A two-component system is required for colonization of host cells by meningococcus

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In order to adapt to changing environments, bacteria have evolved two-component systems (TCSs) that are able to sense and respond to environmental stimuli. The signal perception relies on a sensor protein whose activation allows rapid adaptation through transcriptional regulation achieved by the regulatory protein. The ability to adhere to and grow on the surface of human host cells is an absolute requirement for many pathogens, including Neisseria meningitidis, in order to colonize new hosts and to disseminate inside their host. Among the four TCSs encoded in the meningococcus genome, only the PhoQ (MisS)/PhoP (MisR) system has been shown to constitute a functional signal transduction circuit. To investigate the involvement of this TCS in the adaptation process requisite for host cell colonization, we have tested the ability to grow on host cells of a mutant inactivated for the sensor of the TCS. Our results demonstrate the involvement of the TCS in the adaptation of the meningococcus to growth on host cells. We show that the expression of the PhoQ (MisS)/PhoP (MisR) TCS is cell-contact controlled. Furthermore, this TCS controls the regulation of a group of genes, the REP2 regulon, previously shown to be cell-contact regulated and to encode functions crucial for the adaptation of the bacterium to host cell colonization. Thus, we provide evidence that one of the four TCSs existing in N. meningitidis contributes to the adaptation of the pathogen to growth on host cells.

INTRODUCTION

Neisseria meningitidis is a worldwide cause of septicaemia and meningitis. Paradoxically, the meningococcus asymptptomatically colonizes the upper respiratory tract of about 8–25% of the human population and disseminates from person to person by direct contact (Stephens et al., 2007). In a small percentage of colonized people, the bacterium is able to invade the bloodstream and to cross the blood–brain barrier. The ability of the bacterium to establish an efficient interaction with host cells (epithelial and endothelial cells) is crucial for its behaviour as a commensal and a pathogen, by allowing colonization of the nasopharynx and crossing of the blood–brain barrier. This ability to grow on human cells implies that the bacterium is able to rapidly adapt.

A regulon of 14 genes has been shown to be upregulated as N. meningitidis initially interacts with host cells and downregulated upon prolonged contact with host cells (Morelle et al., 2003). The genes of this regulon encode functions critical for the adaptation of the bacterium to growth onto host cells, such as pilC1, which is required for pilus-mediated adhesion (Taha et al., 1998), and the exonuclease xseB gene, which controls an inducible repair system (Morelle et al., 2005). These genes are characterized by the presence of a REP2 repeat upstream of the open reading frame. The mechanism controlling the expression of this regulon is unknown.

Perception and response to environmental stimuli is frequently mediated by two-component signal transduction systems (TCSs) (Beier & Gross, 2006; García Véscovi et al., 1996). Classical TCSs consist of a membrane-bound sensor kinase and a cytoplasmic response regulator. The histidine kinase is autophosphorylated in response to an environmental signal. The phosphoryl group is then transferred to the response regulator. This ultimately results in modification of gene expression in response to the environmental stimulus (Beier & Gross, 2006). In contrast to Escherichia coli, which possesses 30 TCSs (Blattner et al., 1997; Oshima et al., 2002), N. meningitidis contains only four putative TCSs (Parkhill et al., 2000; Tettelin et al., 2000). Experimental evidence demonstrating a regulatory function is only available for one of these four TCSs, i.e. the NMA0797 (PhoQ, MisS)/0798 (PhoP, MisR)
system (Johnson et al., 2001; Newcombe et al., 2004, 2005; Tzeng et al., 2004, 2006, 2008). A meningococcal knockout mutant in the NMA0798 gene was reported to display an attenuated virulence phenotype in a mouse model of infection (Newcombe et al., 2004). This TCS was also shown to be necessary for the bacterium to cross an epithelial cell layer (Johnson et al., 2001), and was demonstrated to constitute a functional signal transduction system (Tzeng et al., 2006) that modulates the meningococcal virulence factor lipopolysaccharide (Tzeng et al., 2004). In addition, transcriptomic analyses revealed that inactivation of NMA0798 perturbed the expression of a large number of genes (Newcombe et al., 2005), many of which are involved in the synthesis of components of the meningococcal cell surface (Tzeng et al., 2008). Here, we investigated the role of the NMA0797 (PhoQ, MisS)/0798 (PhoP, MisR) system in the adaptation of meningococci to colonization of host cells. We show that this system is required to optimize the growth of the bacteria on the apical surface of human cells. In addition we demonstrate that it controls the expression of the genes of the REP2 regulon involved in the adaptation of the bacterium to growth on host cells.

