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Regulatory networks of *Neisseria meningitidis* and their implications for pathogenesis

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Sessione II

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ATTIVITÀ DI RICERCA

Durante il Dottorato di Ricerca mi sono occupato dello studio della regolazione genica in *Neisseria meningitidis*. In particolare, ho studiato la risposta in termini di espressione genica alla disponibilità di glucosio, identificando un regolatore trascrizionale HexR che coordina il metabolismo centrale di meningococco. Ho caratterizzato le interazioni molecolari tra la proteina HexR e i suoi geni target, e ho studiato l'impatto della regolazione mediata da HexR sulla capacità di *N. meningitidis* di causare batteremia in un modello murino di infezione.
In parallelo, ho indagato il ruolo dei piccoli RNA non codificanti nella regolazione genica di meningococco. Tramite sequenziamento dell'RNA ad alta risoluzione ho identificato e mappato nuovi trascritti potenzialmente regolatori, e ho studiato l'impatto di candidati selezionati sulla batteremia di *N. meningitidis*. Infine, ho iniziato la caratterizzazione molecolare di un nuovo piccolo RNA unico di meningococco, strettamente associato a geni rilevanti per le *Neisseriae* patogene.

Nel periodo del Dottorato di Ricerca sono stato co-autore dei seguenti lavori scientifici:

Antunes A, **Golfieri G**, Ferlicca F, Guadagnuolo S, Scarlato V, Delany I. "Impact of glucose in *Neisseria meningitidis* transcriptome: characterization of the HexR regulon, a glucose-responsive regulator". *manuscript in preparation*

Fagnocchi L, Bottini S, **Golfieri G**, Fantappiè L, Ferlicca F, Antunes A, Guadagnuolo S, Del Tordello E, Siena E, Serruto D, Scarlato V, Muzzi A, Delany I. "Global transcriptomic analysis reveals small RNAs affecting *Neisseria meningitidis* bacteraemia". PLoS One *submitted, under review*

Donnarumma D, **Golfieri G**, Brier S, Castagnini M, Veggi D, Bottomley MJ, Delany I, Norais N. "*Neisseria meningitidis* GNA1030 is a ubiquinone-8 binding protein". FASEB J. 2015 Feb 20. pii: fj.14-263954. [Epub ahead of print]

Contents

1 Abstract

Neisseria meningitidis, the leading cause of bacterial meningitis, can adapt to different host niches during human infection. Both transcriptional and post-transcriptional regulatory networks have been identified as playing a crucial role for bacterial stress responses and virulence. We investigated the *N. meningitidis* transcriptional landscape both by microarray and by RNA sequencing (RNAseq).

Microarray analysis of *N. meningitidis* grown in chemically defined medium in the presence or absence of glucose allowed us to identify genes regulated by carbon source availability. In particular, we identified a glucose-responsive *hexR*-like transcriptional regulator in *N. meningitidis*. Deletion analysis showed that the *hexR* gene is accountable for a subset of the glucose-responsive regulation, and *in vitro* assays with the purified protein showed that HexR binds to the promoters of the central metabolic operons of meningococcus, by targeting a DNA region overlapping putative regulatory sequences. Our results indicate that HexR coordinates the central metabolism of meningococcus in response to the availability of glucose, and *N. meningitidis* strains lacking the *hexR* gene are also deficient in establishing successful bacteremia in a mouse model of infection.

In parallel, RNAseq analysis of *N. meningitidis* cultured under standard or iron-limiting *in vitro* growth conditions allowed us to identify novel small non-coding RNAs (sRNAs) potentially involved in *N. meningitidis* regulatory networks. Manual curation of the RNAseq data generated a list of 51 sRNAs, 8 of which were validated by Northern blotting. Deletion of selected sRNAs caused attenuation of *N. meningitidis* infection in a murine model, leading to the identification of the first sRNAs influencing meningococcal bacteraemia. Furthermore, we describe the identification and initial characterization of a novel sRNA unique to meningococcus, closely associated to genes relevant for the intracellular survival of pathogenic *Neisseriae*.

Taken together, our findings could help unravel the regulation of *N. meningitidis* adaptation to the host environment and its implications for pathogenesis.

2 Introduction

2.1 Meningococcal disease

Neisseria meningitidis is a strictly human pathogen responsible for meningitis and sepsis, two devastating diseases that can kill children and young adults within hours, despite the availability of effective antibiotics. The meningococcal disease was first discovered in 1887 by Anton Weichselbaum, who described the meningococcal infection of the cerebrospinal fluid of a patient (1). The meningococcal disease occurs mainly as sporadic cases in industrialized countries, even if small regions suffer from epidemic outbreaks (e.g. New Zealand). On the contrary, it is largely epidemic in the so-called "meningitidis belt" in the sub-Saharan Africa. Studies performed in Europe (2) have demonstrated that carriage rates are very low in the first few years of life, but sharply rise during adolescence, peaking at 10–35% in 20–24-year olds, then decreasing to less than 10% in older age groups (2, 3). The reported annual incidence of meningococcal disease varies from 0.5 to 10 per 100,000 persons; however, during epidemics the incidence can rise above 1 per 1,000 (4, 5). The case fatality rate ranges from 5 to 15%, and approximately 11 to 19% of individuals surviving the disease often suffer from permanent sequelae, including neurodevelopmental deficits, hearing loss, seizures, ataxia, hemiplegia as well as amputation of limbs (6-10). What changes the colonization state of the organism into a disease state is not entirely clear. Most cases of meningococcal disease occur in otherwise healthy individuals without identified risk factors and for reasons not fully understood. However, certain biological, environmental and social factors have been associated with an increased risk of disease. Infants under 1 year of age, with a peak between 0 and 7 months, are the population at highest risk of infection due to their immature immune systems (6.33-7.08 cases per 100,000). A second peak in incidence is observed in adolescents and young adults (14-24 years; 0.75 cases per 100,000) (11). Microbial virulence factors, environmental conditions facilitating exposure and/or acquisition, impaired immune system, human genetic polymorphisms as well as naso- and oro-pharyngeal irritation caused by smoking and/or respiratory tract infection represent important factors for disease development (6, 12-17). Diagnosis of meningococcal disease can be challenging as its classic signs and symptoms, such as rash, fever, and headache are unspecific especially in the early course of the illness, and may be diagnosed as a more

benign infection. Due to the rapid progression of meningococcal disease, however, if appropriate treatment is delayed it can lead to death within 24 to 48 hours from the first onset of symptoms (7).

2.2 *Neisseria meningitidis*: pathogen and pathogenesis

2.2.1 The pathogen

N. meningitidis is a β-proteobacterium, Gram-negative diplococcus (Figure 1). It is aerobic, non-motile, non-sporulating, usually encapsulated and piliated. It is surrounded by an outer membrane composed of lipids, outer membrane proteins (OMPs), and lipooligosaccharide (LOS), a peptidoglycan layer and an inner membrane. Some meningococcal strains have a polysaccharide capsule attached to their outer membrane, and pathogenic strains are almost always encapsulated. The invasive potential of non-encapsulated disease isolates has recently been reported (18).

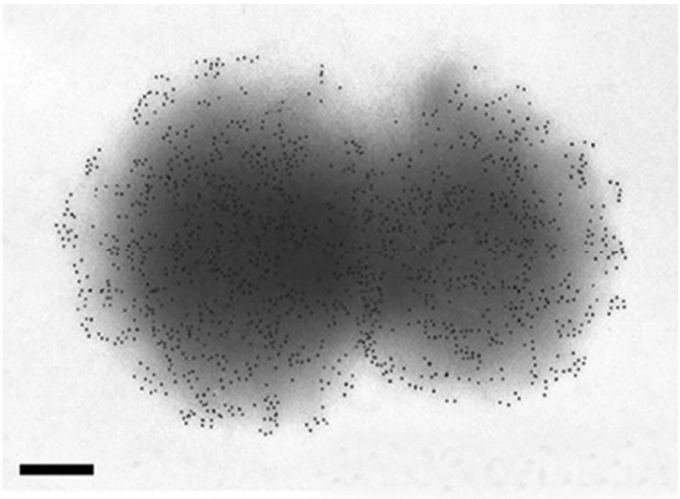

Figure 1 Immuno-gold labelling and transmission electron microscopy of *Neisseria meningitidis*. Analysis of the strain was performed with antisera raised against the NadA adhesin. Scale bars: 200 nm (19).

Traditionally, *N. meningitidis* strains are classified into serogroups according to the immunological reactivity of their capsules. With this method 13 different serogroups have been identified, although only the A, B, C, Y, X and W135 serogroups commonly cause invasive infections (Figure 1). Meningococci are further classified into serotype and serosubtype, based on antigenic differences in their major OMPs, PorA and PorB. The serological classification system, however, is limited due to high frequency of phase and

antigenic variation of outer-membrane structures, which has led to the development of DNA-based approaches to characterize meningococcal strains. A genetic typing system based upon polymorphisms in seven housekeeping genes called Multilocus Sequence Typing (MLST) is now the golden standard for molecular typing and epidemiologic studies (20). Menigococci can thus be classified into lineages, termed clonal complexes. A clonal complex is a group of sequence types (STs) that share at least four of the seven loci in common with a central ancestral genotype (21). MLST technique has shown that the majority of disease associated isolates cluster into a minority of STs called hyperinvasive lineages (22). Why hyperinvasive meningococcal lineages are more pathogenic than others still remains unknown.

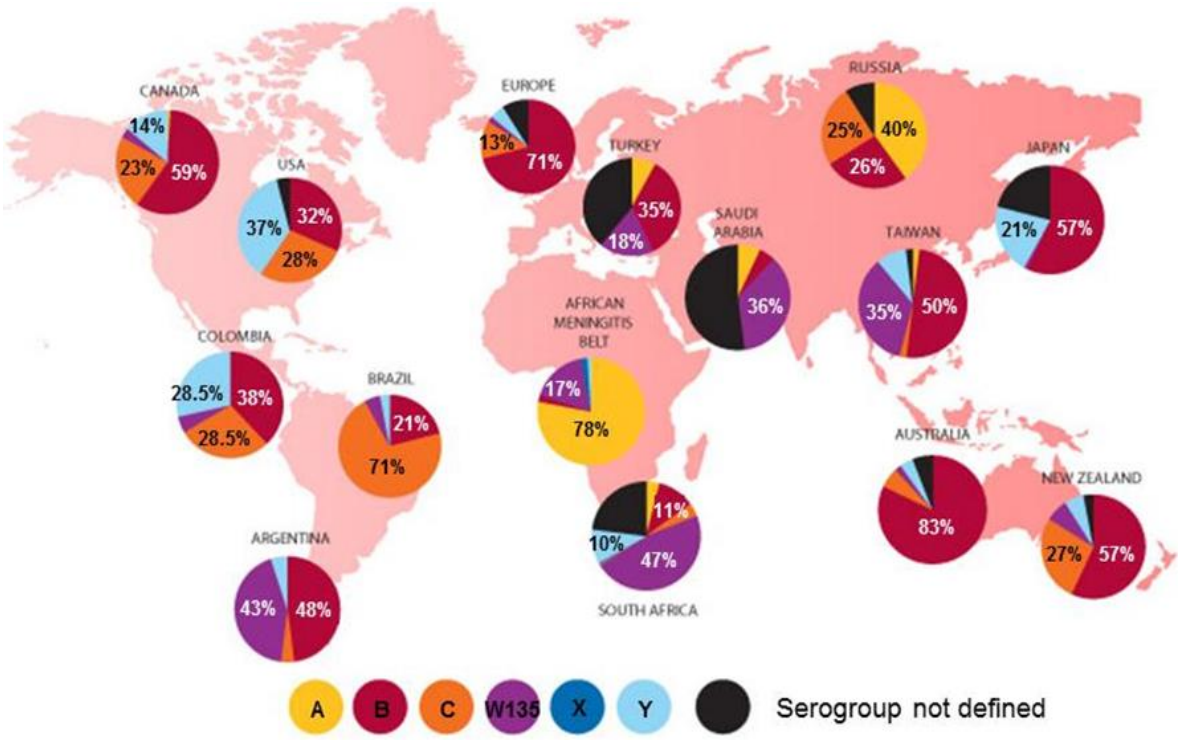

Figure 1 Global distribution of invasive meningococcal serogroups. Graphical representation of serogroup-specific incidence in different geographical areas of the world (adapted from www.meningitisinfo.com).

2.2.2 Colonization and invasion

N. meningitidis colonizes the upper respiratory tract in about 25% of the human population where it can live as commensal. This carrier state represents a successful commensal relationship between the host and the bacterium: it provides the only known reservoir for the human-adapted meningococcal infection and may also contribute to establishing host immunity (23). For largely unknown reasons, in a small subset of carriers meningococcus can invade the pharyngeal mucosal epithelium and disseminate into the bloodstream, causing septicemia. In a subset of cases, the bacteria can also cross the blood-brain barrier and infect the cerebrospinal fluid, causing meningitis.

The pathogenesis of *N. meningitidis* is a complex multi-stage process (Figure 2). Meningococci may be acquired via respiratory droplets or saliva. Colonization of the upper respiratory mucosal surfaces by *N. meningitidis* is the first step in establishment of both the human carrier state and the invasive meningococcal disease (23). The first step in meningococcal colonization is the initial contact with nasopharyngeal epithelial cells mediated by Type IV pili, which may recognize the host receptor CD46 (24), then bacteria proceed to proliferate on the surface of human non-ciliated epithelial cells, forming small microcolonies at the site of initial attachment (23). After the initial colonization, there is a loss or down-regulation of the capsule, whose presence masks the outer membrane proteins by steric hindrance. This event can occur both via cell-contact induced repression (24) and by selection of low or no-capsule expressing bacteria due to phase variation (25). Close adherence of meningococci to host epithelial cells is then mediated by a variety of possibly redundant adhesins that were previously masked by the capsule. One trigger of meningococcal internalization is represented by interaction of the bacterial opacity proteins, Opa and Opc, with CD66/CEACAMs and integrins, respectively, on the surface of the epithelial cell (26). This results in the appearance of cortical plaques and the recruitment of factors leading to the formation and extension of epithelial cell pseudopodia within intracellular vacuoles (27). Once internalized in the epithelial cells, meningococcal survival depends on factors such as the IgA1 protease, which degrades lysosome-associated membrane proteins (28). Meningococci are capable of intracellular replication and this is due in part to the capacity of the organism to acquire iron through specialized transport systems, such as the hemoglobin binding receptor (HmbR), transferring binding protein (TbpAB) and lactoferrin binding protein (LbpAB) (29). This intracellular lifestyle gives the bacteria an

opportunity to evade the host immune response as well as to find new sources of nutrients, and is also a way to cross the epithelium and enter the bloodstream (23). In healthy individuals, bacteria that cross the mucosal epithelium are eliminated by serum bactericidal activity. However, in susceptible individuals meningococcus can occasionally cross the mucosal epithelial barrier, either through transcytosis or directly following damage to the monolayer integrity, or through phagocytes (30). Eventually, bacteria enter the bloodstream and have to evade the host defence mechanisms using strategies such as up-regulation of capsule expression, which can prevent antibody and complement deposition (31) and is both anti-opsonic and anti-phagocytic (30). Meningococcus can also recruit negative regulators of the complement cascade such as factor H, recruited by the factor H-binding protein (fHBP) (32), and complement regulators such as the C4-binding protein (C4bp) bound by PorA porins (33). Once inside the bloodstream, bacteria either multiply slowly, eventually passing across the brain vascular endothelium or the epithelium of the choroid plexus, resulting in infection of the meninges and the cerebrospinal fluid (34), or they undergo rapid multiplication in the bloodstream, resulting in clinical features of bacterial septicemia or meningococcemia (35).

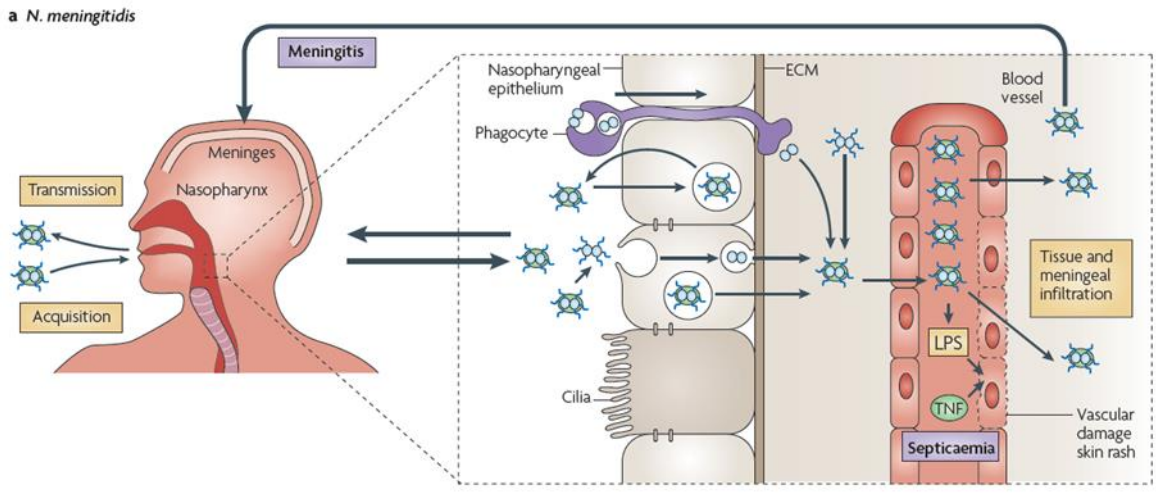

Figure 2 Stages in the pathogenesis of Neisseria meningitidis. Schematic representation of the steps of meningococcal colonization and infection (adapted from (30)).

Meningococcal adaptation to the different host niches also occurs at the level of metabolism (36). Therefore, acquisition of nutrients that enable the bacterium to sustain growth and multiply is critical for the outcome of meningococcal disease. In fact, *N. meningitidis* is capable of adapting to different anatomical compartments of the host (37), where the

availability of key nutrients such as carbon sources is diverse. However, this bacterium requires a restricted variety of substrates such as glucose, lactate or pyruvate as sole carbon sources to initiate growth (38, 39). Glucose is one of the few carbon energy sources that *N. meningitidis* can use as sole energy source, and the preferred carbon substrate for growth of meningococcus in terms of biomass yield (40). Lactate has been shown to be essential for effective colonization and its acquisition has been implicated in the virulence of *N. meningitidis* (41). Studies of *N. gonorrhoeae* have shown that in media containing glucose, lactate stimulates metabolism and that this could affect pathogenicity (42). Nevertheless, glucose is the predominant carbon source in blood as well as in the cerebrospinal fluid (43), the two main niches of meningococcal infection. Moreover, about half of the genes essential for systemic infection encode enzymes involved in the metabolism and transport of nutrients (44). The ability to obtain and synthesize nutrients is therefore essential for meningococcus to survive in the different microenvironments that it encounters within the human host during the course of infection.

Overall, the onset of meningococcal disease can be seen as a failed relationship between the meningococcus and the host. While factors that trigger meningococcal entrance in the bloodstream are not yet fully understood, they are likely dependent on both the host and pathogen sides and include impairing of the integrity of the human nasopharyngeal mucosa, the lack of a protective immune response and microbial factors influencing virulence (4, 5).

2.2.3 Virulence factors

The major virulence factor of *N. meningitidis* is the polysaccharide capsule (Figure 3), which plays a crucial role in meningococcal fitness, protecting the bacterium during airborne transmission between hosts (45), and facilitating colonization and virulence by protecting the meningococcus from desiccation and the host innate and adaptive immune effector mechanisms such as phagocytic killing, opsonization, antimicrobial peptides and complement-mediated bactericidal killing (46, 47). Like many other virulence factors its expression is phase variable (25) and capsule switching between one serogroup to another provides a selective advantage that allows the bacterium to evade opsonization or neutralization by natural or vaccine-induced protective anti-capsular antibodies (48).

The lipo-polysaccharides of meningococcus are more accurately referred to as lipo-oligosaccharide (LOS), because of the presence of repeating short saccharides instead of

long chain saccharides (Figure 3). LOS are the major constituent of the outer leaflet of the meningococcal outer membrane, responsible for the physical integrity and proper functioning of the membrane and required for resistance of *N. meningitidis* to complement (49). Phase and antigenic variations lead to different saccharide chains altering dramatically the antigenic properties of LOS and enabling individual meningococci to display a repertoire of multiple LOS structures simultaneously (50).

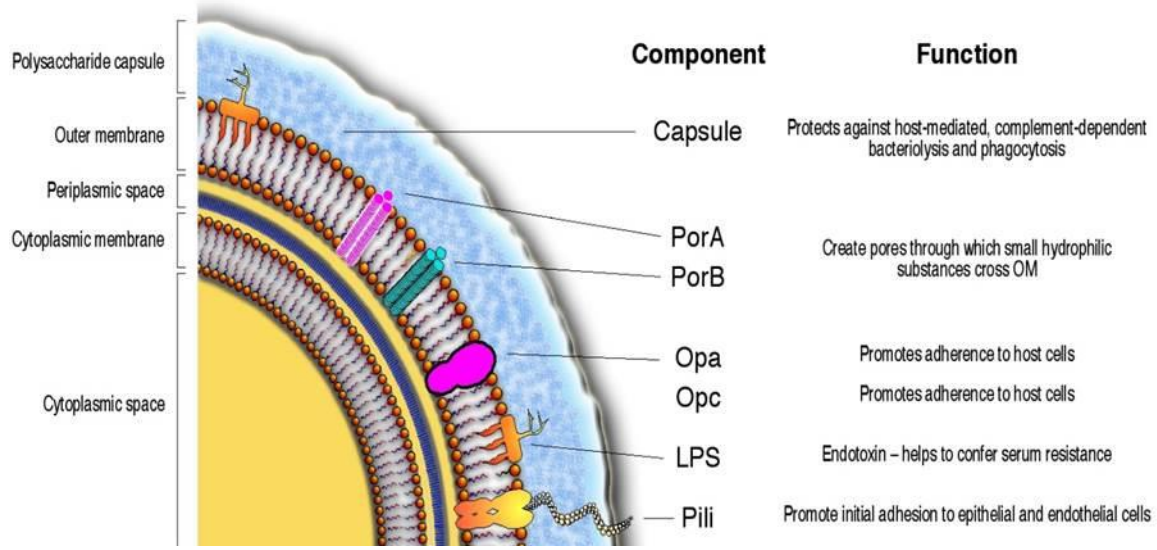

Figure 3 Meningococcal cell compartments. Schematic representation of the different bacterial compartments and of the main components of the outer membrane, together with their known functions (adapted from (6)).

Another group of major virulence factors involved in the interface between the bacterium and the host are the pili (Figure 3), long surface proteins that extend from the bacterial surface beyond the capsule (51, 52). The pilus is composed of identical subunits of pilin, expressed from the *pilE* locus. The *pilE* gene undergoes sequence variation due to homologous recombination with multiple non-expressed truncated *pilS* genes, resulting in different adhesive and immunogenic pili variants (53). Meningococcal pili belong to type IV pilus family, members of which undergo rapid extension and retraction. They represent the major contributor to adhesive property of the capsule and are involved in the initiation of the meningococcus-host cell interaction (54, 55). Together with the outer membrane adhesins, pili facilitate adhesion to host tissues having a crucial role in the initial establishment of encapsulated bacteria on mucosal surfaces, helping the penetration of the negatively charged barrier at the host-pathogen interface (56). In addition to adhesion, pili are involved in several other functions such as facilitating the uptake of foreign DNA from the extracellular

environment, a property that contributes to virulence by promoting genetic adaptability (57). Twitching motility generated by pilus retraction is important for passage through the mucosal layer, movement over epithelial surface and micro-colonies formation (58).

N. meningitidis has evolved a number of other surface structures that mediate interaction with host cells. The two opacity proteins (Opa and Opc) are integral outer membrane proteins that mediate pathogen-host interaction (Figure 3), adhering to and invading of epithelial and endothelial cells (52). Numerous adhesins are generally expressed at low levels during *in vitro* growth but may be important during *in vivo* infections. Furthermore, several adhesins are subject to antigenic variation and/or phase variation, which allow bacteria to generate a broad and variable repertoire of surface structure that facilitates evasion of immune effectors mechanisms and adaptation to different niches (30). The neisserial adhesin A (NadA) was firstly identified during a bioinformatic analysis of the genome of a virulent *N. meningitidis* B strain looking for novel vaccine candidates (19). NadA is a surface-exposed member of the oligomeric coiled-coil adhesin family of bacterial trimeric autotransporter adhesins, such as YadA of *Yersinia* spp. (59) and HadA of *Haemophilus influenzae* biogroup aegyptius (60). It has been shown that NadA mediates adhesion to and invasion of human epithelial cells (61), suggesting a key role of NadA in bacterial adhesion to the naso- and oro-pharyngeal epithelia during meningococcal colonization of the human upper respiratory tract. NadA is a risk factor for the development of meningococcal disease, as it is present in 50% of the disease-associated strains and overrepresented, almost 100%, in hypervirulent meningococcal lineages (62, 63). Several other minor adhesins belong to the family of autotransporter adhesin. Among them, *Neisseria* Hia homologue A (NhhA), mediates low levels of adhesion to epithelial cells and to extracellular matrix components (64). The adhesion penetration protein (App), an autotransporter protein with a highly conserved aminoacid sequence, has been shown to mediate bacterial interaction to epithelial cells during the early stages of colonization. At later stages, App autocleavage may allow bacterial detachment, therefore facilitating bacterial spread (65). Meningococcal serine protease A (MspA) is homologous to App and may also be cleaved and secreted (66). The multiple adhesin family (Maf) is a family of glycolipid adhesins, characterised first in gonococci, which may play a role in Opa-independent cell invasion (67).

