Available carbon source influences the resistance of *Neisseria meningitidis* against complement

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Neisseria meningitidis **is an important cause of septicaemia and meningitis. To cause disease, the bacterium must acquire essential nutrients for replication in the systemic circulation, while avoiding exclusion by host innate immunity. Here we show that the utilization of carbon sources by *N. meningitidis* determines its ability to withstand complement-mediated lysis, through the intimate relationship between metabolism and virulence in the bacterium. The gene encoding the lactate permease, *lctP*, was identified and disrupted. The *lctP* mutant had a reduced growth rate in cerebrospinal fluid compared with the wild type, and was attenuated during bloodstream infection through loss of resistance against complement-mediated killing. The link between lactate and complement was demonstrated by the restoration of virulence of the *lctP* mutant in complement ($C3^{-/-}$)-deficient animals. The underlying mechanism for attenuation is mediated through the sialic acid biosynthesis pathway, which is directly connected to central carbon metabolism. The findings highlight the intimate relationship between bacterial physiology and resistance to innate immune killing in the meningococcus.**

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Abbreviations used: C.I., competitive index; CSF, cerebrospinal fluid; PEP, phosphoenol pyruvate.

Neisseria meningitidis is a commensal of the human nasopharynx. However, the bacterium occasionally enters the bloodstream where it causes a fulminant septicaemic illness with a 10% mortality rate, or spreads to the cerebrospinal fluid (CSF) leading to meningitis (1). Therefore, all the pathological consequences of meningococcal infection depend on the bacterium's ability to acquire nutrients for replication within the systemic circulation while at the same time avoiding exclusion by host innate immune mechanisms. Apart from iron acquisition, little is known about the aspects of microbial physiology that are critical for survival of *N. meningitidis* in vivo. Of the innate immune effectors, the complement system is critical in protection against meningococcal infection, evident from the marked susceptibility of individuals with inherited or acquired complement deficiencies to meningococcal disease (1).

Pathogenic *Neisseria* spp. are fastidious organisms that use a limited number of carbon energy sources including glucose and lactate (2). Both are present within the upper airway, systemic circulation, and CSF (3, 4), niches where *N. meningitidis* resides (1). In *Neisseria gonorrhoeae*, which causes a sexually transmitted disease, addition of lactate at concentrations found in vivo to media containing glucose stimulates growth, and synthesis of protein and LPS (3). This stimulation of metabolism results from lactate being used solely for the production of additional energy via acetyl-CoA and not for gluconeogenesis, which occurs when lactate is the sole carbon source (3). Furthermore, lactate increases the serum resistance of *N. gonorrhoeae* by enhancing sialylation of LPS through greater production of the substrate and the LPS sialyl transferase (4, 5). However, nothing is known about the effect of lactate on

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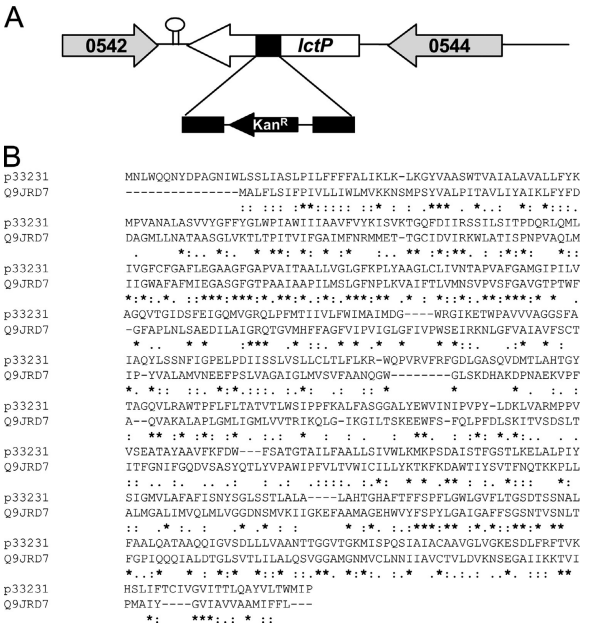

Figure 1. NMB0543 is a homologue of the *E. coli* lactate permease. (A) Organization of predicted open reading frames around NMB0543 and location of the transposon insertion in the mutant, MC58Δ0543, and a putative transcription stop site is marked by ⚲. (B) Alignment of the predicted amino acid sequence of NMB0543 (accession no. Q9JRD7; EMBL) with LctP from *E. coli* (accession no. P33231; SwissProt). Identical residues are shown with an asterisk, conserved substitutions are shown with two dots, and semiconserved substitutions are shown with one dot.

the biology of meningococcal infections. Here we describe the identification of the *N. meningitidis* lactate permease and the importance of lactate for survival of the bacterium within the host.

RESULTS

NMB0543 encodes the *N. meningitidis* lactate permease

The predicted protein encoded by NMB0543 shares 21% amino acid identity and 58% similarity with a lactate permease from *Escherichia coli*, LctP (6; Fig. 1), and is expected to have 14 trans-membrane domains (www.tigr.org), consistent with an integral membrane permease. To investigate the influence of NMB0543 on lactate utilization by *N. meningitidis*, the growth of MC58Δ0543 in single carbon sources was compared against the wild-type strain, MC58 (Fig. 2 A). Whereas the growth of the NMB0543 mutant was indistinguishable from the wild type in 10 mM glucose, growth of the mutant was defective in media with lactate; the initial slight growth of MC58Δ0543 in lactate probably reflects usage of amino acids such as proline as a limited energy source (7).

