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Mutation of *luxS* of *Streptococcus pneumoniae* Affects Virulence in a Mouse Model

Uwe H. Stroeher, Adrienne W. Paton, A. David Ogunniyi, and James C. Paton*

School of Molecular and Biomedical Science, University of Adelaide, Adelaide, South Australia 5005, Australia

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The LuxS protein is required for the biosynthesis of the type 2 autoinducer (AI-2), which is involved in quorum sensing in a wide range of bacterial species. We have determined the effects of a defined *luxS* mutation on the virulence of *Streptococcus pneumoniae*. Although the *luxS* mutant displayed reduced virulence relative to its wild-type parent, the type 2 strain D39, it was by no means avirulent in a mouse model. After intranasal administration, the *luxS* mutant was able to colonize the nasopharynx of the mouse as efficiently as the wild type. However, it was less able to spread from the nasopharynx to the lungs or the blood. Intraperitoneal coadministration studies indicated that the *luxS* mutant was less fit and was readily outcompeted by wild-type D39. However, when administered on its own by this route, the mutant was able to proliferate and cause fatal systemic disease, albeit at a lower rate than the wild type. Western blot analysis of whole-cell lysates of the mutant and its parent did not reveal any differences in the levels of several well-characterized virulence proteins. However, analysis of Coomassie blue-stained protein profiles after separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis showed that mutation of *luxS* had pleiotropic effects on protein expression in all cellular compartments. This is consistent with the product of *luxS* having a regulatory role in *S. pneumoniae*. This is the first report of a direct role for *luxS* (and by inference, AI-2) in the virulence of a gram-positive pathogen. However, the fact that mutagenesis of *luxS* does not completely attenuate *S. pneumoniae* has implications for the possible use of AI-2 antagonists for treatment of pneumococcal infections.

Streptococcus pneumoniae is a major cause of invasive diseases such as pneumonia, meningitis, and bacteremia, as well as less severe but highly prevalent infections such as otitis media (23, 42). The elderly and the very young are most at risk from pneumococcal disease, which causes the deaths of over 1 million children under 5 years of age each year (13, 47). Treatment of patients with pneumococcal disease is being complicated by increasing rates of resistance to penicillin and other antimicrobial drugs and the rapid global spread of multiply resistant *S. pneumoniae* clones. Disease prevention programs have also been frustrated by the low clinical efficacy of pneumococcal polysaccharide vaccines, which are poorly immunogenic in young children and other high-risk groups (9, 31). Recently licensed polysaccharide-protein conjugate vaccines are more immunogenic but are expensive and provide protection against only a limited range of *S. pneumoniae* serotypes (18).

The ongoing threat to human health posed by the pneumococcus has prompted extensive research aimed at understanding its mechanism of pathogenesis, with a view to identifying alternative targets for vaccines and novel drugs. One potential new class of antimicrobial agents includes compounds that interfere with the phenomenon of quorum sensing (21, 32). This is a mechanism whereby bacteria coordinate their gene expression in response to population density. Small autoinducer molecules are secreted by the bacterium, and as the population density increases, cell surface receptors sense the increasing extracellular concentration of autoinducer, triggering alterations in gene expression (2, 16, 46). The functions influenced by quorum sensing in various species are wide ranging and include the production of virulence factors, motility, biofilm formation, and differentiation, as well as bioluminescence (2, 22, 26, 43). The number of organisms which have been shown to regulate some of their key virulence genes by quorum sensing is steadily growing, and the list currently includes the genes for the enterohemorrhagic *Escherichia coli* type III secretion apparatus (15, 34), toxin expression in *Erwinia carotovora* and *Pseudomonas aeruginosa* (17, 28), the hemagglutinin of *Porphyromonas gingivalis* (6), and toxin production in *Clostridium perfringens* (27). The signaling molecule in these organisms is autoinducer 2 (AI-2), which has been studied extensively in *Vibrio harveyi*. The *luxS* gene is required for synthesis of AI-2, which is released from the cell and builds up in the environment. It is then captured on a specific receptor/sensor complex (LuxP/Q), and subsequent phosphorelay via either LuxU or a LuxU homologue leads to modification of the transcriptional activator LuxO. This, in turn, relieves repression of the *Vibrio lux* operon (10, 11).

