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HexR Controls Glucose-Responsive Genes and Central Carbon Metabolism in *Neisseria meningitidis*

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ABSTRACT

***Neisseria meningitidis*, an exclusively human pathogen and the leading cause of bacterial meningitis, must adapt to different host niches during human infection. *N. meningitidis* can utilize a restricted range of carbon sources, including lactate, glucose, and pyruvate, whose concentrations vary in host niches. Microarray analysis of *N. meningitidis* grown in a chemically defined medium in the presence or absence of glucose allowed us to identify genes regulated by carbon source availability. Most such genes are implicated in energy metabolism and transport, and some are implicated in virulence. In particular, genes involved in glucose catabolism were upregulated, whereas genes involved in the tricarboxylic acid cycle were downregulated. Several genes encoding surface-exposed proteins, including the MafA adhesins and *Neisseria* surface protein A, were upregulated in the presence of glucose. Our microarray analysis led to the identification of a glucose-responsive *hexR*-like transcriptional regulator that controls genes of the central carbon metabolism of *N. meningitidis* in response to glucose. We characterized the HexR regulon and showed that the *hexR* gene is accountable for some of the glucose-responsive regulation; *in vitro* assays with the purified protein showed that HexR binds to the promoters of the central metabolic operons of the bacterium. Based on DNA sequence alignment of the target sites, we propose a 17-bp pseudopalindromic consensus HexR binding motif. Furthermore, *N. meningitidis* strains lacking *hexR* expression were deficient in establishing successful bacteremia in an infant rat model of infection, indicating the importance of this regulator for the survival of this pathogen *in vivo*.**

IMPORTANCE

***Neisseria meningitidis* grows on a limited range of nutrients during infection. We analyzed the gene expression of *N. meningitidis* in response to glucose, the main energy source available in human blood, and we found that glucose regulates many genes implicated in energy metabolism and nutrient transport, as well as some implicated in virulence. We identified and characterized a transcriptional regulator (HexR) that controls metabolic genes of *N. meningitidis* in response to glucose. We generated a mutant lacking HexR and found that the mutant was impaired in causing systemic infection in animal models. Since *N. meningitidis* lacks known bacterial regulators of energy metabolism, our findings suggest that HexR plays a major role in its biology by regulating metabolism in response to environmental signals.**

Neisseria meningitidis is a leading cause of meningitis and fulminant septicemia and is a significant public health problem, affecting mainly children and young adults. The annual number of invasive disease cases worldwide is estimated to be at least 1.2 million, with 135,000 deaths related to invasive meningococcal disease (1, 2). Meningococci are classified into 12 serogroups on the basis of the structure of the polysaccharide capsule; the majority of invasive meningococcal infections are caused by serogroups A, B, C, W, Y, and X (3).

N. meningitidis is an encapsulated Gram-negative diplococcal bacterium and a strictly human pathogen. It colonizes about 3 to 30% of the human population, where it resides asymptomatically in the nasopharynx, its only known reservoir (4). For reasons not yet fully understood, some strains of *N. meningitidis* are able to cross the mucosal epithelium and enter the bloodstream, where they evade immune killing by undergoing antigenic variation, by expressing surface antigens that mimic host molecules, and by recruiting human complement regulators (5–7). Furthermore, this pathogen can cross the blood-brain barrier and multiply in the cerebrospinal fluid, causing meningitis (8).

Meningococcal adaptation to the different human host niches also occurs at the level of the metabolism (9), and the acquisition of nutrients that enable the bacterium to sustain growth and to multiply rapidly, causing septicemia, is critical for the outcome of meningococcal disease. *N. meningitidis* is thus capable of adapting to different anatomical compartments of the host, including the nasopharyngeal mucosa, the bloodstream, and the subarachnoid compartment (10), where the available key nutrients, such as carbon sources, are diverse. Moreover, this bacterium can utilize a restricted variety of substrates, such as glucose, lactate, or pyruvate, as sole carbon sources to allow growth (11–13). Glucose is the predominant carbon source in blood and cerebrospinal fluid (14), the two main host niches of infection; therefore, glucose constitutes a crucial carbon source for *N. meningitidis*. Accordingly, about one-half of the genes that are essential for systemic

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TABLE 1 Plasmids and strains used in this study

Plasmid or strain	Description	Antibiotic resistance[a]	Reference or source
Plasmids			
pGEMT	Cloning vector	Amp	Promega
pGEMT-*hexR*KO::Kan	Plasmid for deletion of *N. meningitidis hexR* gene by homologous recombination	Amp, Kan	31
pGEMT-P_{zwf}	Plasmid harboring 321-bp promoter fragment upstream of *zwf* gene	Amp	This study
pGEMT-P_{edd}	Plasmid harboring 386-bp promoter fragment upstream of *edd* gene	Amp	This study
pET15b(+)	Plasmid for inducible expression of histidine-tagged recombinant proteins in *E. coli*	Amp	Life Technologies
pET15b(+)-*hexR*	Plasmid for expression and purification of histidine-tagged HexR in *E. coli*	Amp	This study
pComCmrP$_{ind}$	Plasmid for allelic replacement at chromosomal location between ORFs NMB1428 and NMB1429 and inducible expression under control of P_{tac} promoter and *lacI* repressor	Amp, Clm	33
pComCmrP$_{ind}$-*hexR*	Plasmid for complementation of Δ*hexR* null mutant; derivative of pComCmrP$_{ind}$ containing copy of *hexR* gene	Amp, Clm	This study
Strains			
MC58	Laboratory-adapted *N. meningitidis* reference strain		60
MC58 Δ*hexR*	MC58 derivative lacking *hexR* gene	Kan	31
MC58 Δ*hexR c-hexR*	MC58 derivative lacking *hexR* gene, with copy of *hexR* reintroduced out of locus under control of inducible P_{tac} promoter	Kan, Clm	This study
2996	Clinical isolate		25
2996 Δ*hexR*	2996 derivative lacking *hexR* gene	Kan	This study

[a] Amp, ampicillin; Kan, kanamycin; Clm, chloramphenicol.

infection encode enzymes that are involved in the metabolism and transport of nutrients (15). In addition, several environmental signals have effects on the transcriptional regulation of *N. meningitidis* during host infections, as shown for iron (16, 17), zinc (18), nitric oxide (19), human saliva (20), and human blood (21, 22). Therefore, virulence factors and genes essential for survival need to be regulated tightly and rapidly at the gene expression level in order to respond to the various microenvironments encountered during infection.