To examine the role of NMB0543 in lactate metabolism, uptake studies were performed. The accumulation of ^{14}C lactate by MC58 was rapid (rising to 8.1 nmol/mg of protein in 1 min), whereas uptake by MC58Δ0543 was insignificant (Fig. 2 B), demonstrating that NMB0543 is required for assimilation of lactate by *N. meningitidis*. The orientation of the open reading frames around NMB0543 (Fig. 1 A) indicates that polar effects cannot be responsible for the phenotype of the mutant. Furthermore, growth of MC58Δ0543 in lactate was restored by complementation with a single copy of the gene on the chromosome (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20041548/DC1). Therefore NMB0543 was designated *lctP*, consistent with the *E. coli* ortholog (6).

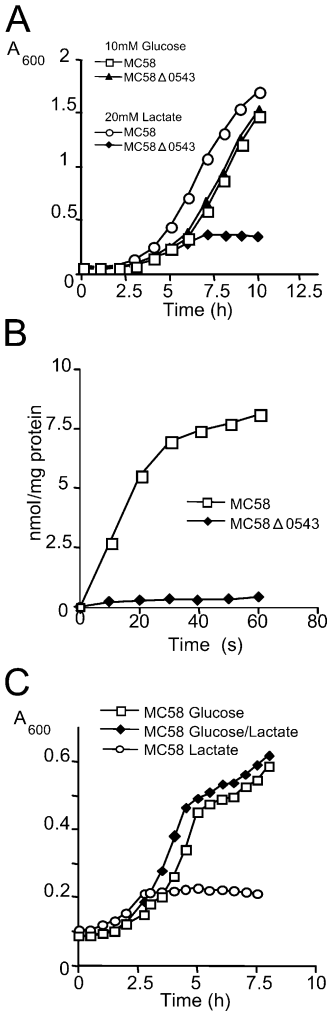

Figure 2. Identification of the *N. meningitidis* lactate permease. (A) Growth of wild-type *N. meningitidis* (MC58), and MC58Δ0543 in the presence of single carbon energy sources as indicated in the key. (B) Uptake of ^{14}C L-lactate by MC58 and MC58Δ0543. (C) Growth of MC58 in defined media with single carbon sources (11 mM glucose or 2 mM lactate), and with both (10 mM glucose or 2 mM lactate).

Lactate stimulation of *N. meningitidis* metabolism

As addition of lactate to medium containing glucose stimulates *N. gonorrhoeae* growth (3, 4, 8), experiments were undertaken to determine whether this also applies to *N. meningitidis*. In the presence of physiological levels of lactate (2

mM) and glucose (10 mM), MC58 consistently emerged from lag phase earlier (on five independent occasions; Fig. S2, available at http://www.jem.org/cgi/content/full/jem.20041548/DC1; $P < 0.017$) and grew more rapidly in early log phase than in glucose only (11 mM to provide the equivalent carbon atoms, Fig. 2 C and Fig. S2); as expected, this stimulation of growth was not evident for MC58Δ*lctP* (unpublished data). Growth in lactate (2 mM) alone was not sustained, presumably as this carbon source was exhausted. However in 20 mM lactate, MC58 emerged from lag phase earlier and had an increased growth rate in log phase compared with when grown in glucose alone (Fig. 2 A).

Lactate enhances the growth of *N. meningitidis* in CSF

A characteristic manifestation of meningococcal disease is spread to and growth in CSF, resulting in meningitis. Therefore, we examined the behavior of *N. meningitidis* wild-type bacteria and the lactate permease mutant during growth in CSF. MC58 grew at a faster rate than MC58Δ*lctP* (Fig. 3 A and Fig. S2), highlighted by the changes in optical density (Fig. 3 B). Furthermore, MC58 reached a larger final biomass than MC58Δ*lctP*. ^{1}H-NMR analysis demonstrated that *N. meningitidis* uses both lactate and glucose in CSF, but that lactate was metabolized more rapidly (Fig. 4, A and B). The lactate concentration fell from 2.2 mM initially to 0.8 mM after 2 h growth, and was absent after 4 h (Fig. 4 C). In contrast, glucose levels reduced gradually, falling from 4.8 mM to 2.4 mM after 8 h. As expected MC58Δ*lctP* was unable to use lactate, but did use glucose, with concentrations reducing from 4.8 mM to 1.6 mM after 8 h growth (Fig. 4 B). Both strains excreted acetate during growth, with 1.5 mM and 0.4 mM acetate being accumulated for MC58 and MC58Δ*lctP*, respectively, indicating activity of phospho-transacetylase acetate kinase pathway (2).

lctP is required for bacteraemia and avoidance of complement-mediated killing

The virulence of MC58Δ*lctP* was examined in the infant rat model of bacteraemia. This model has been used to study the

