Neisseria meningitidis Lactate Permease Is Required for Nasopharyngeal Colonization

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Neisseria meningitidis is a human specific pathogen that is part of the normal nasopharyngeal flora. Little is known about the metabolic constraints on survival of the meningococcus during colonization of the upper airways. Here we show that glucose and lactate, both carbon energy sources for meningococcal growth, are present in millimolar concentrations within nasopharyngeal tissue. We used a mutant defective for the uptake of lactate (C311Δ*lctP*) to investigate the contribution of this energy source during colonization. Explants of nasopharyngeal tissue were inoculated with the wild-type strain (C311) and C311Δ*lctP*; the mutant was recovered at significantly lower levels ($P = 0.01$) than C311 18 h later. This defect was not due to changes in the expression of adhesins or initial adhesion in C311Δ*lctP* to epithelial cells. Instead, lactate appears to be important energy source for the bacterium during colonization and is necessary for growth of the bacterium in nasopharyngeal tissue. Studies with other strains defective for the uptake of specific nutrients should provide valuable information about the environment in which *N. meningitidis* persists during carriage.

Neisseria meningitidis is an important cause of septicemia and meningitis (4, 11). However, in the overwhelming majority of instances infection with this bacterium is entirely asymptomatic (2). *N. meningitidis* colonizes the human nasopharynx in up to 40% of individuals (5, 17), and this is the sole reservoir of the bacterium.

Little is known about the metabolic requirements of the bacterium during colonization, even though the ability to acquire key nutrients is essential for the successful infection of a host. *Neisseria* spp. have strict nutritional requirements and can utilize only a limited number of carbon energy sources, including glucose and lactate (7). These are found in combination in many sites in the body, such as in serum, cerebrospinal fluid, and secretions of the upper airways (18), although the levels of lactate and glucose in tissue from the nasopharynx are unknown. We sought to investigate the role of lactate acquisition by *N. meningitidis* during colonization.

The interaction between *N. meningitidis* and human epithelial cells is an early step in colonization, and has been studied extensively. The principle adhesin expressed by the meningococcus is the type 4 pilus (Tfp), (9), while other surface proteins, including as Opas and Opc, contribute to the initial adhesion of strains, especially those lacking pili and capsule (1, 25). However, *N. meningitidis* is also found internal to the epithelial cell layer in the nasopharynx (17), so we used the organ culture model (OCM) to examine the effect of lactate availability on successful colonization by *N. meningitidis*. In the

OCM, explants of nasopharyngeal tissue are maintained with an air interface and can be successfully infected with pathogenic bacteria (13, 15). The OCM has the major advantage that it includes the full repertoire and organization of cell types and extracellular matrix proteins that *N. meningitidis* encounters during colonization.

We have previously identified the lactate permease of *N. meningitidis* (3a). NMB0543 (20) is predicted to encode a protein with 14 transmembrane domains located in the inner bacterial membrane, which is consistent with a role as a permease. A mutant lacking *lctP* was unable to take up lactate and failed to grow with lactate as the sole carbon source, while growth on glucose was equivalent to that of the wild-type strain; complementation restored the ability of the mutant to grow on lactate and its virulence in the infant rat model. Here, we used the *lctP* mutant to further understand the nutritional requirements of *N. meningitidis* during nasopharyngeal colonization, a critical stage in the pathogenesis of meningococcal infection.

MATERIALS AND METHODS

Bacterial strains and growth. *N. meningitidis* strain C311 is a serogroup B strain isolated from a patient with invasive meningococcal infection (24). C311Δ*lctP* carries a deletion in NMB0543 (19) that encodes the *N. meningitidis* lactate permease (3a). The mutation was backcrossed into the parental strain, and multiple colonies were collected to exclude any influence from phase variation and other second site mutations. In C311Δ*pilE*, *pilE* has been insertionally inactivated with the gene encoding resistance to kanamycin. *N. meningitidis* was grown on brain heart infusion medium with 5% Levanthal's supplement. *Escherichia coli* was propagated on Luria-Bertani media. Kanamycin (Kan) was added at 75 and 25 μg ml^{-1} for *N. meningitidis* and *E. coli*, respectively.