METHODS

Bacterial strains and growth conditions. The strain used in this study is a derivative of the piliated, Opa +, PilC1 + and PilC2 + serogroup C N. meningitidis 8013 strain (Nassif et al., 1993). N. meningitidis strains were grown on GC agar (GCB; Difco), supplemented with Kellogg’s defined supplement (Kellogg et al., 1963) overnight at 37 °C in a moist atmosphere containing 5% CO₂. For selection of transformants, the growth medium was supplemented with kanamycin (100 μg ml⁻¹). E. coli DH5α was grown in Luria–Bertani (LB) broth or on plates at 37 °C and was used for the propagation of plasmids (Sambrook et al., 1989). E. coli BL21(DE3) was used for recombinant protein expression. When necessary, E. coli was grown in the presence of kanamycin (30 μg ml⁻¹) or ampicillin (50 μg ml⁻¹).

Construction of a mutant of the NMA0797 gene. A mutant disrupted for the gene encoding the sensor of the NMA0797/0798 TCS, i.e. NMA0797, was present in the library of signature-tagged mutants of N. meningitidis (Geoffroy et al., 2003) available in the laboratory. This mutation was introduced by transformation into N. meningitidis strain 8013. The transformants were selected in the presence of kanamycin. We showed by RT-PCR that genes NMA0798 and NMA0796 were not co-transcribed. Therefore the insertion of the transposon cannot result in any polar effect.

Adhesion assay. Human umbilical vein endothelial cells (HUVECs) (promoCell) were seeded at 4 × 10⁶ cells cm⁻² in a six-well culture plate and grown overnight at 37 °C in a humidified incubator with 5% CO₂ as previously described (Morelle et al., 2003). Briefly, cell monolayers were infected with bacteria in the exponential growth phase (m.o.i. = 400). Bacteria were left in contact with the cells for 30 min, the supernatant was then removed, and the wells were gently washed with infection medium (t=0 h). Monolayers were then washed every hour and fresh infection medium was added. The adherent bacteria were harvested after 1 h (early stage) and 4 h (late stage) of infection.

Total RNA isolation, real-time RT-PCR. Total RNA isolation and real-time RT-PCR were performed as previously described (Morelle et al., 2003; Yasukawa et al., 2006) from bacteria grown in infection medium and harvested after 1 h and 4 h of adhesion to HUVECs. Two reverse transcription reactions were performed for each RNA sample isolated from three adhesion assays. Primers NMA0612-prom-Up and NMA0612-prom-Down were designed to PCR amplify the NMA0612 promoter region and were used to check the absence of chromosomal contamination in RNA samples (Table 1). The aphA3 gene, which encodes kanamycin resistance, was used as an internal reference. The aphA3 gene is located in the transposon in strain 8013/NMA0797 and inserted in the intergenic region located between divergently transcribed genes NMA2226 and NMA2227 in the wild-type 8013 strain. The ‘induction index’ was calculated for each gene considered at every time point, using non-cell-associated bacteria grown in an identical infection medium as calibrator. Statistical significance was evaluated by using Student’s t-test, with P<0.01 considered significant.

