection model.

**Experimental Procedures**

**Bacterial Strains and Growth Conditions**

The meningococcal strain 2C4-3, isolated as clone 12 from the serogroup C, class I strain 8013 [Nassif et al., 1991] has the relevant phenotype P', Opa', Opc', PilC1'/PilC2'. This strain, as well as the glkP, malY, and gntP deletion mutants derived from it, was grown either in GC medium base (Difco) containing the supple-ments previously described [Kellogg et al., 1963], in RPMI medi-um, and in RPMI medium supplemented with serum. Growth studies of N. meningitidis strains were carried out in glucose-, maltose- or Na+–gluconate-containing RPMI medium lacking glu-tamate. When appropriate, kanamycin or spectinomycin was added at a final concentration of 100 or 75 μg/ml, respectively. Growth of the B. subtilis BSB168-derived gntP mutant and its transformant containing pIC634-gntP-2C4-3 was measured in C MM containing 25 mM of Na+–gluconate in the presence or absence of 1 mM of IPTG. The E. coli strains NM522 (Stratagene) or NM522 transformed with plasmid pREP4-GroES/EL [Amrein et al., 1995] were used for protein overproduction. Strains harboring derivatives of the His-tag expression vector pQE30 (Qiagen) were grown at 37 °C under agitation in LB medium supplemented with 100 μg/ml am-picillin for NM522 and with 50 μg/ml ampicillin and 12.5 μg/ml kanamycin for NM522[pREP4-GroES/EL].

**Plasmids Used in This Study**

The plasmid pSU18c is a pACYC184-derived vector containing the multiple cloning site and the lacZa reporter gene of pUC18 [Bar-tolome et al., 1991]. Plasmid pQE30 (Qiagen) was used for the over-production of His-tagged proteins. For proteins forming inclusion bodies it was used in combination with pREP4-GroES/EL [Amrein et al., 1995], which expresses the genes for the chaperone GroES/EL. The pIC634 plasmid is a derivative of pDG1664 [Guérout-Fleury et al., 1995], which contains the genes for the chaperone GroES/EL. The pIC634 plasmid was transformed with plasmid pREP4-GroES/EL [Amrein et al., 1995] for protein overproduction. Strains harboring 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].

**Conclusions**

The availability of carbon sources is an important fac-tor for the colonization by bacterial pathogens of epithelial surfaces in humans and animals. For example, N. menigitidis strains unable to utilize lactate cannot colonize the nasopharynx [Exley et al., 2005a]. Glucose and maltose are also present in the nasopharynx [Iyer et al., 2005]. N. meningitidis seems to have adapted to this spe-cific environment because lactate, glucose and maltose belong to the few carbon sources utilized by this patho-gen. We here establish that the two carbohydrates, glu-cose and maltose, are taken up by the ion-driven sym-porters GlcP and MalY, respectively. Most striking is the high degree of sequence identity (around 80%) between MalY and presumed maltose symporters of some oral lac-tobacilli (L. salivarius, 82%; L. fermentum, 78%; L. oris, 77%). This finding suggests that N. meningitidis acquired the maltose operon from an oral Lactobacillus strain. Sev-eral enzymes catalyzing the catabolism of glucose and maltose via the PPP or the ED pathway have also been

![Fig. 7. Enhanced virulence of the N. meningitidis ΔNMV_2230 (ΔgntP) 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 ΔNMV_2230 mutant (filled gray 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 4 with the ΔNMV_2230 mutant. Survival of the wild-type strain 2C4-3 and the ΔNMV_2230 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 (error bars) are presented.](image-url)
Table 2. List of primers used in this study