PorA and PorB are the most abundant proteins present in the meningococcal outer membrane (Figure 3). These antigens comprise regions of relatively conserved sequence, which are predicted to form the beta-barrel structure of the proteins, interspersed with more variable regions, which form the putative surface-exposed loops. The monomers associate in trimers creating pores for the passage of small hydrophilic solutes necessary for bacterial metabolism. While not considered adhesins, they interact with numerous human cell types and proteins (68). PorA elicits a protective immune response in humans (69), while the role of PorB in stimulating immune protection is less clear, being immunogenic but poorly accessible for antibodies (70).

The ability to escape the elaborate machinery of the human immune system is a key determinant in the virulence of human pathogens. Many factors contribute to the virulence of *N. meningitidis*, involving mechanism to face antimicrobial peptides, reactive nitrogen and oxygen species, complement-mediated killing and, ultimately, the humoral and cellular components of the immune system. Efflux pump have been shown to have a critical contribution to antimicrobial peptide resistance (71). Enzymes such as catalases (Kat), superoxide dismutase (SodB and SodC), nitrite reductase (AniA) and nitric oxide reductase (NorB) neutralize the toxic effects of reactive oxygen and nitrogen species generated by neutrophilis and macrophages (72-74). Also the complement system, an essential component of the innate immune response, plays a significant role in defence against meningococcal infection, as indicated by the increased susceptibility to *N. meningitidis* infections of patients with complement deficiencies (75). The complement system consists of a well-balanced network of circulating and cell surface-bound proteins that act as substrates, enzymes or modulators of a hierarchical series of extracellular proteolytic cascades. The complement activation is initiated by the classical (CP) or the lectin (LP) pathways and is amplified by the alternative pathway (AP). All of these pathways converge at the level of the C3 complex, leading to cleavage of C3 to C3b by C3 convertases. Deposition of C3b on the surface of an invading pathogen results in its elimination through phagocytosis or lysis following assembly of the membrane attack complex (MAC). *N. meningitidis* uses a variety of mechanisms to survive to the bactericidal action of the complement system, involving its capsule, LOS and other factors (47). One such factor is fHBP, a surface-exposed lipoprotein which binds human factor H (fH), the main inhibitor of the complement AP (76). Sequestering fH allows meningococci to use this down-regulator to limit complement activation on their surface. In

addition, the Neisserial Heparin-Binding Antigen (NHBA) has been described to bind heparin, which may increase bacterial serum resistance due to the potential interactions of heparin with fH (77).

2.3 Meningococcal vaccines

Meningococcal disease progresses rapidly and in its early stages it is easily misdiagnosed (6, 7), making vaccination the best public health option worldwide and the most effective way to prevent outbreaks. No broadly protective vaccine is currently available to provide protection against all serogroups of *N. meningitidis*. Capsular polysaccharides have been successfully used as antigens to produce polysaccharide and glycoconjugate vaccines against four of the five disease-associated serogroups A, C, W-135 and Y. Quadrivalent vaccines against serogroups A, C, W-135 and Y include the conjugate vaccines Menactra (Sanofi Pasteur) and Menveo (Novartis), and the polysaccharide vaccine Menomune (Sanofi Pasteur) and Mencevax (GlaxoSmithKline) (78). A vaccine called MenAfriVac has been developed through a program called the Meningitis Vaccine Project to prevent meningitis group A infections in the African 'meningitidis belt' (79).

In contrast, the group B capsule polysaccharide is not suitable as vaccine antigen because it consists of a homolinear polymer of α(2→8)N-acetyl neuraminic acid, also known as polysialic acid, which is structurally similarity to the sialic acid found in human neural tissue (80, 81). Consequently, it is a poor immunogen in humans and may elicit autoantibodies. Therefore, efforts to develop a group B vaccine focused mainly on non-capsular antigens, such as proteins or LOS. Detergent-extracted Outer Membrane Vesicles (dOMV) have been successfully used in Norway (82), Cuba (83), Chile (84) and New Zealand (85) to control epidemic disease outbreaks caused by specific MenB strains.

A significant limitation of these vaccines is the breadth of coverage provided. The detergent treatment extracts the toxic LOS, but it also extracts other desirable antigens such as fHbp. Consequently, the porin protein PorA results to be the immuno-dominant antigen (86, 87). However, PorA is antigenically variable (88) so the immune response elicited is effective only against strains expressing the same PorA serosubtypes.

The availability of whole genome sequences has contributed radically to change the approach to vaccine development, laying the fundaments for an *in silico* genome-based approach named Reverse Vaccinology (RV). RV aims to identify surface-exposed non-

capsular antigens that are antigenically conserved among strains and that elicit a bactericidal serum response. This approach has led to the development of the recombinant protein vaccine 4CMenB (89, 90). The 4CMenB vaccine contains five genome-derived *Neisseria* antigens (GNA), which are formulated together. The vaccine formulation joins NadA (61, 91), as well as two recombinant fusion proteins of fHbp (92, 93) and NHBA (77, 94) fused to the conserved meningococcal gene products GNA2091 and GNA1030, respectively. The vaccine formulation also includes detergent-extracted outer membrane vesicles (dOMVs) from the NZ98/254 strain (87). The 4CMenB vaccine was licensed as Bexsero in 2013, following its progression through clinical trials that have demonstrated its safety (95, 96) and its efficacy in inducing a protective immune response in infants, children, adolescents and adults against the majority of MenB strains (97, 98).

Another licensed vaccine against meningococcal serogroup B is the recombinant protein-based vaccine composed of equal amounts of lipidated fHbp variants from different subfamilies. This vaccine was licensed in October 2014 in the US for a target population of adolescents and young adults. In preclinical study, the bivalent vaccine elicited high bactericidal titers against different MenB strains, suggesting a good breadth of coverage (99). In a phase I trial, it was assessed that the vaccine was well tolerated in adults, adolescents and young children (99). However, it is not suitable for use in infants considering that it consists or purified lipoproteins known as TLR-2 agonists (100). In addition, the *in vivo* level of fHbp expression strongly affects the effectiveness of the bivalent vaccine.

2.4 Genetics of N. meningitidis

Genome sequences are available for a growing number of *N. meningitidis* strains. These data show that the meningococcal chromosome is between 2.0 and 2.2 megabases in size and contains about 2000 genes (101-104). The meningococcus shares about 90% nucleotide homology with either *N. gonorrhoeae* or the commensal *N. lactamica*. While approximately 70% of the genome encodes for essential metabolic functions, about 10% of the genome is represented by mobile elements such as IS elements and prophage sequences (101), leading to DNA transfer between meningococci, gonococci, commensal spp. as well as other bacteria (105). Another evident characteristic of the neisserial genome is the high abundance of repetitive DNA sequences, polymorphic regions and genetic switch mechanisms (e.g.

slipped-strand mispairing) which lead to genetic instability, facilitating duplication or deletion of regions in the genome, as well as recombination (105). Except for the IHT-A1 capsule locus, no specific core pathogenome has been identified (106), suggesting that virulence may be dependent on multiple redundant genes. The GC percentage is variable along the chromosome with an average of 51.63%, with defined regions of low GC content that likely have been acquired by relatively recent horizontal gene transfer events (107). These events are relatively common in *N. meningitidis* due to its natural transformation competence (108). For example, the acquisition of the capsule locus by horizontal gene transfer, possibly from *Pasteurella multocida* or *P. hemolytica (104)*, appears to be a major event in the evolution of the pathogenicity of the meningococcus from an un-encapsulated ancestor (23). A central characteristic of the genome is its plasticity contributing to the non-clonal behavior of meningococcus and its phenotypic diversity, which allow the bacteria to successfully adapt to the host.

2.5 Gene regulation and adaptation to the host environment

During infection, *N. meningitidis* can invade diverse sites within the human host, which represent different niches with respect to nutrient availability, environmental stress factors and competing microorganisms. Therefore meningococcus is subjected to constant selective pressures and its ability to rapidly adapt its metabolism and cellular composition to environmental changes is essential for its survival (109). Bacteria achieve adaptation to the environment either by changing their genotype (genome plasticity) or by transient alterations in gene expression. These two mechanisms are complementary and both lead to phenotypic variations.

2.5.1 Genome plasticity

The high natural competence of meningococci is a leading cause of horizontal gene transfer and therefore genome variability (110). In addition, the abundance of repetitive DNA sequences contributes to meningococcal genome plasticity. The most frequent repeat sequence element is the neisserial DNA uptake sequence (DUS). Nearly 2000 copies of this 12-bp sequence involved in recognition and uptake of DNA from the environment (111) are found in the *N. meningitidis* genome. On the other hand, the 20-bp long dRS3 elements

promote both permanent genomic changes, such as insertions and chromosomal rearrangements (104) and recombination with exogenous DNA (103). Correia elements are mobile elements of 100-150 bp, which carry transcription initiation sequences as well as binding sites for DNA bending protein, suggesting that they may have a role in modulating the expression of nearby genes (112). Finally, the meningococcal genome is also littered with insertion sequences (IS) and other repeat sequences whose function has not been completely determined yet, such as AT-rich repeats (101) and REP2 repeats (113).

Another major source of genome plasticity is phase variation (PV), the adaptive process by which bacteria undergo frequent and reversible phenotypic changes resulting from genetic alterations in specific loci of their genomes. Short tandem sequence repeats are the basis for PV, which can occur during replication through slipped-strand mispairing, altering the unit number of these repeats. The presence of repeat units may cause a slippage during replication of either the synthesis strand, leading to addition events, or the template strand, leading to deletions in the newly synthesized filament (105). When occurring in the coding sequence of a gene or within its promoter region, PV can change the transcriptional and translational state of the gene by introducing frameshift mutations or changing the spacing between critical promoter elements. It has been proposed that in *N. meningitidis* over 100 genes are potentially phase variable, altering mainly virulence-associated, surface-exposed molecules such as outer membrane proteins PorA, Opc, Opa, pili and adhesins, as well as LOS and capsule (105, 114-116). Meningococcal strains associated with disease have high frequency of PV, indicating that varying surface-exposed components provides substantial benefits during transmission between hosts (117).

Distinct from phase variation, antigenic variation is a mechanism of immune evasion where bacteria express different moieties of functionally conserved molecules that are antigenically distinct within a clonal population. This process is distinct from phase variation, as only one variant is expressed at any given time, although the cell still contains the genetic information to produce a whole range of antigenic variants. In the pathogenic *Neisseria* species, antigenic variation occurs in several surface components, including type IV pili, LOS and Opa proteins (105).

2.5.2 Transcriptional regulators

Survival under the rapidly changing conditions encountered within the host requires timely alterations in gene expression. Several environmental signals have been shown to have an impact on the *N. meningitidis* transcriptional regulation during host infection, such as iron (118, 119), zinc (120), nitric oxide (121) human saliva (122) and human blood (123, 124). At the transcriptional level, these alterations could be controlled by global factors, for example through changes in associations between different alternative sigma factors and core RNA polymerase, which reprogram the specificity of promoter recognition by the enzyme to allow expression of entirely new sets of target genes (125). In addition, different transcriptional regulators activated by various stresses can regulate the transcription of many genes important for survival and virulence. Although extensive transcriptional regulation is expected to accompany the infection process of *N. meningitidis*, only 36 putative transcriptional regulators are encoded by the meningococcal genome. This number is especially striking if compared to *Escherichia coli*, which harbors more than 200 transcriptional regulators. This striking limitation for transcriptional regulation is possibly related to the restricted ecological niches of the *Neisseriaceae* (126). Only 5 of the predicted regulators have been characterized so far, and the regulons of 4 have been dissected in detail.
Bacterial pathogenesis and survival are dependent on the ability to acquire iron (127), which is limiting during human infection being sequestrated by host iron-binding proteins. Although *N. meningitidis* does not produce siderophores for iron acquisition, it possesses outer membrane receptors that have been postulated to scavenge the iron-loaded siderophores secreted by other bacteria colonizing the nasopharyngeal tract (128) such as the hemoglobin binding receptor (HmbR), transferring binding protein (TbpAB) and lactoferrin binding protein (LbpAB) (29). However, since iron overload results in toxicity for the bacterium, meningococcus tightly regulates iron uptake by the ferric uptake regulator Fur (129). The Fur protein senses the intracellular iron concentration and binds to and represses iron uptake genes using ferrous iron as a co-repressor (118, 119, 130). Fur has been also reported to act positively in the expression of certain genes. The regulon of Fur comprises more than 200 genes (131) regulated either directly or by an indirect mechanism which involves a Fur-repressed small regulatory RNA named NrrF (132, 133).
During colonization and infection, *N. meningitidis* is also exposed to highly divergent partial pressures of oxygen (72). The fumarate and nitrate reductase regulator protein (FNR) is a

transcriptional activator that enables meningococci to survive under oxygen limitation by inducing sugar fermentation and denitrification pathways, utilizing nitrite and nitric oxide as electron acceptors (134, 135). Under oxygen limitation, FNR binds to DNA and activates target genes as a dimer containing a [4Fe-4S] cluster. This cluster dissociates in the presence of oxygen, destabilizing the dimer, with loss of FNR activity (135, 136). Interestingly, the mediator of complement evasion fHBP has been shown to be positively regulated by oxygen limitation through a FNR dedicated promoter (137).

N. meningitidis is often exposed to the gaseous free-radical nitric oxide (NO), generated both internally by its own metabolism and externally by the human host tissue, which is rich in macrophages, a potent source of NO during infection (138). The nitric oxide sensitive repressor (NsrR) is the major NO-responsive transcriptional regulator, repressing a small regulon of 4 genes. As NO concentration increases, NsrR is specifically inactivated through the alteration of its iron-sulfur cluster, thus leading to up-regulation of denitrifying genes (121, 139). Another regulator potentially relevant to the infection process is the Neisserial adhesin Regulator (NadR), a MarR-like protein demonstrated to regulate expression of both the phase-variable meningococcal adhesin NadA (116, 140, 141) and the MafA1 and MafA2 adhesins, shown to adhere to glycolipid receptors on human cells (142, 143). These genes are affected by NadR in opposing ways, depending on their specific promoter architectures (122). As typical of MarR-like proteins, a small molecule ligand, the 4-hydroxyphenylacetic acid (4HPA) catabolite of aromatic amino acids found in human saliva (144), acts as signal to alter the DNA binding activity of NadR *in vivo*, leading to repression or activation of its target genes (122, 145). Other transcriptional regulators of *N. meningitidis* include the LysR-type regulator CrgA, that is upregulated upon contact with human epithelial cells and acts as a repressor of its own transcription and type IV pili subunits (146, 147); AsnC is a global regulator that controls the response to poor nutrient conditions by binding to leucine and methionine, two amino acids representing general nutrient abundance (148); the Zinc uptake regulator (Zur) is a Fur-like regulator that responds specifically to zinc and controls zinc uptake by regulating a TonB-dependent receptor that allows high affinity zinc acquisition (149, 150).

2.5.3 Small regulatory RNAs

Small non coding RNAs (sRNAs) are regulatory elements whose essential role is well-established in all organisms (151). In pathogenic bacteria, regulatory sRNAs are a heterogeneous group of transcripts, which modulate a wide range of physiological processes through different mechanisms (152, 153). These regulators often function as coordinators of adaptation and/or virulence, integrating environmental signals and controlling target gene expression (154-156). sRNAs are commonly classified according to their mechanism of action within bacterial cells. Riboswitches are RNA sequences within the 5'-untranslated region (UTR) of the messenger RNAs (mRNAs) they regulate, that can adopt different conformations in response to environmental changes or the binding of metabolites (157). RNA thermometers are structured *cis*-regulatory elements that alter the efficiency of translational initiation in response to temperature (158, 159). The CRISPR (clustered regularly interspaced short palindromic repeats) RNAs contain short regions of homology to foreign DNA sequences and can interfere with bacteriophage infection, plasmid conjugation and natural transformation (160-162). In a recent publication, an archaeal CRISPR system has been reported to target both RNA and DNA molecules (163). Some sRNAs can bind proteins and alter their functions (164). Finally, the most extensively studied class are sRNAs modulating translation and stability of target mRNAs through direct base pairing. This class of regulatory RNAs can be further divided into two distinct broad classes: the *cis*-acting antisense sRNAs, encoded from the strand of DNA opposite to their mRNA targets and having extensive complementarity to these (165), and the *trans*-acting sRNAs, acting on multiple distal targets with a limited complementarity (153). RNA base-pairing interactions are usually in the 5-UTR of the target mRNA and have been shown to alter mRNA structure ultimately leading to changes in translation efficiency and, as a consequence, mRNA stability (154, 164). However, sRNAs can also interact with coding regions, regulating their targets not by translational control but by accelerating decay of the sRNA-mRNA duplex through RNase E, often in concert with the RNA chaperone Hfq (166-169). The majority of the regulation by the known trans-encoded sRNAs is negative (155, 170): base pairing with the target mRNA usually leads to repression of protein levels through translational inhibition, mRNA degradation, or both (171-173). However, activation of gene expression by sRNAs has also been reported (174). In such cases, base pairing of the sRNA disrupt an inhibitory secondary structure which sequesters the ribosome binding site (155, 175, 176). In

addition to their function as post-transcriptional regulators, some sRNAs may also encode for protein functions, providing for a dual role within the cell (177).

The role of many different sRNAs in pathogenic bacteria has been elucidated so far, and sRNAs have been found to be involved in a number of cellular mechanisms, from remodelling of the metabolism and regulation of homeostasis (178), to repression of outer membrane protein synthesis (179-183), to adaptation and resistance to stress (184-186), to virulence and pathogenesis (184, 187-190), as well as biofilm formation (191) and quorum sensing system regulation (184, 192). Interestingly, in recent years evidence of regulation of host transcripts by pathogen- or symbiont-encoded sRNAs had been arising (163, 193), indicating that sRNA-mediated regulation could extend outside the bacterial cell and play a role in cross-communication between invading bacteria and their host.

Activity of *trans*-encoded base-pairing sRNAs in Gram-negative bacteria often depends on the hexameric RNA chaperone Hfq, a homologue of the Sm-like proteins involved in splicing and mRNA decay in eukaryotes (168, 170). The Hfq protein is conserved in a wide range of bacteria and ranges in length from 70 to 100 amino acids (194). In all cases, the Sm motif is located in the N-terminal region of the molecule. The C-terminal domain seems not to play a significant role in the major functions of Hfq. In fact, a C-terminal truncated form of the *E. coli* Hfq lacking the C-terminal amino acids can replace the intact *E. coli* Hfq (195). The main role of Hfq in assisting sRNA regulation is to promote sRNA-mRNA base pairing via multiple mechanisms: it increases the annealing rates of RNA molecules (196-198), stabilizes cognate sRNA-mRNA duplexes (199), promotes the structural remodelling of sRNA and target mRNAs (200), and increases the local concentration of both RNA species (201). The centrality of Hfq in assisting regulatory circuits involved in fitness and virulence is highlighted by the pleiotropic effects of Hfq-KO in many pathogens, whose phenotypes include increased sensitivity to host defence mechanisms and attenuation in animal models (133, 202-205). Hfq–independent post-transcriptional regulation by sRNAs has been shown to occur in Gram-positive bacteria such as *Staphylococcus aureus* and *Bacillus subtilis*, even though Hfq is present in these organisms (206, 207).

The role of sRNA-mediated regulation in *N. meningitidis* has been investigated only in the last decade. Even as more and more high-resolution transcriptomic analyses of pathogenic *Neisseriae* become available (208-211), only few sRNAs have been characterized to date in meningococcus or the closely related gonococcus, and are involved in a number of critical

pathogenic processes including regulation of gene expression, natural transformation and antigenic variation (133, 162, 212-214). The *trans*-acting sRNA NrrF is synthesized during iron starvation and is involved in controlling iron metabolism and maintaining homeostasis (132, 133, 215). AniS, another *trans*-acting sRNA, was identified by a microarray screening of differentially expressed transcripts between *N. meningitidis* wild type and Δ*hfq* mutant strains, is induced under anoxia and may be involved in down regulation of FNR-repressed genes (212). A third sRNA had been identified in *N. meningitidis* strain H44/76 by RNAsequencing of a mutant in which σ^E is highly expressed (209). This was subsequently clarified to be the tracrRNA of the Type II CRISPR/Cas system of meningococcus, which has been recently shown to limit natural transformation of the bacterium (162). In addition, RNA thermometers have been identified in the 5'-UTRs of three meningococcal genes that are essential for resistance against immune killing (213). Interestingly, another novel sRNA has been proposed to facilitate the formation of a G-quadruplex DNA structure involved in antigenic variation of the pilus in gonococcus (214).

2.5.4 Identification of novel sRNAs

Despite the critical regulatory roles they play in many bacterial processes, non-coding regulatory sRNAs have not been readily identified and annotated within available bacterial genome sequences. Because of this, experimental strategies paired with bioinformatics analyses have become increasingly important for sRNA discovery (216, 217). The first sRNAs were fortuitously discovered using genetic screens, or through radiolabeling of total RNA and subsequent isolation of short-length fractions from gels (218). It was only very recently that many new sRNAs have been identified and characterized in a wide range of bacterial species. This was mainly possible thanks to the increased availability of novel technologies such as computational predictions of sRNAs (219, 220), high density (tiling) microarrays (208) and high-throughput cDNA sequencing (RNAseq) that are used to study sRNAs at the genome-wide level (221, 222). These latter techniques not only allow the identification of novel sRNAs, but also the analysis of the whole transcriptome of bacteria under different growth conditions. Tiling arrays carry up to hundreds of thousands of DNA oligonucleotides systematically covering the sense and antisense strand of a genome, including the intergenic regions (IGRs) from which most known sRNAs are expressed (219). An important issue with this kind of technique, as well as with RNAseq, is choosing

physiologically significant conditions to assess expression of sRNAs. Genomic tiling arrays have been successfully used to study the transcriptome of *Neisseria meningitidis* (208), *Listeria monocytogenes* (223), *Bacillus subtilis* (224), *Halobacterium salinarum* (225) and *Mycoplasma pneumoniae* (226), as well as specific genomic features in *Escherichia coli* (227) and *Caulobacter crescentus* (228). However, array-based approaches require hundreds of thousands probes and are limited by background noise and cross hybridization, and therefore requires extensive normalization (229). On the other hand, the RNAseq approach directly determines the cDNA sequence. A population of RNA is converted to a library of cDNA fragments with appropriate adaptors attached to one or both ends. Each molecule is then sequenced at a high-throughput rate, generating extremely high numbers of short reads, which are subsequently mapped to the reference genome to assemble a transcriptome map. In principle, any high-throughput sequencing technology can be used for RNAseq, and the Illumina, SOLiD and Roche 454 Life Science systems have already been applied for this purpose in bacteria (210, 230-235). A transcriptome analysis at nucleotide resolution can be used to improve genome annotation by facilitating the discovery of new genes or transcripts, the correction of gene annotation, the detection of UTRs and transcription start sites and the determination of operon structure (229). Furthermore, whole-transcriptome analysis now allows the global interrogation of sRNA abundance and antisense RNAs by allowing detection of transcripts arising from non-coding regions. For example, Perkins *et al.* detected 55 intergenic regions that are likely to encode new sRNAs in *Salmonella* Typhi Ty2 (236), and the number of known sRNAs in *L. monocytogenes* has been more than doubled by a tiling-array based study (223). Recently, high-density arrays together with a new bioinformatic tool named chipSAD revealed the presence of 91 differentially expressed putative sRNAs after incubation of *N. meningitidis* in whole human blood (208). RNAseq studies have become increasingly widespread in recent years, allowing the identification of more putative sRNAs in various bacterial species ranging from *B. subtilis* (237) to *Helicobacter pylori* (232) and the related *Campylobacter jejuni* (238), to *Vibrio cholerae* (191) *L. monocytogenes* (239) and *N. gonorrhoeae* (210). Interestingly, RNAseq also allowed for the exploration of the relationship between sRNAs and the Hfq protein by co-immunoprecipitation (231, 240, 241).

In conclusion, the technical evolution of whole-transcriptome analyses during recent years made possible to study in detail, possibly down to single-nucleotide resolution, the

involvement of elements such as transcriptional regulators, sRNAs, riboswitches and *cis*-antisense regulators in the physiology and pathogenicity of any prokaryote.