Expression and purification of recombinant NMA0798 protein. The open reading frame of NMA0798 devoid of the stop codon was amplified by PCR using genomic DNA from N. meningitidis and primers NMA0798-NcoI/NMA0798-XhoI (Table 1), which contain restriction sites for Ncol and Xhol. This PCR product was digested with Ncol and Xhol, purified with the QIAEXII gel extraction kit and subcloned into pET28a(+) (Novagen) which had been previously digested with Ncol and Xhol. This introduced a six-

Table 1. Oligonucleotides used in the study

<table>
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<th>Sequence (5’ to 3’)</th>
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<tr>
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<tr>
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<tr>
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histidine tag at the C-terminus of the recombinant protein. The protein was expressed in E. coli BL21(DE3) and purified using Ni-NTA agarose (Qiagen).

Electrophoretic mobility shift assay (EMSA). The experiment was performed as described by Tzeng et al. (2006), using as probes PCR products generated using genomic DNA from N. meningitidis as a template and primers indicated in Table 1.

DNase I protection. The experiment was performed as described by Tzeng et al. (2006), using primers FootprintREP2-xseB-F and FootprintREP2-xseB-R (Table 1), corresponding to the coding or the non-coding strand.

RESULTS

The NMA0797 (PhoQ, MisS)/0798 (PhoP, MisR) system is required to optimize the growth of N. meningitidis onto host cells

We investigated the role of the NMA0797/0798 TCS in meningococcal colonization of host cells. A mutant of the sensor was engineered, thus abolishing the perception of an environmental signal by the bacterium. The ability of this mutant to grow onto the apical surface of host cells was evaluated by determining a competitive index with the mutant to grow onto the apical surface of host cells was evaluated by determining a competitive index with the parental wild-type (WT) strain 8013. HUVEC monolayers were infected with a mix of WT strain (kanamycin sensitive), and mutant 8013NMA0797 strain (kanamycin resistant). The number of bacteria adhering at $t=0\,$h (30 min after the infection), $t=1\,$h (early adhesion step) and $t=4\,$h (late adhesion step) was determined. The ratio of WT and mutant strains interacting with the monolayer was determined. At $t=0\,$h, the initial change in the natural logarithm of the ratio quantified the difference in adhesiveness. Subsequent changes in the logarithm of the ratio, measured at $t=1\,$h and $t=4\,$h, quantified the difference in fitness, i.e. the ability of the bacteria to grow onto the monolayer. This revealed that the mutant disrupted for the sensor kinase displayed an altered phenotype (Fig. 1). The 8013NMA0797 strain did not display any adhesiveness defect ($t=0\,$h). However, at $t=4\,$h, the number of mutant bacteria interacting with the cells was drastically reduced ($P<0.01$, Fig. 1a). Mutant 8013NMA0797 also displayed a growth defect in broth compared to that of the WT strain (doubling time of 60 min vs 40 min for the WT; Fig. 1b), when both strains were cultured together. However, although a maximal 1:9 ratio was measured after 5 h of culture in broth, a 1:15 ratio was calculated after 4 h of adhesion to host cells. Therefore, the deformity of fitness displayed by strain 8013NMA0797 was significantly more pronounced on cells than in broth (Fig. 1). These data demonstrated that an efficient meningococcus–host cells interaction was dependent on the fully functional NMA0797/0798 TCS. Thus, efficient colonization of N. meningitidis relies on the ability of the sensor to perceive an environmental signal during the early step of adhesion.

The expression of the NMA0797/0798 TCS is cell-contact regulated

We investigated whether the expression of the TCS was regulated upon contact with host cells. The level of transcription of genes NMA0797 and NMA0798 was measured by quantitative RT-PCR in WT strain 8013 grown in infection medium and from adherent bacteria harvested after 1 h and 4 h of adhesion to HUVECs (Fig. 2). A modest but significant 1.5–1.8-fold increase ($P<0.01$) of expression of the NMA0797 and NMA0798 genes was measured after 1 h of adhesion to host cells, whereas after 4 h of adhesion the level of transcription of both genes returned to the non-induced state (Fig. 2). These data
indicate that the NMA0797/0798 system is transiently expressed upon initial contact with host cells.