In this work, we assessed for the first time the effects of glucose on *N. meningitidis* at the transcriptional level. We observed that, besides an increase in energy metabolism through the Entner-Doudoroff (ED) pathway, there is upregulation of genes encoding surface-exposed proteins that have been implicated in adhesion and immune evasion, such as the MafA proteins and NspA (23, 24), respectively. Moreover, we identified an RpiR-like transcriptional regulator, HexR, that is responsible for part of the glucose-responsive regulation and that affects the fitness of this bacterium *in vivo*.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *N. meningitidis* strains MC58 (25) and 2996 (26) (Table 1) were routinely cultured in GC medium-based agar (Difco) supplemented with Kellogg's supplement I (27). Liquid cultures were grown at 37°C in a 5% CO_2 atmosphere. For determination of growth curves, strains were grown to the stationary phase in GC medium-based medium with Kellogg's supplement I. For transcriptomic experiments, strains were grown to the mid-logarithmic phase in modified Catlin 6 (C6) medium (28). Because the concentration of glucose in human blood has been reported to be 4.5 mM (14), we decided to assay the effects of glucose on gene transcription *in vitro* by adding a 12-fold excess of glucose (55 mM) to the growth medium (1% [wt/vol]). Strains were stocked in GC medium with 15% glycerol and were stored at −80°C. When required, chloramphenicol (5 μg/ml), kanamycin (100 μg/ml), and/or isopropyl-β-D-1-thiogalactopyranoside (IPTG) (1 mM) were added to culture media at the indicated final concentrations. *Escherichia coli* DH5α (29) and BL21(DE3) (30) strains were grown in Luria-Bertani medium; when required, ampicillin and/or IPTG was added to achieve final concentrations of 100 μg/ml and 1 mM, respectively.

Construction of mutant and complemented strains. DNA manipulations were carried out routinely as described for standard laboratory methods (31). *N. meningitidis hexR* mutants of the MC58 and 2996 strains were constructed as described previously (32). For complementation of the MC58 Δ*hexR* null mutant, the *hexR* gene under the control of the P_{tac} promoter and the *lacI* repressor was reinserted into the intergenic region between the converging open reading frames (ORFs) NMB1428 and NMB1429 by transformation with pComCmrP$_{ind}$-*hexR* (Table 1), a derivative plasmid of pSLComCm^R (33) in which the *hexR* gene was amplified from the MC58 strain with the primers GG006 and GG007 (Table 2) and cloned as a 849-bp NdeI/NsiI fragment downstream of the P_{tac} promoter. This plasmid was transformed into the MC58 Δ*hexR* strain, and transformants were selected on chloramphenicol. All transformants were verified by PCR analysis for the correct insertion through a double homologous recombination event.

RNA preparation. Bacterial cultures were grown in liquid medium to an optical density at 600 nm (OD_{600}) of 0.5 to 0.7 and then were added to an equal volume of frozen medium to bring the temperature immediately to 4°C. Cells were harvested by centrifugation at 3,400 × *g* for 20 min. In preparation for transcriptome experiments, total RNA was isolated using an RNeasy minikit (Qiagen), following the manufacturer's instructions. Total RNA was extracted from three independent bacterial cultures, and 15-μg aliquots of the samples were pooled. Three independent RNA pools were prepared for each condition tested.

Microarray procedures, hybridization, and analysis. DNA microarray analysis was performed using Agilent custom-designed oligonucleotide arrays (34). cDNA probes were prepared from 5 μg of RNA pools and hybridized as described previously (34). Three hybridizations per experiment were performed, using cDNA probes from three independent pools, as detailed above. Changes in transcript levels were assessed by grouping all $\log_2$ ratios of the Cy5 and Cy3 values corresponding to each gene within experimental replicas and spot replicas and were filtered by comparing them against the zero value by Student's *t* test (one tailed). Validation of

TABLE 2 Oligonucleotides used in this study

Oligonucleotide	Sequence[a]	Restriction site	Application
GG006	ATATCATATGTTAAGCAAAATCAGCGAATCACTG	NdeI	*hexR* complementation
GG007	ATATATGCATTCAATCTTTGTCGTAATCGATGTGC	NsiI	*hexR* complementation
GG012	CTGTACTTCCAGGGCTTAAGCAAAATCAGCGAATCACTG		PIPE cloning of *hexR*
GG013	AATTAAGTCGCGTTAATCTTTGTCGTAATCGATGTGC		PIPE cloning of *hexR*
GG034	CTCGAGCGTCTGAAAGTGGGAAGCGG	XhoI	*zwf* promoter probe
GG035	GGATCCGTACTCATCGTATTATCTCGTCAGG	BamHI	*zwf* promoter probe
GG036	CTCGAGCCCCTATTCCGTTACAACAATCG	XhoI	*edd* promoter probe
GG037	GGATCCTTCACGGTCGGTCTCCTGTC	BamHI	*edd* promoter probe
0089RT-F	GAAACGATGCTGGTGGAAC		qRT-PCR
0089RT-R	CCGCTGGTAATGATGTATTGG		qRT-PCR
0207RT-F	TGACCAAATTCGACACCGT		qRT-PCR
0207RT-R	ATCGACACCGAGTTCTTTCC		qRT-PCR
0334RT-F	ATTTTGATTGACCGCCTCAC		qRT-PCR
0334RT-R	CACTGATCGAAGGGGTTGAC		qRT-PCR
0663RT-F	TATGCCGTTACCCCGAATGT		qRT-PCR
0663RT-R	CAGTGTTGACTTTGCCGATG		qRT-PCR
1389RT-F	ATGGTTTCCCGCCTCTTG		qRT-PCR
1389RT-R	CGATGTGCTTGTTGTGTATGCT		qRT-PCR
1392RT-F	AGCCTGTGAAAACCTTGCTG		qRT-PCR
1392RT-R	TTGATTTGCTGGGAAGAAGC		qRT-PCR
1393RT-F	TTGAAAAGCGAAATGGGTTC		qRT-PCR
1393RT-R	GGTGTAAGGGTGGACGAAGG		qRT-PCR
1476RT-F	ACGTTGCCATTTACAACGAA		qRT-PCR
1476RT-R	GTTCGGCGTTGGTAATTTCT		qRT-PCR
1710RT-F	GCAAATGAGTTCCGCCATC		qRT-PCR
1710RT-R	ATAGGCAGGGTGGTCAAGG		qRT-PCR
1968RT-F	TCAAACAAGGTGCGAAATTG		qRT-PCR
1968RT-R	CCATACTGTTGTCGGTGTCG		qRT-PCR
2159RT-F	GTTATCTCCGCCGCTTCCT		qRT-PCR
2159RT-R	GCTGTTGGGCACGATGTT		qRT-PCR
16SRT-F	ACGTAGGGTGCGAGCGTTAATC		qRT-PCR
16SRT-R	CTGCCTTCGCCATCGGTATTCCT		qRT-PCR

[a] Underlined letters indicate restriction enzyme sites.

microarray results was performed by real-time quantitative (qRT)-PCR analysis of individual RNA samples unrelated to the microarray RNA pools, as detailed below.

Real-time quantitative PCR experiments. For qRT-PCR experiments, 2 μg of total RNA treated with Turbo DNA-free DNase (Ambion) was reverse transcribed using random hexamer primers and Moloney murine leukemia virus reverse transcriptase (Promega), following the manufacturer's instructions. qRT-PCR assays were performed in triplicate on distinct RNA preparations, in 25-μl reaction mixtures containing 80 ng of cDNA, 1× Brilliant II SYBR green quantitative PCR master mixture (Agilent), and 0.2 μM gene-specific primers (Table 2). Amplification and detection of specific products were performed with a LightCycler 480 real-time PCR system (Roche), using the following procedure: 95°C for 10 min; 40 cycles of 95°C for 30 s, 55°C for 1 min, and 72°C for 30 s; and then dissociation curve analysis. The 16S rRNA gene was used as an internal reference for normalization, and relative transcript changes were determined by using the $2^{-\Delta\Delta CT}$ relative quantification method (35). Relative transcript changes for each gene were plotted with the standard deviation of the three replicates.