2.6 Objective of the study

The aim of this work is to investigate the *N. meningitidis* transcriptome and global regulation under infection-relevant conditions, both at the transcriptional and post-transcriptional level.
Here we assess for the first time the effect of glucose on *N. meningitidis* at the transcriptional level. Following this, we identify and characterize a HexR-like transcriptional regulator implicated in the glucose-responsive regulation, and we show that this regulator has an impact on the fitness of *N. meningitidis* during infection.
In order to identify novel post-transcriptional regulators in *N. meningitidis*, we perform curated RNAseq analysis of meningococci grown under *in vitro* conditions. From this approach we derive a list of 42 putative small non-coding RNAs potentially involved in *N. meningitidis* regulatory networks. Deletion analysis of selected candidates leads us to the identification of the first sRNAs influencing meningococcal bacteraemia. Furthermore, we describe the initial characterization of a novel sRNA unique to meningococcus, closely associated to genes relevant for the intracellular survival of pathogenic *Neisseriae*.

3 Results I

3.1 Global analysis of *Neisseria meningitidis* expression in response to glucose

In order to investigate the effect of glucose on global transcription in *Neisseria meningitidis* and its involvement in the regulation of metabolic and cellular processes, we compared the expression profiles of bacteria grown in the presence or absence of glucose to exponential growth phase using custom Agilent oligonucleotide microarrays (212). A gene was considered differentially expressed when it displayed more than two-fold induction or repression in the glucose samples compared to the reference samples (*t*-test $p \leq 0.05$). The global gene expression could be grouped in 13 functional categories (Figure 5). Four categories are found over-represented: energy metabolism (30%), hypothetical proteins (26%), transport and binding proteins (11%) and cell envelope (9%).

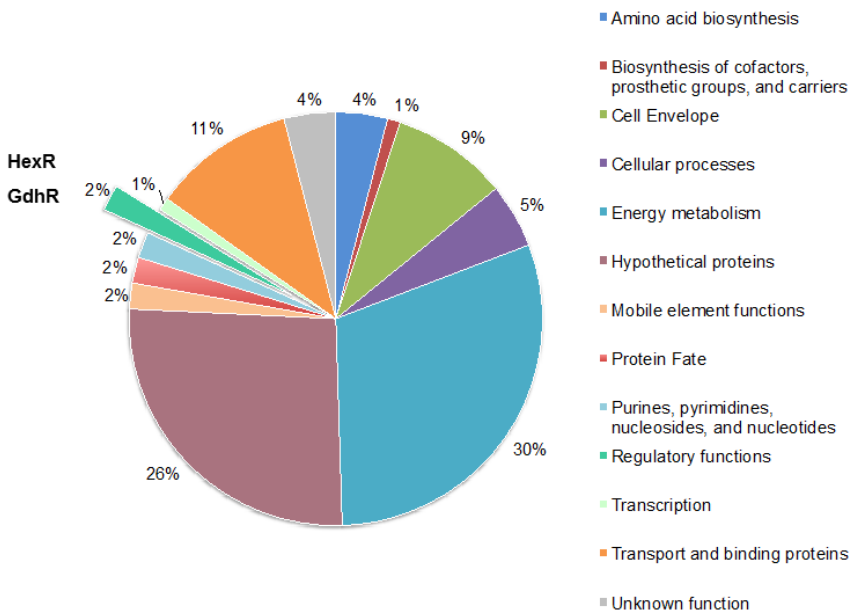

Figure 4 Graphical representation of genes differently expressed in presence of glucose. Genes are grouped in functional categories according to the classification of TIGRfams. Transcriptional regulators found differently expressed are highlighted.

Growth of *N. meningitidis* MC58 strain in the presence of glucose altered the expression of 82 genes (3.8% of the MC58 genome). Among these, 49 and 33 genes were up- and down-regulated, respectively (Figure 5A). Those contiguous genes present in the same orientation and that exhibit similar regulation have been grouped into likely operons. The majority of the genes found to respond to glucose belong to energy metabolism. We found genes belonging to the Entner-Doudoroff (ED) pathway (*zwf, pgl, edd, eda*), the pentose phosphate pathway (*tal*) and the catabolic branch of the Embden–Meyerhof–Parnas (EMP) pathway (*pgi1*, *gapB, pykA*) to be highly up-regulated. On the other hand, genes belonging to the anabolic branch of the EMP pathway (*pgi2, gapA*) were found to be down-regulated. The presence of glucose therefore up-regulates genes that encode functions leading to sugar catabolism, and down-regulates genes whose products catalyze the inverse reactions, thus promoting the utilization of available sugar energy sources. We also observed down-regulation of all genes involved in the tricarboxylic acids cycle (*aldA, prpB, prpC, lpdA3, sdhABCD, sucCD*) as well as in acetate production (*ackA2*). Genes related to aminoacid metabolism and transport were also differentially expressed. The NADPH-specific glutamate dehydrogenase (*gdhA*) was found up-regulated, together with genes related to aminoacid metabolism (*trpF, purF*). On the other hand, the NADH-specific counterpart to *gdhA* (*gluD*) was found downregulated, as well as the proline importer protein (*putP*). Coherently with the availability of a highly energetic carbon source such as glucose, we also found the lactate importer protein (*lctP*) to be down-regulated, together with a gene encoding a putative uracil permease (NMB1048), possibly the first step in the pyrimidine salvage pathway.

In addition to metabolic changes, we also observed genes related to meningococcal pathogenesis being induced by glucose (Figure 5A). For instance, *nspA* encoding the Neisseria surface protein A implicated in binding of factor H and therefore immune evasion, was up-regulated by glucose as well as the capsule gene NMB0067 (*siaD*). Furthermore, several surface exposed proteins with immunogenic properties and proposed as vaccine candidates such as NMB0390 and NMB1468 were up- and down-regulated in the presence of glucose, respectively. Genes coding for proteins involved in the contact and interaction with the host were also differentially expressed in presence of glucose. As an example, the loci NMB0375 and NMB0652 encoding for the *mafA* (multiple adhesion family A), were up-regulated by glucose. Interestingly, also NMB1214 encoding for a hemagglutinin/hemolysin-related protein (Hrp), an adhesin highly immunogenic (242) was up-regulated in presence of

glucose. Other factors related to the cell envelope were found down-regulated in presence of glucose, such as NMB1807 (*ponA*) encoding penicillin-binding protein 1 and NMB0342 (*ispA*) encoding intracellular septation protein A. Interestingly, both these genes have been found down-regulated in the glucose-rich human blood (124).

Taken together, our data indicate that glucose is not only involved in the regulation of gene expression related to metabolism, but it also impacts on other majors pathways that are important during *N. meningitidis* interaction with the host.

In order to confirm the results obtained in the microarrays expression profiling, we selected a subset of eight genes with fold change values ranging from highly up-regulated to down-regulated and performed real-time quantitative PCR (qRT-PCR) (Figure 5B). The results obtained are similar to the microarray data with a good coefficient of correlation (r^2=0.82) (Figure 5C).

3.2 NMB1389, a HexR transcriptional regulator

Having mapped the transcriptomic profile of *N. meningitidis* in response to glucose, we moved to an *in silico* analysis looking for potential glucose-responsive regulators involved in this molecular mechanism. Analysis of the *N. meningitidis* strain MC58 genome identified two potential carbon-related transcriptional regulators that were also differentially expressed in response to glucose: NMB1711 (*gdhR*) was down-regulated by glucose and has been previously described to be involved in the regulation of glutamate transport (243), and NMB1389 was up-regulated in presence of glucose (Figure 5 and Figure 6). We also searched for potential orthologues of the cAMP receptor protein (Crp) and the catabolic repressor/activator protein (Cra) in the *N. meningitidis* genome, however orthologues of these major carbon-source responsive regulators were not found.

The NMB1389 gene encodes for a HexR-like transcriptional regulator from the RpiR family, whose members are often involved in sugar catabolism regulation in proteobacteria. It contains two domains, a helix-turn-helix (HTH) binding domain at the N-terminal region and a Sugar Isomerase (SIS) domain at the C-terminal region, which is predicted to bind phosphosugars. The NMB1389 (*hexR*) nucleotide sequence is highly conserved among the available *N. meningitidis* genome sequences and is present in several species of the *Neisseria* genus such as *N. gonorrhoeae, N. lactamica, N. macacae, N. sicca, N. mucosa, N, flavescens* and *N. elongata.*

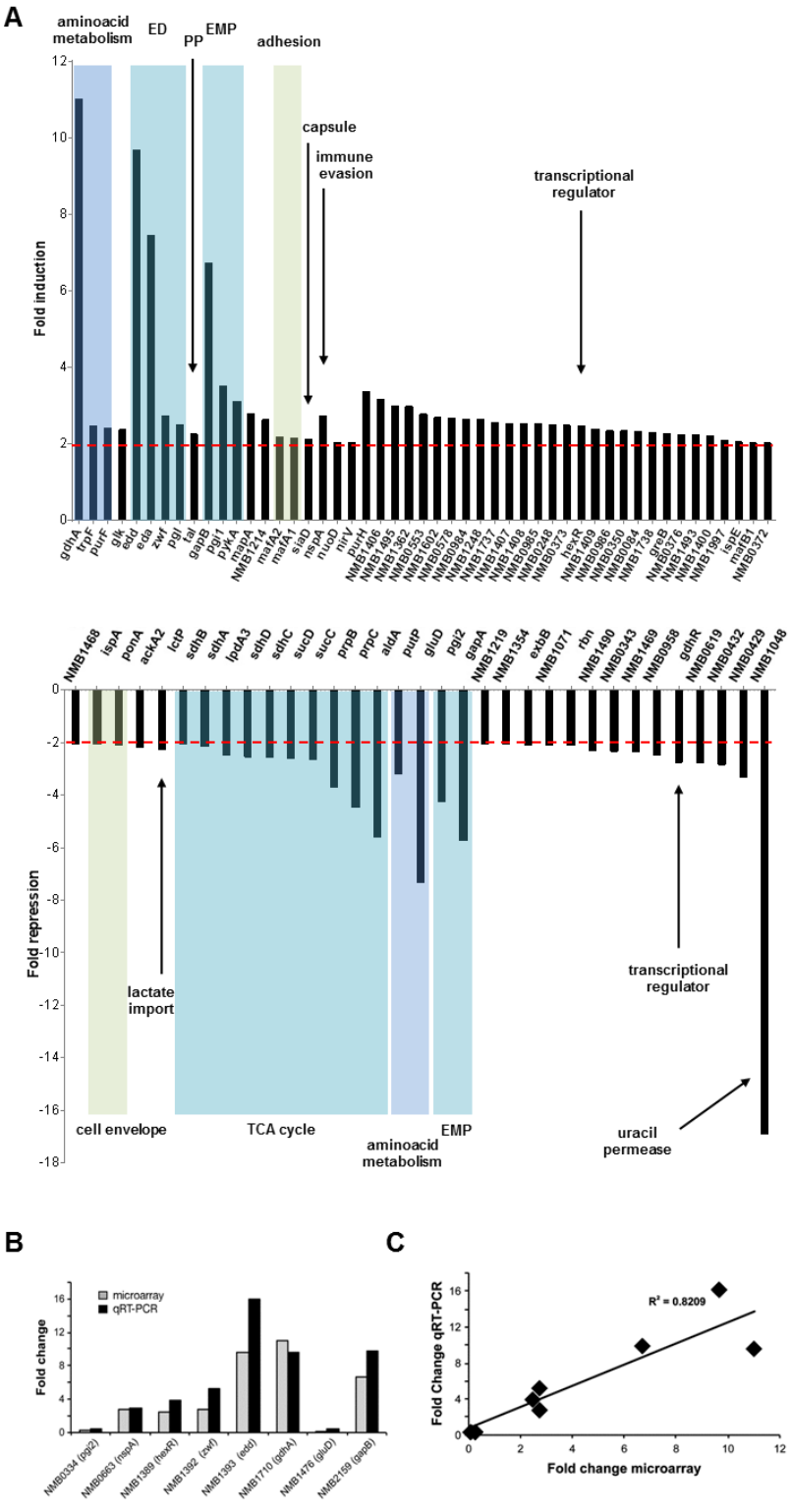

Figure 5 Transcriptional profile of *N. meningitidis* MC58 in response to glucose. (A) The relative ratios of the microarray competitive hybridizations are shown for glucose-responsive expression (MC58 +Glc versus MC58 -Glc). Differentially expressed genes are shown with fold change larger than 2-fold and $p \leq 0.05$. ED, Entner-Doudoroff pathway; PP, pentose phosphate pathway; EMP, Embden–Meyerhof–Parnas pathway; TCA, tri-carboxylic acids. **(B)** Comparison of microarray (grey bars) and qRT-PCR (black bars) expression data for eight selected genes. **(C)** Correlation between microarray and qRT-PCR results for the eight genes shown in (B).

In order to assess the role of HexR in meningococcal carbon metabolism regulation, we generated a knockout Δ*hexR* strain and we performed a global transcriptional analysis of wild type MC58 strain and its isogenic Δ*hexR* mutant strain grown in modified Catlin 6 medium (C6) or in C6 with 1% glucose (C6+Glc) until mid-logarithmic growth phase. Thirty-six genes were found to be differentially expressed in the Δ*hexR* strain compared to the wild type in at least one condition, with a fold change threshold of two-fold and *t*-test $p \leq 0.05$ (Table 1). Although only 27% 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 "energy metabolism". Interestingly, all genes differently expressed in the Δ*hexR* mutant grown in presence of glucose were found up-regulated (with the exception of *pgi1*, that is in operon with *hexR* and therefore susceptible to polar effects of the deletion), suggesting that HexR represses a variety of genes in response to glucose (Table 1). On the other hand, in the absence of glucose within the growth medium, the lack of *hexR* induced both up- and down-regulation of its target genes. Finally, we could observe 13 genes regulated by HexR and found with a fold change below the threshold in the wild type strain grown in presence or in absence of glucose, therefore suggesting that their control by HexR is not glucose-mediated (Table 1).

In order to validate the results obtained in the *hexR* microarray experiments, we selected six genes showing diverse fold change levels across the two experimental conditions (C6 in the presence or absence of glucose) and performed qRT-PCR on RNAs extracted from the Δ*hexR* and the WT strains under those conditions. Results show good agreement with the microarray data (Figure 6A). We also performed the same analysis comparing the Δ*hexR* strain to either the WT 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, whether comparing it to the WT or the c-*hexR* strain (Figure 6B). Taken together, these results confirm that the expression of these genes is regulated by HexR.

Table 1 List of genes differentially expressed in the ΔhexR mutant strain in the absence or in the presence of glucose.

				Transcriptome analysis[a]					
				ΔhexR vs MC58 in C6		ΔhexR vs MC58 in C6+Glc		MC58 C6+Glc vs.C6	
Gene ID	Gene name	Function	Putative Operon	fold change	*p*	fold change	*p*	fold change	*p*
NMB1393	*edd*	phosphogluconate dehydratase	NMB1393-1394	52,2	3,1E-10	4,8	2,2E-06	9,7	4,0E-05
NMB1394	*eda*	4-hydroxy-2-oxoglutarate aldolase	NMB1393-1394	45,9	4,6E-04	5,9	4,8E-02	7,4	3,2E-02
NMB1392	*zwf*	glucose-6-phosphate 1-dehydrogenase	NMB1392-1387	8,5	0,0E+00	3,1	0,0E+00	2,7	0,0E+00
NMB2159	*gapB*	glyceraldehyde 3-phosphate dehydrogenase	NMB2159	7,2	4,2E-10	1,8	2,0E-05	6,7	1,4E-07
NMB0089	*pykA*	pyruvate kinase	NMB0089-0088	6,6	1,4E-07	1,6	8,4E-04	3,1	2,5E-05
NMB1391	*pgl*	6-phosphogluconolactonase	NMB1392-1387	6,2	5,6E-05	2,0	1,0E-02	2,5	3,0E-04
NMB1390	*glk*	glucokinase	NMB1392-1387	4,5	2,3E-03	1,4	4,0E-01	2,4	1,4E-02
NMB0351	*tal*	transaldolase	NMB0351-0349	4,0	3,3E-08	1,5	7,8E-04	2,3	4,6E-06
NMB0350		hypothetical protein	NMB0351-0349	2,4	2,2E-02	1,4	4,7E-01	2,3	1,0E-02
NMB1624	*nirV*	putative nitrite reductase	NMB1623-1624	2,2	4,5E-02	-1,2	6,8E-01	2,0	1,7E-02
NMB0207	*gapA*	glyceraldehyde 3-phosphate dehydrogenase	NMB0207	1,9	1,9E-02	11,6	2,0E-05	-5,7	1,3E-03
NMB1476	*gluD*	glutamate dehydrogenase, NAD-specific	NMB1477-1476	1,2	3,9E-01	3,2	2,0E-04	-7,3	6,0E-07
NMB0429		hypothetical protein	NMB0429-0434	1,1	6,7E-01	7,1	2,5E-06	-3,3	3,6E-13
NMB0334	*pgi2*	glucose-6-phosphate isomerase	NMB0334-0336	1,1	6,2E-01	2,1	9,8E-04	-4,2	1,3E-08
NMB1968	*aldA*	aldehyde dehydrogenase A	NMB1968	-1,2	2,6E-01	3,2	8,2E-05	-5,6	4,5E-07
NMB0430	*prpB*	2-methylisocitrate lyase	NMB0429-0434	-1,4	7,2E-02	6,1	7,8E-05	-3,7	1,1E-06
NMB0432		conserved hypothetical protein	NMB0429-0434	-1,4	1,3E-01	4,0	5,5E-05	-2,8	3,6E-04
NMB0431	*prpC*	methylcitrate synthase	NMB0429-0434	-1,6	3,6E-02	7,0	7,5E-05	-4,5	1,2E-06
NMB0433	*acnA*	aconitate hydratase	NMB0429-0434	-1,7	2,5E-01	3,6	2,0E-02	-2,4	9,2E-02
NMB0435	*ackA2*	acetate kinase	NMB0435	-2,6	3,1E-02	3,0	1,5E-02	-2,2	4,1E-03
NMB1048		hypothetical protein, putative integral membrane protein	NMB1048	-2,9	4,5E-02	-1,5	4,2E-01	-16,9	9,5E-05
NMB0792		transporter, NadC family	NMB0794-0792	2,2	2,0E-03	3,6	1,5E-06	-1,9	6,4E-02
NMB0791	*ppiB*	peptidyl-prolyl cis-trans isomerase	NMB0791-0790	1,5	4,1E-02	2,2	2,0E-03	1,1	5,1E-01
NMB0401	*putA*	proline dehydrogenase	NMB0401	1,1	6,2E-01	2,2	1,4E-04	-1,7	1,8E-02
NMB0434	*prpF*	putative AcnD-accessory protein	NMB0429-0434	-1,4	5,0E-01	2,4	1,3E-02	-1,8	1,0E-01
NMB1088		conserved hypothetical protein	NMB1088	-2,0	1,0E-02	1,2	3,6E-01	-1,5	1,8E-03
NMB2095		putative adhesin complex protein	NMB2095	-2,0	1,1E-03	-1,1	6,8E-01	-1,6	5,9E-03
NMB0866		hypothetical protein, putative periplasmic protein	NMB0866-0864	-2,0	4,4E-02	1,2	5,1E-01	-1,2	5,3E-01
NMB0763	*cysK*	cysteine synthase	NMB0761-0763	-2,1	6,5E-03	-1,2	2,6E-01	-1,7	4,4E-02
NMB1154	*cysD*	sulfate adenylyltransferase, subunit 2	NMB1158-1151	-2,2	1,2E-04	-1,3	1,2E-01	-1,2	4,8E-01
NMB1191	*cysN*	sulfate adenylyltransferase, subunit 1	NMB1196-1189	-2,2	7,5E-07	-1,2	3,0E-01	-1,2	4,7E-01
NMB1189	*cysI*	sulfite reductase hemoprotein, beta-component	NMB1196-1189	-2,3	1,6E-08	-1,2	2,6E-01	-1,8	1,7E-05
NMB1151	*cysI*	sulfite reductase hemoprotein, beta-component	NMB1158-1151	-2,4	3,8E-08	-1,2	8,3E-02	-1,7	2,3E-05
NMB0865		hypothetical protein	NMB0866-0864	-2,5	5,0E-03	1,1	6,7E-01	-1,9	6,4E-02
NMB1388	*pgi1*	glucose-6-phosphate isomerase	NMB1392-1387	-1,9	2,5E-02	-3,9	4,4E-04	3,5	4,0E-04
NMB1389	*hexR*	RpiR/YebK/YfhH family protein transcriptional regulator	NMB1392-1387	-154,1	2,3E-09	-166,7	6,6E-12	2,5	1,7E-05

[a]Microarray results are average values from three separate experiments ($p \leq 0.05$).

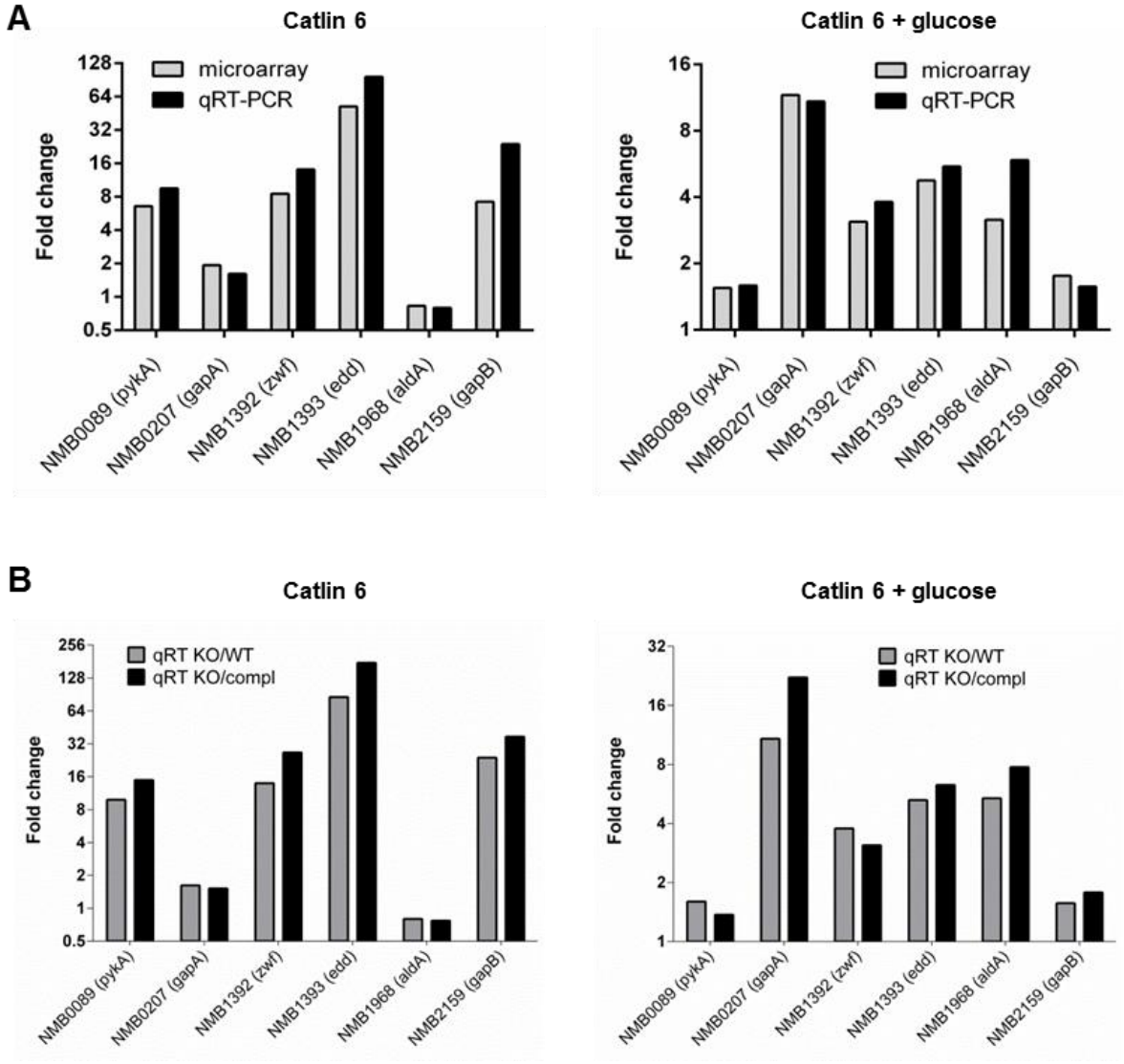

Figure 6 Validation of the Δ*hexR* microarray data. **(A)** Comparison of microarray (grey bars) and qRT-PCR (black bars) expression data for six selected genes, under glucose-lacking (left) or glucose-replete (right) conditions. **(B)** Comparison of qRT-PCR expression levels for the same genes as in (A), in the Δ*hexR* strain versus the WT strain (grey bars) and in the Δ*hexR* strain versus the complemented strain (black bars).