The NMA0797/0798 TCS is involved in the regulation of the expression of the host cell-contact regulated REP2 regulon

Since the expression of the TCS is slightly upregulated upon initial contact with host cells, we investigated whether this TCS could be involved in the regulation of the REP2 regulon, whose expression is also induced upon initial contact with host cells (Morelle et al., 2003, 2005). The level of transcription of a subset of REP2 genes, i.e. pilC1, xseB and parC, was measured (Fig. 3). In WT strain, a 3.2-, 2.4- and 4.6-fold increased level of transcription was respectively measured for the pilC1, xseB and parC genes (P<0.01; Fig. 3a) after 1 h of adhesion to host cells, whereas the expression of the three genes was non-induced after 4 h of adhesion. This transient induction was consistent with data from a previous report (Morelle et al., 2003). In contrast, strain 8013NMA0797 did not show any transient induction of the expression of the three genes investigated (Fig. 3b). This suggests that the NMA0797/0798 TCS may be involved in the control of the expression of the cell-contact-regulated REP2 regulon upon early contact with host cells.

The regulator of the NMA0797/0798 TCS interacts with the REP2 sequence

The above data suggest that the expression of the genes of the REP2 regulon is controlled directly or indirectly by the NMA0797/0798 TCS. Considering that the REP2 repeats have been shown to be the location of the promoter region of the genes of this regulon (Morelle et al., 2003, 2005), we investigated the putative interaction of the regulatory NMA0798 protein with the REP2 sequence. The neisserial NMA0798 protein was overexpressed in E. coli, purified and used in EMSAs (Fig. 4).

The promoter of the NMA0797/0798 TCS has previously been reported to bind the NMA0798 protein (Tzeng et al., 2006). As expected, following incubation of the NMA0798 protein with the NMA0798 promoter probe, we detected a shift in the DNA probe, in a dose-dependent manner (Fig. 4a). We then tested whether NMA0798 protein bound to the REP2 sequence. Since the REP2 sequence was

Fig. 2. Cell-contact regulation of the transcription of the NMA0797/798 TCS in N. meningitidis. The transcriptional level of each gene during early (1 h) and late (4 h) adhesion was measured by real-time quantitative RT-PCR analysis from strain 8013. A chromosomal kanamycin resistance aphA3 cassette was used as reference. For each gene, the induction ratio compares the transcription of the gene in cell-associated bacteria with that of bacteria grown in the absence of human cells (IM, infection medium alone). Data represent means ± SEM, resulting from at least six independent measurements carried out on three different batches of RNA.

Fig. 3. Cell-contact regulation of the transcription of three REP2-associated genes in N. meningitidis. The transcriptional level of each gene during early (1 h) and late (4 h) adhesion was measured by real-time quantitative RT-PCR analysis from strains 8013 (a) and 8013NMA0797 (b). A chromosomal kanamycin resistance aphA3 cassette was used as reference. For each gene, the induction ratio compares the transcription of the gene in cell-associated bacteria with that of bacteria grown in the absence of human cells (IM, infection medium alone). Data represent means ± SEM, resulting from at least six independent measurements carried out on three different batches of RNA.
previously shown to display sequence polymorphism (Morelle et al., 2003), three REP2 sequences were included in the assay. EMSAs showed that migration of the three probes was retarded when incubated with the NMA0798 protein (Fig. 4b–d). No retardation was observed when the NMA0798 protein was incubated with an internal fragment of the coding region of the parE gene used as a negative control (Fig. 4e). These findings provided evidence that the NMA0798 regulatory protein binds to both its own promoter, as usually described for classical TCSs, and the REP2 sequences.