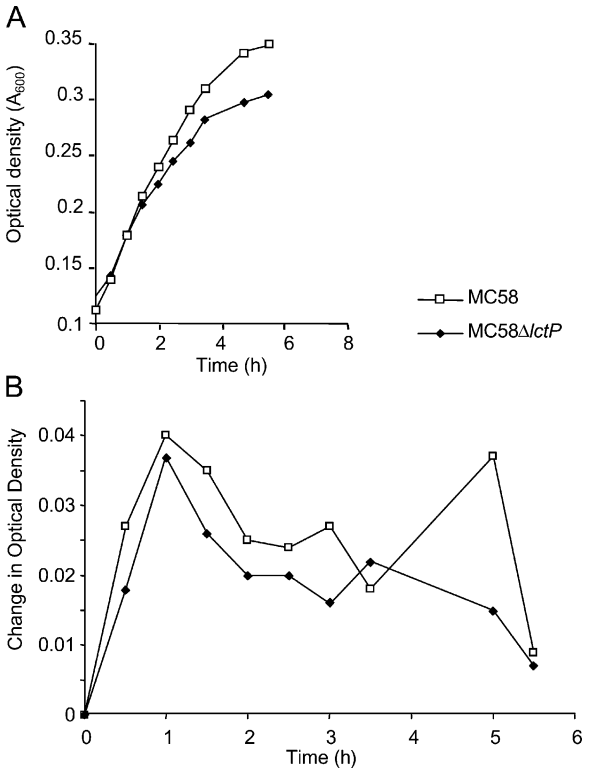

Figure 3. Growth of *N. meningitidis* in CSF. (A) Growth of wild-type *N. meningitidis* MC58 (open squares) and MC58Δ*lctP* (solid diamonds) in CSF. (B) The half hourly changes in optical density of cultures (A_{600}) are shown. The difference in the growth of the strains is statistically significant (Student's *t* test comparing changes in OD of the strains, $P = 0.025$).

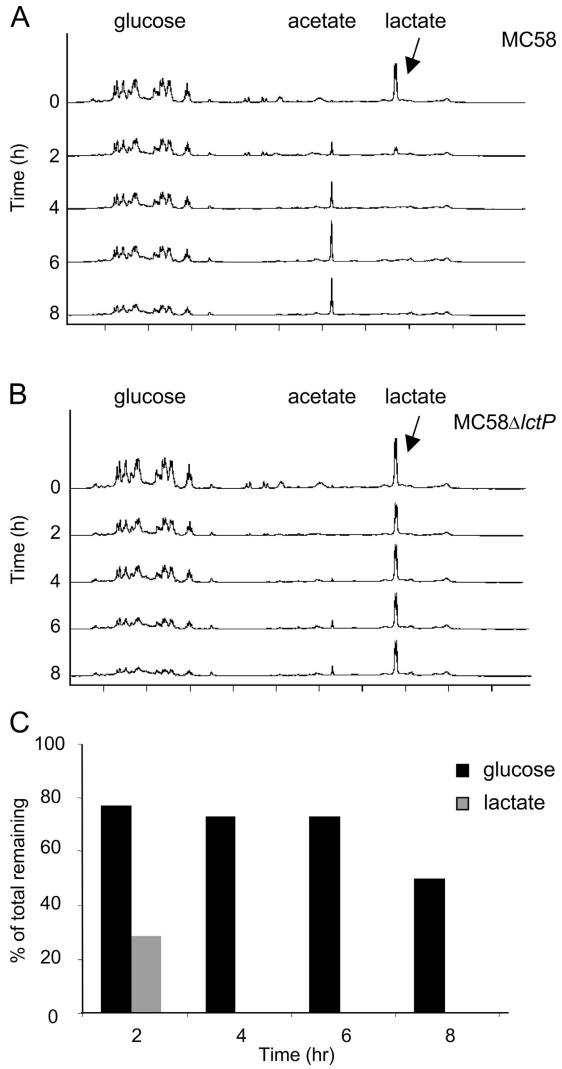

Figure 4. Utilization of carbon sources by *N. meningitidis* in CSF. (A) MC58 and (B) MC58Δ*lctP* were grown in CSF and the effect on carbon content was analyzed by ^{1}H-NMR. The peaks representing individual carbon sources are labeled. The time (in hours) when samples were taken during growth is indicated. (C) The percentage of lactate and glucose remaining in CSF during growth of MC58.

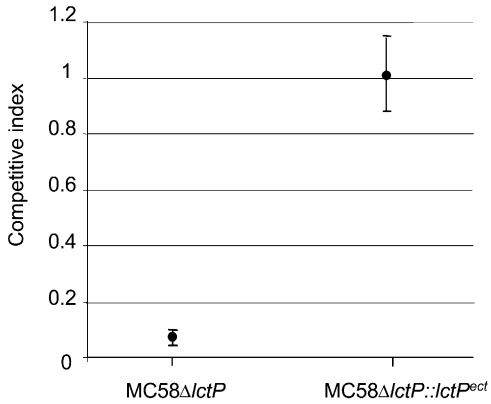

Figure 5. The lactate permease mutant is attenuated during bloodstream infection. Competitive indices (C.I.) of the lactate permease mutant (MC58Δ*lctP*) and the complemented strain (MC58Δ*lctP*::*lctP*^{ect}) in the infant rat model. Error bars show the standard deviation.

role of meningococcal genes during systemic infection, a critical step in pathogenesis. The competitive index (C.I.) of MC58Δ*lctP* (0.108, $P < 0.01$; Fig. 5) demonstrates that this strain is markedly attenuated in its ability to cause sustained bacteraemia compared against the wild-type strain. The virulence of the mutant was restored in the complemented strain, MC58Δ*lctP*::*lctP*^{ect} (Fig. 5).

To proliferate in the systemic circulation *N. meningitidis* must resist innate immune killing mechanisms. Therefore, we examined the sensitivity of MC58Δ*lctP* to complement. The survival of MC58Δ*lctP* in normal human sera was only 16% that of the wild type, whereas there was no significant difference in the survival of strains in heat-treated serum (Fig. 6 A and Fig. S3, available at http://www.jem.org/cgi/content/full/jem.20041548/DC1). Complementation of MC58Δ*lctP* with the wild-type allele of the gene demonstrated that loss of LctP was solely responsible for the serum sensitivity of MC58Δ*lctP* (Fig. 6 B).