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Restriction sites are in bold letters and the *N. meningitidis* DNA uptake sequence is written in italics.
To purify *E. coli* 2-dehydro-3-deoxyphosphogluconate aldolase, the *eda* gene was PCR amplified by using genomic DNA of *E. coli* strain NM522 as the matrix and the primers edaFcoliBam and edaRcoliSal (table 2). The resulting amplicon was cut with *Bam*HI and *Sal*I, 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 the enzyme was carried out as previously described [Galiner et al., 1997]. Maltose phosphorylase of *Enterococcus faecalis* and ribitol-5-P 2-dehydrogenase from *L. casei* were purified as described in Mokhtari et al. [2013] and Bourand et al. [2013], 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 at 450 nm was continuously followed with a Kontron Bio-Tek spectrophotometer using the Autorate program. All mixtures (except for PgcM) contained 50 mM of Tris/HCl, pH 7.4, and 10 mM of MgCl₂. To measure the activity of the various enzymes we added the following: for Glk, 10 mM of glucose, 1 mM of NADP, 1 mM of ATP, 10 μg of glucose-6-phosphate 1-dehydrogenase (Sigma), and 8 μg of purified Glk; for GntK, 1 mM of glucose, 1 mM of NADP, 1 mM of ATP, 25 μg of 6-phosphogluconate dehydrogenase (Sigma), and 2 μg of purified GntK; for Gnd, 1 mM of gluconate-6-P, 1 mM of NADP, and 10 μg of purified Gnd; for Edd, 1 mM of gluconate-6-P, 1 mM of NADH, 30 μg of *E. coli* 2-dehydro-3-deoxyphosphogluconate aldolase, 20 μg of triosephosphate isomerase (Sigma), 24 μg of glyceral-3-P dehydrogenase (Sigma), and 40 μg of purified Edd; for Rpe, 0.5 mM of D-xylulose-5-P, 1 mM of NADH, and 18 μg of *L. casei* D-ribitol-5-P 2-dehydrogenase [Bourand et al., 2013], and 10 μg of purified Rpe; for D-ribose-5-P isomerase, 1 mM of ribose-5-P, 1 μM of NADH, 18 μg of *L. casei* ribitol-5-P 2-dehydrogenase, and 30 μg of purified D-ribose-5-P isomerase; for FBP aldolase, 5 mM of FBP, 1 mM of NADH, 20 μg of triosephosphate isomerase, 24 μg of glyceral-3-P dehydrogenase and 5 μg of purified FBP aldolase. For PgcM we used 50 mM of phosphate buffer, pH 7.4, and either 10 μg of purified Rpe, 0.5 mM of D-xylulose-5-P, 1 mM of NADH, 18 μg of *L. casei* ribitol-5-P 2-dehydrogenase, and 30 μg of purified D-ribose-5-P isomerase; for *EDA* aldolase, 1 mM of ribose-5-P, 1 mM of NADH, 18 μg of *L. casei* ribitol-5-P 2-dehydrogenase, and 30 μg of purified D-ribose-5-P isomerase; for FBP aldolase, 5 mM of FBP, 1 mM of NADH, 20 μg of triosephosphate isomerase, 24 μg of glyceral-3-P dehydrogenase and 5 μg of purified FBP aldolase.

The three deletion mutants were constructed by first amplifying upstream and downstream regions of the targeted gene by using the corresponding primer pairs listed in table 2. The two upstream and downstream DNA fragments of the *glcP* and the *malY* genes were cloned into plasmid pQE30 cut with *Bam*HI and *Hind*III for *glcP* and *Bam*HI and *Kpn*I for *malY*. The *glcP*-related fragments were cut with *Bam*HI, *Eco*RV and *Eco*RV/*Hind*III and the *malY* fragments with *Bam*HI/*Eco*RV and *Eco*RV/*Kpn*I. In each case, the simultaneous insertion of the two amplified fragments into pQE30 created an *Eco*RV site. A spectromycycin cassette was inserted in the *Eco*RV site of the *glcP*-related fragments and a kanamycin cassette in the *Eco*RV site of the *malY* construct. The two antibiotic resistance cassettes were amplified with primers, which created *Sma*I sites at their ends and thus allowed blunt end ligation. For *gntP* deletion, a *Bam*HI/*Eco*RV upstream fragment was first cloned into pBluescript KS+ followed by a kanamycin resistance cassette amplified with *Eco*RI and *Hind*III sites. Finally, the downstream *gntP* fragment containing *Hind*III and *Sal*I sites was added. The three deletion plasmids also contained the uptake sequence for *N. meningitidis* allowing the transformation of 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 *gntP* mutant derived from it were grown in CTA medium containing 1% glucose. Similarly, the wild-type strain and the *malY* mutant were grown in CTA medium containing 1% maltose. The medium also contained 17 mg/l of phenol red as a pH indicator, which changes its color from red to yellow owing to the acidification of the medium during utilization of the added sugar [Sáez-Nieto et al., 1982].