Expression and purification of recombinant HexR. The *hexR* gene was amplified from the MC58 genome using primers GG012 and GG013 (Table 2) and was cloned as a 843-bp fragment into the pET15b(+) vector (Life Technologies) by using the polymerase-incomplete primer extension (PIPE) enzyme-free cloning method (36), generating the pET15b(+)-*hexR* plasmid (Table 1). This plasmid was transformed into the *E. coli* BL21(DE3) strain, the expression of a recombinant HexR protein containing an N-terminal histidine tag was induced by the addition of 1 mM IPTG and growth at 25°C for 6 h, and the protein was purified by Ni-nitrilotriacetic acid (NTA) (Qiagen) affinity chromatography under nondenaturing conditions, according to the manufacturer's instructions. In brief, IPTG-induced *E. coli* cultures were concentrated in lysis buffer (20 mM Tris, 500 mM NaCl, 10 mM imidazole, 1 mg/ml chicken egg lysozyme) supplemented with complete EDTA-free protease inhibitor cocktail (Roche) and were incubated for 30 min at 4°C. Lysis was then performed by sonication, and cleared filtered supernatants were applied to the column for Ni-NTA affinity purification. After washing of the Ni-NTA resin with 12.5 volumes of wash buffer (20 mM Tris, 500 mM NaCl, 25 mM imidazole), the His-HexR protein was eluted with 2.5 volumes of elution buffer (20 mM Tris, 500 mM NaCl, 250 mM imidazole). Eluted fractions were collected and pooled, and protein concentrations were determined by the Bradford colorimetric method (Bio-Rad). Pooled eluates were then dialyzed four times against 150 volumes of storage buffer (20 mM Tris-HCl [pH 8.0], 100 mM NaCl, 1 mM dithiothreitol [DTT], 10 to 50% glycerol); the concentration of glycerol was increased in a stepwise manner up to a final concentration of 50%.

DNase I footprinting. The *zwf* and *edd* promoter regions were amplified with the primer pairs GG034/GG035 and GG036/GG037, respectively (Table 2). The PCR products were purified and cloned into the pGEMT vector (Promega) as 321-bp and 386-bp fragments, generating pGEMT-P_{zwf} and pGEMT-P_{edd}, respectively (Table 1). Two-picomole samples of each plasmid were end labeled with [γ-^{32}P]ATP by T4 polynucleotide kinase, after digestion at either the XhoI or BamHI site introduced by PCR with the oligonucleotides described above. Following a second digestion with either BamHI or XhoI, the labeled probes were purified by polyacrylamide gel electrophoresis (PAGE) as described previously (37). DNA-protein binding reactions were carried out for 15 min at room tempera-

TABLE 3 Numbers of genes differently expressed in the presence of glucose, grouped in functional categories according to the TIGR family classifications

Functional category	No. of genes		% of regulated genes
	Downregulated	Upregulated	
Amino acid biosynthesis	1	2	4
Biosynthesis of cofactors, prosthetic groups, and carriers	0	1	1
Cell envelope	2	5	9
Cellular processes	2	2	5
Energy metabolism	12	13	31
Hypothetical proteins	8	13	26
Mobile element functions	0	2	2
Protein fate	1	1	2
Purines, pyrimidines, nucleosides, and nucleotides	0	2	2
Regulatory functions	1	1	2
Transcription	0	1	1
Transport and binding proteins	5	4	11
Unknown function	1	2	4
Total	33	49	100

ture in footprinting buffer (20 mM Tris-HCl [pH 8], 50 mM KCl, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM DTT, 0.05% Nonidet P-40) containing 60 fmol of labeled probe, 100 ng of salmon sperm DNA, and a range of HexR concentrations, as indicated (see Fig. 4B). Samples were then treated with 0.3 U of DNase I (Roche) for 2 min at room temperature. DNase I digestions were stopped and samples were purified, loaded, and run on 8 M urea-6% polyacrylamide gels as described previously (38). A G+A sequence reaction (39) was performed for each probe, in parallel with the footprinting reactions.

Bioinformatic analysis of the HexR binding site. The HexR binding consensus sequence was derived by aligning the three 17-bp sites mapped on the *zwf* and *edd* promoters by DNase I footprinting. The sequence of each intergenic region from the MC58 strain genome was extracted and scanned *in silico* for the presence of HexR binding motifs, using the EMBOSS fuzznuc algorithm (A. Bleasby, European Bioinformatics Institute).

***In vivo* infant rat model.** A competitive index (CI) assay was performed in infant rats to determine the *in vivo* fitness of the Δ*hexR* mutant strain, relative to the wild-type (WT) strain, as described by Fagnocchi and coworkers (32). *N. meningitidis* strain 2996 was used because it requires significantly smaller inocula (10^3 to 10^4 CFU) for infection than does the MC58 strain (10^8 CFU). Briefly, bacteria of *N. meningitidis* strain 2996 were grown to the mid-logarithmic phase in Mueller-Hinton medium supplemented with 0.25% (wt/vol) glucose and were diluted to the desired concentration in phosphate-buffered saline (PBS). Six- to 8-day-old pups from litters of outbred Wistar rats (Charles River) were challenged intraperitoneally with wild-type strain 2996 and the isogenic knockout mutant strains, at a 1:1 ratio, to establish mixed infections. Groups of infant rats were used for the infectious doses of 4.5×10^3 and 4.5×10^4 CFU. A control group of 9 infant rats was injected with PBS. Eighteen hours after the bacterial challenge, blood samples were obtained by cheek puncture and aliquots were plated for viable cell counting. In parallel, the experiment was reproduced *in vitro* by establishing mixed inocula of the wild-type and mutant strains, at a 1:1 ratio, with the same infectious doses as described above. The number of CFU per milliliter retrieved after each experiment was determined after overnight incubation at 37°C, in a 5% CO_2 atmosphere, on Columbia agar supplemented with 5% horse blood, with or without kanamycin. Enumeration of wild-type bacteria and mutant bacteria allowed determination of the CI ratio using the following formula: CI = (WT output/mutant output)/(WT input/mutant input). Observed CIs for the two infectious doses followed the same statistical distribution and were pooled to increase the power of the analysis. Statistical significance was assessed with the Mann-Whitney test.

Ethics statement. All animal trials were carried out in compliance with current Italian legislation on the care and use of animals in experimentation (legislative decree 116/92) and with Novartis animal welfare policies and standards. Protocols were approved by the Italian Ministry of Health (authorization no. DM 166/2012-B) and by the local Novartis animal welfare body (AWB) (research project AWB 201202). Following infection, animals were clinically monitored daily for criteria related to their feeding ability, reactivity, and motility, as well as cutaneous redness. After 18 h, all animals were alive and normally reactive and were euthanized by cervical dislocation, as preestablished in agreement with Novartis animal welfare policies.