All pathways of complement activation lead to cleavage of C3 to C3a and C3b, which binds to cell surfaces leading to opsonization and/or assembly of the membrane attack complex and bacteriolysis. We therefore compared the amount of C3 binding to wild-type *N. meningitidis* and the lactate permease mutant. There was a significantly increased C3 binding to the lactate permease mutant ($P < 0.001$; Fig.

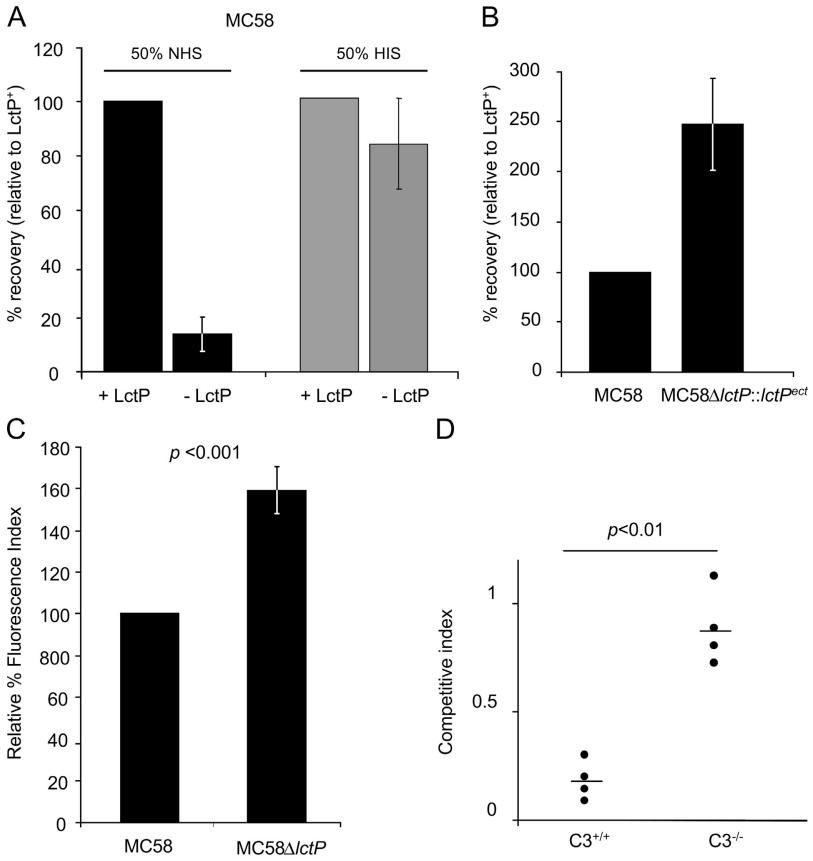

Figure 6. LctP is required for resistance against complement-mediated lysis. (A) Relative resistance of MC58Δ*lctP* to complement-mediated lysis compared with MC58 in 50% normal human serum (NHS) or heat inactivated serum (HIS). The error bars show the standard error of the mean. (B) Complementation of MC58Δ*lctP* restores resistance against serum-mediated killing. (C) FACS analysis demonstrates that C3 deposition on MC58Δ*lctP* is higher than MC58. (D), The competitive index (C.I.) of wild type and lactate permease strain in wild-type and C3$^{-/-}$ mice. The virulence of MC58Δ*lctP* is restored in complement deficient animals.

6 C and Fig. S4, available at http://www.jem.org/cgi/content/full/jem.20041548/DC1), consistent with its enhanced sensitivity to complement-mediated lysis.

The *lctP* mutant is attenuated through its inability to withstand complement-mediated killing

The attenuation of the *lctP* mutant during bacteraemic infection could either have been due to a defect in growth (as in the CSF; Fig. 3 A), or increased susceptibility to complement-mediated lysis (as in the serum killing assays, Fig. 6 A). To differentiate between these possibilities, the virulence of the *lctP* mutant was determined in animals lacking an intact complement system. We measured the C.I. of MC58Δ*lctP* in congenic $C3^{-/-}$ and $C3^{+/+}$ mice 20 h after infection, the time of maximum bacteraemia. The results (Fig. 6 D) demonstrate that the virulence of the *lctP* mutant is restored to wild-type levels in the absence of C3. Thus, during bacteraemia, *lctP* influences the behavior of *N. meningitidis* through its effect in preventing complement-mediated killing and not bacterial growth.

Sensitivity of the *lctP* mutant to complement remains in the absence of capsule

A major mediator of complement resistance in serogroup B *N. meningitidis* is the polysaccharide capsule (9, 10). Therefore the effect of loss of *lctP* was evaluated in a strain lacking *siaD*, which encodes the polysialyltransferase required for capsule biogenesis (11). Loss of encapsulation resulted in a marked increase in susceptibility to complement-mediated killing, therefore assays with unencapsulated strains were performed with lower concentrations of serum (3%) than encapsulated strains (50%); introduction of the wild-type allele of *siaD* restored resistance to complement to the level of MC58 (unpublished data). Nevertheless, the difference in complement sensitivity of a strain with (MC58Δ*siaD*) or without *lctP* (MC58Δ*siaD*Δ*lctP*) was still evident in a capsule negative background (Fig. 7 and Fig. S5, available at http://www.jem.org/cgi/content/full/jem.20041548/DC1), demonstrating that *lctP* influences serum resistance even in the absence of the polysaccharide capsule.