To identify the precise location of NMA0798 binding sites in the REP2 sequence, DNase I protection assays were performed (Fig. 5). Two areas of protection were observed on addition of increasing concentrations of the NMA0798 protein (Fig. 5a, b), one region on the coding strand (Fig. 5a) and one region on the non-coding strand (Fig. 5b). In conclusion, two distinct NMA0798 binding sites were identified in the REP2 sequence, located on both sides of the transcription start site of the xseB gene (Fig. 5c).

**DISCUSSION**

In this work we explored the molecular mechanism the meningococcus has evolved to adapt to growth on host cells, through the investigation of the only meningococcal TCS previously described as a functional system. Adhesion assays revealed that the sensor kinase of the NMA0797/0798 TCS is involved in the adaptation to growth on host cells. A mutation in the gene encoding the sensor results in a defect in the perception of an external signal occurring during the colonization process, and in the prevention of the TCS activation. Thus, the sensor is involved in the perception of environmental variations that take place during host cell colonization.

The TCS participates in a regulation pathway resulting in the transcriptional control of a group of genes that are coordinately and transiently upregulated upon contact with host cells. Since the expression of these genes, i.e. the REP2 regulon, is induced when the bacteria interact with host cells, the encoded functions are likely to be important for the adaptation process. We hypothesize that during the early adhesion step a signal is sensed by the NMA0797/0798 TCS, which results in the activation of the TCS, in its auto-induction, and in the induction of the NMA0797/0798 regulon, which includes the REP2 genes.

The NMA0797/0798 TCS shares homology with several TCSs present in *E. coli* and *Salmonella enterica*: OmpR/EnvZ, PhoP/PhoQ and CpxR/CpxA. Newcombe et al. (2005) renamed the NMA0797/0798 TCS PhoP/PhoQ based on similar phenotypes of the two TCSs in response to Mg$^{2+}$. However, the transcriptomic analysis that the authors performed did not identify any gene involved in the transport of Mg$^{2+}$ as being transcriptionally altered in a NMA0797 background. In contrast, in the same study the gene whose expression was the most strongly repressed was homologous to *cpxP* (20% identity at the amino acid level), which is one of the most strongly induced members of the CpxR/CpxA regulon (DiGiuseppe & Silhavy, 2003). The *cpxP* gene was also reported to be regulated by
NMA0798 in the transcriptomic analysis carried out by Tzeng et al. (2008). Together with the fact that the CpxR/CpxA pathway plays a key role in the regulation of adhesion-induced gene expression (Otto & Silhavy, 2002), these data suggest that the NMA0797/0798 TCS could combine the function of the different TCSs that it shares homologies with.

In this work we assume that the altered adhesion process and transcriptional patterns of the REP2 genes observed in mutant 8013NMA0797 resulted from the absence of activation of the regulatory NMA0798 protein. In Salmonella Typhimurium, deletion of the phoP gene or constitutive expression of the PhoP regulatory protein both lead to attenuated virulence in mice (Miller & Mekalanos, 1990; Shin et al., 2006). These findings demonstrate that the surge conferred by PhoP’s positive feedback loop, which is dependent on the activation of the sensor PhoQ, is necessary to trigger salmonella’s virulence programme (Shin et al., 2006). This highlights how crucial the perception of the environmental signal is and hence the key role of the sensors that allow the auto-induction of the system. It must be underlined that although many bacteria contain dozens of these signalling pathways, cross-talk between different TCSs at the level of phosphorylation is extremely rare (Laub & Goulian, 2007).

Two independent transcriptomic analyses reported lists of genes whose expression was altered in meningococcal strains inactivated for the NMA0798 gene (Newcombe et al., 2005; Tzeng et al., 2008). Only 29 genes were identified in both studies and among them none of the REP2 genes. The RNAs used in these two studies were isolated from bacteria grown on agar plates and in broth, respectively. We hypothesize that in the absence of host cells, i.e. in the absence of stimulating signal, the expression of the NMA0798 subregulon that comprises genes specifically induced upon contact with host cells remains unchanged.