Interestingly, the LuxS protein appears to play a dual role in many bacterial species; in addition to its involvement in AI-2 synthesis, it is important in the methyl cycle (44). LuxS, together with the methylthioadenosine/*S*-adenosylhomocysteine nuclease (MTA/SAHase), converts *S*-adenosylhomocysteine to homocysteine and adenosine via a two-step process. The exact role LuxS plays in the production of AI-2 is still unclear, but AI-2 may be a spontaneous by-product of the conversion of

* Corresponding author. Mailing address: School of Molecular and Biomedical Science, University of Adelaide, Adelaide, South Australia 5005, Australia. Phone: 61-8-83035929. Fax: 61-8-83033262. E-mail: james.paton@ adelaide.edu.au.

4,5-dihydroxy-2,3-pentanedione to 4-hydroxy-5-methyl-3(2*H*) furanone (44, 45). The AI-2 molecule produced by *V. harveyi* has been reported to be a furanosyl borate diester (8), but whether this holds for AI-2 molecules produced by other organisms is still unclear. What is known is that the periplasmic AI-2 binding protein proposed to be involved in quorum sensing in *Salmonella enterica* serovar Typhimurium (LsrB) shows little homology to the AI-2 binding protein of *V. harveyi* (35).

S. pneumoniae has a well characterized quorum-sensing system involved in regulation of competence for genetic transformation. Indeed, this was the first such system to be described in bacteria (37). The signaling molecule is a peptide which is encoded by *comC*, exported from the cell by ComAB and then sensed by the two-component signal transduction system ComDE (reviewed by Morrison and Lee [22]). The pneumococcus also has a *luxS* homologue, but whether this is involved in a second quorum-sensing circuit has not previously been investigated. Mutation of *luxS* in *Streptococcus pyogenes* was recently shown to have marked effects on the expression of several virulence-related genes, resulting in increased hemolytic activity and reduced secretion of SpeB; the in vitro growth rate in certain media was also affected (20). In the present study, we have examined the role of LuxS in the pathogenesis of *S. pneumoniae* infection with a view to assessing the potential of AI-2 antagonists for treatment of infections caused by this organism.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *S. pneumoniae* strain used in this study was the virulent type 2 strain D39 (1). Bacteria were grown overnight on blood agar and were inoculated into either serum broth (meat extract broth supplemented with 10% horse serum) or Todd-Hewitt broth with 0.5% yeast extract (THY) for growth prior to inoculation or challenge. *S. pneumoniae* D39 was transformed as described previously (4, 49), and transformants were selected on blood agar containing 0.2 μg of erythromycin/ml. *V. harveyi* strains BB152 and BB170 (3) were obtained from S. Kjelleberg, University of New South Wales, Australia. They were grown at 30°C either on Luria-Bertani agar containing 0.16 M NaCl or in autoinducer bioassay (AB) medium (0.3 M NaCl, 0.05 M $MgSO_4$, and 0.2% vitamin-free Casamino Acids [Difco], adjusted to pH 7.5 with KOH, sterilized, and supplemented with 10 ml of 1 M potassium phosphate [pH 7.0], 10 ml of 0.1 M L-arginine, 20 ml of glycerol, 1 ml of 10-μg/ml riboflavin and 1 ml of 1-mg/ml thiamine per liter).

DNA manipulations. *S. pneumoniae* chromosomal DNA used in Southern hybridization experiments was extracted and purified with the Wizard genomic DNA purification kit (Promega Corporation, Madison, Wis.). Chromosomal DNA was purified according to the manufacturer's instructions, except that cell lysis was induced by the addition of 0.1% (wt/vol) deoxycholate followed by incubation at 37°C for 10 min. PCR amplification was performed in a Hybaid Touchdown thermal cycler, and the 50-μl reaction volume contained PCR buffer (1.5 mM $MgCl_2$, 10 mM Tris [pH 8.4], 50 mM KCl, and 100 μg of gelatin/ml), 1.5 U of *Taq* polymerase, 1 μM (each) primer, 100 ng of DNA template, and 200 μM (each) four deoxynucleoside triphosphates. The program comprised 25 cycles consisting of denaturation at 95°C for 30 s, annealing at 50°C for 30 s, and elongation at 72°C for 1 to 4 min (1 min for each kilobase of expected product). A 5-μl sample of the PCR product was analyzed by agarose gel electrophoresis.