LctP contributes to LPS sialylation

Sialylation of LPS contributes to serum resistance of pathogenic *Neisseria* spp. (3–5). We examined the effect of lactate availability in unencapsulated strains that are unable to sialylate LPS (MC58Δ*siaD*Δ*lst* and MC58Δ*siaD*Δ*lst*Δ*lctP*). Loss of LctP had no significant impact on resistance to complement-mediated killing in strains that are unable to sialylate their LPS (Fig. 8 A and Fig. S6, available at http://www.jem.org/cgi/content/full/jem.20041548/DC1) indicating that *lctP* contributes to resistance to complement killing through LPS sialylation. Therefore, we examined the extent of LPS sialylation of strains. Whole cell extracts from MC58, MC58Δ*lst* and MC58Δ*lctP* were analyzed by SDS-PAGE and immunoblotting using an antibody against LPS (Fig. 8 B) and a mAb, 3F11 (Fig. 8 C), which recognizes the terminal Gal1-4GlcNAc epitope in the lacto-*N*-neotetraose moiety of LPS and therefore detects unsialylated LPS (12). The binding of the antibody against LPS was similar for all strains, as was the binding of 3F11 to neuraminidase-treated samples (Fig. 8 D). However, there was increased binding of 3F11 to the *lst* and *lctP* mutant compared with MC58, demonstrating that the LPS of MC58Δ*lctP* is less sialylated than in the wild-type strain, consistent with its increased susceptibility to complement.

Available lactate affects resistance to complement-mediated lysis through a link between the sialic acid biosynthesis pathway and intermediary metabolism

We next investigated the basis by which lack of lactate results in the increased sensitivity to complement. In *N. menin-*

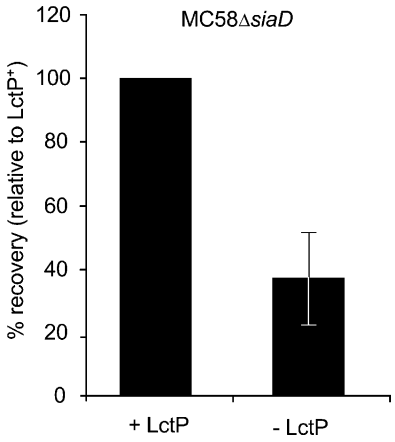

Figure 7. LctP contributes to complement sensitivity in the absence of capsule. Relative resistance to complement of strains lacking capsule with (strain MC58Δ*siaD*, shown by +LctP) or without (MC58Δ*siaD*Δ*lctP*, indicated with −LctP) the lactate permease. Assays were performed in the presence of human serum (3%, error bars indicate the standard error of the mean).

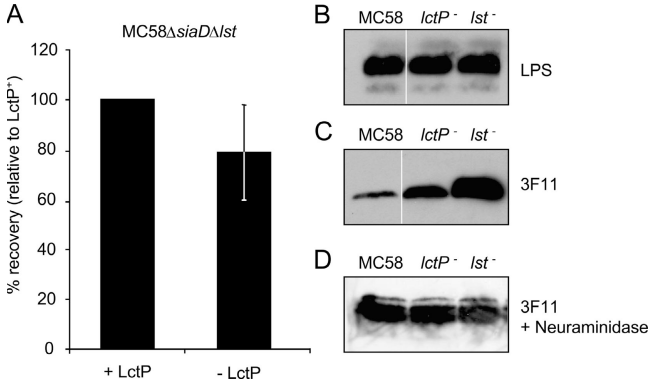

Figure 8. LctP influences LPS sialylation. (A) Relative resistance to complement of strains lacking capsule and unable to sialylate their LPS with (MC58Δ*siaD*Δ*lst*) or without (MC58Δ*siaD*Δ*lst*Δ*lctP*) the ability to utilize lactate. Error bars indicate the standard error of the mean. Western blot of cell lysates of strains (shown above each lane) probed with mAbs against (B) total LPS (L3,7,9), and a mAb against unsialylated LPS (3F11) before (C) or after (D) neuraminidase treatment. White lines indicate that intervening lanes have been spliced out.

