Role of *Neisseria meningitidis luxS* in Cell-to-Cell Signaling and Bacteremic Infection

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Numerous pathogenic bacteria contain *luxS*, which is required for autoinducer-2 production. Here, we demonstrate that *Neisseria meningitidis* contains a functional copy of *luxS* that is necessary for full meningococcal virulence; strains with a *luxS* deletion are defective for bacteremia, a prerequisite of meningococcal pathogenesis.

Bacteria have evolved mechanisms to coordinate gene expression in response to population density. Known as quorum sensing, these mechanisms involve the production and detection of signaling molecules (called autoinducers or pheromones), which modulate critical functions including virulence factor production, plasmid conjugal transfer, activation of secretion systems, swarming, swimming and twitching motility, biofilm differentiation, and bioluminescence (for reviews, see references 2, 13, 15, 28, and 30). Thus, bacteria in populations display properties denied individual cells.

Gram-positive and gram-negative bacteria are known to utilize distinct signal molecules for quorum sensing. Gram-positive bacteria export and detect small peptides, while gram-negative bacteria produce membrane-permeative *N*-acyl homoserine lactones (13, 15). Recently, however, potential quorum sensing systems based on a signal molecule called autoinducer-2 (AI-2) were shown to be present in both gram-positive and gram-negative species (2, 23, 24). The *luxS* gene product is required for AI-2 production by *Salmonella enterica* serovar Typhimurium (24), *Escherichia coli* (19, 23), *Shigella flexneri* (6), *Helicobacter pylori* (9, 11), *Porphyromonas gingivalis* (4, 5), *Streptococcus pyogenes* (12), and *Actinobacillus actinomycetemcomitans* (8). The chemical structure of AI-2 appears to be conserved, possibly allowing interspecies communication (2, 3, 18).

Neisseria meningitidis colonizes the human nasopharynx and can cause meningitis and/or septicemia (16). Here, we demonstrate that *N. meningitidis* possesses a functional *luxS* necessary for AI-2 production and full meningococcal virulence.

Serogroup B *N. meningitidis* possesses a functional *luxS* gene. Investigation of the MC58 genome sequence (25) using *LuxS* from *Vibrio harveyi* (*LuxS_{Vh}*) led to the identification of NMB1981 (*N. meningitidis* serogroup B annotated sequence), a 504-bp open reading frame (ORF) with 80% identity to *LuxS_{Vh}* which is annotated as being of unknown function. To establish whether NMB1981 encodes a functional product, the

gene was amplified with rTth DNA polymerase XL (Perkin-Elmer, Warrington, United Kingdom), ligated in both orientations into pGEM T-Easy (Promega, Madison, Wis.), and then transferred into *Escherichia coli* DH5 α , which cannot produce AI-2 due to a mutation in *luxS* (24). Strains were grown at 37°C in Luria-Bertani medium, and AI-2 production was assayed using *V. harveyi* BB170 as the reporter (3). Exogenous AI-2 induces premature bioluminescence by this reporter strain. Only clones containing NMB1981 expressed through the vector-encoded promoter (pGEM*luxS*-F) produced culture supernatants with AI-2 activity (Fig. 1A); no activity was detected if the insert was in the opposite orientation (pGEM*luxS*-R). Identical results were obtained with the NMB1981 homologue from strain SD (data not shown). Thus, NMB1981 complements the defective *luxS* in DH5 α and was designated *luxS_{Nm}*.