The oligonucleotides used for PCR amplification of *luxS* were designed with reference to the *luxS* sequence in the genome of *S. pneumoniae* R6 (a derivative of D39) (accession number NC_003098). The primers were LuxSI (GCCCTGCAGGGACCAAAAGGAGACATC) and LuxSII (GGCGGATCCAACAGCTGCGATTTTAGCACT), which contain *Pst*I and *Bam*HI sites, respectively (underlined). The resultant 220-bp PCR product was cloned into the *Pst*I and *Bam*HI sites in the suicide vector pACH74 (14). All restriction enzymes and *Taq* DNA polymerase were purchased from Roche Molecular Biochemicals (Rotkreuz, Switzerland) and used according to the manufacturer's recommendations.

Southern hybridization analysis. Chromosomal DNA (2.5 μg) was digested with appropriate restriction enzymes, and the digests were electrophoresed on agarose gels in Tris-borate-EDTA buffer. DNA was then transferred to a positively charged nylon membrane (Hybond N^+; Amersham, Little Chalfont, England) as described by Southern (33), hybridized to digoxigenin (DIG)-labeled probe DNA, washed, and then developed with anti-DIG-alkaline phosphatase conjugate (Roche) and 4-nitro blue tetrazolium–X-phosphate substrate according to the manufacturer's instructions. DIG-labeled lambda DNA, restricted with *Hin*dIII, was used as a DNA molecular size marker.

Cell fractionation and SDS-PAGE analysis. Cells were grown in THY to an A_{600} of approximately 0.5 and harvested by centrifugation at 5,000 × *g* for 10 min. The culture supernatant was collected, and proteins were precipitated in 12% trichloroacetic acid. After centrifugation, precipitated proteins were redissolved in sodium dodecyl sulfate (SDS) loading buffer and stored at −20°C. Meanwhile, the bacterial cells were resuspended in phosphate-buffered saline (PBS) and lysed with a French pressure cell (SLM Aminco Instruments) at 12,000 lb/in^2. Unbroken cells were removed by centrifugation at 5,000 × *g* for 10 min, and the supernatant was centrifuged at 100,000 × *g* for 1 h at 4°C. The high-speed supernatant (soluble fraction) and the pellet (insoluble fraction) were stored at −20°C prior to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) analysis. Protein concentrations in the various fractions were determined by using the method of Bradford (5). Samples were dissolved in SDS loading buffer and subjected to SDS-PAGE as described by Laemmli (19). Loadings were adjusted so that fractions from D39 and D39*luxS* contained the same amount of total protein. Gels were stained with Coomassie brilliant blue R250.

Western immunoblotting. Bacterial cell lysates from 1 ml of log-phase culture (A_{600}, 0.25; 5 × 10^8 CFU/ml) were separated on 12% SDS–PAGE gels (17) prior to transfer onto nitrocellulose (41). Filters were probed with mouse antibodies specific for various pneumococcal virulence proteins, used at a dilution of 1:3,000, followed by a 1:5,000 dilution of goat anti-mouse immunoglobulin G conjugated to alkaline phosphatase (Bio-Rad Laboratories, Hercules, Calif.). Enzyme-labeled bands were visualized with the 4-nitro blue tetrazolium–X-phosphate substrate system (Roche). A benchmark prestained protein ladder (Life Technologies, Gaithersburg, Md.) was used as molecular size markers.