3.2.1 HexR directly binds to *edd* and *zwf* promoter regions

The meningococcal *hexR* gene co-localizes with genes of the central carbohydrate metabolism (Figure 8A). We first expressed HexR as a fusion protein to a N-terminal histidine tag, and purified it by nickel affinity chromatography. We obtained good purity of the His-HexR protein and stability across dialysis steps as assessed by SDS-PAGE (Figure 8B). We then analyzed the binding of purified HexR to the promoter regions of the central carbon metabolism genes we had found differently expressed in the Δ*hexR* transcriptional analysis. DNase I footprinting experiments showed that HexR is able to bind the promoter regions of the two divergent operons that control central carbohydrate metabolism in *N. meningitidis* . Specifically, HexR is able to protect a region inside the promoter of *zwf*, as well as two regions inside the promoter of *edd* (Figure 8A and C). However, the affinity of HexR for the mapped sites seems to be different between the two promoters. In fact, we identified a higher affinity site in the *zwf* promoter overlapping a putative -10 box and transcriptional start site (protected at 100 nM HexR) as well as a similar affinity site in the -35 box of the *edd* promoter (protected at 128 nM); on the other hand, higher concentrations of HexR were needed to protect the region overlapping the -10 box and the +1 site of the *edd* promoter (640 nM) (Figure 8C).

3.2.2 *In* silico prediction of *N. meningitidis* HexR DNA-binding consensus sequence

From DNase I footprinting analysis we were able to identify three HexR-binding sites. These three operators were used to define a HexR-binding consensus sequence with a 17-bp pseudopalindromic motif (K**TGTA**NTWWWAN**TACA**M) (Figure 8D). This consensus sequence resembles the *in-silico* predicted HexR binding motif for *Betaproteobacteria* in the RegPrecise database (245). We then used this motif to search *in silico* for similar HexR-binding sites in the *N. meningitidis* MC58 genome and correlated the results with our transcriptome data (Table 1). We identified five HexR-regulated operons with a potential HexR-binding site overlapping their promoter region (Figure 8E). This is the case of the central carbon metabolism genes *zwf* and *edd* (part of the ED pathway), *gapB* and *pykA* (part of the glycolytic branch of the EMP pathway) and *tal* (part of the pentose phosphate pathway).

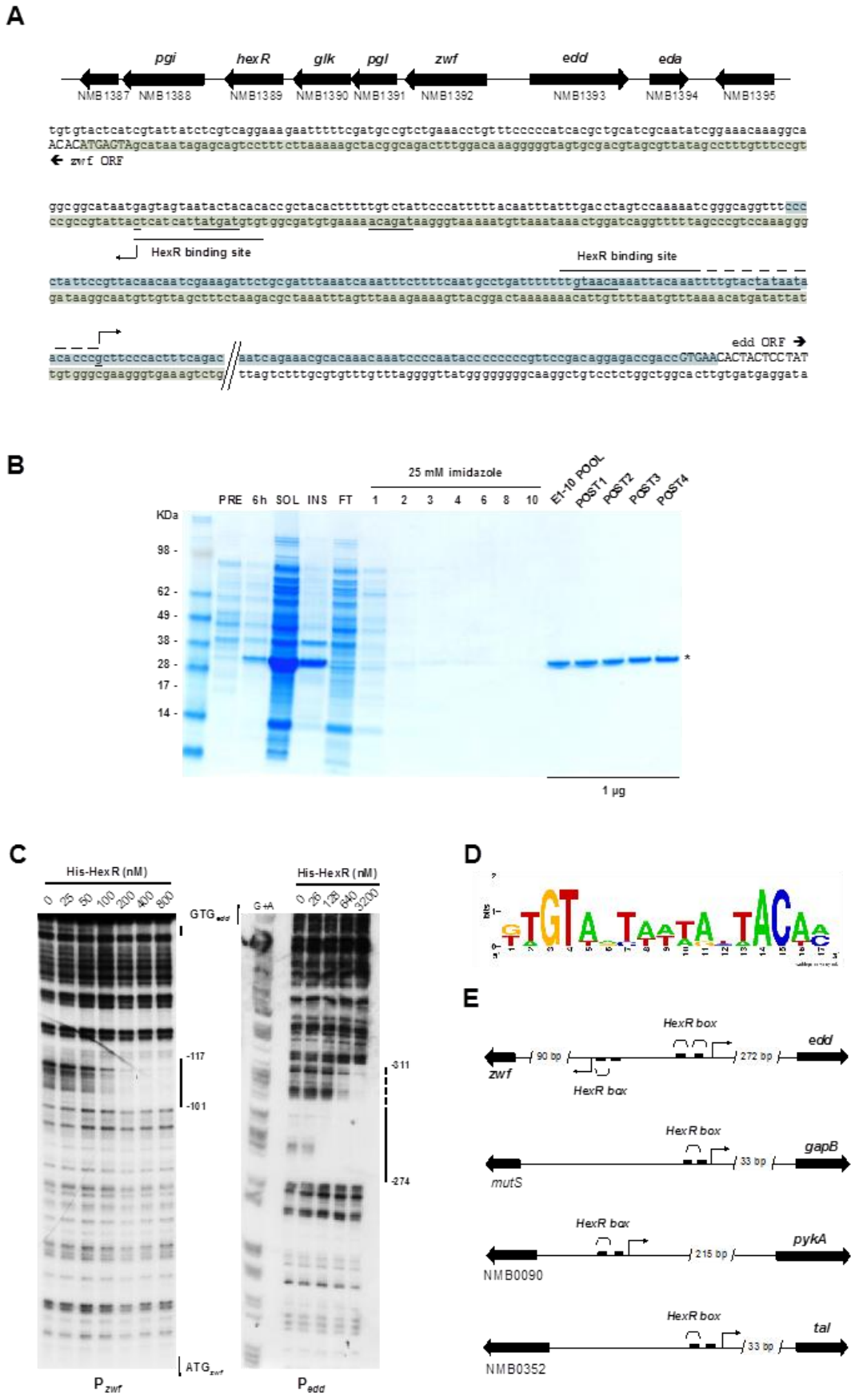
A
pgi
hexR
glk
pgl
zwf
edd
eda
NMB1387
NMB1388
NMB1389
NMB1390
NMB1391
NMB1392
NMB1393
NMB1394
NMB1395
zwf ORF
HexR binding site
HexR binding site
edd ORF
B
25 mM imidazole
PRE
6h
SOL
INS
FT
1
2
3
4
6
8
10
Et-10 POOL
POST1
POST2
POST3
POST4
KDa
98 -
62 -
49 -
38 -
28 -
17 -
14 -
1 µg
C
His-HexR (nM)
0
25
50
100
200
400
800
GTG
-117
-101
ATG
His-HexR (nM)
G+A
0
26
128
640
3200
-311
-274
D
E
HexR box
edd
90 bp
272 bp
zwf
HexR box
HexR box
gapB
33 bp
mutS
HexR box
pykA
215 bp
NMB0090
HexR box
tal
33 bp
NMB0352

Figure 7 (cont.) HexR directly binds to the promoter regions of genes involved in the central carbon metabolism. (A) Genetic organization of hexR locus and sequence of zwf-edd intergenic region. Bent arrows indicate operon transcriptional start sites identified by RNA sequencing experiment (data not shown). Putative promoter sequences are underscored. Probes used for DNase I footprinting experiments are highlighted. Bars indicate regions protected against DNase I digestion by HexR. **(B)** SDS-PAGE analysis of fractions from nickel-affinity chromatography of the HexR recombinant protein. SOL, soluble fraction after sonication; INS, insoluble pellet after sonication; FT, flow-through; 1-10, washing step fractions; E1-10 POOL, pooled eluates; POST1-4, pooled eluates after each dialysis step. **(C)** DNase I footprinting of HexR binding to zwf and edd promoter regions. **(D)** HexR-binding consensus sequence derived from mapped sites on zwf and edd promoters (WebLogo 3.2). **(E)** Sequences matching HexR-binding consensus found upstream of HexR-regulated operons (fuzznuc, EMBOSS). Brackets indicate predicted HexR-binding sequences overlapping putative promoter elements.

3.2.3 HexR binding affinity is not altered by KDPG (2-keto-3-deoxy-6-phosphogluconate)

Since HexR mainly regulates gene expression encoding for the central carbon metabolism, it is reasonable to speculate that one or more of the intermediate products of carbon metabolism could be an effector for HexR action. Therefore we tested several carbon metabolic intermediates such as keto-deoxy-6-phosphogluconate (KDPG), glucose-6-phosphate, fructose-1,6-diphosphate and 6-phosphogluconic acid as putative effectors for *N. meningitidis* HexR binding *in vitro*. However, we could not observe any difference on the HexR binding affinity to its cognate targets in the presence of any of these molecules (Figure 9). On the other hand, in *P. putida* and *S. oneidensis*, KDPG was reported to dissociate HexR from the target DNA (244). When we compared protein sequences, *N. meningitidis* HexR shows 41% identity with its orthologue in *P. putida* and 40% with *S. oneidensis* (strain MR-1), whereas *P. putida* and *S. oneidensis* proteins have higher reciprocal identity (58%) (Figure 9). This difference may explain why we did not observe an effect for KDPG on HexR DNA-binding affinity under the *in-vitro* conditions used. Differences in the phosphosugar-binding C-terminal region of the protein could imply that *N. meningitidis* HexR may use a different effector than other proteobacteria.

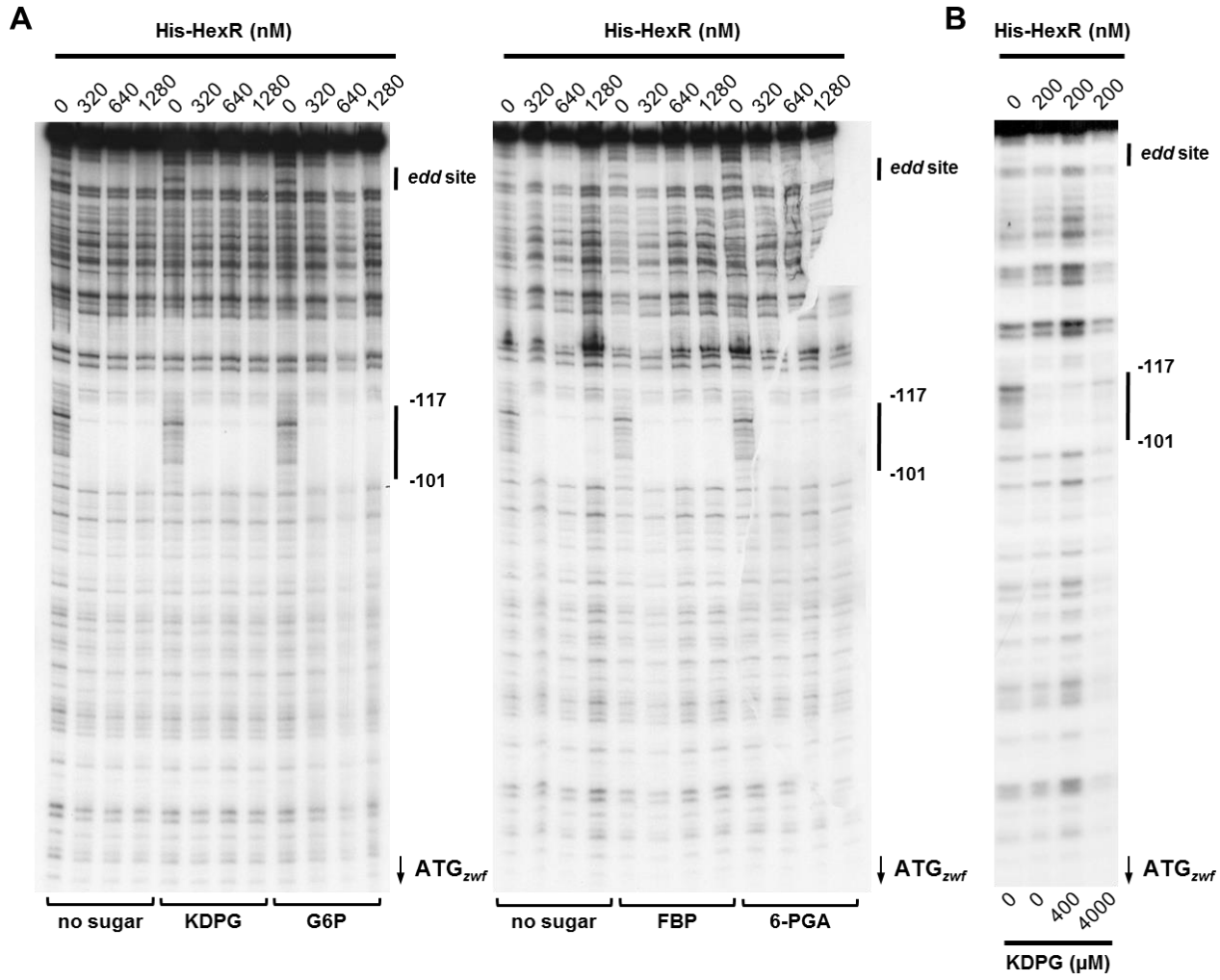

Figure 8 HexR binding to the promoter region of *zwf* is unaffected by addition of phosphosugars. (A) DNase I footprinting of HexR binding to *zwf* promoter region. Bars indicate regions protected against DNase I digestion by HexR. Increasing concentrations of HexR protein were incubated with radiolabeled DNA in the presence of 400 μM of the indicated phosphosugars, representing intermediates of the central carbon metabolism pathways. KDPG, 3-Deoxy-2-keto-6-phosphogluconic acid; G6P, glucose 6-phosphate; FBP, fructose 1,6-diphosphate; 6-PGA, 6-phosphogluconic acid. **(B)** Increasing concentrations of KDPG were incubated with radiolabeled DNA in the presence of a binding concentration of HexR protein.

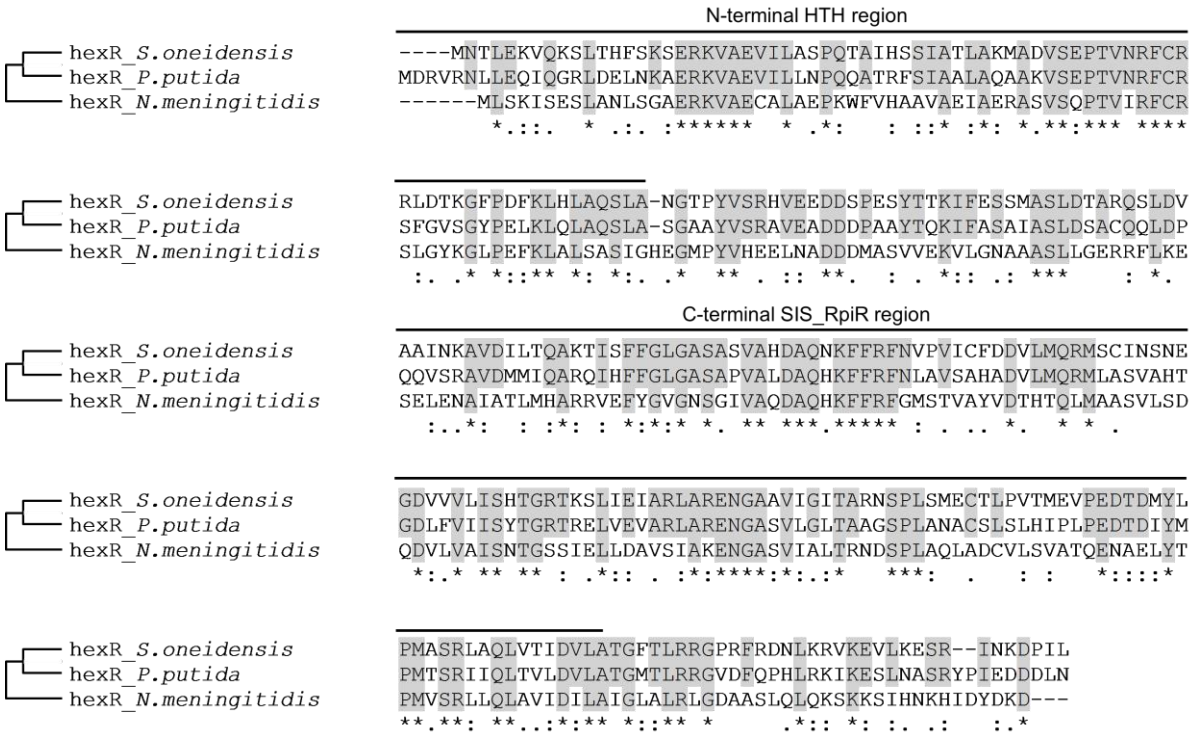

Figure 9 Comparison of HexR proteins in proteobacteria. Multiple alignment of HexR protein sequences (Clustal Omega). Common amino acids among the three protein sequences are shaded in grey.

3.2.4 HexR deletion does not alter *N. meningitidis* resistance to oxidative stress

The expression of *nirV* (NMB1624), encoding for a putative nitrite reductase, was found up-regulated by glucose through HexR. Furthermore, upstream of NMB1624 it is located *aniA* (copper-containing nitrite reductase), and the *aniA-nirV* operon has been shown to be involved in *N. meningitidis* anaerobic respiration (121). Since anaerobic respiration also contributes to the ability of *N. meningitidis* to tolerate oxidative stress (121), we decided to test if the absence of HexR could affect sensitivity to oxidative stress agents, like H_2O_2 or paraquat. We performed disc-diffusion stress assays following the Kirby-Bauer method, but we did not observe any difference between the wild type and the Δ*hexR* mutant strains under the conditions used (data not shown).

3.2.5 HexR deletion impairs survival of *N. meningitidis* during infection *in vivo*

In order to assess the viability of the Δ*hexR* mutant strain *in vivo*, a competitive index (CI) assay was performed in infant rats to determine the fitness of the mutant relative to the wild type strain. 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 as compared to the wild type strain (data not shown). The median CI observed for the challenged infant rats in the *hexR* experiment is larger than 1, indicating that more wild type bacteria (approximately 10-fold) survived in the animal model than Δ*hexR* mutant bacteria. As a comparison, the median CI for an unrelated KO strain that has no effect on meningococcal fitness *in vivo* is very close to 1 (Figure 10). This suggests that the lack of HexR expression significantly affects the survival of *N. meningitidis* during *in vivo* infection.

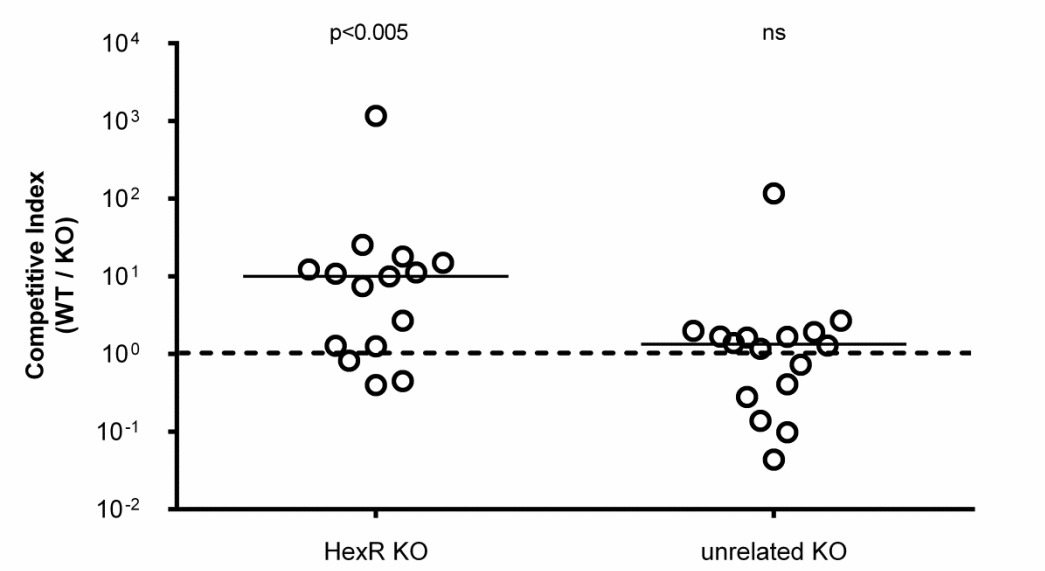

Figure 10 Deletion of *hexR* impairs survival during infection *in vivo*. Individual competitive indices (CI) from intraperitoneal challenge of infant rats with *N. meningitidis* WT and *hexR*-KO strains at a 1:1 ratio are shown. Circles indicate individual animals. Solid line indicates median, dashed line indicates CI=1. A CI >1 means the WT is more competitive than the mutant. Statistical significance was assessed with the Mann-Whitney test (ns, not significant).

4 Results II

4.1 Deep sequencing analysis of *N. meningitidis* identifies novel putative small RNAs

In order to explore the transcriptional landscape of *N. meningitidis* at nucleotide resolution, we performed RNAsequencing analysis (RNAseq) on total RNAs extracted from *N. meningitidis* MC58 strain cultured under standard or iron-limiting *in vitro* growth conditions, using a custom Illumina pipeline (ref. Materials and Methods).

After read mapping and assembly, the resulting dataset was manually curated in search of novel small transcripts. As a proof of concept, we could detect the known iron-responsive regulatory small RNA (sRNA) NrrF (132) being more expressed under iron limitation (Figure 12A, left panel), as well as another meningococcal sRNA regulator, AniS (212), being repressed under iron limitation (Figure 12B, left panel). These findings are in line with previous reports and with Northern blot experiments performed under the same conditions as our RNAseq (Figure 12A-B, right panels). We could also detect the expected expression profiles of various housekeeping bacterial sRNAs, such as the 6S RNA known to regulate RNA polymerase activity (246) (Figure 12C) and the transfer-messenger RNA involved in the rescue of stalled ribosomes (247) (Figure 12D). Taken together, these findings highlighted the power of our RNAseq analysis in identifying sRNAs.

In curating our dataset, we selected areas of transcription longer than 50 base pairs (bp) that did not fall entirely within annotated open reading frames (ORFs) or tRNA genes on the same strand. Then we looked for promoter-like elements in the vicinity of each putative transcriptional start site, and for sequences capable of rho-independent termination near the end of identified transcripts. We also annotated the presence of known neisserial repeat sequences within each area of transcription. In total, we found 51 non-ORF, non-tRNA associated transcripts expressed in the MC58 strain of *N. meningitidis* (Table 2). These include transcripts of known function such as the 4.5S signal recognition particle RNA (248), the RNase P ribozyme (249) and the tracrRNA of the minimal Type II CRISPR/cas system of *N. meningitidis*, which has been shown to limit natural transformation of the bacterium (162). We also found known meningococcal sRNAs within our analysis, such as the abovementioned NrrF and AniS (Table 2). Interestingly, we found two sRNAs (BNS1

and BNS2) reported to be induced upon exposure of *N. meningitidis* to human whole blood (208) to be also synthesized under standard *in vitro* conditions (Table 2).

Overall, this analysis identified 42 previously unreported transcripts expressed by *N. meningitidis* during *in vitro* growth.