RESULTS

Global analysis of *Neisseria meningitidis* gene expression in response to glucose. In order to investigate the effects of glucose on global transcripts in *N. meningitidis*, we compared the RNA profiles of bacteria grown to the exponential growth phase in the presence or absence of glucose, using custom Agilent oligonucleotide microarrays (34). The ratios of fluorescence values in the red and green channels corresponding to each gene were grouped and then filtered to select only genes that displayed the same changes in fluorescence across three independent replicates ($P \leq 0.05$). A gene was defined as being differentially transcribed when it displayed >2-fold changes in fluorescence between the glucose samples and the reference samples (see Table S1 in the supplemental material). Growth of the *N. meningitidis* MC58 strain in the presence of glucose altered the transcript levels of 82 genes (3.8% of the MC58 genome). Among these, transcript levels of 49 genes increased and transcript levels of 33 genes decreased; we proposed to refer to these sets of genes as upregulated and downregulated, respectively (Table 3). The global gene expression could be grouped into 13 functional protein categories, some of which are overrepresented, including energy metabolism (30%), hypothetical proteins (26%), transport and binding proteins (11%), and cell envelope (9%) (Table 3). Genes belonging to the Entner-Doudoroff (ED) pathway (*zwf*, *pgl*, *edd*, and *eda*), the pentose phosphate (PP) pathway (*tal*), and the catabolic branch of the Embden-Meyerhof-Parnas (EMP) pathway (*pgi1*, *gapB*, and *pykA*) were found to be highly upregulated (Fig. 1; also see Table S1 in the supplemental material). In contrast, genes belonging to the

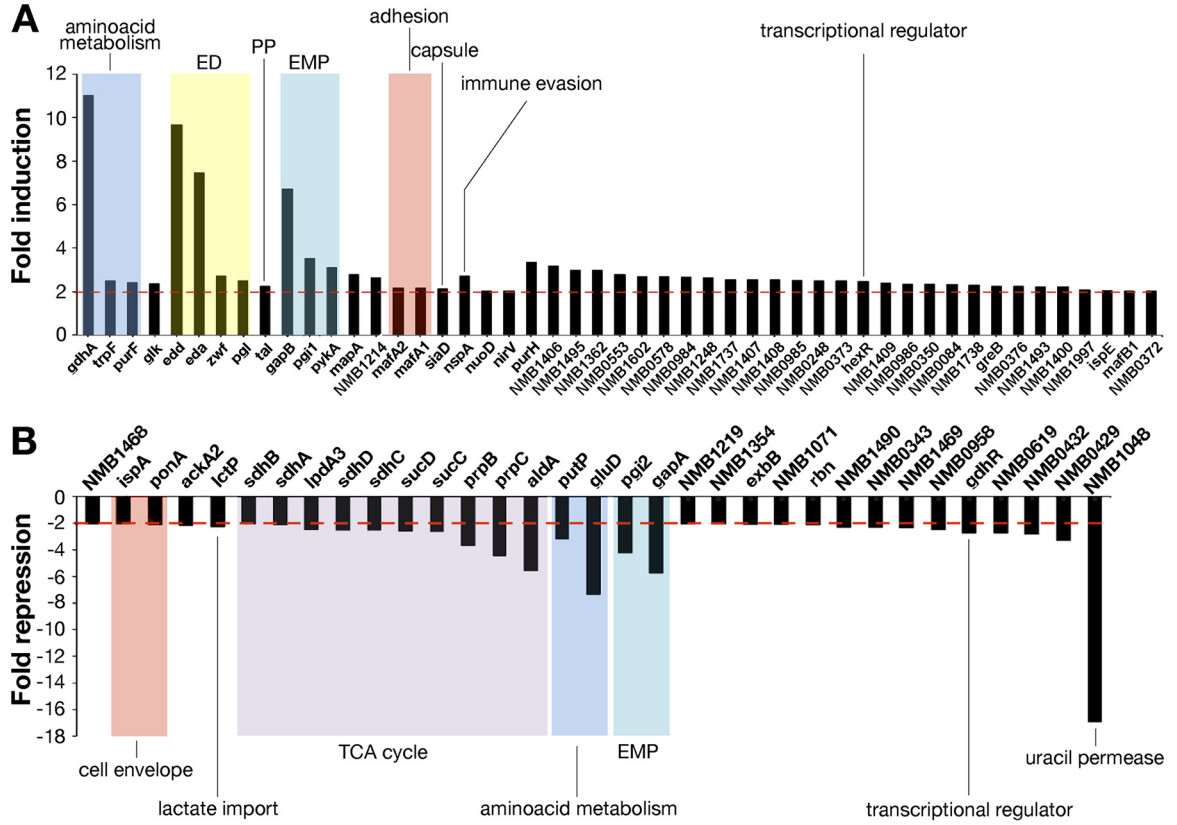

FIG 1 Transcriptional profile of *N. meningitidis* strain MC58 in response to glucose. The relative ratios of the microarray competitive hybridizations are shown for glucose-responsive expression (MC58 with glucose versus MC58 without glucose). Differentially expressed genes, with *P* values of ≤0.05 and >2-fold induction (A) or >2-fold repression (B), are shown. ED, Entner-Doudoroff pathway; PP, pentose phosphate pathway; EMP, Embden-Meyerhof-Parnas pathway; TCA, tricarboxylic acid.

anabolic branch of the EMP pathway (*pgi2* and *gapA*) were found to be downregulated. Therefore, the presence of glucose upregulates genes that encode functions leading to glucose catabolism (glycolysis) and downregulates genes whose products catalyze the inverse reactions (gluconeogenesis), thus promoting the utilization of available sugar energy sources. We also observed downregulation of all genes involved in the tricarboxylic acid (TCA) cycle (*aldA*, *prpB*, *prpC*, *lpdA3*, *sdhABCD*, and *sucCD*), as well as acetate production (*ackA2*). Genes related to amino acid metabolism and transport were also differentially expressed. The NADPH-specific glutamate dehydrogenase gene (*gdhA*) was upregulated, together with genes related to amino acid metabolism (*trpF* and *purF*). In contrast, the NADH-specific counterpart to *gdhA* (*gluD*) was downregulated, as was the proline importer gene (*putP*). Consistent with the availability of a carbon source such as glucose, the lactate importer gene (*lctP*) was downregulated, together with a gene encoding a putative uracil permease (NMB1048).

In addition to metabolic changes, genes related to *N. meningitidis* pathogenesis were induced by glucose (Fig. 1; also see Table S1 in the supplemental material). For instance, *nspA* (encoding NspA, a factor H binding protein) was upregulated by glucose, as was the capsule gene NMB0067 (*siaD*). Furthermore, several genes coding for surface-exposed proteins that exhibit immunogenic properties and were proposed previously as vaccine candidates, such as NMB0390 and NMB1468, were upregulated and downregulated, respectively, in the presence of glucose. Genes coding for proteins involved in contact and interaction with the host were also differentially transcribed in the presence of glucose. The *mafA* (multiple adhesion family A) genes NMB0375 and NMB0652, encoding polymorphic glycolipid-binding lipoproteins implicated in adhesion (23), were upregulated by glucose. Interestingly, NMB1214, encoding the effector protein (HrpA) in a two-partner secretion system implicated in adhesion and intracellular survival (40), also was upregulated in the presence of glucose. Genes coding for proteins related to the cell envelope, such as NMB1807 (*ponA*, encoding penicillin-binding protein 1) and NMB0342 (*ispA*, encoding intracellular septation protein A), were downregulated in the presence of glucose. Interestingly, both of these genes have been found to be downregulated in glucose-rich human blood (22).