Organ culture model. Human nasopharyngeal mucosa explants were derived from adenoids resected from children with noninfective conditions after informed consent from parents or guardians was obtained. The study was approved by the South Sheffield Research Ethics Committee. Tissue was immersed in antibiotic-containing minimal essential medium (MEM; Gibco, Uxbridge, United Kingdom) for 4 h and dissected to produce 3 to 4 mm blocks of mucosa

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(13, 14, 15). The tissue was then transferred to MEM without antibiotics for 1 h and then supported in a 4-cm petri dish with the epithelial surface projecting above a bed of non-nutrient agar. The technique of culturing explants with an air interface was then used (13, 22). The petri dish with the agar-embedded explant was placed inside a 10-cm petri dish containing a reservoir of MEM that was perfused into the agar with a wick. The OCM was incubated in a humidified atmosphere of 5% CO_2 in air at 37°C.

Measurement of meningococcal survival within nasopharyngeal tissue. To measure meningococcal survival within the tissue, 100 μl of phosphate-buffered saline (PBS) containing ca. 1.5×10^8 CFU of *N. meningitidis* was placed on the surface of the explant. After 4 h and 18 h of incubation, explants were washed, weighed, and then homogenized in a modified French press (Constant Systems, Warwick, United Kingdom) at 10 lb/in². Viable bacteria within the homogenized tissue were enumerated by plating onto solid media. Each strain was inoculated into eight OCMs from different individuals.

Measurement of lactate and glucose. Nasopharyngeal tissue ($n = 5$) was lysed by using a cell disruptor as described above and then passed through 0.4-μm pore filter. The amount of lactate and glucose in the lysates was measured on an automated Beckman Synchron LX20 analyzer and adjusted for the volume of tissue. Lactate was assayed by its conversion to pyruvate by lactate dehydrogenase and modification of the NAD to NADH coenzyme (monitored by absorbance at 340 nm). Glucose levels were determined by its metabolism by glucose oxidase in the presence of oxygen. Each sample was loaded into a reaction cup containing a Beckman oxygen electrode (Beckman-Coulter, High Wycombe, United Kingdom). The rate of oxygen consumption was measured, and the glucose concentration within the samples was calculated from the standard curve.

Western analysis. Whole-cell lysates were prepared from overnight cultures of bacteria, and the cell density was adjusted to 10^{10} CFU ml^{-1} and mixed with an equal volume of dissociation buffer (50 mM Tris [pH 6.8], 2% sodium dodecyl sulfate [SDS], 0.1% bromophenol blue, 10% glycerol). Samples were denatured by boiling for 5 min prior to fractionation by SDS–16% polyacrylamide gel electrophoresis before transfer to polyvinylidene difluoride membranes (Immobilon-P; Millipore) for 90 min at 70 V. Membranes were washed in 0.5% milk in PBS and then incubated for 2 h at room temperature with monoclonal antibody SM1 (anti-pilin) (23), B306 (anti-Opc) (26), A222 (anti-Opc) (8), or B33 (anti-Opa) (21) at a 1:10,000 dilution, followed by 1 h of incubation with the secondary antibody (Dako) at the same dilution. Cross-reaction was detected by using the ECL chemiluminescence rapid detection kit (Amersham)

Interactions with epithelial cells. For adhesion to epithelial cells, bacteria were harvested from solid media into PBS, adjusted to the desired concentration, and then added to Chang epithelial cells at a multiplicity of infection of 100:1 (bacteria to epithelial cells). Adhesion to the cells was assayed by standard protocols (27); cells were infected for 3 h and washed three times to remove nonadherent bacteria, and then the adherent bacteria were recovered after lysis of host cells in saponin (0.1%) for 10 min. Assays were carried out in triplicate and on three independent occasions.

Statistical analysis. Data were collected from experiments by using explants from multiple donors. Comparisons between mutant and wild-type bacteria were initially screened by Kruskal-Wallis analysis and, where the null hypothesis was rejected, comparisons between the *lctP* mutant and wild-type were tested by Mann-Whitney U using the SPSS statistical package (version 11) after adjustment for variation in the inocula. Kruskal-Wallis analysis was used to analyze levels of lactate and glucose in explants.