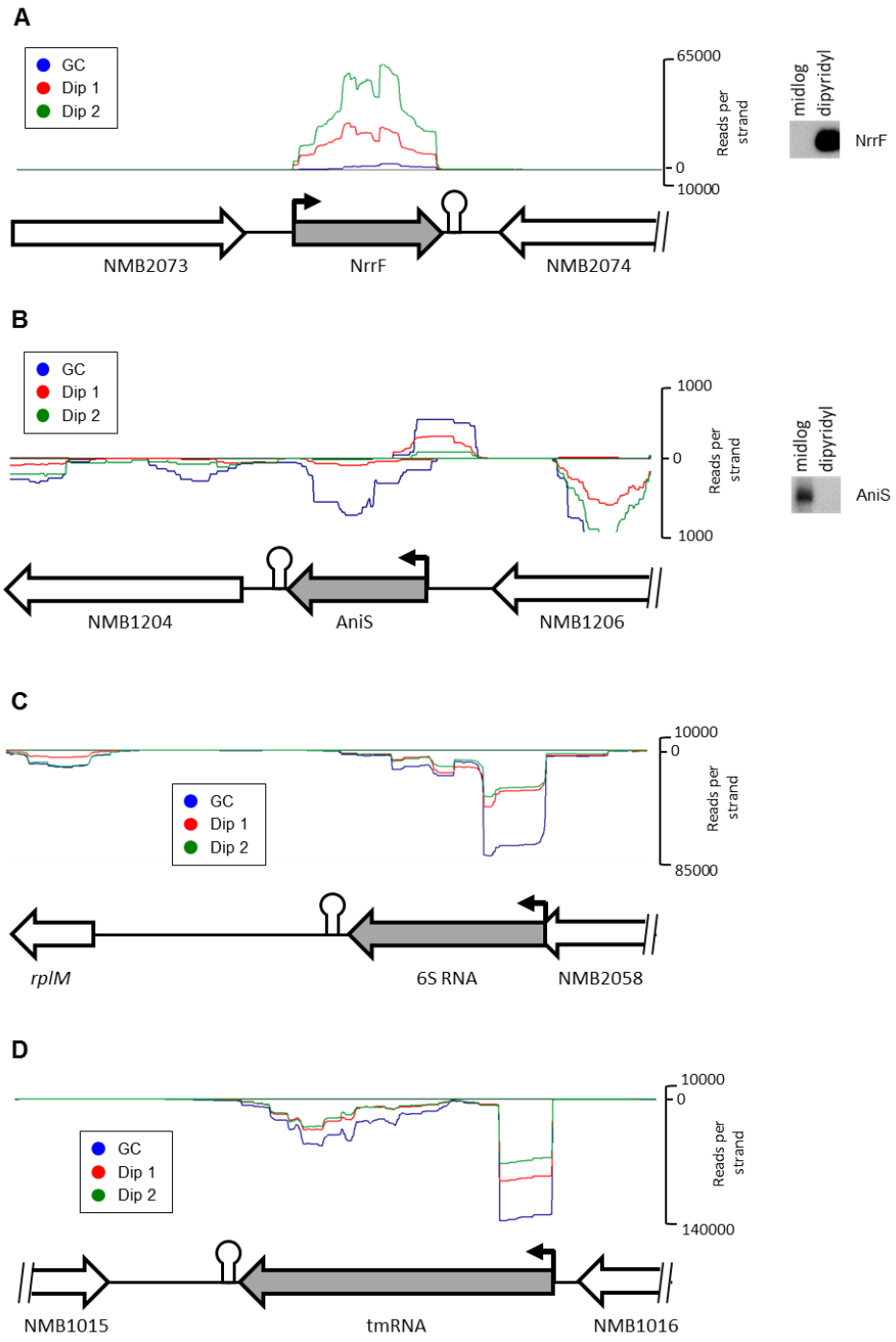

Figure 11 Examples of RNA sequencing profiles for known small RNAs. Read mapping (upper panel), schematic representation of locus (lower panel) and Northern blot (right panel) for the regulatory sRNAs NrrF **(A)** and AniS **(B)**, as well as the two housekeeping sRNAs 6S **(C)** and tmRNA **(D)**. GC, mid-log sample; Dip 1-2, dipyridyl-treated samples.

Table 2 List of sRNAs identified by manual curation of the RNA sequencing dataset.

No.	UP ORF	DOWN ORF	Orientation[a]	Signal start[b]	Signal End[c]	Length (nt)	Terminator	Notes	Common to tiling microarray analysis[d]
1	**0011**	**0012**	<<<	9156	8896	260	Y		Y
2	**0018**	**0019**	<><	18388	18686	298	N	NIME-associated	
3	**0019**	**0020**	<><	18958	19276	318	Y	NIME-associated	
4	**0021**	**0022**	<><	19660	19730	70	N	NIME-associated	
5	**0022**	**0023**	<><	21179	21281	102	N	NIME-associated	
6	**0049**	**0050**	<><	51720	52011	291	Y	NIME-associated	
7	**0049**	**0050**	<><	52504	52835	331	N	NIME-associated	
8	**0064**	**0065**	<>>	74158	74482	324	N	long 5'UTR	
9	**0224**	**0225**	>><	233537	233291	246	Y		
10	**0225**	**0226**	<><	234513	234974	461	Y		
11	**0322**	**0323**	>><	333019	333143	124	Y		
12	**0444**	**0445**	>>>	458754	458970	216	Y		
13	**0754**	**0755**	><>	783335	783130	205	Y		
14	**0837**	**0838**	>>>	864414	864529	115	N	long 5'UTR	Y
15	**0882**	**0883**	><>	903783	903587	196	N		
16	**0898**	**0899**	>>>	918175	918349	174	Y		Y
17	**0899**	**0900**	>>>	919118	919351	233	Y		Y
18	**0914**	**0915**	<><	928073	928413	340	N		
19	**0997**	**0998**	<<>	1014714	1014620	94	Y	= 4.5S RNA	
20	**0999**	**1000**	><>	1020376	1020304	72	N		Y
21	**1000**	**1001**	>><	1022632	1021864	768	Y		Y
22	**1015**	**1016**	>><	1031165	1030805	360	Y	= tmRNA	
23	**1022**	**1023**	<><	1038476	1038565	89	Y		
24	**1039**	**1040**	><>	1054878	1054824	54	Y		
25	**1049**	**1050**	>><	1067603	1067433	170	N		
26	**1069**	**1070**	<>>	1090231	1090427	196	N		
27	**1205**	**1205**	<<<	1210463	1210346	117	Y	= AniS	Y
28	**1205**	**1206**	<><	1210426	1210498	72	Y		
29	**1251**	**1252**	<><	1258764	1258851	87	Y		
30	**1400**	**1401**	<><	1431963	1432411	448	N		Y
31	**1410**	**1411**	>><	1439977	1439888	89	N		Y
32	**1563**	**1564**	<><	1624303	1624382	79	Y	= BNS1	Y
33	**1649**	**1650**	<><	1717408	1717469	61	Y		
34	**1649**	**1650**	<><	1717607	1717669	62	Y		
35	**1650**	**1651**	<>>	1718351	1718539	188	N	long 5'UTR	Y
36	**1752**	**1753**	<>>	1834475	1834684	209	N	long 5'UTR	Y
37	**1787**	**1788**	>><	1876028	1875800	228	N	NIME-associated	
38	**1787**	**1788**	>><	1875788	1875559	229	N	NIME-associated	
39	**1787**	**1788**	>><	1875507	1875299	208	Y	NIME-associated	
40	**1826**	**1827**	<>>	1919690	1919756	66	Y	= tracrRNA (CRISPR)	Y
41	**1880**	**1881**	><>	1985617	1985247	370	N		Y
42	**1923**	**1924**	>><	2022033	2021848	185	Y		Y
43	**1941**	**1942**	<<<	2038729	2038619	110	N		Y
44	**1969**	**1970**	><>	2064856	2064740	116	Y		
45	**1982**	**1983**	>>>	2081647	2082159	512	Y	NIME-associated	
46	**2057**	**2058**	<<<	2179866	2179624	242	Y	= 6S RNA	
47	**2062**	**2063**	>><	2185395	2185493	98	Y	= BNS2	Y
48	**2073**	**2074**	>><	2195867	2195996	129	Y	= NrrF	Y
49	**2132**	**2133**	<<<	2240181	2240094	87	Y		
50	**2137**	**2138**	>><	2251791	2251410	381	Y	= RNase P	
51	**2149**	**2150**	<<<	2260429	2260231	198	N		

[a] : > and < indicate the forward and the reverse strand, respectively .

[b] : genomic coordinate of the first nucleotide giving a signal peak in the RNA sequencing dataset (MC58 genome)

[c] : genomic coordinate of the last nucleotide giving a signal peak in the RNA sequencing dataset (MC58 genome).

[d] : Fagnocchi et al., PLoS One submitted

4.1.1 Validation of novel meningococcal small RNAs

In a parallel study to our RNAseq, we performed tiling microarray analysis of *in vitro* cultured meningococcus to assess differential expression of transcripts arising from intergenic regions (IGRs) under seven conditions mimicking physiologically relevant stresses (Fagnocchi et al., *submitted*). Interestingly, 18 out of the 51 putative sRNAs identified by RNAseq were also found in the tiling microarray dataset (Table 2), suggesting that these transcripts are being synthesized during *in vitro* growth, and further up-regulated under specific stress conditions.

We selected 8 putative sRNAs for validation, comprising 2 BNS previously validated by RACE (208) as well as 6 putative new transcripts. From the tiling microarray dataset we derived the conditions that induced maximal expression of each of these sRNAs and validated their presence by Northern blot (Figure 13). We obtained positive signals for all of the candidate sRNAs analyzed, corresponding to small transcripts in the range of 100-400 nucleotides (nt) in length (Figure 13, left panel). As expected from the microarray data, the signal from sRNA0837-0838 was detected only in RNA extracted from logarithmic phase, while the other signals from sRNA0898-0899, sRNA0899-0900, sRNA1400-1401 and BNS2 were significantly induced in stationary phase (Figure 13, left panel). sRNA1880-1881 and sRNA1923-1924 instead showed similar expression levels both in logarithmic and in stationary phase samples. A signal for BNS1 was detected only in Northern blot performed on total RNA from C6 minimal medium supplemented with glucose (Figure 13, left panel), a condition in which it shows induction in the microarray results. All of the validated sRNAs appear to be short intergenic transcripts having their putative promoters, transcriptional start sites and putative terminator sequences located in the IGR between two flanking ORFs. The exception to this is sRNA0837-0838, which is detected as a specific transcript of about 100 nt possibly arising from the processing of a longer primary transcript that encompasses also NMB0838 (Figure 13, right panel).

Putative ORFs have been identified only within the sequence of validated sRNA0899-0900, raising the question whether this transcript may code for a small protein or peptide. However, no ribosomal binding site could be identified upstream of the start codon for this ORF, and no function could be inferred from the predicted amino acid sequence (data not shown).

Altogether, experimental validation by Northern blot confirmed the identification by RNAseq of 6 novel and 2 previously identified meningococcal sRNAs.

4.1.2 Deletion of selected sRNAs impairs meningococcal fitness *in vivo*

In order to assess the role of validated sRNAs in meningococcal fitness *in vivo*, we generated knockout mutants in *N. meningitidis* strain 2996 and performed a competitive index (CI) assay was performed in infant rats to determine the viability of each mutant relative to the wild-type strain. We successfully deleted six validated sRNAs, comprising the two BNS (Figure 14A). Growth curves in GC medium showed no significant differences for the sRNA-KO mutants as compared to the wild type strain (data not shown). The median CI observed for the challenged infant rats in 3 out of 6 sRNA-KO experiments (BNS1, sRNA0898-0899 and sRNA1400-1401) is significantly larger than 1, indicating that more wild type bacteria survived in the infant rat blood than sRNA-KO bacteria (Figure 14B). This suggests that lack of expression of these sRNAs affects *in vivo* survival of *N. meningitidis* in the bacteraemia model. The remaining 3 sRNA-KO strains (BNS2, sRNA0899-0900, sRNA1923-1934) exhibit CIs not significantly different from 1, indicating that lack of their expression is not sufficient to alter meningococcal fitness *in vivo*.

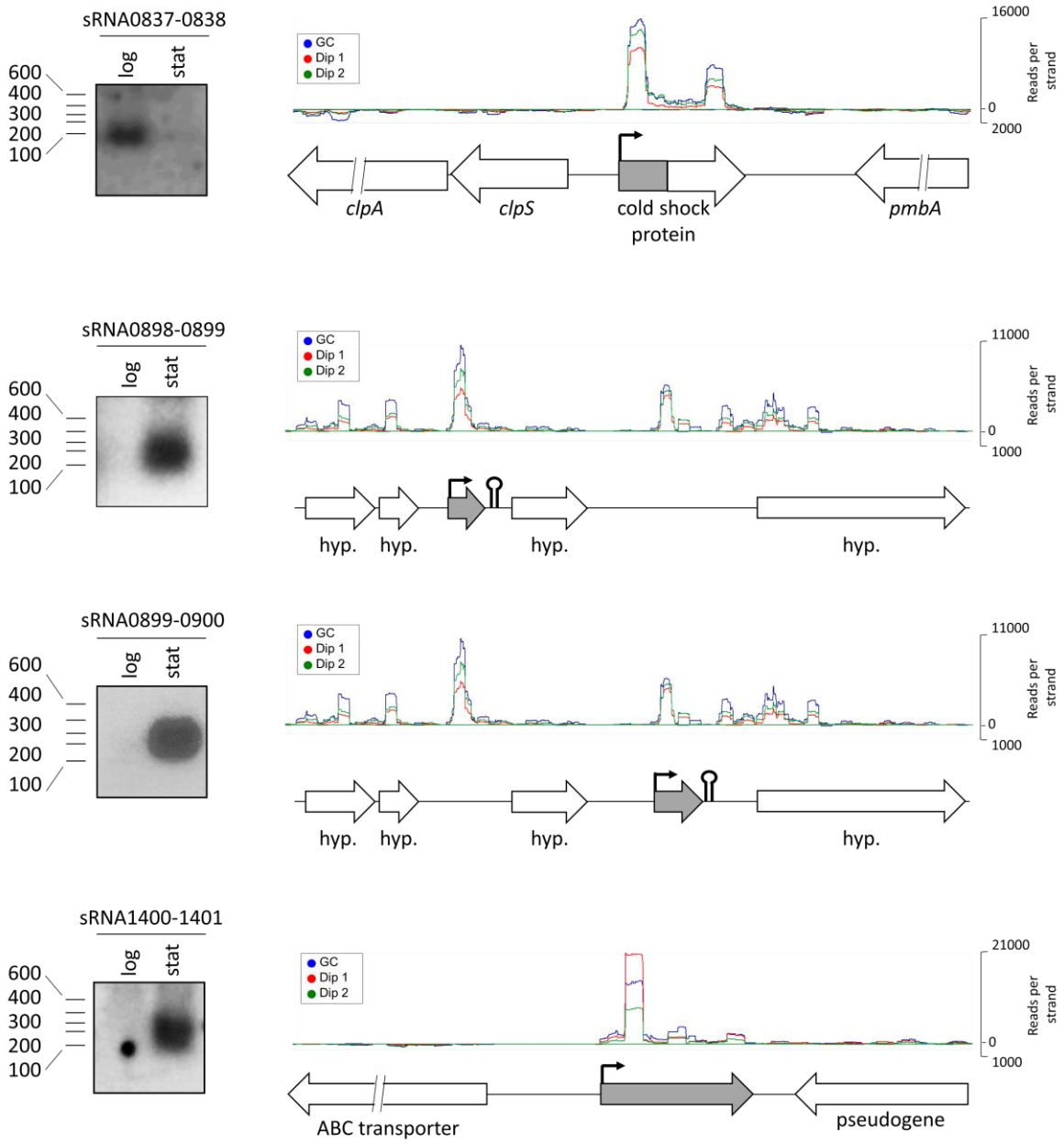

Figure 13 Experimental validation of novel meningococcal small RNAs. Northern blot analysis (left), read mapping (upper right) and schematic representation of locus (lower right) for newly discovered *N. meningitidis* MC58 sRNAs. GC, mid-log sample; Dip 1-2, dipyridyl-treated samples. Conditions of maximum sRNA expression used for Northern blot validation were derived from a parallel analysis of the MC58 strain using tiling microarrays (Fagnocchi et al., PLoS One *submitted*).

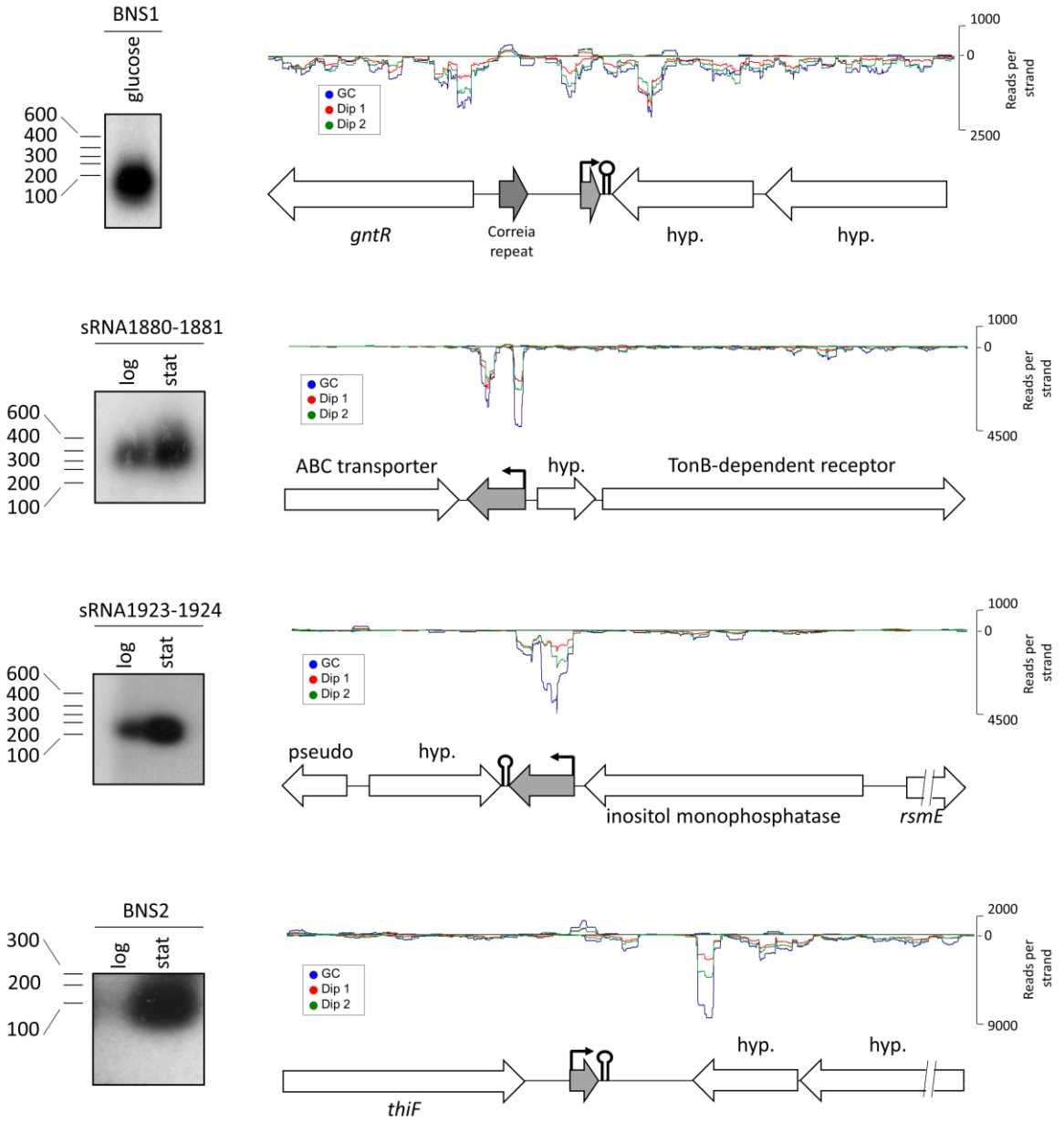

Figure 13 (continued) Experimental validation of novel meningococcal small RNAs. Northern blot analysis (left), read mapping (upper right) and schematic representation of locus (lower right) for newly discovered *N. meningitidis* MC58 sRNAs. GC, mid-log sample; Dip 1-2, dipyridyl-treated samples. Conditions of maximum sRNA expression used for Northern blot validation were derived from a parallel analysis of the MC58 strain using tiling microarrays (Fagnocchi et al., PLoS One *submitted*).

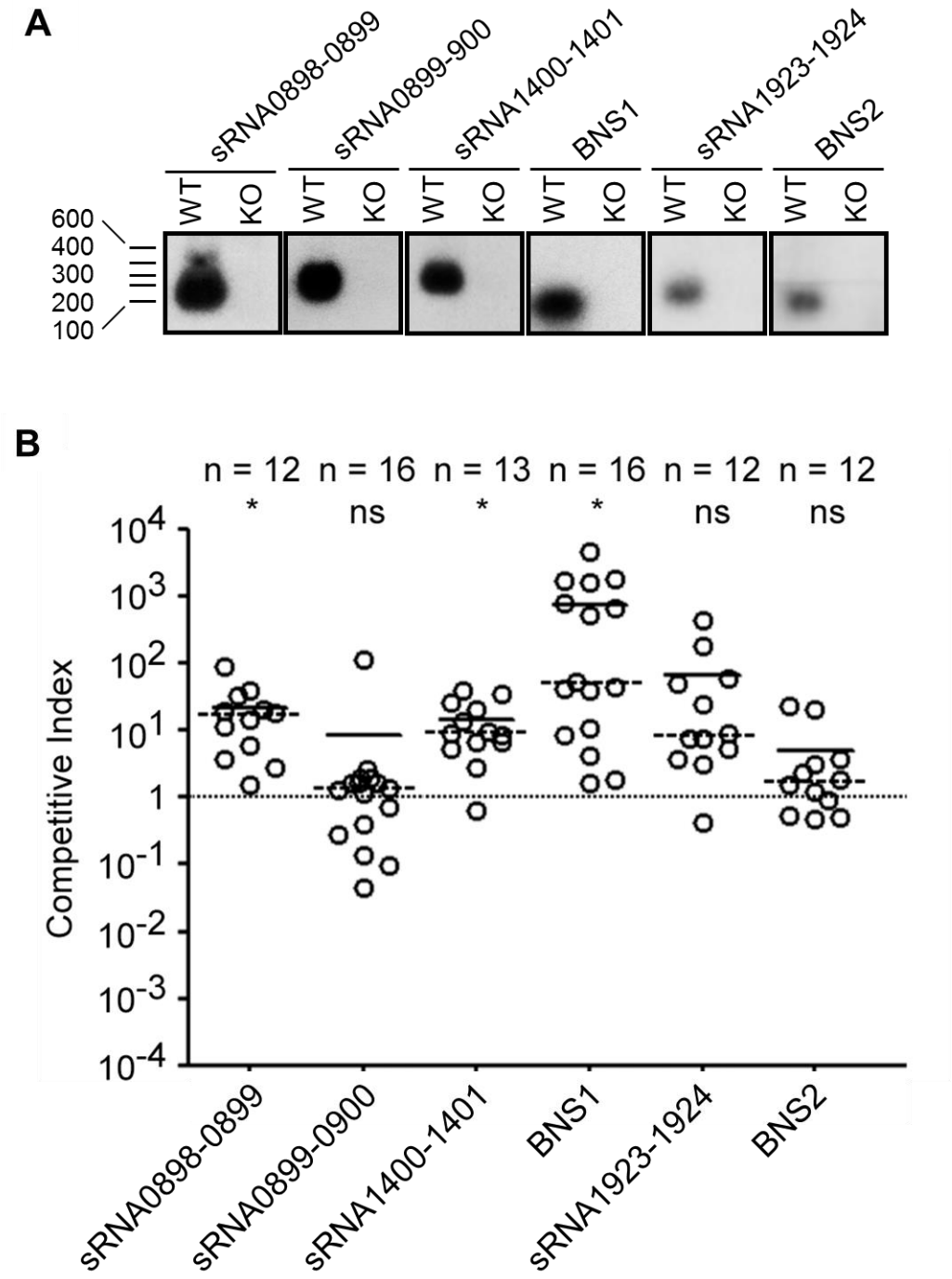

Figure 14 Deletion of sRNAs impairs meningococcal fitness *in vivo*. (A) Northern blot validation of the indicated sRNAs KO strains. RNAs were extracted from *N. meningitidis* WT and relative KO strains each grown in the condition of maximal sRNA expression (ref. Figure 13). **(B)** Competitive indices (CI) from intraperitoneal infection of infant rats with *N. meningitidis* WT and the indicated sRNA KO strains at a 1:1 ratio. Circles indicate individual animals. Solid line indicates median, dashed line indicates mean. A CI >1 means the WT is more competitive than the mutant. The numerosity of each group and the results of statistical analysis are shown above the graph (*, $p \leq 0.05$; ns, not significant).

4.2 sRNA1880-1881 is a novel small RNA unique to meningococcus that closely associates with pathogen-specific genes

In our RNAseq results, the novel sRNA1880-1881 appears as two peaks next to each other of approximately 160 nt in length, suggesting that the NMB1880-1881 IGR might actually harbor more than one sRNA transcript (Figure 15A). However, Northern blot validation of sRNA1880-1881 detected a single signal close to 400 nt in length (Figure 13). To elucidate the nature of the transcript arising from this IGR, we generated a knockout mutant of sRNA1880-1881 in the MC58 strain of *N. meningitidis*, and probed total RNAs from both the mutant and the wild type strain with two different probes, each specific for one of the peaks detected in the RNAseq. Our results show that both probes detect the same specific signal close to 400 nt, a length compatible with the 370 nt that a transcript spanning both peaks would cover according to RNAseq (Figure 15B). Sequence analysis of the validated sRNA1880-1881 shows that this sRNA has putative promoter elements in the vicinity of its transcriptional start site as defined by RNAseq, and its sequence harbors two long complementary GC-rich stretches that are predicted to form a highly stable structure (Figure 15C). The operon surrounding sRNA1880-1881 is absent in commensal *Neisseriae* and horizontally acquired by pathogenic species (250) and has been shown to be associated with the intracellular survival of invading gonococci (251). Analysis of the conservation of the locus in available neisserial genomes showed that the sRNA sequence is not present in *N. gonorrhoeae* and is exclusive to *N. meningitidis*, and is often subject to duplication across meningococcal strains (Figure 16). Interestingly, duplication of sRNA1880-1881 is always associated with sequence changes upstream of the +1 site of the newly inserted sRNA copy (Figure 16). These changes disrupt the predicted sRNA promoter by altering the spacing between its -10 and -35 elements (Figure 15C), possibly affecting expression of the duplicated sRNA copy in these strains. The sRNA1880-1881 gene also shows sequence variability across meningococcal genomes, as short deletions of 12-55 bp in length that are not exclusively associated with duplication events (Figure 16). Interestingly, deletions always affect the region between the two complementary GC-rich stretches located at the opposite ends of the sRNA sequence (Figure 15C), raising the question whether conservation of structural elements may be relevant for the function of sRNA1880-1881. However, searches for sRNA1880-1881 against the Rfam database of RNA families (252) returned no

results (data not shown), indicating that this small transcript is not found in any other bacterium.