**Complementation of a Glucose-Negative *E. coli* Strain with the NMV_1892 Gene**

Heterologous complementation assays were carried out following a previously described method [Pimentel-Schmitt et al., 2009]. First, the *N. meningitidis glcP* gene NMV_1892 together with its ribosome binding site was amplified by PCR with *N. meningitidis* 2C4-3 genomic DNA as the template and the primers SP219 and SP220, which introduce *Bam*HI and *Hind*III restriction sites (table 2). 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 [Chandler, 1991], thus putting the NMV_1892 gene under control of *Plac*. 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 insert 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 [Lévy et al., 1990]. 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**

The approach described above was also used to complement the *E. coli* strain GSJ100 [Blaudeck et al., 2003], which lacks the periplasmic maltose binding protein MalE and therefore cannot utilize maltose, with the *N. meningitidis malY* gene. The *malY* gene together with its ribosome binding site was amplified by PCR with the primers SP236 and SP237. The amplicon was cloned into pSU18c cut with *Bam*HI and *Hind*III. The resulting plasmid was used to transform the *E. coli* *malE* mutant GSJ100 [Blaudeck et al., 2003]. Fermentation assays with the complemented mutant and control strains were carried out on MacConkey agar as described above.
Complementation of a B. subtilis gntP Mutant with the NMV_2230 Gene

In order to test whether the GntP-like NMV_2230 can complement a gntP mutant, we deleted the gntP gene of B. subtilis strain BSB168. First, the upstream region of gntP and the apha33 kana-
mycin resistance gene were amplified by using B. subtilis genomic DNA and plasmid pGEM-T-apha33 [Joyer et al., 2010], respectively, as the template and appropriate primer pairs (table 2). The forward primer for apha33 overlapped the reverse primer for the gntP upstream region, which in the second step allowed the fusion of the two DNA fragments by PCR amplification by using the two amplics as the template and the two distal oligonucleotides as primers. The fused DNA was cut with Apal 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 the template and appropriate primers (table 2). The resulting amplicon was cut with SpeI and SalI and cloned into pGEM-T Easy containing apha33 and the upstream region of gntP. The resulting plasmid pGEM-T-AgtP-
Kan was used to transform the B. subtilis strain BSB168 and a dou-
ble recombination allowed the replacement of gntP with the kana-
mycin-resistance gene apha33. 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 the template and the primers D-GntP-HindIII and R-GntP-SphI (table 2). The resulting DNA fragment was cut with HindIII and SphI and inserted into plasmid pIC634, which is a derivative of pDG1664 [Guérout-Fleury et al., 1997], containing the E. coli lacI gene and the spac promoter. In one of the resulting plasmids 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 down-
stream regions of the B. subtilis thrC gene and thus allowed the si-
multaneous integration of lacI, erm, and the Pspac-controlled gntP gene at the thrC locus of the B. subtilis wild-type strain BSB168. Growth studies with the wild-type strain, gntP mutant and the complemented strain were carried out in CSK MM containing gluconate as the sole carbon source [Galíner et al., 1997].

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 Pas-
teur Review Board. Bacterial loads in infected 6-week-old female BALB/c mice (Janvier, France) were determined from blood samples taken at 2, 6 and 24 h after intraperitoneal challenge with stan-
dardized inocula of 10^7 CFU per mouse for each tested strain. Bac-
terial counts were determined by plating serial dilutions of blood on GC medium base plates.

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