RESULTS

Measurement of carbon sources in nasopharyngeal tissue. To evaluate the carbon sources available to *N. meningitidis* during colonization, levels of glucose and lactate were measured in homogenates of nasopharyngeal tissue. Glucose and lactate were present in all five samples analyzed (Table 1), with levels similar to those present in serum (reference serum levels of 0.6 to 2.4 mmol/liter and 3.9 to 5.8 mmol/liter for lactate and glucose, respectively). The amount of lactate or glucose did not change significantly over time and was not affected by infection either with the wild-type strain or *lctP*-deficient bacteria.

The lactate permease is required for effective colonization of the organ culture model. Next, the ability of C311Δ*lctP* to colonize the OCM was compared to the wild-type strain; both

TABLE 1. Levels of lactate and glucose in nasopharyngeal tissue

Strain	Concn(mM)^a			
	Lactate		Glucose	
	4 h	18 h	4 h	18 h
Uninfected	1.50 (1.10)	1.00 (0.68)	6.84 (2.83)	6.20 (1.60)
C311	2.64 (1.21)	1.00 (1.06)	7.12 (1.05)	6.70 (1.85)
C311Δ*lctP*	1.74 (0.70)	0.64 (0.47)	7.14 (2.78)	5.88 (1.17)

^{a} Results are shown with standard deviations in parentheses. There was no significant difference in concentrations between infected (with either C311 or C311Δ*lctP*) and noninfected tissues or at the two time points (Kruskal-Wallis analysis).

strains were assayed in explants from eight patients. After 18 h of incubation, C311Δ*lctP* was at a significant disadvantage compared to C311, with the mutant colonizing at approximately a tenth of the level of the wild-type bacterium (Fig. 1, $P = 0.01$). The organization of open reading frames around *lctP* indicates that the colonization defect of C311Δ*lctP* could not have arisen due to polar effects but resulted from the loss of the lactate permease.

Loss of the lactate permease does not affect the expression of known adhesins. Strains unable to express Tfp are defective for survival in the OCM (12; R. M. Exley, R. B. Sim, L. Goodwin, Y. Li, E. N. Mowe, R. C. Read, and C. M. Tang, Abstr. 14th International Pathogenic *Neisseria* Conference, p. 275, 2004). To determine whether the diminished colonization capacity of the *lctP* mutant resulted from alterations in adhesins at the bacterial surface, the expression of Tfp, Opa, and Opc was examined. Western analysis was performed on whole-cell lysates of the wild-type strain, C311Δ*lctP*, and a *pilE* mutant. Loss of *lctP* was not associated with a detectable change in the production of Tfp, Opa, and Opc by *N. meningitidis* (Fig. 2).

Bacteria lacking *lctP* have enhanced adherence with isolated epithelial cells. To examine whether *lctP* might contribute to colonization during the interaction of the bacterium with epithelial cells, Chang conjunctival cells were infected with C311 and C311Δ*lctP*, and the adhesion of the strains was compared. As a negative control, cells were infected with bacteria unable
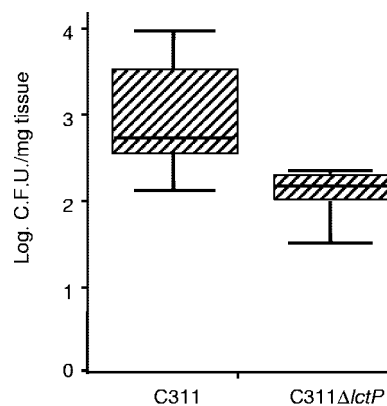