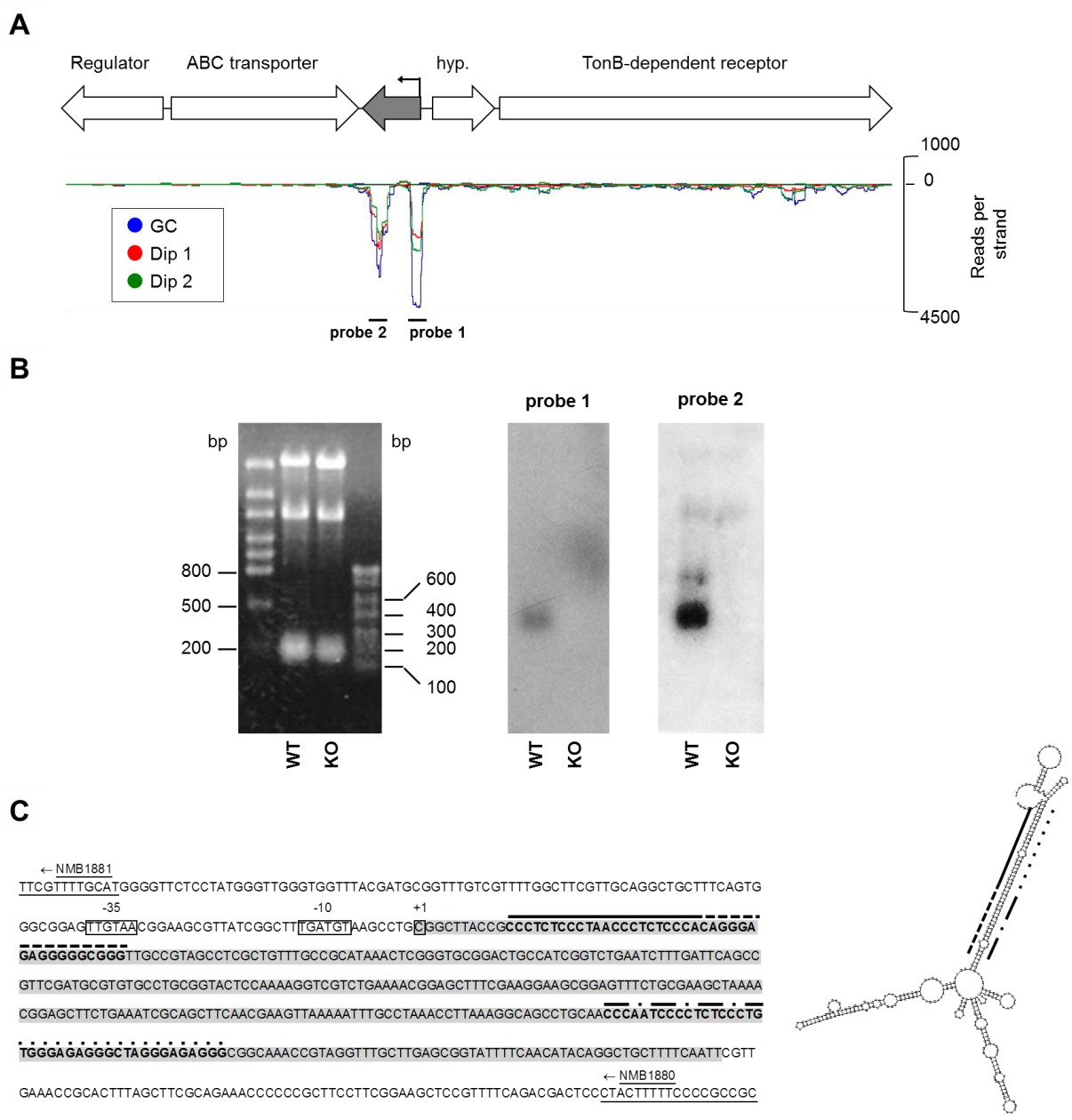

Figure 15 Validation of sRNA1880-1881 and generation of sRNA KO strain. (**A**) Schematic representation of sRNA1880-1881 locus and its expression profile on RNAseq. White and grey arrows indicate ORFs and sRNAs, respectively. Putative promoters are indicated by a bent arrow. Approximate positions of probes used for Northern blot experiments are highlighted. (**B**) Northern blot for sRNA1880-1881 expression in *N. meningitidis* WT and sRNA KO strains, using probes mapping on different regions of the RNAseq signal area. (**C**) Sequence (left) and predicted structure (right) of sRNA1880-1881. Putative regulatory and structural elements are highlighted.

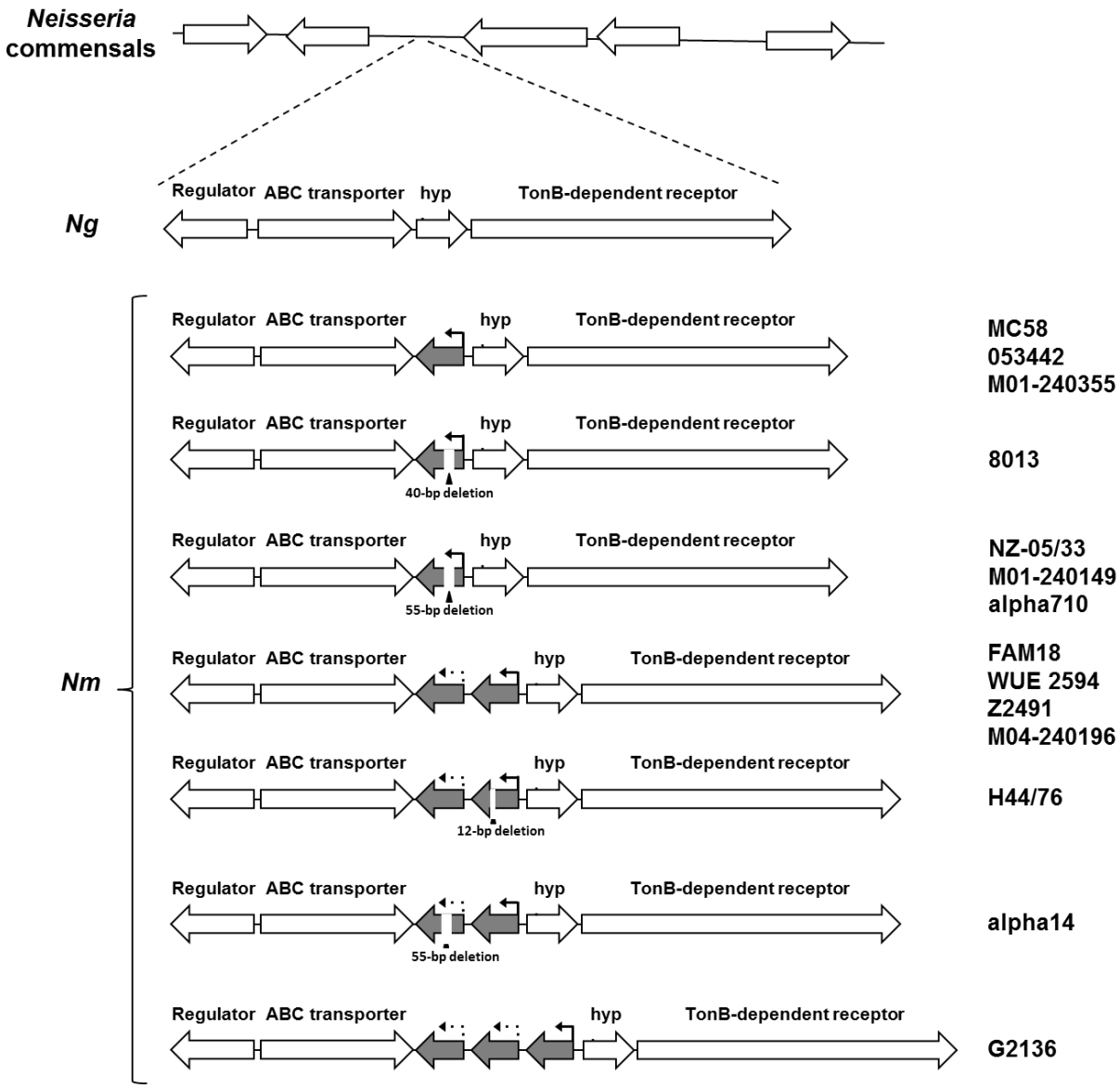

Figure 16 sRNA1880-1881 is unique to meningococcus and closely associated with pathogen-specific genes. Schematic representation of sRNA1880-1881 locus and its conservation across *Neisseria* species. White and grey arrows indicate ORFs and sRNAs, respectively. Putative promoters are indicated by a bent arrow. Deletions in the sRNA sequence and changes in the putative promoter sequences are highlighted.

When amplifying across the NMB1880-1881 IGR, multiple PCR bands could be observed arising from *N. meningitidis* MC58 genomic DNA (Figure 17A). This suggests that amplicons of different sizes are present within the DNA population, reflecting variability in the IGR structure. Sequencing of cloned PCR products from NMB1880-1881 IGR amplification confirmed the presence of a duplicated sRNA1880-1881 sequence in our MC58 strain (Figure 17B), including the changes in the promoter sequence that are specific to strains harboring duplications (Figure 16). These findings indicate that the sRNA-encoding NMB1880-1881 IGR is a locus of high plasticity and subject to both in-strain and between-strain duplication events.

Taking our results together, we identified and validated a unique meningococcal sRNA that is closely associated to pathogen-specific genes and is prone to genetic rearrangement.

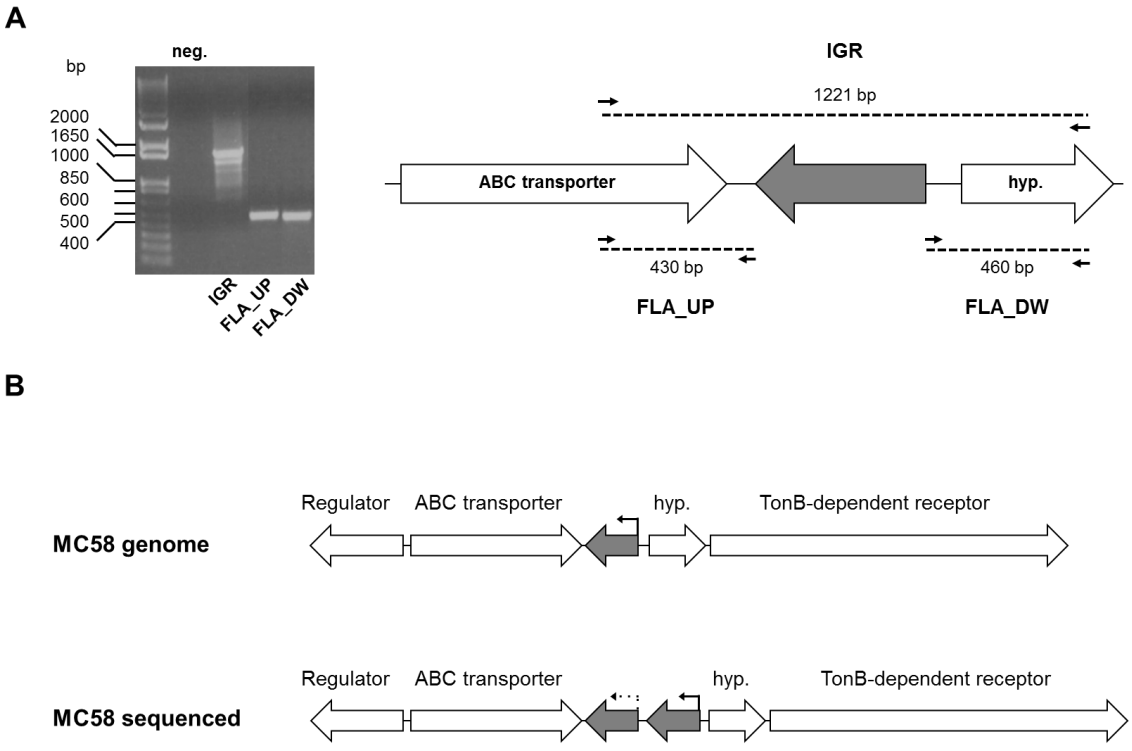

Figure 17 Evidence for in-strain duplication of sRNA1880-1881. (A) PCR amplification strategy for sRNA1880-1881 locus. **(B)** Schematic representation of expected locus sequence based on MC58 genome data and experimental results for sequencing of main IGR PCR band obtained in (A). White and grey arrows indicate ORFs and sRNAs, respectively. Putative promoters are indicated by a bent arrow. Changes in the putative promoter sequences are highlighted.

4.2.1 sRNA1880-1881 is not part of a regulatory network under *in vitro* conditions

To better understand the role of sRNA1880-1881 within the regulatory networks of meningococcus, we tested its expression by Northern blot on total RNAs from *N. meningitidis* exposed to different *in vitro* conditions mimicking physiologically relevant stresses, or from mutant strains lacking known regulators or factors involved in sRNA activity and stability. Our results indicate that sRNA1880-1881 is stably transcribed across all conditions tested, and shows little or no changes in expression compared to known regulated sRNAs such as NrrF (Figure 18A). Having already generated a knockout mutant of sRNA1880-1881 in the MC58 strain of *N. meningitidis* (Figure 15B and Figure 18B), we compared the expression profiles of the sRNA1880-1881 KO to the wild type strain using custom Agilent oligonucleotide microarrays (212). However, deletion of this sRNA did not impact global gene expression at either logarithmic or stationary phase of growth, apart from a polar effect of the resistance marker used in engineering the KO strain (Figure 18C).

Taken together, these results indicate that during *in vitro* growth sRNA1880-1881 does not integrate into a global regulatory network, either upstream or downstream of itself. Therefore, we hypothesized that this sRNA may have functional interactions with the pathogen-specific operon it is closely associated with.

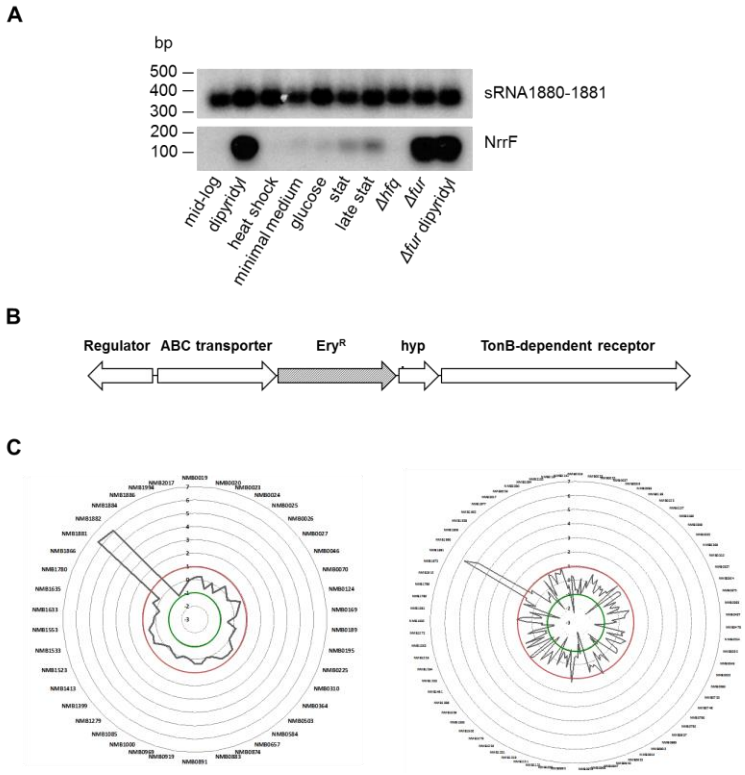

Figure 18 sRNA1880-1881 is not part of a regulatory network under *in vitro* conditions. (**A**) Northern blot showing expression of sRNA1880-1881 compared to the known regulated sRNA NrrF. (**B**) Genetic makeup of the MC58 sRNA KO strain used for microarray experiments. (**C**) Representation of microarray results comparing MC58 WT and sRNA KO strains at logarithmic or stationary phase of growth ($p \leq 0.05$). Red and green circles indicate the threshold used for calling genes up- and down-regulated, respectively.

4.2.2 Expression of sRNA1880-1881 limits transcription of neighboring genes *in vitro*.

As the NMB1880-1881-1882 operon is not expressed *in vitro* according to our RNAseq (Figure 19A), any effect of the sRNA1880-1881 deletion on the surrounding genes is difficult to measure. To elucidate the relationship between the sRNA and its neighboring genes, we generated a set of isogenic mutants of *N. meningitidis* strain MC58 driving inducible expression of the operon surrounding the sRNA. All the mutants in our panel express the NMB1880-1881-1882 genes under the control of a *lac*-repressed P_{tac} promoter, and they differ only by the architecture of the NMB1880-1881 IGR. In addition to reconstructing the wild-type IGR (sRNA wt strain) and generating a complete deletion mutant of sRNA1880-1881 (sRNA null strain), we also introduced engineered copies of sRNA1880-1881, carrying multiple point mutations in the putative promoter (sRNA mutP strain) or in the GC-rich stretches (sRNA mutGC strain) which are predicted to disrupt transcription of the sRNA and its structural features, respectively (Figure 19B). We induced expression of the operon from the isogenic mutants and compared the level of steady-state RNA for each gene to the wild type MC58 strain by qRT-PCR. Interestingly, in the sRNA wt strain we observed operon and sRNA expression at a very similar level to that of MC58, confirming that our isogenic mutants are correctly reproducing the functional architecture of this region in meningococcus (Fig. 19C, first and second cluster). Changing the putative promoter sequence from the optimal consensus reduces sRNA transcription almost as dramatically as a complete deletion, as we observed in strains sRNA mutP and sRNA null, respectively (Figure 19C, third and fourth cluster). This result highlights the relevance of the identified promoter elements for sRNA expression. On the other hand, disrupting the GC-rich structural features as in the sRNA mutGC strain does not seem to impact the steady-state level of sRNA1880-1881 in a significant way (Figure 19C, fifth cluster). Regarding NMB1880-1881-1882 expression, we observe that lack of transcription of the sRNA increases IPTG induction of the operon RNA (Figure 19C, third and fourth cluster), suggesting that active transcription of sRNA1880-1881 may limit operon expression, either by interfering with the transcription machinery on the opposite DNA strand or by modulating the steady-state level of the operon messenger RNA.

Further studies will be needed to elucidate the molecular mechanisms governing the functional interaction of sRNA1880-1881 with its neighboring genes, and to identify the physiological relevance of this regulation for meningococcus.

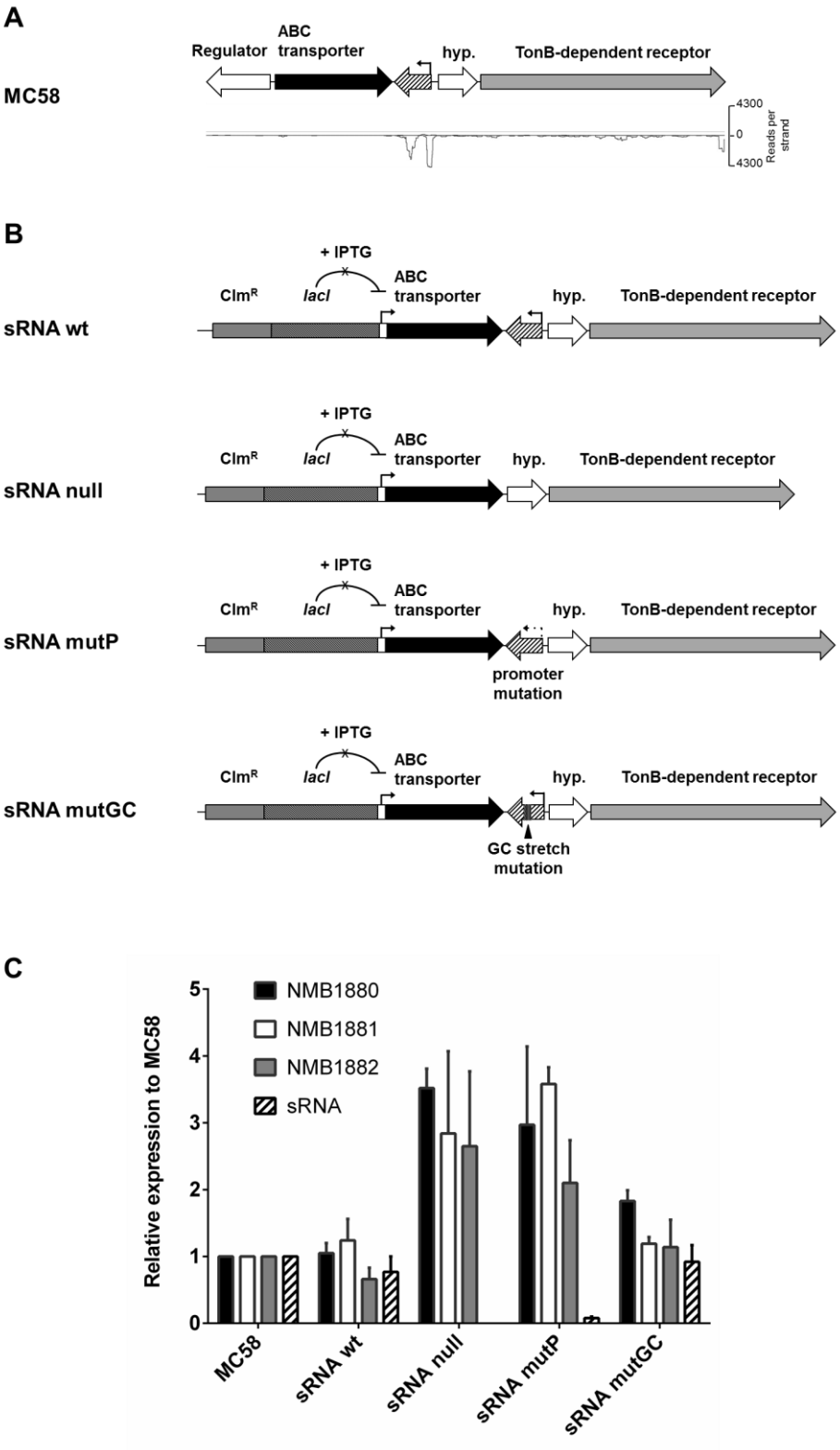

Figure 19 Expression of sRNA1880-1881 limits transcription of neighboring genes *in vitro*. (A) Schematic representation of sRNA1880-1881 locus and its expression profile on RNAseq. Solid and striped arrows indicate ORFs and sRNA, respectively. **(B)** Schematic representation of MC58 isogenic mutants for inducible expression of the NMB1880-1881-1882 operon. Squares indicate elements of the inducible P_{ind} promoter system. Engineered mutations in the sRNA promoter and in the GC-rich sequence are highlighted. **(C)** qRT-PCR expression levels for sRNA1880-1881 and the surrounding genes in the same panel of strains as in (B), upon induction of the P_{ind} promoter with 1 µM IPTG.

5 Discussion

The ability of microorganisms to detect and respond to variable external conditions, such as environmental stress and carbon sources availability in different niches, requires a coordination of sensing mechanisms and regulatory circuits (253) and is often crucial for the adaptation of pathogenic bacteria to the host environment.

In the first part of this work, we show the transcriptional profile of *N. meningitidis* in response to glucose, one of the main carbon sources that meningococcus encounters in its different niches of colonization. Glucose induces the differential expression of a high number of genes, and interestingly some of them are found to have the same trend of regulation in response to human blood (123), where glucose is abundant. In *N. meningitidis*, glucose is mainly metabolized through the Entner–Doudoroff (ED) pathway and to a lesser extent by the pentose phosphate (PP) pathway (254, 255), while the Embden-Meyerhof-Parnas (EMP) pathway is not fully functional, because it lacks the phosphofructokinase gene (40). Similarly, in *Pseudomonas putida* the ED pathway synthesizes the major part of the pyruvate (67-87%) and the PP pathway accounts for the remaining part (256). Accordingly, in our microarray analysis we found genes driving glucose catabolism through these pathways (*glk, zwf, edd, eda, pgl, pgi1, gapB, pykA)* to be highly up-regulated by the presence of glucose (Figure 20). On the other hand, genes from the gluconeogenesis (*pgi2, gapA*) are down-regulated in presence of glucose (Figure 20). This can have physiologically relevant consequences since *gapA* was shown to play a role also in adhesion (257) and is controlled by the repressor NadR (122). Furthermore, also the acetate kinase *ackA2*, involved in acetate production from acetyl-coenzyme-A (acetyl-CoA), was down-regulated by glucose (Figure 20). Since the catabolism of glucose results in the accumulation of acetate, a feedback effect of acetate surplus could explain this observation. Similarly, other pathways that would be directly affected by an overabundance of acetyl-CoA are found repressed in presence of glucose, such as the tricarboxylic acid (TCA) cycle enzymes *prpB, prpC, lpdA3, sdhABCD* and *sucCD* (Figure 20). Indeed, growth on glucose has been reported to reduce the levels of TCA cycle enzymes also in gonococci (258, 259). Interestingly, the glucose-repressed genes encoding for the TCA cycle enzymes are also repressed in the glucose-rich human blood (123, 124) and in a recent report, the TCA cycle *prp* operon has been suggested

to help *N. meningitidis* in colonization of the propionate-rich and glucose-poor oral cavity of adults (260). Also the NADH-specific glutamate dehydrogenase *gludD* was found repressed in presence of glucose as well as the *putP-putA* operon involved in glutamate degradation and proline utilization. This suggests an interconnection between the carbon catabolism and the nitrogen metabolism in response to carbon source availability. On the other hand, the NADPH-specific *gdhA* was up-regulated 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 glucose concentration is higher than that of lactate (261), such as in human whole blood, where *gdhA* has been found upregulated (123).