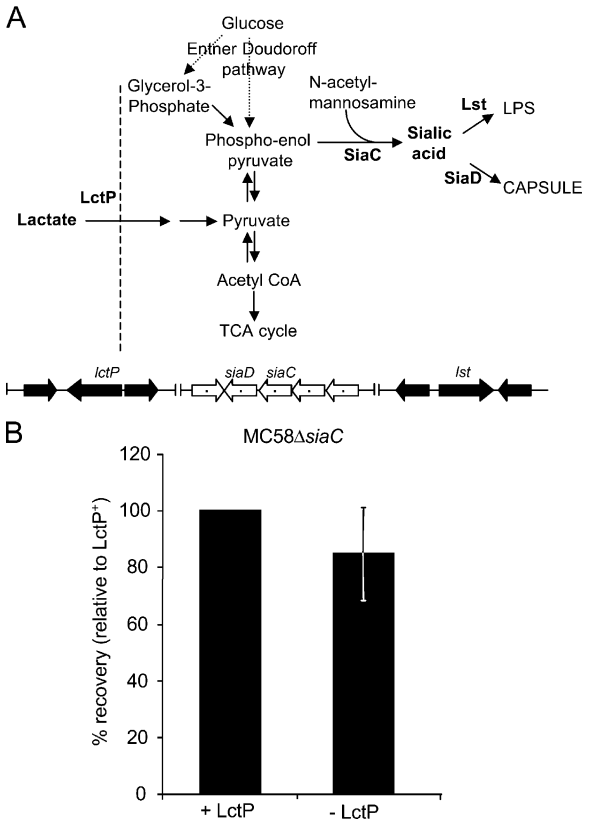

Figure 9. The metabolic fate of lactate in *N. meningitidis*. (A) Intermediary metabolism in MC58 with enzymes encoded on the horizontally acquired, capsule biosynthesis locus shown in dotted arrows. (B) Relative resistance to complement of MC58Δ*siaC* and MC58Δ*siaC*Δ*lctP*. Assays were performed in 3% human serum, and the error bar shows the standard error of the mean.

gitidis, sialic acid is predicted to be synthesized from *N*-acetyl mannosamine and phosphoenol pyruvate (PEP; Fig. 9 A), a reaction catalyzed by the enzyme SiaC, encoded on the horizontally acquired capsule biosynthesis locus (13). PEP can be formed by a circuitous route from glucose via the Entner Doudoroff pathway (2). In contrast, PEP is generated directly from lactate after conversion to pyruvate (Fig. 9 A), providing a potential connection between lactate availability and resistance against complement-mediated lysis. Therefore, the *lctP* mutation was introduced into a strain lacking *siaC*. There was no significant difference in complement susceptibility between strains with and without LctP in a *siaC*-negative background (Fig. 9 B and Fig. S7, available at http://www.jem.org/cgi/content/full/jem.20041548/DC1), consistent with the sialic acid biosynthesis pathway acting as a link between lactate availability, and resistance against complement-mediated lysis.

DISCUSSION

To produce disease, *N. meningitidis* and *N. gonorrhoeae* must replicate in environments that contain mixtures of lactate and glucose. This report is the first description of a mutant of *Neisseria* that is unable to assimilate or use lactate. Attempts to investigate lactate metabolism through inactivation of lactate dehydrogenase would be difficult given the multiple isoforms of this enzyme in *N. meningitidis* and *N. gonorrhoeae* (5, 14–16). Thus, the identification of the lactate permease mutant is valuable for investigating the influence of this carbon source on pathogenesis, particularly when it cannot be excluded from virulence assays such as experiments in vivo.

As *N. gonorrhoeae* (3), the meningococcus emerges earlier from lag phase and grows more rapidly when lactate is added to media containing glucose. In the CSF, lactate and glucose are both metabolized by *N. meningitidis* with lactate used preferentially. In addition, we observed an increased growth rate of the wild-type strain compared with the *lctP* mutant in CSF. During meningeal infection, acquisition of lactate may enhance meningococcal growth. This effect might be more pronounced during active meningitis when CSF lactate levels are replenished and rise to over 3.8 mM whereas glucose concentrations fall (17). The activity of the phospho-transacetylase acetate kinase pathway (2), shown by the production of acetate, may also be relevant as the pathway results in the production of acetyl phosphate, a phosphate donor to two component regulatory systems such as PhoP/PhoQ, which is involved in gene regulation during pathogenesis (18, 19).

During bloodstream infection, the *lctP* mutant is significantly attenuated. As the virulence of the *lctP* mutant was restored to wild-type levels in complement deficient animals, we demonstrate that the lack of resistance to innate immune killing is responsible for the attenuation of the *lctP* mutant, rather than reduced growth. Therefore lactate availability appears to have a dual role during pathogenesis. In the CSF where there are few complement components, lactate contributes to bacterial growth, while in contrast, available lactate contributes to resistance against complement during bacteraemic infection. The sensitivity of the lactate permease mutant to complement was still observed in a capsule-negative mutant. Our results demonstrate that inability to import lactate reduces LPS sialylation, and suggest that this is responsible for the increased susceptibility to complement-mediated lysis. In *N. gonorrhoeae*, sialylation is important for conferring resistance against complement (20) by blocking deposition of complement components and/or antibodies to the bacterial cell surface (21), or by influencing factor H binding, inhibiting the alternate pathway of complement activation (22). There is conflicting evidence about the role of LPS sialylation in *N. meningitidis*. Although there is a clear correlation between resistance to complement and LPS sialylation among clinical isolates (23), mutants lacking *lst* do not exhibit enhanced sensitivity to this important innate immune mechanism (24).

Our findings with the *siaC*-negative strain (Fig. 8 B) indicate that available lactate is directed through the sialic acid biosynthesis pathway to enhance resistance against complement-mediated lysis. Although glucose can also be converted into PEP (and thence sialic acid), this occurs via the Entner Doudoroff pathway (2), and not through a direct route as when lactate is the carbon source. This provides an

explanation of how lactate contributes to resistance against complement-mediated killing. Lactate utilization could also lead to other changes in the bacterium aside from LPS sialylation, such as enhanced capsulation, although the effect of lactate on resistance against complement was still observed in nonencapsulated strains. In the gonococcus, additional lactate induces changes in the amount and structure of LPS (3, 5). Full structural analysis is required to detail all the effects of lactate on *N. meningitidis* LPS.