Determination of AI-2 activity. The method used is essentially that described previously (3). Cell-free supernatants were prepared as follows. *V. harveyi* strain BB152 was grown in AB medium overnight and then subcultured into fresh AB medium and grown to an A_{600} of approximately 0.9; bacterial cells were removed by centrifugation, and the supernatant was subsequently filter sterilized. *S. pneumoniae* was grown overnight on blood agar plates and subcultured into THY and then grown to an A_{600} of approximately 0.45; the culture was subsequently treated as described above. The indicator strain B172 was grown overnight in AB medium and diluted 1:5,000 in fresh AB medium. The cell-free supernatants were added to the diluted BB172 cells at a 1:10 ratio, and the luminescence was monitored over a period of 5 h using a TD-20/20 illuminometer (Turner Designs, Sunnyvale Calif.).

Intranasal colonization model. The method used was a modification of that described by Wu et al. (48). Bacterial strains used for the intranasal challenge were grown in THY broth to an A_{600} of approximately 0.25. Ten mice were anaesthetized prior to challenge by intraperitoneal injection with Nembutal (pentobarbitone sodium; Rhone-Merieux) at a dose of 66 μg per gram of body weight. Subsequently, 20 μl of *S. pneumoniae* D39 or its *luxS*-negative derivative, at concentrations of approximately 1 × 10^8 CFU/ml, was pipetted into the nares and involuntarily inhaled. Five mice were sacrificed by carbon dioxide asphyxiation at both 24 and 48 h postchallenge. Blood (75 μl) was collected from each mouse by retro-orbital bleeding with heparinized capillary tubing prior to sacrifice, of which 45 μl was diluted in 160 μl of sterile PBS. After exposure of the trachea, the nasopharynx was washed with 1 ml of buffer (0.5% trypsin–0.02% EDTA in sterile PBS) by insertion of a 26-gauge needle sheathed in tubing into the tracheal end of the upper respiratory tract. Buffer was allowed to drip into the nasopharynx slowly and was collected from the nose, with each wash taking approximately 40 seconds. Both lungs were entirely removed, rinsed twice in PBS to remove excess blood, weighed, and placed in 2 ml of sterile PBS. The lungs were then homogenized with a CAT X120 homogenizer (CAT, M, Zipperer GmbH, Staufen, Germany) at 30,000 rpm for approximately 10 s. All blood, lung, and nasal washout samples were then serially diluted and plated on blood agar (supplemented with 0.2 μg of erythromycin/ml in the case of the *luxS* mutant) to determine the number of viable pneumococci. Differences in the levels of colonization were analyzed using Student's unpaired *t* test (two-tailed).

Intraperitoneal challenge. Bacterial strains were grown in serum broth to an A_{600} of 0.08. Groups of mice were then injected intraperitoneally with 0.1 ml of diluted cultures containing approximately 1 × 10^5 CFU of *S. pneumoniae* D39 or its *luxS*-negative derivative (confirmed by viable count). Mice were monitored at approximately 4-h intervals, and the survival time of each mouse was recorded.

The statistical significance of differences in median survival times between groups was analyzed using the Mann-Whitney U test (two-tailed).

Intraperitoneal competition model. Ten mice were injected intraperitoneally with 0.1 ml of serum broth containing 1×10^4 CFU each of *S. pneumoniae* D39 and its *luxS*-negative derivative. Five mice were sacrificed by carbon dioxide asphyxiation at each of 24 and 48 h postchallenge. Blood (75 μl) was removed from each mouse by retro-orbital bleeding by heparinized capillary tubing prior to sacrifice, of which 45 μl was diluted in 160 μl of sterile PBS. The peritoneal cavity was also washed by injection of 4 ml of sterile PBS, and the washout was collected to determine the number of CFU in the peritoneum. The peritoneal cavity was then opened, and the spleen was removed and placed in 2 ml of sterile PBS. The spleens were then homogenized at 30,000 rpm for approximately 10 s. All blood, spleen, and peritoneal washout samples were serially diluted and plated onto blood agar or blood agar supplemented with erythromycin to determine the numbers of wild-type and *luxS* mutant *S. pneumoniae*.