**Fig. 5.** DNase I protection of the xseB-associated REP2 sequence by NMA0798. The coding (a) or non-coding (b) strand of 32P-end-labelled REP2 sequence fragment between positions -146 and +285 with respect to the xseB transcription start site was incubated with increasing amounts of NMA0798 for 20 min at 30 °C and then subjected to DNase I digestion. The amount of added protein was 0, 600 and 1200 ng. Dideoxy chain termination sequences are shown. Black bars indicate regions protected. (c) Sequence of the xseB-associated REP2 sequence. The protected regions for NMA0798 are shaded. The transcription start site is labelled +1. The translation start codon of the xseB gene is indicated in bold. The core consensus sequence for the NMA0798 binding site previously reported (Tzeng et al., 2008) is underlined.
We showed that the NMA0798 regulatory protein directly interacts with the REP2 sequence and that the NMA0798 protein exhibits a higher affinity for the NMA0798 promoter region compared to the REP2 sequences. The very high concentration of protein used for EMSA studies could be suggestive of concern about the physiological relevance of the binding. Alternatively, differential binding affinity for different operators that correlate with differential gene regulation is a well-known feature of global regulators (Sebastian et al., 2002; Wosten et al., 2006). Besides, a discrepancy was previously reported between the binding affinities of PhoP determined in vivo and in vitro (Miyashiro & Goulian, 2007). The order of promoter activation in vivo was indeed qualitatively different from the order expected from in vitro measurements. This suggests that the observed differences between the in vitro and in vivo data are due to additional factors that affect PhoP binding affinity and are only present in vivo (Miyashiro & Goulian, 2007).

A previous study demonstrated that PhoP overexpression can substitute for PhoQ- and phosphorylation-dependent activation. Either a high concentration of PhoP or activation via phosphorylation stimulates PhoP self-association (Lejona et al., 2004). In addition, high-level expression of PhoP in vivo in the absence of phosphorylation shows the same ordering of promoter activation (Miyashiro & Goulian, 2007). Besides, the auto-phosphorylation of the NMA0797 protein and the subsequent transfer of the phosphoryl group to the NMA0798 regulatory protein has been previously demonstrated (Tzeng et al., 2006). This led us not to investigate the role of phosphorylation in the binding affinity of the regulatory protein NMA0798 for its binding sites.

Tzeng et al. (2008) proposed a consensus sequence for the NMA0798 DNA binding site, i.e. (A/T)(A/T)TGTA(A/G/C)/G. In our study, DNase I footprinting mapping of the NMA0798 binding sites in the REP2 sequence identified sequences similar to the core consensus sequence for the NMA0798 binding site previously reported (Tzeng et al., 2008). The differences in affinity between the NMA0798 promoter and the REP2 sequences for the NMA0798 protein evidenced in EMSAs could be related to differences in sequence from the consensus proposed by Tzeng et al. (2008). Moreover, the two binding sites are located upstream and downstream of the transcription start site in the REP2 sequence. A binding site located downstream of the transcription start site for a transcriptional activator has previously been reported for the regulatory proteins Rns from E. coli (Munson & Scott, 2000), and SlyA from S. enterica (Shi et al., 2004).

The contribution of TCSs to bacterial virulence is poorly known for many pathogens. This is particularly true regarding the nature of the environmental signals sensed by the TCSs, which have been verified experimentally in very few cases (Beier & Gross, 2006). A few studies have involved contact with host cells as a stimulus sensed by TCSs, i.e. in biofilm formation by Pseudomonas aeruginosa (Goodman et al., 2004; Kulasekara et al., 2005) or in staphylococcal adhesion (Liang et al., 2006). Although the stimulus sensed by the NMA0797/0798 TCS still needs to be identified to explain the human host specificity that is a feature of N. meningitidis, this study represents a significant advance in our understanding of how the ability of the meningococcus to colonize human cells is regulated.

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