Overall, we observed that *N. meningitidis* response to glucose modulates a higher number of genes compared to its response to other environmental signals such as zinc (17 genes, (120)), lactate (23 genes, data not shown) or iron (83 genes, (118, 119)). Furthermore, 11 genes found differentially expressed in presence of glucose show a similar pattern of expression when *N. meningitidis* is exposed to lactate (data 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 for hypothetical proteins, but also proteins related to cell envelope such as NMB0342 (*ispA*), NMB1729 (*exbB*) and NMB0543 (*lctP*).

This work identified a RpiR-family HexR regulator controlling the central carbon metabolism of *N. meningitidis* in response to glucose (Figure 20). The number of genes differentially expressed in a Δ*hexR* strain of *N. meningitidis* is similar to what has beeen reported for other proteobacteria (262). In other species, members of this family can be repressors such as RpiR *in Escherichia coli* (263), transcriptional activators like the GlvR of *B. subtilis* that modulates maltose metabolism (264) or dual-purpose transcriptional factors like HexR in *Pseudomonas putida* (244), *P. aeruginosa* (265) and *Shewanella oneidensis* (262). 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 within the promoter of *zwf* as well as a similar affinity site (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 - 3μM of HexR were necessary to bind to the operators within the *zwf* and *edd*/*gap-1* promoter regions by DNA footprinting (244). Perhaps interestingly, when looking at the transcriptome data the fold change for *edd* containing two HexR operators is

almost double that for *zwf*, where only one operator was identified. Overall, our *in vitro* data agree with what has been reported for HexR in other proteobacteria species, where it directly regulates the transcription of central carbon metabolism encoding genes (262). This is the case of *P. putida*, where HexR binds to the promoter region of the *zwf-1* gene and functions as a repressor (266) or in *S. oneidensis*, where HexR was shown to bind to DNA regions of several genes including the *zwf-pgl-edd-eda* operon (262). In other species, HexR glucose-responsive binding to the promoters of its target genes has been shown to be mediated by the ED pathway metabolite KDPG. Since the *N. meningitidis* HexR protein is divergent from its homologs in *P. putida* and *S. oneidensis*, this could explain why we did not observe an effect for KDPG on HexR DNA-binding affinity under the *in vitro* conditions used. Nevertheless, the HexR-binding DNA consensus is very similar between *N. meningitidis*, *P. putida* and *Shewanella spp.* , and common HexR-responsive genes are found between these species (244).

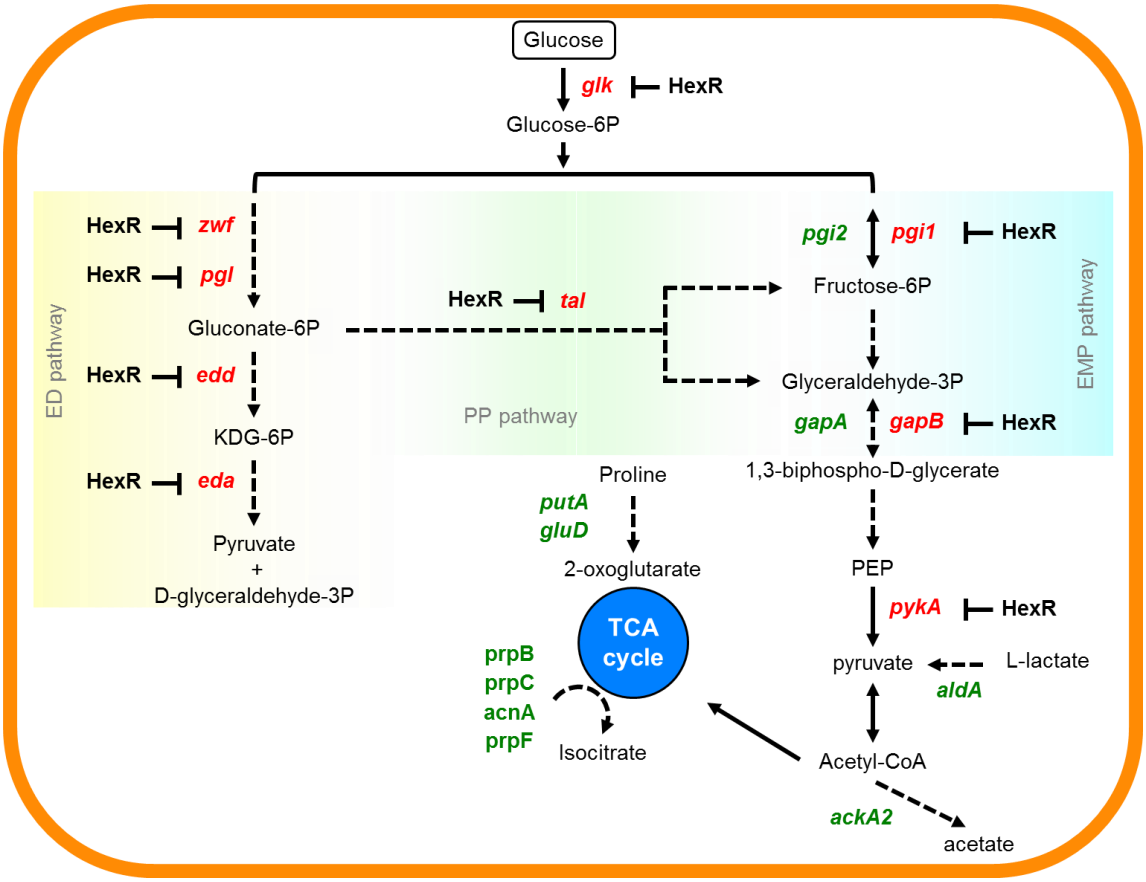

Figure 20 Model of glucose- and HexR-mediated regulation in *N. meningitidis*. Schematic representation of the main metabolic pathways affected by glucose availability. Genes significantly up- (red) and down-regulated (green) by glucose are shown. Genes subject to glucose-responsive HexR repression are highlighted. ED, Entner-Doudoroff pathway; PP, pentose phosphate pathway; EMP, Embden–Meyerhof–Parnas pathway; TCA, tri-carboxylic acids cycle.

In *E. coli*, expression of the enzymes in the ED pathway is essential for the colonization of the gastrointestinal tract (267), whereas in *P. putida* the ED pathway plays an important role for the generation of redox currency that is required to counteract oxidative stress (268). Similarly, our results indicate that a meningococcal strain lacking *hexR* shows reduced fitness during *in vivo* infection, indicating the importance of this transcriptional regulator not only in the metabolic adaptation but also in the survival of *N. meningitidis* within the host. It would be interesting to investigate during which steps of the infection, such as adhesion, colonization, and/or multiplication is HexR 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 or meninges) where nutrient availability is very diverse, it uses a restrict 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 meningococcus does not follow the same paradigm of carbon catabolite repression (CCR) 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, such as *nspA*, suggesting that other mechanisms either transcriptional or post-transcriptional could impact gene expression in response to glucose.

In the second part of this work, we report the curated identification of novel non-coding transcripts in *N. meningitidis*. RNAseq experiments are the *de facto* golden standard for discovery of novel small non-coding RNAs (221, 269, 270). We chose to explore the transcriptome of meningococci grown either under standard *in vitro* conditions or under iron limitation. Iron-regulated genes of *N. meningitidis* are both relevant to the infection process (109) and well characterized in the literature (118, 119, 132), providing us with solid benchmarks against which to validate our analyses. The number of small intergenic transcripts identified by curation of our RNAseq data is in line with recent findings on *in vitro* cultured *Neisseria* (210). In another recent report, a notably larger number of candidate sRNAs has been reported in gonococcus (211), highlighting the stringency of the criteria applied to our RNAseq curation. We identified different classes of sRNA transcripts, the most numerous being intergenic sRNAs, but we also identified 4 particularly long (100-300

nt) 5'-untranslated regions (UTRs) of genes. These may have regulatory functions *in cis* as riboswitches or thermosensors (158, 271) or even act as independent sRNAs after processing of a primary longer transcript (272). Interestingly, we also identified 10 putative transcripts of diverse lengths (70-500 nt) arising from the NIME repeat regions of the meningococcal genome (101). In order to validate our candidates, we derived information from a microarray analysis of meningococcal sRNAs differentially expressed under diverse *in vitro* conditions (Fagnocchi et al., *submitted*). In this report, the stationary phase of growth represents the condition in which most differentially expressed intergenic transcripts were identified, comprising 68% and 76% of total up- and down-regulated putative sRNAs, respectively. Accordingly, in our Northern blot experiments we could confirm the identification of 5 novel and 1 previously reported meningococcal sRNAs being induced in stationary phase, 1 novel sRNA arising from the processing of a 5'-UTR being repressed in stationary phase, as well as 1 previously identified sRNA expressed in presence of glucose. It should be noted that while putative sRNAs were identified based on the RNAseq profile of a standard mid-logarithmic *in vitro* growth, Northern blot experiments for 4 out of the 8 tested sRNAs did not detect any signal from log phase total RNA samples. Due to the large difference in sensitivity between the two techniques, even a high number of reads for a sRNA in the mid-logarithmic RNAseq dataset does not necessarily correlate to a commensurate Northern blot signal, in particular when the level of expression in the condition of maximum induction (i.e. stationary phase) is many fold higher than in the reference condition.

Our *in vivo* experiments using a murine model of bacteremia showed that knocking out three sRNAs (BNS1, sRNA0898-0899 and sRNA1400-1401) affects the *in vivo* survival of *N. meningitidis*. Interestingly, the knockout of BNS1 generated the lergest CI values, suggesting a relevant role for this sRNA on meningococcal survival during infection. A genome-wide screening of insertional mutants of *N. meningitidis* (44) identified 73 genes that are essential for bacteremia, comprising genes involved in the same processes in which BNS1 has been implicated through microarray experiments: energy metabolism and transport of metabolic molecules, amino acid biosynthesis and purine, pyrimidine, nucleosides and nucleotides biosynthesis (Fagnocchi et al., *submitted*). Taken together with the results of HexR deletion in the same model of bacteremia, this highlights the tight correlation between the carbon metabolism of *N. meningitidis* and its survival in the host environment during infection. Several studies describe the emergence of sRNAs as

regulators of metabolism, with several found to act at the interface of bacterial metabolism and virulence factor expression. For instance, the *E. coli* Spot42 sRNA selectively turns off the synthesis of enzymes required for galactose metabolism when a preferred carbon source is available (273) and synergizes with the global transcriptional regulator Crp to contribute to the overall efficiency of CCR (274). Since there is no evidence of CCR regulators in the *N. meningitidis* genome, this highlights the importance of other mechanisms for regulation of metabolism, such as the HexR and BNS1/GntR regulatory networks. Furthermore, although deletion of three more sRNAs (BNS2, sRNA0899-0900, sRNA1923-1934) does not alter meningococcal fitness in this model, we cannot exclude that they may be involved in functions related to meningococcal pathogenesis that are not assessed by the model used.

In this study we also report the discovery and initial characterization of a novel sRNA unique to meningococcus, transcribed antisense to an operon silent *in vitro*. The sequence of sRNA1880-1881 harbors two long complementary GC-rich stretches and is highly susceptible to copy number variation (CNV) both between different *N. meningitidis* strains as well as within different clones of the same strain. Recently, another sRNA harboring a GC-rich sequence capable of forming a complex secondary structure has been implicated in the antigenic variation of the gonococcal pilus via a recombination-dependent mechanism (214). The gonococcal *pilE* sRNA and the novel sRNA1880-1881 share no similarities apart from the presence of one or more GC-rich sequences. However, since these sequences are highly conserved across meningococcal genomes even when other parts of sRNA1880-1881 may differ, it would be interesting to investigate whether CNV of this sRNA depends on the formation of secondary structures similarly to what has been reported for *N. gonorrhoeae*, or meningococcus uses a different mechanism altogether.

The sRNA locus includes two operons: the NMB1880-1881-1882 operon comprises two genes encoding a putative iron-uptake system and a short hypothetical protein, while the diverging NMB1878/1879 gene is a member of the AraC family of transcriptional regulators (275). The homologue of the NMB1882 gene in *N. gonorrhoeae* has been shown to associate with the intracellular survival of invading gonococci (251). On the other hand, the AraC-like regulator is a homologue of MpeR in *N. gonorrhoeae*, which has been shown to activate expression of genes involved in iron uptake and antibiotic resistance (276, 277). The meningococcal MpeR has been shown to bind to the divergent promoter region of NMB1880, however transcriptional profiling could not detect any gene differentially

expressed upon deletion or overexpression of MpeR (278). This strongly suggests that the meningococcal MpeR protein is not active in regulating gene expression under *in vitro* conditions. On the contrary, sRNA1880-1881 is stably expressed under all the *in vitro* conditions tested, including iron limitation and lack of the Fur iron-responsive regulator. Furthermore, the steady-state transcript level of sRNA1880-1881 is not affected by deletion of the *hfq* gene, indicating that Hfq is likely not involved in the stability and/or turnover of this sRNA (168). Transcriptome profiling indicates that sRNA1880-1881 is not affecting global gene expression *in vitro*, however expression of this sRNA limits IPTG-induced transcription of the NMB1880-1881-1882 operon on the opposite strand. This suggests that the sRNA may have a role in tightly controlling the expression of the neighboring genes it is closely associated to, under conditions that do not require their activity. Since this operon is required for intracellular survival of invasive *Neisseriae* (251), it is tempting to speculate that the lack of activity of the MpeR regulator and the stable expression of sRNA1880-1881 observed under *in vitro* conditions may be reversed during invasion of cells in the host, possibly leading to activation of an intracellular iron scavenging system. Further experiments will be needed to elucidate during which steps of the infection, such as adhesion and/or invasion of epithelial cells, may expression of sRNA1880-1881 and its surrounding genes be regulated.

6 Materials and Methods

6.1 Bacterial strains and culture conditions

N. meningitidis strains (Table 3) were routinely cultured in GC-based (Difco) agar medium supplemented with Kellogg's supplement I (279). Liquid cultures were grown to mid-logarithmic or stationary phase in GC-based medium with Kellogg's supplement I or in Catlin 6 modified medium (C6) with/without the addition of 1% glucose (w/v) (280) at 37°C in a 5% CO_2 atmosphere. Strains were stocked in GC medium with 15% glycerol and stored at −80°C. When required, erythromycin (5 μg/ml), chloramphenicol (5 μg/ml), kanamycin (100 μg/ml) and/or isopropylβ-D-1-thiogalactopyranoside (IPTG) (1 mM) were added to culture media at the indicated final concentrations. For RNA sampling under different stress conditions, *N. meningitidis* was exposed to stresses as follows: 1) iron limitation: mid-logarithmic cultures in GC medium exposed for 5 min to 250 μM 2,2-dipyridyl (Sigma), 2) heat shock: mid-logarithmic GC cultures exposed for 10 min to 44°C in a water bath. 3) minimal medium: mid-logarithmic cultures grown in C6 minimal medium, 4) glucose availability: mid-logarithmic cultures in C6 medium with the addition of 1% glucose (w/v), 5) stationary phase: RNA collected from cultures 3 hours past mid-logarithmic phase, 6) late stationary phase: RNA collected from cultures 6 hours past mid-logarithmic phase.
Escherichia coli DH5α (281) and BL21 (DE3) strains (282) were grown in Luria-Bertani medium, and when required, ampicillin and/or IPTG were added to achieve a final concentration of 100 μg/ml and 1 mM, respectively.

6.2 Construction of mutant and complementation strains

DNA manipulations were carried out routinely as described for standard laboratory methods (283). In order to obtain a *hexR* mutant of the MC58 and 2996 strains by replacing it with a kanamycin cassette, the pGEMT-*hexR*KO::Kan plasmid was constructed. The downstream region of the *hexR* gene containing 176 bp of NMB1388 and 119 bp of the *hexR* gene was amplified by PCR with primers HexR1/HexR2 generating a XbaI/BamHI fragment (Table 4). Then the upstream region of *hexR* containing 311 bp of NMB 1390 and 90 bp of the *hexR* gene was amplified by PCR with primers HexR3/HexR4 generating a *Bam*HI/*Hind*III

fragment (Table 4). Both fragments were inserted into pGEMT vector (Promega), and a kanamycin cassette was inserted into the *Bam*HI site, between the flanking regions, generating pGEMT-*hexR*KO::Kan (Table 3). The plasmid was then linearized and used for transformation to make a *hexR* knockout mutant by selection on kanamycin leading to the generation of MC58 Δ*hexR* and 2996 Δ*hexR* strains, respectively (Table 3).

For the complementation of the MC58 Δ*hexR* null mutant, the *hexR* gene under the control of the P_{tac} promoter and the *lacI* repressor was re-inserted into the intergenic region between the converging open reading frames (ORFs) NMB1428 and NMB1429, by transforming with pComCmrP_{ind}-*hex*R (Table 3), a derivative plasmid of the pSLComCm^R (284), in which the *hexR* gene was amplified from the MC58 strain with the primers GG006/GG007 (Table 4) and cloned as a 849 bp *Nde*I/*Nsi*I fragment downstream of the P_{tac} promoter. This plasmid was transformed into the MC58 Δ*hexR* strain and transformants were selected on chloramphenicol.

To generate sRNA-KO mutant strains, the upstream and downstream flanking regions of each sRNA were amplified by PCR with specific primer pairs (Table 4). Then the respective upstream and downstream flanking regions were fused through self-priming PCR, amplified with external primer pairs and cloned as PCR products carrying a *BamHI* or *XmaI* restriction site between upstream and downstream flanking regions in the pGEMT (Promega) or pBluescript (Novagen) vector. The plasmids containing the sRNA flanking regions were digested with *BamHI* or *XmaI* and an erythromycin cassette was inserted generating pGEMT- or pBluescript-sRNAKO plasmids (Table 3). Following linearization these plasmids were used to transform MC58 and 2996 strains, generating the corresponding ΔsRNA strains (Table 4).

In order to construct MC58 isogenic mutants for inducible expression of the NMB1880-1881-1882 operon, a KO mutant of the region of interest was generated. The pGEMT-1880KO::Ery plasmid was constructed as follows: flanking regions comprising the NMB1878-1879 and NMB1881-1882 sequences were amplified from genomic DNA with oligos GG180/GG181 and GG186/GG188 respectively (Table 4), then fused through self-priming PCR, amplified with external primer pairs and cloned as a PCR product carrying a *BamHI* restriction site between upstream and downstream flanking regions into pGEMT vector (Promega), generating pGEMT-FLA-1880 (Table 3); then an erythromycin cassette was inserted into the *BamHI* site, between the flanking regions, generating pGEMT-

1880KO::Ery (Table 3). The plasmid was linearized and used for transformation to generate a MC58 1880KO knockout mutant (Table 3) by selection on erythromycin. To construct the isogenic complementation mutants, first the pCOM1880-P_{ind}-null construct was generated. Flanking regions comprising the NMB1878-1879 and NMB1881-1882 sequences were amplified from genomic DNA with oligos GG180/GG185 and GG187/GG188 respectively (Table 4), while the NMB1880 gene was amplified with oligos GG189/GG190 (Table 4). The three PCR products were then cloned into pComCmrP_{ind} as *XmaI/XbaI*, *NsiI/SpeI* and *NdeI/NsiI* fragments respectively, generating pCOM1880-P_{ind}-null (Table 3). *In vitro* synthesis (Life Technologies) produced plasmids pMK-sRNAwt, pMK-sRNAmutP and pMK-sRNAmutGC, each harboring one copy of sRNA1880-1881 with either the wild type sequence, or a sequence with mutations in the promoter elements or in the GC-rich stretches (Table 3). Subcloning of these sequences into pCOM1880-P_{ind}-null as *Xho*I/*Nsi*I fragments generated pCOM1880-P_{ind}-sRNAwt, pCOM1880-P_{ind}-sRNAmutP and pCOM1880-P_{ind}-sRNAmutGC respectively (Table 3). The four plasmid were linearized and used for transformation to generate the MC58 isogenic mutants of the same name (Table 3) by selection on chloramphenicol. All transformants were verified by PCR analysis for the correct insertion by a double homologous recombination event.

6.3 RNA preparation

Bacterial cultures were grown in liquid medium to an OD_{600} of 0.5-0.7 and then added to equal volume of frozen medium to bring the temperature immediately to 4ºC. Cells were harvested by centrifugation at 3400 g for 20 minutes. In preparation for transcriptome experiments, total RNA was isolated using the RNeasy Mini kit (Qiagen) following the manufacturer's instructions. Total RNA was extracted from three independent bacterial cultures and 15 μg of each sample were pooled together. Three independent RNA pools were prepared for each condition tested.

For Northern blot analysis of sRNA expression, total RNA was isolated using TRIzol reagent (Life Technologies) following the manufacturer's instructions. Briefly, bacterial pellets were resuspended in 1 ml of TRIzol reagent and incubated at room temperature for 5 minutes. Then the aqueous phase was extracted by adding 0.2 ml of chloroform (Sigma), thouroughly mixing, and centrifuging at 12000 x g for 15 minutes. Nucleic acids were then precipitated from the aqueous phase by adding 1 volume of 100% ethanol and 0.1 volumes

of 3 M sodium acetate (Sigma) (pH 5.2) and incubating 30 minutes on dry ice. After incubation, samples were centrifuged again at 12000 x g for 30 minutes, washed with 1 volume of 70% ethanol in water (v/v), and dried at room temperature. Pellets containing nucleic acids were added of 80 µl of DEPC-treated water (Ambion) and left to resuspend overnight, then treated with 10 µl RQ1 DNase (Promega) for 1 hour at 37°C. The DNA-free RNA was then extracted with phenol:chloroform:isoamylalcohol (Sigma) and precipitated as above. Dried RNA pellets were left to resuspend overnight in DEPC-treated water, then stored at -80°C.

6.4 Northern blot

Northern blot analysis was carried out using the Northern-Max kit (Ambion) according to the manufacturer's instructions. In brief, 3-5 µg of total DNA-free RNA were fractionated on 1% agarose-formaldehyde gel and transferred onto an Hybond XL nylon membrane (GE Healthcare) through capillary blotting. Then 5 pmol of radioactively labeled primers (Table 4) were used as probes. Hybridization was performed at 37°C overnight, low-stringency washes at room temperature.

6.5 Microarray procedures, hybridization and analysis

DNA microarray analysis was performed using an Agilent custom-designed oligonucleotide arrays. Briefly, cDNA probes were prepared from 5 µg of RNA pools and hybridized as described previously (212). Three hybridizations were performed using cDNA probes from three independent pools. Differentially expressed genes 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 comparing them against the zero value by Student's *t* test statistics (one tail).

6.6 RNA sequencing

Whole transcriptome pair-ended cDNA libraries were synthesized using the Ambion RNAseq Library Construction Kit (Life Technologies) from total RNAs extracted from *N. meningitidis* cultures grown to mid-logarithmic phase under standard *in vitro* conditions

or exposed to iron limitation. Libraries were sequenced with a HiSeq2000 platform (Illumina). Reads were mapped with bowtie 0.12.7 (285) on the *Neisseria meningitidis* MC58 reference genome NC_003112.2 (NCBI), then visualized on the Artemis genome browser (286).

6.7 Quantitative real-time PCR (qRT-PCR) experiments

2 μg of total RNA treated with Tubo-free DNase (Ambion) was reverse transcribed using random hexamer primers and M-MLV reverse transcriptase (Promega) following the manufacturer's instructions. Real-time quantitative RT-PCR was performed with triplicate biological samples in a 25 μl reaction mixture containing 80 ng of cDNA, 1X Brilliant II SYBR green quantitative PCR master mixture (Agilent) and 0.2 μM of gene-specific primers (Table 3). Amplification and detection of specific products were performed with an LightCycler 480 Real-Time PCR system (Roche) using the following procedure: 95ºC for 10min, followed by 40 cycles of 95ºC for 30 s, 55ºC for 1 min and 72ºC for 30 s then ending with a dissociation curve analysis. The 16S rRNA gene was used as the endogenous reference control and the relative transcript change was determined using the $2^{-\Delta\Delta Ct}$ relative quantification method (287). Student's *t*-test was used to calculate statistical significance ($p < 0.05$).