In order to confirm the results obtained in the microarray expression profiling, we selected a subset of 8 genes with changes ranging from highly upregulated to downregulated and we performed real-time quantitative (qRT)-PCR (see Fig. S1A in the supplemental material). The results obtained are similar to the microarray data, with a good coefficient of correlation ($r^2 = 0.82$) (see Fig. S1B in the supplemental material). Therefore, we conclude that glucose is involved in the control of gene expression related to metabolism and to functions relevant for *N. meningitidis* interactions with the host.

NMB1389, a HexR transcriptional regulator. Searching the

N. meningitidis genome for potential orthologues of the major bacterial carbon-source-responsive regulators, i.e., the cAMP receptor protein (Crp) and the catabolic repressor/activator protein (Cra) (41), we identified no orthologues of these regulators. In contrast, two potential carbon-related transcriptional regulators were differentially transcribed in response to glucose within the microarray data set; NMB1711 (*gdhR*) was downregulated by glucose and was described previously as being involved in the regulation of glutamate transport (42), and NMB1389 was upregulated in the presence of glucose (Fig. 1).

The NMB1389 gene encodes a protein with 41% amino acid identity and 58% amino acid similarity to the HexR protein of *Pseudomonas putida* (43). HexR-like transcriptional regulators from the RpiR family are often involved in sugar catabolism regulation in proteobacteria (43, 44). HexR contains two domains, namely, a helix-turn-helix (HTH) binding domain in the N-terminal region and a sugar isomerase (SIS) domain in the C-terminal region that is predicted to bind phosphosugars. In the gammaproteobacteria *P. putida* and *Shewanella oneidensis*, binding of the 2-dehydro-3-deoxyphosphogluconate (KDPG) metabolic intermediate of glycolysis was reported to dissociate HexR from its target DNA (43, 44). The NMB1389 (*hexR*) nucleotide sequence is highly conserved (>98%) among the available *N. meningitidis* genome sequences, and it is present in all species of the *Neisseria* genus, such as *Neisseria gonorrhoeae*, *Neisseria lactamica*, *Neisseria cinerea*, *Neisseria flavescens*, *Neisseria mucosa*, *Neisseria sicca*, and *Neisseria subflava*.

In order to assess the role of HexR in *N. meningitidis* carbon metabolism regulation, we generated a knockout Δ*hexR* strain and we performed microarray analysis of the wild-type MC58 strain and its isogenic Δ*hexR* mutant strain grown to the exponential growth phase in the presence or absence of glucose. In the absence of glucose in the growth medium, 23 genes showed changes in transcript levels in the Δ*hexR* strain versus the wild-type strain, with a 2-fold change threshold and *t* test *P* values of ≤0.05 (see Table S2 in the supplemental material). When the same strains were grown in the presence of glucose, *hexR* deletion altered transcript levels of 19 genes (see Table S3 in the supplemental material). Transcripts of 6 genes (*zwf*, *edd*, *eda*, *hexR*, *ackA2*, and NMB0792) were altered under both conditions, and consequently the deletion of *hexR* resulted in the upregulation or downregulation of 36 genes in total. Among these, upregulated genes were compared for their transcripts profiles under both conditions, as well as in our glucose data set (Fig. 2). Interestingly, all genes that were upregulated in the Δ*hexR* mutant in the absence of glucose displayed the same trend when the wild-type strain was exposed to glucose. This observation indicated that HexR repressed these genes in the absence of glucose and that HexR mediated the glucose-induced response in the transcription of these genes (Fig. 2A). In contrast, when bacteria were grown in the presence of glucose, the lack of *hexR* induced the upregulation of genes that were repressed by glucose in the wild-type strain (Fig. 2B). Furthermore, such regulation was lost when the strain lacking *hexR* was not exposed to glucose (Fig. 2B), indicating that glucose regulation of these genes is independent of HexR.

In order to validate the results obtained in the *hexR* microarray experiments, we selected 6 genes that showed different transcript levels under the two experimental conditions (C6 medium in the presence or absence of glucose), and we performed qRT-PCR analyses of RNA extracted from the Δ*hexR* and WT strains under

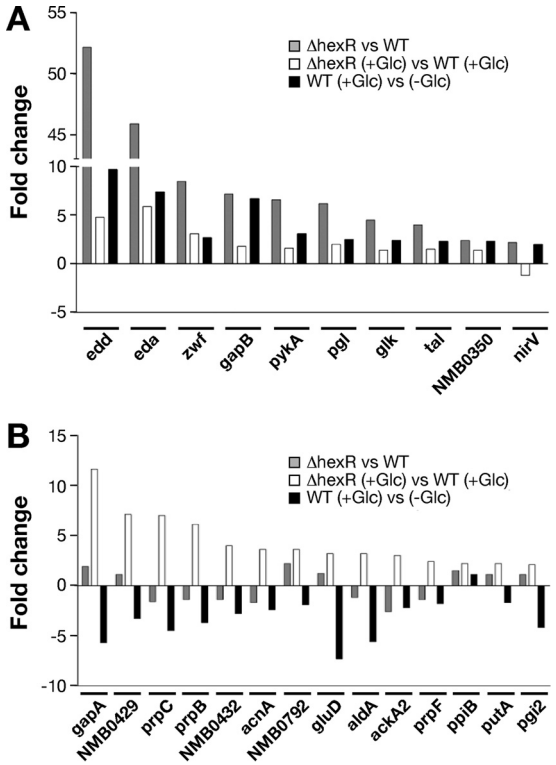

FIG 2 Comparison of the relative ratios of microarray competitive hybridizations for *hexR*-dependent expression in the absence or presence of glucose (Glc), showing that HexR represses a variety of genes in response to glucose. The relative ratios for glucose-responsive expression in the WT strain are also shown. Only genes that were upregulated in the Δ*hexR* strain versus the WT strain, with >2-fold changes in the absence (A) versus the presence (B) of glucose, are shown.

those conditions. The results obtained were in agreement with the microarray data (see Fig. S1C and D in the supplemental material). We also performed a similar analysis comparing the Δ*hexR* strain with either the wild-type strain or the complemented c-*hexR* strain, in which expression of HexR had been induced with 1 mM IPTG. We observed similar trends and expression levels for the selected genes in the Δ*hexR* strain in comparison with the wild-type strain or the c-*hexR* strain (Fig. 3). In conclusion, these results confirm that expression of these genes is under the direct or indirect control of HexR.