FIG. 1. The lactate permease is required for efficient nasopharyngeal colonization. Nasopharyngeal tissue explants (eight for each strain) received 1.5×10^8 CFU of bacteria, followed by incubation for 18 h. The number of recovered bacteria is shown (the 25th and 75th percentiles are indicated by the shaded box). The wild type is recovered in 10-fold-greater levels than the mutant (Mann-Whitney, $P = 0.01$).
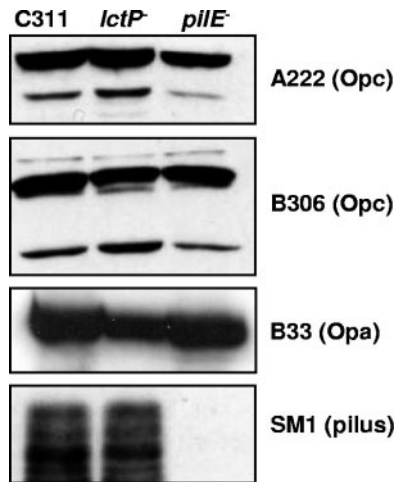
FIG. 2. Expression of adhesins by the *lctP* mutant. Whole-cell extracts were obtained from the strains, separated by SDS–16% polyacrylamide gel electrophoresis, and transferred to membranes. The reactivities of extracts with the monoclonal antibodies SM1 (α-pili), B306 (α-Opc), A222 (α-Opc), and B33 (α-Opa) are shown.

FIG. 4. The lactate permease is not necessary for the early stage of nasopharyngeal colonization. Bacteria were harvested from nasopharyngeal tissue explants 4 h after receiving 1.5×10^8 CFU of *N. meningitidis*. The numbers of recovered bacteria are shown (the 25th and 75th percentiles are indicated by the shaded box; Mann-Whitney, $P > 0.05$).

to express Tfp (C311Δ*pilE*), the principle meningococcal adhesin. As expected, the *pilE* mutant strain adhered to Chang cells at reduced rates compared to C311. The *lctP* mutant did not have a defect for host cell adhesion, but instead exhibited enhanced association with epithelial cells compared to the wild-type strain (Fig. 3). Infected epithelial cells were also examined by immunofluorescence microscopy; there was no evidence that clumping of the *lctP* mutant that could account for its enhanced recovery in the adhesion assays (not shown).

The *lctP* mutant does not exhibit an early colonization defect. Although the *lctP* mutant was not defective during interactions with isolated epithelial cells, these cells may not be fully representative of the cells to which *N. meningitidis* adheres in the OCM during the initial stage of colonization. Therefore, to provide further insights into the role of LctP on meningococcal colonization, OCMs were infected with the wild-type strain or

C311Δ*lctP*, and bacteria were recovered only 4 h later. At this early time point, there was no detectable difference in the level of C311Δ*lctP* and C311 recovered from the tissue (Fig. 4).

DISCUSSION

Colonization of the nasopharynx by *N. meningitidis* is a complex series of events that requires efficient scavenging of vital nutrients from human tissues, evasion of innate immune killing, and tropism to specific host cells. Here, we used a mutant defective for the acquisition of lactate to demonstrate that *N. meningitidis* must acquire this carbon source to colonize nasopharyngeal tissue successfully. Rather than only studying the interaction of the bacterium with isolated epithelial cells, we utilized the OCM, which provides the broad range of cellular environments and metabolic constraints encountered by the bacterium in the upper airway.

We found that the levels of both glucose and lactate in nasopharyngeal tissue are comparable to those in serum. Both are known to be effective carbon sources for *N. meningitidis*. However, levels of glucose and lactate did not alter appreciably during infection with the wild-type or *lctP* mutant bacteria. This may be because *N. meningitidis* utilizes only a fraction of the total carbon sources available in the upper airway. Alternatively, there might be mechanisms in the tissue that replenish the levels of glucose and lactate.