Expression of *N. meningitidis* AI-2 activity is dependent on *luxS_{Nm}*. Next, we investigated whether *N. meningitidis* initiates *luxS_{Nm}*-dependent AI-2 production. A total of 432 bp of *luxS_{Nm}* (nucleotides 4 to 436 of the ORF) was deleted by inverse PCR and replaced with the kanamycin resistance gene from Tn903, yielding pGEM Δ *luxS*-kan. The insert of pGEM Δ *luxS*-kan was then cloned into pGIT5.3, a vector containing the *Neisseria* uptake sequence, and the resulting plasmid (pGIT5.3 Δ *luxS*-kan) was introduced into *N. meningitidis* by transformation; Southern hybridization confirmed the integration of the deleted allele in MC58 Δ *luxS* and B16/B6 Δ *luxS*. A complemented strain, MC58 Δ *luxS*^{Ect}, was constructed to enable the attribution of functions to *luxS_{Nm}*. *luxS_{Nm}*, under the control of an *opa* promoter, was introduced in the intergenic region between NMB102 and NMB103 (genes oriented in a tail-to-tail fashion), with *ermC* downstream of *luxS*. The construct (pYH204) was introduced into MC58 Δ *luxS* by transformation, generating MC58 Δ *luxS*^{Ect}; Southern analysis confirmed that integration into the NMB102/NMB103 intergenic region had occurred. *N. meningitidis* bacteria were grown at 37°C on brain heart infusion plates with Levinthal's supplement or in Mueller-Hinton medium for 24 h with shaking for AI-2 production. A list of the bacterial strains and plasmids used in this study is presented in Table 1.

AI-2 activity was detected in MC58 culture supernatants

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FIG. 1. (A) Complementation of *E. coli* DH5α with *luxS_{Nm}*. The growth profiles and extracellular AI-2 activity of *E. coli* DH5α carrying either pGEM T-Easy or the same vector containing *luxS_{Nm}* in the forward or reverse orientation were measured. Growth profiles: empty vector, closed squares; *luxS_{Nm}* in forward orientation, closed circles; *luxS_{Nm}* in reverse orientation, open circles. AI-2 activity: *V. harveyi* BB120 (wild type, after 24 h), black bar; *luxS_{Nm}* in forward orientation, grey bars; *luxS_{Nm}* in reverse orientation, white bars. Activities are presented as the fold increase of bioluminescence in relation to *E. coli* DH5α carrying an empty vector at the respective time point. (B) Growth profiles and AI-2 activity of *N. meningitidis*. *N. meningitidis* strains MC58 (closed circles) and MC58Δ*luxS* (open circles) were grown, and the optical densities at 600 nm of cultures were measured. Culture supernatants of MC58 (grey bars), MC58Δ*luxS* (white bars), and *V. harveyi* BB120 (black bar) were assayed for AI-2 activity. Activities are presented as the fold increase of bioluminescence in relation to the control (sterile medium) at the respective time points. The results for MC58Δ*luxS*^{Ect} were identical to those for the wild type and are not shown. For panels A and B, single representative experiments are shown, although similar results were obtained on three occasions for both assays.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype and/or details	Source or reference
Bacterial strains		
<i>E. coli</i> DH5α	F' <i>endA1 supE44 thi-1 hsdR17</i> (r _K ⁻ m _K ⁺) <i>recA1 gyrA relA1</i> Δ(<i>lacIZYA-argF</i>)U169 <i>deoR</i> [φ80d <i>lac</i> Δ(<i>lacZ</i>)M15]	Gibco-BRL
<i>N. meningitidis</i>		
MC58	Serogroup B; ET-5	25
B16/B6	Serogroup B; ET-37	17
SD	Serogroup B: B:15:P1.16	1
MC58Δ <i>luxS</i>	Deleted <i>luxS_{Nm}</i> replaced with kan	This study
MC58Δ <i>luxS</i> ^{Ect}	Wild-type <i>luxS_{Nm}</i> located in the NMB102/103 intergenic region of MC58Δ <i>luxS</i>	This study
<i>V. harveyi</i>		
BB120	Wild type	3
BB170	Sensor AI-1 ⁻ , sensor AI-2 ⁺	3
Plasmids		
pGEM T-Easy	Cloning vector	Promega
pGEM <i>luxS</i> -F	1.5-kb fragment encoding <i>luxS</i> of <i>N. meningitidis</i> MC58 and flanking region in pGEM-T Easy, forward orientation	This study
pGEM <i>luxS</i> -R	1.5-kb fragment encoding <i>luxS</i> of <i>N. meningitidis</i> MC58 and flanking region in pGEM-T Easy, reverse orientation	This study
pGEMΔ <i>luxS</i> -kan	<i>luxS_{Nm}</i> with internal deletion and Tn903 kanamycin resistance gene	This study
pGIT5.3	Derived from pCRII; containing a chloramphenicol resistance cassette and <i>Neisseria</i> DNA uptake sequence	L. Barber and T. Baldwin, unpublished data
pGIT5.3Δ <i>luxS</i> -kan	pGIT5.3 derivative containing the insert of pGEMΔ <i>luxS</i> -kan	This study
pYH204	<i>luxS_{Nm}</i> in the NMB102/103 intergenic region with <i>ermC</i>	This study