HexR binds to *edd* and *zwf* promoter regions. The *N. meningitidis hexR* gene maps within a genomic region that includes genes of central carbohydrate metabolism (Fig. 4A). Because these genes showed different transcript levels in the Δ*hexR* mutant, we decided to investigate whether transcription could be regulated by HexR. Therefore, DNase I footprinting experiments were performed with the P_{zwf} and P_{edd} promoter probes to identify the binding sites of the HexR protein, with results shown in Fig. 4B. With increasing concentrations of recombinant HexR protein, we observed the appearance of defined protected areas. The presence of 100 nM HexR resulted in a partially protected region of the P_{zwf} probe, whose boundaries became clear with protein concentrations of ≥200 nM. The protected region spans position +1 to position −16 with respect to the transcriptional start site, thus overlapping the −10 promoter element. In a similar footprint experiment with the P_{edd} promoter probe, 128 nM HexR resulted

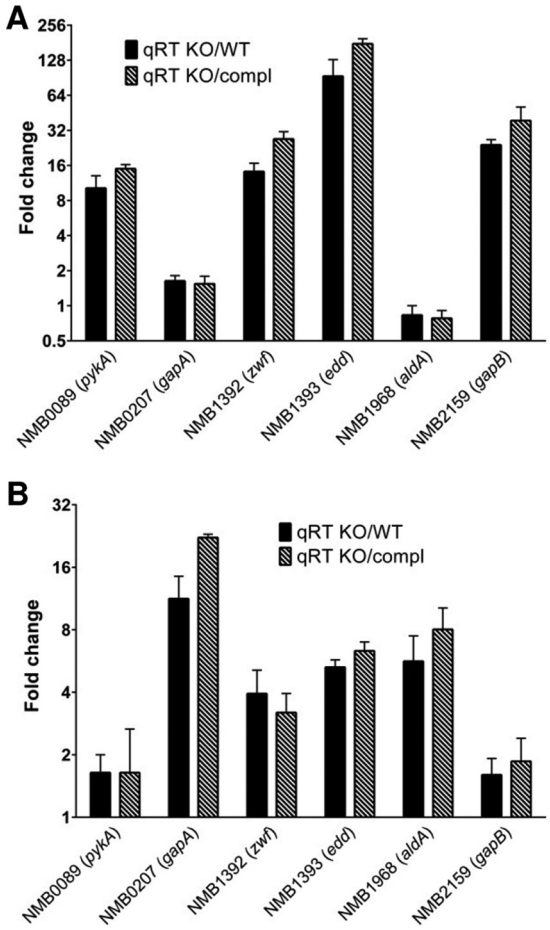

FIG 3 Comparison of qRT-PCR expression levels for 6 selected genes in the Δ*hexR* knockout (KO) strain versus the WT strain and in the Δ*hexR* strain versus the complemented (compl) strain, when grown in C6 medium in the absence (A) or presence (B) of glucose. Bars, fold changes for selected genes; error bars, standard deviations of three qRT-PCR biological replicates.

in an area of DNase I protection, whose boundaries were extended with 640 and 3,200 nM protein. The protected region spans position −38 to position +1, thus overlapping the −35 promoter region with high affinity and the −10 region with low affinity. This region of protection likely is constituted by two adjacent HexR binding sites with different affinities (Fig. 3B).

In gammaproteobacteria, the KDPG metabolic intermediate of glycolysis was reported to abrogate the affinity of HexR for its target DNA (43, 44). Therefore, we tested several metabolic intermediates (KDPG, glucose-6-phosphate, fructose-1,6-biphosphate, and 6-phosphogluconic acid) as putative effectors for *N. meningitidis* HexR binding *in vitro*. However, we did not observe differences in the HexR binding affinity for its cognate targets in the presence of the tested molecules (data not shown). Differences in the amino acid sequence of the phosphosugar-binding C-terminal region of the protein may account for a still unknown HexR effector in response to glucose in *N. meningitidis*.

***In silico* prediction of *N. meningitidis* HexR DNA-binding consensus sequence.** From DNase I footprinting analysis, we identified three HexR binding sites. These sequences were used to grasp information on the HexR binding consensus sequence, identifying a 17-bp pseudopalindromic motif (KTGTANTWWW ANTACAM) (Fig. 4C). This sequence resembles the HexR binding motif predicted *in silico* for betaproteobacteria in the RegPrecise database (45). We then used this motif to screen the *N. meningitidis* MC58 genome *in silico* and obtained 428 hits, among which 5 overlapped the promoter regions of HexR-regulated operons (Fig. 4D). Besides the central carbon metabolism genes *zwf* and *edd* (part of the ED pathway), the same motif was identified upstream of the *gapB* and *pykA* (part of the glycolytic branch of the EMP pathway) and *tal* (part of the pentose phosphate pathway) genes. We hypothesize that HexR regulates transcription of these genes through direct binding to their promoter regions.

HexR deletion impairs survival of *N. meningitidis* during *in vivo* infection. In order to assess the viability of the Δ*hexR* mutant strain *in vivo*, a competitive index (CI) assay was performed with *N. meningitidis* strain 2996 in infant rats, to determine the fitness of the mutant relative to the wild-type strain, as described by Fagnocchi and coworkers (32). Growth curves in GC-rich medium as well as in C6 medium with and without glucose showed no significant differences for the Δ*hexR* mutant versus the wild-type strain (data not shown). The median CI observed for the challenged infant rats in the *hexR* experiment was >1, indicating that more wild-type bacteria (approximately 10-fold more) survived in the animal model than did Δ*hexR* mutant bacteria (Fig. 5). When the same competition assay was performed under *in vitro* conditions, no difference in survival was observed between wild-type and mutant bacteria (Fig. 5). This suggests that the lack of HexR expression significantly affects the survival of *N. meningitidis* during *in vivo* infection.

DISCUSSION

The ability of microorganisms to detect and to respond to variable external conditions, such as environmental stress and the availability of carbon sources in different niches, requires a coordination of sensing mechanisms and regulatory circuits (46) and often is crucial for the adaptation of pathogenic bacteria to the host environment. Here we show the transcriptional profile of *N. meningitidis* in response to glucose, one of the main carbon sources that the bacterium encounters in its different niches of colonization. Glucose induces the differential expression of a large number of genes; interestingly, some of them are found to have the same trend of regulation in response to human blood (21), where glucose is abundant. In *N. meningitidis*, glucose is metabolized mainly through the Entner-Doudoroff (ED) pathway and to a lesser extent through the pentose phosphate (PP) pathway (47, 48), while the Embden-Meyerhof-Parnas (EMP) pathway is not fully functional, because it lacks the phosphofructokinase gene (49). Similarly, in *P. putida* the ED pathway synthesizes the major part of the pyruvate (67 to 87%) and the PP pathway accounts for the remaining part (50). Accordingly, *N. meningitidis* microarray analysis revealed that genes driving glucose catabolism through these pathways (*glk*, *zwf*, *edd*, *eda*, *pgl*, *pgi1*, *gapB*, and *pykA*) were highly upregulated by the presence of glucose (Fig. 6). In contrast, genes from the gluconeogenesis pathway (*pgi2* and *gapA*) were downregulated in the presence of glucose (Fig. 6). This can have physiologically relevant consequences, since *gapA* was shown to play a role in adhesion (51) and its expression is controlled by the NadR repressor (20). Furthermore, the acetate kinase gene *ackA2*, which is involved in the production of acetate from acetyl coenzyme A (acetyl-CoA), was downregulated by glucose (Fig. 6). Since the catabolism of glucose results in the accumulation of