Within the host, pathogenic bacteria must successfully acquire key nutrients from the microenvironment that are required for their growth and integrity in vivo. Our results provide an example of the intimate relationship between carbon source availability and the expression of specific virulence determinants by *N. meningitidis*. This relationship involves a link between intermediary metabolism and enzymes encoded by the capsule biosynthesis locus, a pathogenicity island, resulting in evasion of innate immune killing by the complement system. This raises three important points. First, functions encoded by horizontally acquired elements can confer significant benefit to a pathogen through integration with intermediary metabolism; this may be crucial for providing the bacterium scope to explore further niches in the host. Second, metabolism and virulence cannot be considered as distinct functions within the bacterial cell. Finally, a novel class of antimicrobial compounds and vaccines could be designed that block nonessential metabolic pathways and render the bacterium more sensitive to innate immune killing.

MATERIALS AND METHODS

Bacterial strains and growth. The bacterial strains and plasmids used in this study are shown in Table I. *N. meningitidis* was grown on Brain Heart Infusion medium with Levanthal's supplement in the presence of 5% CO_2 at 37°C. *E. coli* was grown on Luria Bertani medium. Antibiotics were: 50 and 75 $\mu g/ml^{-1}$ kanamycin; 200 and 2 $\mu g/ml^{-1}$ erythromycin; and 25 and 2.5 μg ml^{-1} tetracycline for *E. coli* and *N. meningitidis*, respectively. For studies on lactate metabolism, strains were grown in meningococcal chemically defined medium consisting of modified Jyssums minimal media (2) with 1.4 mM arginine, 0.06 mM cysteine, 1 mM glutamate, and 1 mM glycine. Glucose and lactate were added to the media as specified. Growth was measured by reading optical density at 600 nm, and growth rates were calculated as the difference in values over specified intervals and shown as changes in optical density.

Generation of *N. meningitidis* mutants. An NMB0543 (www.tigr.org) mutant was constructed in MC58 by amplifying the defective allele from

Table I. Bacterial strains and plasmids used in this study

Strains/plasmid	Genotype/description	Reference
N. meningitidis		
MC58	Wild-type serogroup B	12
MC58Δ0543		This study
MC58Δ*lctP*	Insertional inactivation of *lctP*	
MC58Δ*lst*	Insertional inactivation of *lst*	This study
MC58Δ*siaD*	Insertional inactivation of *siaD*	This study
MC58Δ*siaD*Δ*lctP*	Insertional inactivation of *lctP*, *siaD*	This study
MC58ΔsiaDΔlstΔlctP	Insertional inactivation of *lctP*, *lst*, *siaD*	This study
MC58Δ*siaC*	Insertional inactivation of *siaC*	This study
MC58Δ*siaC*Δ*lctP*	Insertional inactivation of *siaC* and *lctP*	This study
MC58Δ*lctP*::*lctP*^{ect}	Complemented MC58Δ*lctP*	
E. coli		
DH5α	F′ endA1 supE44 thi-1 hsdR17(r_K^- m_K^+) recA1 gyrA relA1 Δ(lacIZYA-argF) U169 deoR (ϕ80dlacΔ[lacZ]M15)	GIBCO-BRL
Plasmids		
pCR TOPO 2.1	Cloning vector	Invitrogen
pIP10	siaDΔery	28
pYH*lctP*	Cloned *lctP* of *N. meningitidis*	This study
pSTΔ*lst*	*lst* interrupted by *tetM*	This study
pSTΔ*siaC*	*siaC* interrupted by *tetM*	This study
pYHS25	Vector for complementation	29
pRME104	pYHS25 containing *lctP*	This study
pRME105	pYHS25 containing *siaD*	This study
pRME106	pYHS25 containing *lst*	This study
pRME107	pYHS25 containing *siaC*	This study

mutant 9B10 identified by signature-tagged mutagenesis (25), and using the 2.9-kb product to transform MC58. To generate an *lst* mutant, ~500 b.p. upstream and downstream fragments of NMB0922, which encodes the α-2, 3 LPS sialyl transferase (26), were amplified from strain MC58, ligated into pST-Blue (Novagen); the *tetM* cassette (encoding tetracycline resistance) was obtained from pJS1845 (gift from D. Stephens, Emory University, Atlanta, GA; reference 27) and introduced between the up- and downstream fragments. The *siaC* mutant was constructed using the same strategy but using pCRTopo2.1 as the vector. Details of the oligonucleotides used in this study are given in Table S1 available at http://www.jem.org/cgi/content/full/jem.20041548/DC1. Capsule-negative mutants were constructed by transforming strains with pIP10 (28), which contains an internal deletion of *siaD* (encoding the α-2, 8 polysialyl transferase) replaced with an erythromycin-resistance cassette.

For complementation, *lctP, lst*, *siaC*, and *siaD* were amplified from MC58 with High Fidelity Expand Taq (Boehringer), and the products ligated into pCR Topo 2.1, excised and then introduced into a multiple cloning site in pYHS25, downstream of an *opa* promoter. This vector contains the promoter, multiple cloning site and *ermC* flanked by fragments of NMB0102 and NMB0103 (29). After transformation of *N. meningitidis*, integration of the vector by double crossover leads to a single chromosomal copy of the complementing gene in the intergenic region between NMB0102 and NMB0103, which are open reading frames orientated in a tail-to-tail fashion. The inserts in plasmids used for complementation were verified by nucleotide sequencing. All transformants were analyzed by Southern hybridization (unpublished data). Each mutation was backcrossed into the parental strain, and multiple colonies were pooled to exclude any effects of phase variation. Complementation of *siaC* and *siaD* restored resistance against complement to wild-type levels (unpublished data) whereas complementation of *lst* restored sialylation of LPS (Fig. S9, available at http://www.jem.org/cgi/content/full/jem.20041548/DC1).