from early logarithmic growth onward (Fig. 1B). While no AI-2 activity was observed in supernatants of MC58Δ*luxS*, the single ectopic *luxS* allele in MC58Δ*luxS*^{Ect} restored AI-2 activity to wild-type levels. Identical results were obtained with strain B16/B6 and its corresponding mutants (not shown).

***N. meningitidis luxS* mutants are attenuated for bacteremic infection.** To investigate the influence of *luxS_{Nm}* on meningococcal pathogenesis, the ability of MC58Δ*luxS* to cause disseminated disease was compared directly against that of MC58. Bacteria were grown overnight on brain heart infusion agar and suspended in phosphate-buffered saline, and the number of CFU was determined. Bacteria (100 μl) in phosphate-buffered saline were injected intraperitoneally into five-day-old infant rats (Wistar; Harlan, Bicester, United Kingdom). The virulence of strains was assessed using groups of at least three

FIG. 2. MC58Δ*luxS* is defective for bacteremic infection compared with the parental strain, MC58. This defect is restored by supplying a single, intact copy of *luxS* in *trans* to MC58Δ*luxS* (MC58Δ*luxS*^{Ect}); the difference in the virulences of MC58Δ*luxS* and MC58Δ*luxS*^{Ect} is highly significant ($P < 0.001$). The CI is the proportion of the strains in the bloodstream 21 to 24 h following infection with a 1:1 ratio of the strains.

animals in each of three experiments by determining their competitive index (CI). The CI is the proportion of mutant to wild-type bacteria recovered from the bloodstream following inoculation with a 1:1 ratio of the strains (26); results were compared by using a one-tailed Student's *t* test. MC58Δ*luxS* showed decreased survival in infected animals when compared with MC58 ($P < 0.01$; Fig. 2). Similar results were obtained with B16/B6 (not shown). Providing *luxS*_{Nm} in *trans* (MC58Δ*luxS*^{Ect}) restored the virulence to wild-type levels, demonstrating that the loss of *luxS*, not any polar effect, is responsible for attenuation.

Complete genome sequences of bacterial pathogens provide an important resource for microbial research (21), though the annotations may miss biologically important homologies. While a *luxS* homologue was identified in the annotated Z2491 serogroup A strain sequence (14), the annotated MC58 genome (25) does not contain a *luxS* homologue. However, investigation of the MC58 database revealed an ORF closely related to *luxS*_{Nm}. We demonstrate that *luxS*_{Nm} complements DH5α and that *N. meningitidis* expresses AI-2 in a *luxS*-dependent fashion with maximal production during late exponential growth, consistent with a role in quorum sensing.

Studies with the infant rat model show that *luxS*_{Nm} contributes to the ability of the bacterium to cause bacteremia, a prerequisite for meningococcal pathogenicity. This model has been used to demonstrate the influence of other well-characterized virulence determinants on disseminated infection (20, 27). Although CIs provide a sensitive measure of a strain's virulence by eliminating host-to-host variation (22, 26), our findings may underestimate the impact of *luxS*_{Nm} on pathogenesis. AI-2 produced by the wild type could partially cross-feed a *luxS*_{Nm} mutant in mixed-inoculum experiments. However, the results are also consistent with a metabolic function of the LuxS protein, which is distinct from quorum sensing (18, 29).

Little is known about the regulatory networks governing the expression of *N. meningitidis* virulence factors apart from that of a LysR homologue, required for entry into host cells (7). Identification of the response elements and the genes con-

trolled by AI-2-dependent signaling may help elucidate this aspect of pathogenesis and identify novel virulence determinants for this important human pathogen. Furthermore, the demonstration that *luxS*_{Nm} is required for full virulence suggests that interrupting this pathway may provide a means for preventing meningococcal disease (10).

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