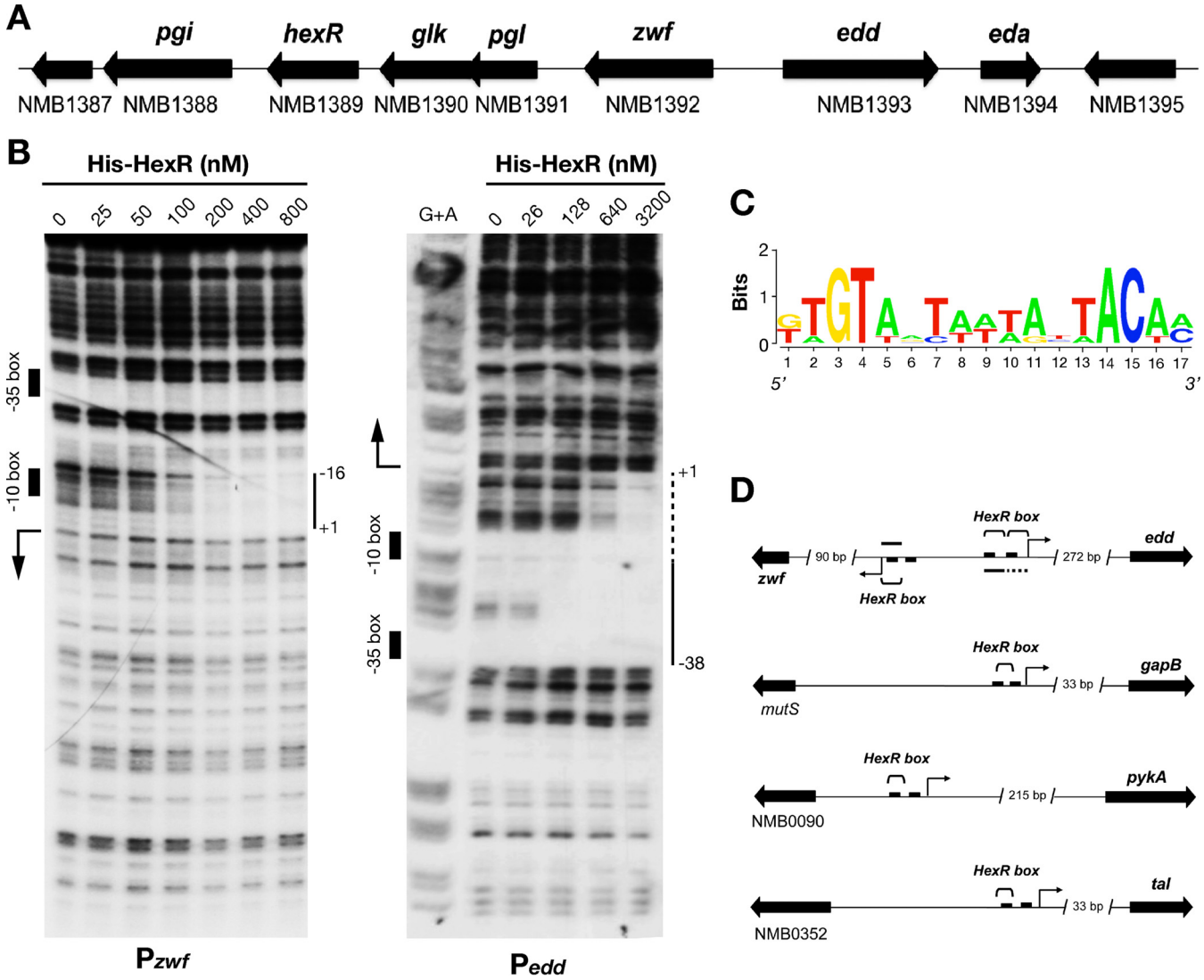

FIG 4 Direct binding of HexR to the promoter regions of genes involved in central carbon metabolism. (A) Genetic organization of the *hexR* locus with respect to central carbon metabolism genes. (B) DNase I footprinting analysis of HexR binding to the *zwf* and *edd* promoter regions. Bars, regions protected against DNase I digestion by HexR; arrows, operon transcriptional start sites identified by total RNA sequencing experiments (data not shown); boxes, putative promoter elements. (C) HexR binding consensus sequence derived from mapped sites on the *zwf* and *edd* promoters (WebLogo 3.2). (D) Sequences matching the HexR binding consensus sequence found upstream of HexR-regulated operons (EMBOSS fuzznuc algorithm). Brackets, predicted HexR binding sequences overlapping putative promoter elements.

acetate, a feedback effect of an acetate surplus could explain this observation. Similarly, other pathways that would be directly affected by an overabundance of acetyl-CoA, such as the TCA cycle enzymes encoded by *prpB*, *prpC*, *lpdA3*, *sdhABCD*, and *sucCD*, were found to be repressed in the presence of glucose (Fig. 6). Indeed, growth on glucose has been reported to reduce the levels of TCA cycle enzymes also in gonococci (52, 53). Interestingly, the glucose-repressed genes encoding the TCA cycle enzymes were also repressed in glucose-rich human blood (21, 22) and, in a recent report, the TCA cycle *prp* operon was suggested to help *N. meningitidis* in the colonization of the propionate-rich and glucose-poor oral cavities of adults (54). Also, the NADH-specific glutamate dehydrogenase gene *gludD* was found to be repressed in the presence of glucose, as was the *putP-putA* operon, which is involved in glutamate degradation and proline utilization. This suggests an interconnection between carbon catabolism and nitrogen metabolism in response to carbon source availability. In contrast, NADPH-specific *gdhA* was upregulated by glucose. High-level expression of *gdhA* is dependent on ammonia assimilation from the TCA cycle intermediate 2-oxoglutarate and may result in growth advantages when the glucose concentration is higher than that of lactate (55), such as in human whole blood, where *gdhA* has been found to be upregulated (21).

Overall, we observed that the *N. meningitidis* response to glucose modulates a large number of genes, compared to the responses to other environmental signals, such as zinc (17 genes [18]), iron (83 genes [16, 17]), or lactate (23 genes [data not shown]). Furthermore, 11 genes that were found to be differentially expressed in the presence of glucose showed similar patterns of expression when *N. meningitidis* was exposed to lactate (data

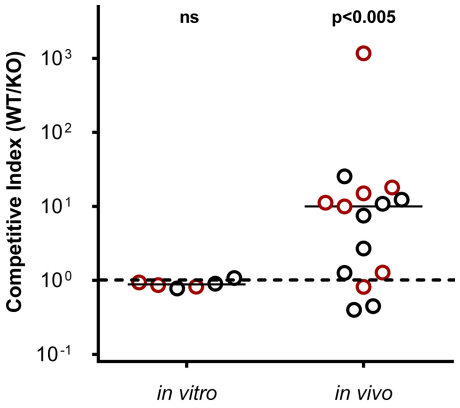

FIG 5 Deletion of *hexR* impairs survival during infection *in vivo*. Individual competitive index (CI) values from *in vitro* growth assays or intraperitoneal challenge of infant rats (*in vivo*) with *N. meningitidis* WT and Δ*hexR* knockout (KO) strains in a 1:1 ratio are shown. Circles, individual samples. Two infectious doses were used, i.e., 4.5×10^3 CFU (red) and 4.5×10^4 CFU (black). Solid line, median; dashed line, CI of 1. A CI of >1 indicates that the WT strain is more competitive than the mutant. CIs observed with the two infectious doses followed the same statistical distribution and were pooled to increase the power of the analysis. Statistical significance was assessed with the Mann-Whitney test. ns, not significant.

not shown), suggesting that these genes respond to the availability of a carbon source rather than to the type of sugar added. Most of these genes encode hypothetical proteins but also proteins related to the cell envelope, such as NMB0342 (*ispA*), NMB1729 (*exbB*), and NMB0543 (*lctP*).