6.8 Expression and purification of recombinant HexR

The *hexR* gene was amplified from the MC58 genome using primers GG012/GG013 (Table 3) and cloned as a 843 bp fragment into pET15b(+) (Life Technologies) vector via the polymerase-incomplete primer extension (PIPE) enzyme-free cloning method (288), generating pET15b-HexR plasmid (Table 4). This plasmid was transformed into the *E. coli* BL21(DE3) strain and the expression of a recombinant HexR protein containing a N-terminal histidine tag (His-tag), was induced by the addition of 1 mM IPTG and grown at 25°C for 6 h, and the protein was purified by Ni-NTA (Qiagen) affinity chromatography under non-denaturing conditions according to the manufacturer's instructions. In brief, IPTG-induced *E. coli* cultures from above were concentrated in Lysis Buffer (20 mM Tris, 500 mM NaCl, 10 mM imidazole, chicken egg lysozyme 1 mg/ml), supplemented with Complete EDTA-Free Protease Inhibitor Cocktail (Roche) and incubated 30 min at 4°C.

Lysis was then performed by sonication, and cleared, filtered supernatants were applied to the column for nickel-affinity purification. After washing 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 together, 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 DTT, 10-50% glycerol) increasing the concentration of glycerol stepwise, up to 50% final glycerol.

6.9 DNase I Footprinting

The *zwf* and *edd* promoter regions were amplified with the primer pairs GG034/GG035 and GG036/GG037 respectively. The PCR products were purified and cloned into pGEMT vector (Promega) as 321 bp and 386 bp fragments generating pGEMT-P_{zwf} and pGEMT-P_{edd} respectively. Two pmol sample of each plasmid was end labeled by T4 polynucleotide kinase with [γ^{-32}] ATP after digestion at either the *XhoI* or *BamHI* site introduced by PCR with the oligos above. Following a second digestion with either *BamHI* or *XhoI*, the labeled probes were purified by polyacrylamide gel electrophoresis (PAGE) as described previously (116). DNA-protein binding reactions were carried out for 15min at room temperature 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-40) containing 60 fmol of labeled probe, 100 ng of salmon sperm DNA and a range of HexR concentrations as indicated in the figures. Samples were then treated with 0.3 U of DNase I (Roche) for 2 min at room temperature. DNase I digestion were stopped and samples purified loaded and run on a 8 M urea 6% polyacrylamide gel as described previously (289). Where indicated, reactions were supplemented with the phosphosugars 3-Deoxy-2-keto-6-phosphogluconic acid (Sigma), glucose 6-phosphate (Sigma), fructose 1,6-diphosphate (Sigma) or 6-phosphogluconic acid (Sigma) at concentrations of 400 – 4000 µM prior to addition of the labeled probe. A G+A sequence reaction (290) was performed for each probe and run in parallel to the footprinting reactions.

6.10 Bioinformatic analysis of the HexR binding site

The HexR-binding consensus sequence was derived from 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 via the EMBOSS fuzznuc algorithm (Alan Bleasby, 2000). Structure of operons and putative transcriptional start sites were determined based on the RNA sequencing experiment.

6.11 Bioinformatic analyses of small RNAs

Open reading frames were predicted by submitting the sRNA sequence to the NCBI ORF Finder. Results were then manually curated by analyzing the sequence upstream of each putative ORF looking for matches to the consensus Shine-Dalgarno ribosomal binding sequence (AGGAGGU) around 6 bp upstream of the predicted starting codon. ORFs lacking a good RBS or predicted to be shorter than 10 aminoacids were discarded from the analysis. RNA secondary structures were predicted with the mfold web server (291). Conservation analysis of sRNA1880-1881 was performed by aligning the NMB1877-NMB1882 genomic region from MC58 strain with the homologous regions in available neisserial genomes. Results were visualized and interpreted by means of Geneious software (BioMatters).

6.12 Amplification and sequencing of NMB1880-1881 intergenic region

The intergenic region harboring sRNA1880-1881 was amplified from the MC58 strain with the primers GG103/GG106 (Table 4). Multiple products arising from *Taq* PCR amplification were separated on agarose gel, purified and TA-cloned into the pGEM-T vector (Promega). Clones positive for the presence of an insert in the pGEM-T multiple cloning site were sequenced with different oligos spanning across the NMB1880-1881 IGR (Table 4) and the resulting reads were assembled to reconstruct the IGR sequence of our MC58 clone.

6.13 Disc diffusion viability assays

Sensitivity of *N. meningitidis* strains to different stress agents was assayed by means of a modified Kirby-Bauer disc diffusion method (292). In brief, approximately 5 x 10^8 bacteria from mid-logarithmic phase cultures were inoculated in 6 ml of GC soft agar (8 g/l) and poured onto square GC plates (25 ml). After 30 min, up to five Whatman paper discs (6-mm diameter; GE Healthcare) were applied to the plate and 10 µl of the agent to be tested was applied to each disc. The plates were incubated for 24 h at 37°C, 5% CO2 and then the diameter of growth inhibition was recorded. At least three plates were prepared for each agent tested, and we measured the growth inhibition zone twice for each disc. Each experiment was performed at least two times on different days. The results were averaged, and the error bars represent the standard deviation of the mean. All the agents tested were obtained from Sigma-Aldrich.

6.14 *In vivo* infant rat model

The infant rat model was used as previously described (293). Briefly, bacteria were grown to mid-log phase in GC medium, washed, and resuspended at the desired concentration in PBS. Six to eight-day-old pups from litters of outbred Wistar rats (Charles River) were challenged intraperitoneally with the 2996 wild-type and the isogenic knockout mutant strains at a 1:1 ratio to establish mixed infections. Groups of infant rats were used for each infectious dose of 4.5 x 10^3 or 4.5 x 10^4 CFUs respectively. A control group of 9 infant rats was injected with PBS. After 18h post bacterial challenge, blood samples were obtained by cheek puncture, and aliquots (100 µl of undiluted sera as well as 1/10 and 1/100 dilutions) were plated onto columbia agar supplemented with 5% horse blood with or without kanamycin for viable cell counting. The number of CFU/ml found in blood was determined after overnight incubation of the plates at 37 ◦C in a 5% CO2 atmosphere. Enumeration of wild-type bacteria and mutant bacteria allowed to determine the competitive index (CI) ratio using the following formula: CI=(WT output/mutant output)/(WT input/mutant input). Observed CIs from the two infectious doses follow the same statistical distribution and were pooled to increase the power of the analysis. Statistical significance was assessed with the Mann-Whitney test.

6.15 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 the Novartis Animal Welfare Policy and Standards. Protocols were approved by the Italian Ministry of Health (Authorization D.M. n. 166/2012 - B) and by the local Novartis Animal Welfare Body (Research Project AWB 201202). Following infection, animals were clinically monitored daily for criteria related to their ability to feed, reactivity and motility, and cutaneous redness. After 18 hours all animals were alive and normally reactive, and were euthanized by cervical dislocation, as pre-established in agreement with Novartis Animal Welfare Policies.

Table 3 Plasmids and strains used in this study.

Name	Description	Antibiotic resistance	Reference
pGEMT	Cloning vector	Ampicillin	Promega
pGEMT-*hexR*KO::Kan	Plasmid for deletion of *Nm hexR* gene by homologous recombination	Ampicillin, Kanamycin	This study
pGEMT-P_{zwf}	Plasmid harboring 321 bp promoter fragment upstream of *zwf* gene	Ampicillin	This study
pGEMT-P_{edd}	Plasmid harboring 386 bp promoter fragment upstream of *edd* gene	Ampicillin	This study
pGEMT-FLA-s17	pGEMT containing the flanking region of sRNA0899-0900 with a BamHI site in the middle	Ampicillin	This study
pGEMT-s17KO::Ery	pGEMT-FLA-s17 derivative in which a 1200bp Ery cassette was cloned as a BamHI fragment between flanking regions	Ampicillin, Erythromycin	This study
pGEMT-FLA-s27	pGEMT containing the flanking region of sRNA1400-1401 with a BamHI site in the middle	Ampicillin	This study
pGEMT-s27KO::Ery	pGEMT-FLA-s27 derivative in which a 1200bp Ery cassette was cloned as a BamHI fragment between flanking regions	Ampicillin, Erythromycin	This study
pGEMT-FLA-s38	pGEMT containing the flanking region of sRNA1923-1924 with a BamHI site in the middle	Ampicillin	This study
pGEMT-s38KO::Ery	pGEMT-FLA-s38 derivative in which a 1200bp Ery cassette was cloned as a BamHI fragment between flanking regions	Ampicillin, Erythromycin	This study
pGEMT-FLA-SRS3	pGEMT containing the flanking region of sRNA1880-1801 with a BamHI site in the middle	Ampicillin	This study
pGEMT-SRS3::Ery	pGEMT-FLA-SRS3 derivative in which a 1200bp Ery cassette was cloned as a BamHI fragment between flanking regions	Ampicillin, Erythromycin	This study
pGEMT-FLA-1880	pGEMT containing the flanking regions of NMB1880 and sRNA1880-1881 with a BamHI site in the middle	Ampicillin	This study
pGEMT-1880KO::Ery	pGEMT-FLA-1880 derivative in which a 1200bp Ery cassette was cloned as a BamHI fragment between flanking regions	Ampicillin, Erythromycin	This study
pBluescript (pBS-KS)	Cloning vector	Ampicillin	Novagen
pBS-KS-FLA-IG26	pBS-KS containing the flanking region of sRNA0898-0899 with a XmaI site in the middle	Ampicillin	This study
pBS-KS-IG26::Ery	pBS-KS-FLA-IG26 derivative in which a 1200bp Ery cassette was cloned as a XmaI fragment between flanking regions	Ampicillin, Erythromycin	This study
pBS-KS-FLA-BNS1	pBS-KS containing the flanking region of BNS1 with a XmaI site in the middle	Ampicillin	This study
pBS-KS-BNS1::Ery	pBS-KS-FLA-BNS1 derivative in which a 1200bp Ery cassette was cloned as a XmaI fragment between flanking regions	Ampicillin, Erythromycin	This study
pBS-KS-FLA-BNS2	pBS-KS containing the flanking region of BNS2 with a XmaI site in the middle	Ampicillin	This study
pBS-KS-BNS2::Ery	pBS-KS-FLA-BNS2 derivative in which a 1200bp Ery cassette was cloned as a XmaI fragment between flanking regions	Ampicillin, Erythromycin	This study
pMK	Cloning vector	Kanamycin	Life Technologies
pMK-sRNAwt	Vector harboring one copy of sRNA1880-1881, derived from *in vitro* synthesis	Kanamycin	This study
pMK-sRNAmutP	Vector harboring one copy of sRNA1880-1881 with mutations disrupting its promoter sequence, derived from *in vitro* synthesis	Kanamycin	This study
pMK-sRNAmutGC	Vector harboring one copy of sRNA1880-1881 with mutations disrupting pairing of its GC-rich stretches, derived from *in vitro* synthesis	Kanamycin	This study
pET15b(+)	Plasmid for inducible expression of histidine-tagged recombinant proteins in *E. coli*	Ampicillin	Life Technologies
pET15b(+)-*hexR*	Plasmid for expression and purification of histidine-tagged HexR in *E. coli*	Ampicillin	This study
pComCmrP$_{ind}$	Plasmid for allelic replacement at a chromosomal location between ORFs NMB1428 and NMB1429 and inducible expression under the control of the P_{tac} promoter and the *lacI* repressor	Ampicillin, Chloramphenicol	Ieva et al., J Bacteriol 2005
pComCmrP$_{ind}$-*hexR*	Plasmid for complementation of HexR null mutant, derivative of pComCmrP$_{ind}$ containing a copy of *hexR* gene	Ampicillin, Chloramphenicol	This study
pCOM1880-P$_{ind}$-null	Plasmid for complementation of NMB1880 + sRNA1880-1881 null mutant, derivative of pComCmrP$_{ind}$ containing a copy of NMB1880 gene and no copy of sRNA1880-1881	Ampicillin, Chloramphenicol	This study
pCOM1880-P$_{ind}$-sRNAwt	Plasmid for complementation of NMB1880 + sRNA1880-1881 null mutant, derivative of pComCmrP$_{ind}$ containing a copy of NMB1880 gene and one copy of sRNA1880-1881	Ampicillin, Chloramphenicol	This study
pCOM1880-P$_{ind}$-sRNAmutP	Plasmid for complementation of NMB1880 + sRNA1880-1881 null mutant, derivative of pComCmrP$_{ind}$ containing a copy of NMB1880 gene and one copy of sRNA1880-1881 with mutations disrupting its promoter sequence	Ampicillin, Chloramphenicol	This study
pCOM1880-P$_{ind}$-sRNAmutGC	Plasmid for complementation of NMB1880 + sRNA1880-1881 null mutant, derivative of pComCmrP$_{ind}$ containing a copy of NMB1880 gene and one copy of sRNA1880-1881 with mutations disrupting pairing of its GC-rich stretches	Ampicillin, Chloramphenicol	This study
MC58	*Nm* laboratory-adapted reference strain	-	Tettelin et al., Science 2000
MC58 ΔFur	MC58 derivative, lacking *fur* gene	Kanamycin	Delany et al., J Bacteriol 2003
MC58 Δhfq	MC58 derivative, lacking *hfq* gene	Chloramphenicol	Fantappiè et al., Infect Immun 2009
MC58 Δ*hexR*	MC58 derivative, lacking *hexR* gene	Kanamycin	This study
MC58 Δ*hexR c-hexR*	MC58 derivative, lacking *hexR* gene, with a copy of *hexR* reintroduced out-of-locus under control of inducible P_{tac} promoter	Kanamycin, Chloramphenicol	This study
MC58 ΔsRNA1880-1881	MC58 derivative, lacking sRNA1880-1881	Erythromycin	This study
MC58 1880KO	MC58 derivative, lacking NMB1880 gene and sRNA1880-1881	Erythromycin	This study
MC58 sRNAnull	MC58 derivative, with the NMB1880 gene under control of inducible P_{tac} promoter, no sRNA in NMB1880-1881 IGR	Chloramphenicol	This study
MC58 sRNAwt	MC58 derivative, with the NMB1880 gene under control of inducible P_{tac} promoter, one wt copy of sRNA in NMB1880-1881 IGR	Chloramphenicol	This study
MC58 sRNAmutP	MC58 derivative, with the NMB1880 gene under control of inducible P_{tac} promoter, one copy of sRNA in NMB1880-1881 IGR with mutations disrupting its promoter sequence	Chloramphenicol	This study
MC58 sRNAmutGC	MC58 derivative, with the NMB1880 gene under control of inducible P_{tac} promoter, one copy of sRNA in NMB1880-1881 IGR with mutations disrupting pairing of its GC-rich stretches	Chloramphenicol	This study
2996	Clinical isolate	-	Comanducci et al., J Exp Med. 2002
2996 ΔsRNA0898-0899	2996 derivative, lacking sRNA0898-0899	Erythromycin	This study
2996 ΔsRNA0899-0900	2996 derivative, lacking sRNA0899-0900	Erythromycin	This study
2996 ΔsRNA1400-1401	2996 derivative, lacking sRNA1400-1401	Erythromycin	This study
2996 ΔBNS1	2996 derivative, lacking BNS1	Kanamycin	This study
2996 ΔsRNA1923-1924	2996 derivative, lacking sRNA1923-1924	Erythromycin	This study
2996 ΔBNS2	2996 derivative, lacking BNS2	Erythromycin	This study

Table 4 Oligonucleotides used in this study.

Name	Sequence[a]	Restriction Sites	Application
Hex-R1	ATTCG TCTAGA GGTTTCGTCGTTGATGCGGTTTTTG	XbaI	*hexR* KO
Hex-R2	CAAATGGTTCG GGATCC GTTGCCACACAGGAAAATG	BamHI	
Hex-R3	CTGTGTGGCAAC GGATCC CGAACCATTTGGGTTCCGC	BamHI	
Hex-R4	ATTCG AAGCTT TCACGGAAAAGGCTTTGAGC	HindIII	
GG006	ATATCATATGTTAAGCAAAATCAGCGAATCACTG	NdeI	*hexR* complementation
GG007	ATATATGCATTCAATCTTTGTCGTAATCGATGTGC	NsiI	
GG012	CTGTACTTCCAGGGCTTAAGCAAAATCAGCGAATCACTG	-	PIPE cloning of *hexR*
GG013	AATTAAGTCGCGTTAATCTTTGTCGTAATCGATGTGC	-	
GG034	CTCGAGCGTCTGAAAGTGGGAAGCGG	XhoI	*zwf* promoter probe
GG035	GGATCCGTACTCATCGTATTATCTCGTCAGG	BamHI	
GG036	CTCGAGCCCCTATTCCGTTACAACAATCG	XhoI	*edd* promoter probe
GG037	GGATCCTTCACGGTCGGTCTCCTGTC	BamHI	
0089RT-F	GAAACGATGCTGGTGGAAC	-	qRT-PCR
0089RT-R	CCGCTGGTAATGATGTATTGG	-	
0207RT-F	TGACCAAATTCGACACCGT	-	
0207RT-R	ATCGACACCGAGTTCTTTCC	-	
0334RT-F	ATTTTGATTGACCGCCTCAC	-	
0334RT-R	CACTGATCGAAGGGGTTGAC	-	
0663RT-F	TATGCCGTTACCCCGAATGT	-	
0663RT-R	CAGTGTTGACTTTGCCGATG	-	
1389RT-F	ATGGTTTCCCGCCTCTTG	-	
1389RT-R	CGATGTGCTTGTTGTGTATGCT	-	
1392RT-F	AGCCTGTGAAAACCTTGCTG	-	
1392RT-R	TTGATTTGCTGGGAAGAAGC	-	
1393RT-F	TTGAAAAGCGAAATGGGTTC	-	
1393RT-R	GGTGTAAGGGTGGACGAAGG	-	
1476RT-F	ACGTTGCCATTTACAACGAA	-	
1476RT-R	GTTCGGCGTTGGTAATTTCT	-	
1710RT-F	GCAAATGAGTTCCGCCATC	-	
1710RT-R	ATAGGCAGGGTGGTCAAGG	-	
1968RT-F	TCAAACAAGGTGCGAAATTG	-	
1968RT-R	CCATACTGTTGTCGGTGTCG	-	
2159RT-F	GTTATCTCCGCCGCTTCCT	-	
2159RT-R	GCTGTTGGGCACGATGTT	-	
1880RT-F	ATTGAAGGCGCAGATTGAC	-	
1880RT-R	CGTGTATCCAACTTGCCAAC	-	
1881RT-F	ACGGATACAGCGACAAAGTG	-	
1881RT-R	CTCGCGTATCGTCATGCT	-	
1882RT-F	CAACGACGGCTACACTGTTT	-	
1882RT-R	CATTTGTTGCGATGTGATGA	-	
RT-SRS3NEW-F	GACTGCCATCGGTCTGAATC	-	
RT-SRS3NEW-R	CTCCGTTTTAGCTTCGCAGA	-	
16SRT-F	ACGTAGGGTGCGAGCGTTAATC	-	
16SRT-R	CTGCCTTCGCCATCGGTATTCCT	-	

[a]: underscored letters indicate restriction enzyme sites.

Table 4 (continued) Oligonucleotides used in this study.

Name	Sequence[a]	Restriction Sites	Application
nrrF-NB	GTATGTCTCGTATATGCCGACTCCAAGTGTGAAAGTGATGATGGGGAAAT	-	NrrF NB
1205-3	GGCAGCCACACCCAAACAC	-	AniS NB
sRNA10-R	CCAGCTTGATATACTCGGCGGAT	-	sRNA0837-0838 NB
SRS19-R4	GAATAAATATGTCCCATTGTCATCCCCTTAAGCTGATG	-	sRNA0898-0899 NB
sRNA17-R2	ATCGTGTGGATATACGCTGTTTGTCAGGTGTTTTCAAGCACCGTGGGAAA	-	sRNA0899-0900 NB
GG075	GCTCCATAAGACATAATCAACTGTG	-	sRNA1400-1401 NB
Bns1_p	CCTCCCGAATATATCTGCCTGCTGTTTCCTCTTTATTCAG	-	BNS1 NB
GG052	cacccgagtttatgcggcaaacagcg	-	sRNA1880-1881 NB
GG054	ttaacttcgttgaagctgcgatttcagaagct	-	
GG087	AGTATGAATGGTCAATACATTGCGG	-	sRNA1923-1924 NB
SRS17-R4	CACATTACGGGGAAAACGTTTTACTCAATGAG	-	BNS2 NB
UP_IG26F	GCTCTAGAGAAACAGGCACAACGGCAAA	XbaI	sRNA0898-0899 KO
UP_IG26R	TCCCCCGGGCTTAAATCGCCCGTTAAGGC	XmaI	
DW_IG26F	TCCCCCGGGTTTATTTTCAACATCAGCTTAAGG	XmaI	
DW_IG26R	CCGCTCGAGCTTTTTCTTCCATTTTCGGGCT	XhoI	
FLA-UP17F	CGAATACTCCATGCTGTTACGTG	-	sRNA0899-0900 KO
FLA-UP17R	TTTCTGCAAGCGGATCCGGAAATGTGTCAAGAGAATTAGCCC	BamHI	
FLA-DO17F	GACACATTTCCGGATCCGCTTGCAGAAAATAGAAAGATTGG	BamHI	
FLA-DO17R	CACGATAACTATTTGATTTGCTTCCG	-	
flaUP1400F	CCAAAGATTTGGCGGCTAACAGCC	-	sRNA1400-1401 KO
flaUP1400R	GTCCCGACAGCGGATCCATATACAGATATTTCAGGCTGCCTG	BamHI	
flaDO1401F	TATCTGTATATGGATCCGCTGTCGGGACGGTGTGCCGAAG	BamHI	
flaDO1401R	TTCGGCAGTCCTGTTCTACCGC	-	
UP_bns1F	GCTCTAGACGACAATCTTGTCGTGCG	XbaI	BNS1 KO
UP_bns1R	TCCCCCGGGGAGAATCCCGTTATTTTAAG	XmaI	
DW_bns1F	TCCCCCGGGCTTCAGACGGTATCAGCC	XmaI	
DW_bns1R	CCGCTCGAGCCGTTTTGTCCATATTTCTGC	XhoI	
GG103	GCGGTTGGCAAGTTGGATACAC	-	sRNA1880-1881 KO
GG104	GGATCCttgaaaccgcactttagcttcgc	BamHI	
GG105	gcggtttcaaGGATCCaaagcagcctgcaacgaagcc	BamHI	
GG106	CGGACGGGTCGGACAACTC	-	
flaUP1923F	TTGTACTGTCTTCGGCTTCGTCG	-	sRNA1923-1924 KO
flaUP1923R	AAACCAATAGGGGATCCGCACGTTGAAAATGCCGTCTGAAC	BamHI	
flaDO1924F	TTTCAACGTGCGGATCCCCTATTGGTTTTCCCGTATCCAC	BamHI	
flaDO1924R	GTCGATCCGATAGACGGGACGAAC	-	
UP_bns2F	GCTCTAGACGCCTGAAACGCATCAACC	XbaI	BNS2 KO
UP_bns2R	TCCCCCGGGGATGCCGTCTGAAACGGC	XmaI	
DW_bns2F	TCCCCCGGGGTTCCATCGGATAAAAGGC	XmaI	
DW_bns2R	CCGCTCGAGGATGGTGCTGTAAATGGACG	XhoI	
GG149	TTAAAAAGGCAGAACCCGTTGCG	-	Sequencing of NMB1880-1881 IGR
GG150	AGGTGCTGTTTCAGGTTGATGG	-	
GG172	GGCTTACCGCCCTCTCCCTAAC	-	
GG173	CGAATTGAAAAGCAGCCTGTATGTTG	-	
GG180	aaCCCGGGGCGTTTTGCGCCAGCCGTTGCAG	XmaI	Isogenic mutants of NMB1880-1881-1882 operon and sRNA
GG181	GGATCCATGAACACCGCCGCCATCTACCG	BamHI	
GG186	CGGTGTTCATGGATCCACCCAACCCATAGGAGAACCCCATG	BamHI	
GG188	aaACTAGTTCTGAAACAGAAACATCGGCCTG	SpeI	
GG185	aaTCTAGAATGAACACCGCCGCCATCTACCG	XbaI	
GG187	aaATGCATACCCAACCCATAGGAGAACCCCATG	NsiI	
GG189	aaCATATGAAACCGCGTTTTTATTGGGCAGC	NdeI	
GG190	aaATGCATaaaCTCGAGCTACTTTTTCCCCGCCGCAACGG	NsiI, XhoI	

[a]: underscored letters indicate restriction enzyme sites.

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