Uptake of lactate and metabolism in CSF. The uptake of lactate by bacteria was measured as described previously (30) in bacteria grown to mid-exponential phase in meningococcal chemically defined medium containing 10 mM glucose and 1 mM DL-lactate. CSF was obtained from uninfected patients (provided by Microbiology Laboratories, Royal Hallamshire Hospital with approval of the Local Ethical Committee). Bacteria (10^8 CFU) were inoculated from overnight growth into 10 ml of CSF and incubated at 37°C in 5% CO_2. Samples were removed at intervals, the bacteria pelleted by centrifugation (10,000 *g* for 2 min), and the supernatant filtered (0.45 μm pore) before analysis by ^{1}H-NMR (at 500 MHz; DRX500 spectrometer; Bruker). Signals were integrated using FELIX 2000 (Accelrys Inc.).

Virulence assays. To examine the effect of *lctP* on virulence, mixed litters of 5-d-old rats (Wistar) were inoculated i.p. with 10^7 CFU of *N. meningitidis* in 100 μl PBS. Bacteria were grown overnight on solid media, resuspended in PBS, and then enumerated by measuring the A_{260} of an aliquot of the suspension in lysis buffer (1% SDS/0.1 M NaOH). The virulence of the *lctP* mutant was compared directly with MC58 in individual animals given a 1:1 ratio of wild-type to mutant bacteria. The number of mutant (kan^R) and wild-type bacteria recovered from the blood of animals 24 h later was established by plating to media with and without kanamycin. The C.I. was calculated as the (number of mutant/wild-type bacteria recovered from animals)/(number of mutant/wild-type bacteria in the inoculum).

C.I.s were also determined in the murine model of bacteraemia (31) as no $C3^{-/-}$ infant rats are available. $C3^{+/+}$ and $C3^{-/-}$ mice were backcrossed to the C57BL/6 background for over 10 generations. Female, 6-wk-old mice received 10^4 CFU of each strain i.p. in Brain Heart Infusion/0.5% iron dextran, and bacteria recovered by tail vein bleeding 20–24 h later. Studies with animals were approved under a Home Office project licence.

Complement-mediated bacteriolysis, C3 binding, and LPS analysis. Bacteria were harvested from solid media, and 5×10^8 CFU were inoculated in 10 ml DMEM with glucose (25 mM), lactate (2 mM), and 100 μM CMP-neuraminic acid (CMP-NANA; Sigma) and grown for 2 h at 37°C in the presence of 5% CO_2 with gentle agitation. For complement-mediated bacteriolysis assays, the cells were harvested by centrifugation at 20,000 *g*, and then 100 μl aliquots containing 10^4 CFU were incubated with serial dilutions of normal human sera for 1 h at 37°C. The number of bacteria in the inoculum and after incubation with serum was determined by plating to solid media; assays were performed in duplicate and at least on three independent occasions. Heat-inactivated sera was used in control assays. Figures show the percentage survival of bacteria lacking the lactate permease against the corresponding strain expressing the permease. Student's *t* test analysis was performed to indicate significant differences.

To measure C3 deposition, bacteria were grown as above, collected by centrifugation at 20,000 *g*, fixed in 3% paraformaldehyde for 15 min, and then washed twice in PBS. Next, 10^7 bacteria in 100 μl were incubated with dilutions of serum for 30 min at 37°C, washed twice then resuspended in PBS/0.1% Tween 20 containing a FITC-conjugated goat anti-C3 antibody (1:300 dilution; ICN Biomedicals) and incubated for 30 min. After washing in PBS-Tween 0.1%, fluorescence was measured using a FACS Calibur analyzer (Becton Dickinson). Results are presented as the relative fluorescence index (the percentage of positive cells multiplied by the geometric mean fluorescence) of MC58Δ*lctP* with respect to wild-type bacteria; significant difference was examined with a Student's *t* test.

For LPS analysis, cells were grown in liquid media as above and cell extracts prepared as follows. Bacteria were enumerated, pelleted by centrifugation and resuspended in SDS-PAGE loading buffer (50 mM Tris HCl pH 6.8, 2% SDS-PAGE, 10% glycerol, 1.1% bromophenol blue, 100 mM β-mercaptoethanol) to a concentration of 10^{10} CFU ml^{-1}. Extracts were boiled for 10 min before SDS-PAGE analysis. LPS was detected using α-L3,7,9 mAb (at a dilution of 1:500; NIBSC), and unsialylated LPS detected with mAb 3F11 (1:100; gift from M. Apicella, University of Iowa, Iowa City, IA). Binding of a secondary antibody (1:1,000, antimurine immunoglobulin conjugated to HRP; DakoCytomation) was detected using the ECL system (Amersham Biosciences). For neuraminidase-treated extracts, the same procedure was followed except bacteria were resuspended in 50 mM sodium citrate, pH 6.0, at a concentration of 10^{10} CFU ml^{-1}, and then incubated for 1 h at 37°C with 50 U neuraminidase (New England Biolabs) before lysis in SDS-PAGE buffer.

Online supplemental material. The results of individual bacteriolysis assays and C3 binding are shown as well data relating to bacterial growth. The orientation of reading frames around relevant genes, list of oligonucleotides, and details of complementation are also provided. Available at http://www.jem.org/cgi/content/full/jem.20041548/DC1.

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