This study identified an RpiR family HexR regulator controlling the central carbon metabolism of *N. meningitidis* in response to glucose (Fig. 6). Although only 28% of the total glucose-regulated genes in *N. meningitidis* are also controlled by HexR, this regulator controls the majority (60%) of the glucose-regulated genes belonging to the functional category of energy metabolism. The number of genes differentially expressed in a Δ*hexR* mutant strain of *N. meningitidis* is similar to values reported for other proteobacteria (44). In other species, members of this family can be repressors such as RpiR *in Escherichia coli* (56), transcriptional activators like GlvR of *Bacillus subtilis*, which modulates maltose metabolism (57), or dual-purpose transcriptional factors like HexR in *P. putida* (43), *Pseudomonas aeruginosa* (58), and *S. oneidensis* (44). The *N. meningitidis* HexR acts as a repressor by binding specific DNA sequences within the promoters of its target genes, albeit with different affinities. In our DNA-protein footprinting experiments, we identified a 100 nM HexR affinity site in the *zwf* promoter and a site with similar affinity (128 nM) in the *edd* promoter, while a second site within the *edd* promoter required 640 nM HexR for protection. Similar results were obtained in *P. putida*, where 100 nM to 3 μM HexR was necessary to bind to the operators within the *zwf* and *edd/gap-1* promoter regions in DNA footprinting (43). Interestingly, in the transcriptome data, the fold change for *edd*, containing two HexR operators, was almost double that for *zwf*, for which only one operator was identified. Overall, our *in vitro* data agree with findings reported for HexR in other proteobacterial species, where it directly regulates the transcription of central carbon metabolism genes (44). Fur-

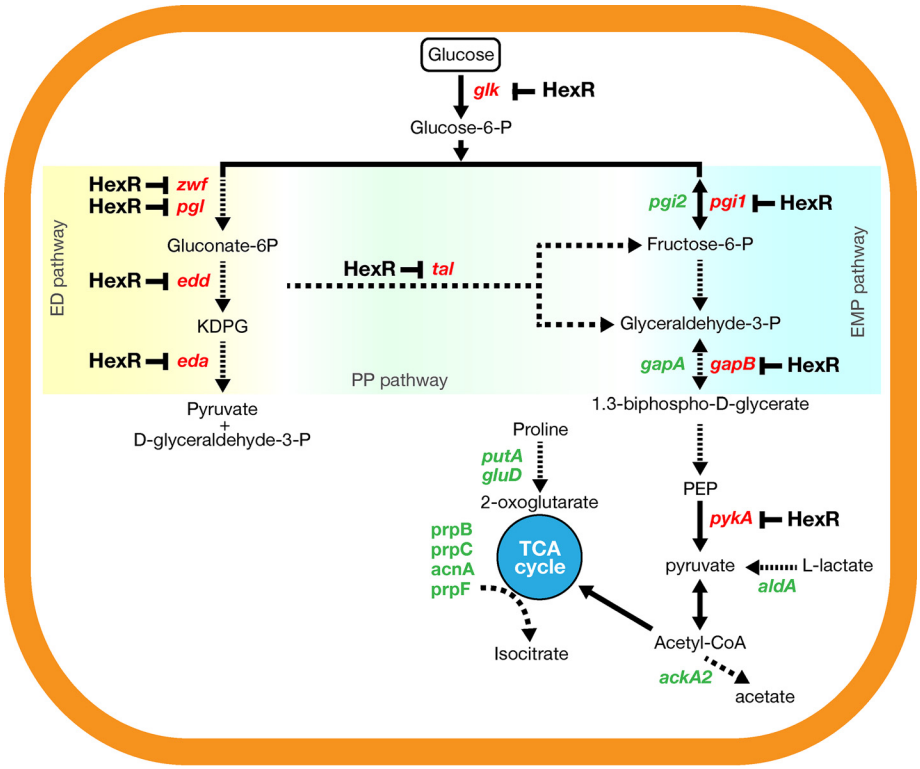

FIG 6 Model of glucose- and HexR-mediated regulation in *N. meningitidis*, showing the main metabolic pathways affected by glucose availability. Genes that are significantly upregulated (red) and downregulated (green) by glucose are shown. Genes subject to glucose-responsive HexR repression are highlighted. ED, Entner-Doudoroff; PP, pentose phosphate; EMP, Embden-Meyerhof-Parnas; TCA, tricarboxylic acid; P, phosphate; PEP, phosphoenolpyruvate.

thermore, the HexR binding DNA consensus sequences are very similar for *N. meningitidis*, *P. putida*, and *Shewanella* spp., and common HexR-responsive genes are found among these species (43).

Our results show that HexR not only controls the expression of genes from central carbon metabolism but also regulates genes involved in nitrogen metabolism, redox potential, and denitrification, therefore suggesting that HexR may play an important role in connecting these major pathways in responses to environmental adaptation. In *E. coli*, expression of the enzymes in the ED pathway is essential for colonization of the gastrointestinal tract (59); in *P. putida*, the ED pathway plays an important role in the generation of redox currency, which is required to counteract oxidative stress (60). Similarly, a meningococcal strain lacking *hexR* showed reduced fitness during *in vivo* infection, which indicates the importance of this transcriptional regulator not only in metabolic adaptation but also in the survival of *N. meningitidis* within the host. It would be interesting to investigate the steps of infection, such as adhesion, colonization, and/or multiplication, during which HexR is mostly expressed *in vivo* and therefore regulating its targets.

It is interesting to note that, although *N. meningitidis* inhabits different niches in the host (such as the nasopharynx, blood, and meninges), where nutrient availability is very diverse, it uses a restricted range of carbon sources, does not have a complete EMP pathway for carbon metabolism, and has no equivalent to known global carbon catabolite regulators. This means that *N. meningitidis* does not follow the same paradigm of carbon catabolite repression as reported for enterobacteria or Gram-positive low-G+C bacteria and that HexR plays a major role in the biology of *N. meningitidis* by regulating its central carbon metabolism in response to environmental signals. However, we have also shown that not all glucose-responsive genes are regulated through HexR (e.g., *nspA*), suggesting that other mechanisms, either transcriptional or posttranscriptional, could affect gene expression in response to glucose.

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