There are several potential explanations for the colonization defect of the *lctP* mutant, including (i) enhanced susceptibility to innate immune effectors, (ii) failure to interact with certain host cells, and (iii) inability to acquire nutrients for growth. We have recently shown that the *N. meningitidis lctP* mutant is susceptible to complement-mediated lysis, a finding consistent with the effect of adding lactate to the gonococcus (10, 18). However, there is little or no proinflammatory response to *N. meningitidis* in the upper airway, with colonization being asymptomatic in the overwhelming majority of cases. In the
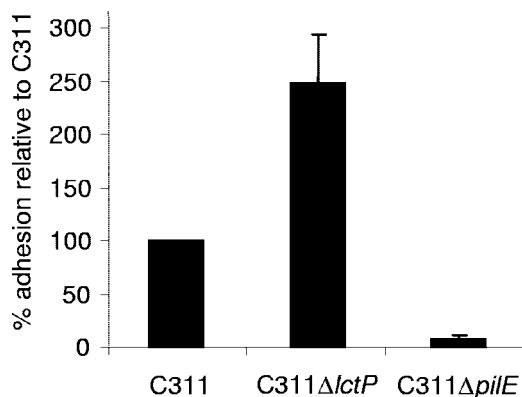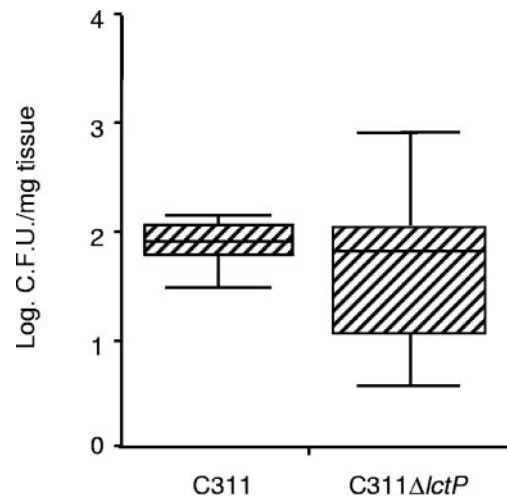

FIG. 3. The lactate permease is not defective for adhesion to epithelial cells. Bacteria were incubated with Chang cells at a multiplicity of infection of 100:1 for 3 h, and adherent bacteria were enumerated by plating. C311Δ*lctP* associates with epithelial cells at higher levels than the wild-type strain (Student *t* test, $P < 0.01$; error bars show the standard error of the mean).

absence of active inflammation, complement levels at the mucosal surface are negligible (6), and therefore serum-resistant strains would not have a selective advantage for survival in the OCM. Alternatively, the mutant might be selectively eliminated by other components of the innate immune response such as antimicrobial peptides or during interactions with phagocytic cells. To colonize the nasopharynx effectively, *N. meningitidis* needs to express adhesins to bind host cells in the upper airways. We found that the expression of Tfp and other adhesins was not affected in the *lctP* mutant, which actually exhibited enhanced association with epithelial cells. The likeliest reason for this increased adhesion is that the lipopolysaccharide of wild-type bacteria is more extensively sialylated than in an *lctP* mutant (Exley et al., in press), which may inhibit bacterial association with epithelial cells (3).

We propose that the reason for the attenuation of C311Δ*lctP* is a failure to replicate at wild-type levels through an inability to utilize available lactate for growth. This is supported by our finding that the lactate permease mutant colonized at wild-type levels early on after infection (at 4 h). If the mutant was excluded either through enhanced susceptibility to complement or failure to adhere to epithelial cells, C311Δ*lctP* should be at a competitive disadvantage soon after infection. Between 4 and 18 h, the number of wild-type bacteria increased in the OCMs by an order of magnitude, whereas the level of infection with C311Δ*lctP* remained almost constant. We have shown previously that lactate is utilized more rapidly than glucose in the cerebrospinal fluid and that additional lactate enhances the growth rate of the bacterium in the media containing glucose. Therefore, the inability to utilize lactate will affect the growth of the *lctP* mutant in an environment with mixed carbon energy sources such as the nasopharynx (Table 1).

Meningococcal infection and disease show a pronounced seasonal variation, with the highest rates observed during winter. There is also an association between influenza and invasive meningococcal infection (16, 28). This could be explained by viral infection increasing the presence of activated phagocytic cells at the epithelial surface, increasing local lactate concentrations. The lactate could then be used as a substrate for meningococcal growth and promote resistance to complement-mediated lysis, favoring survival of the bacterium in the bloodstream, and increasing the likelihood that infection will lead to disease.

Colonization of the nasopharynx is a prerequisite for meningococcal disease, and therefore detailed knowledge of the growth requirements and metabolic state of the bacterium in this location will have important implications on the design novel interventions for protecting individuals against this pathogen. The approach of evaluating mutants with specific defects in the acquisition of key nutrients in the OCM could be used to further understand the role of other aspects of microbial physiology during colonization and provide insights into the microenvironment where *N. meningitidis* survives and replicates during this critical stage in pathogenesis.

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