Adherence assay. Adherence of pneumococci to A549 cells (human type II pneumocytes) and HEp-2 cells (human laryngeal carcinoma) was determined essentially as described previously (36). Briefly, pneumococci were suspended at a density of 1×10^6 CFU/ml (confirmed by viable count) in Dulbecco's modified Eagle's medium buffered with 20 mM HEPES and supplemented with 2 mM L-glutamine. Washed A549 or HEp-2 monolayers in 24-well tissue culture plates were infected with 1-ml aliquots of bacterial suspension. After incubation at 37°C for 2 h, the culture medium was removed, and the monolayers were washed four times with PBS to remove nonadherent bacteria. The cell monolayers were then detached from the plate by treatment with 100 μl of 0.25% trypsin–0.02% EDTA. Cells were then lysed by addition of 400 μl of 0.025% Triton X-100, and 50-μl aliquots (and serial tenfold dilutions thereof) were plated on blood agar to determine the total number of adherent bacteria per well. Assays were carried out in quadruplicate, and results were expressed as the mean CFU/well ± standard error. The significance of differences in adherence was analyzed using Student's unpaired *t* test (two-tailed).

RESULTS

Construction of *S. pneumoniae* with an insertion-duplication mutation in *luxS*. A 220-bp fragment of the *luxS* gene was PCR amplified from *S. pneumoniae* D39 chromosomal DNA by using oligonucleotides LuxSI and LuxSII, digested with *Pst*I and *Bam*HI, and cloned into the suicide vector pACH74. The resulting plasmid pLuxS01 was then transformed into D39. Interruption of the *luxS* open reading frame by insertion-duplication in a selected erythromycin-resistant transformant (designated D39*luxS*) was confirmed by PCR amplification using a combination of oligonucleotides LuxSI, LuxSII, and the forward and reverse M13 sequencing primers that anneal to pACH74 (result not shown). The mutation in D39*luxS* was also confirmed by Southern hybridization analysis of genomic DNA using the 220-bp DIG-labeled LuxSI/LuxSII PCR product as probe. This labeled a single 12-kb fragment in *Bam*HI digests of D39 chromosomal DNA but labeled 10- and 8-kb fragments in *Bam*HI digests of D39*luxS* DNA (result not shown). Examination of the DNA sequence flanking *luxS* in the genome sequence of *S. pneumoniae* R6 (a derivative of D39) (accession number NC_003098) indicated that *clpL* (which encodes a heat shock protease) is located 295 nucleotides downstream of *luxS*, but it is on the opposite DNA strand. This intergenic region also contains a weak stem-loop structure ($\Delta G = -10.3$ kcal/mol). Thus, insertion-duplication mutagenesis of *luxS* is unlikely to have polar effects.

In vitro growth. LuxS appears to be involved in the activated methyl cycle within the cell (45), and hence mutations in *luxS* might impact upon growth rate. Furthermore, if LuxS plays a role in quorum sensing, it may influence the growth of *S. pneumoniae* at higher culture densities. The growth rate of D39*luxS* was compared with that of wild-type D39 using various starting densities of cells resuspended in either serum broth or THY medium after growth overnight on blood agar. Experiments were carried out at least twice and showed no significant difference in the growth rates between the two strains, regardless of the culture medium or initial inoculum (data not shown).

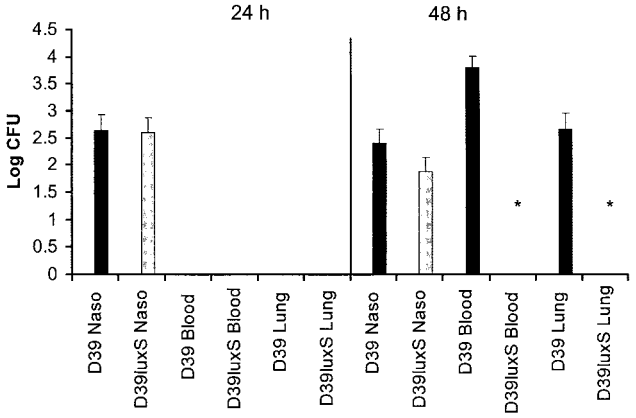

FIG. 1. Numbers of D39 and D39*luxS* organisms in the nasopharynx, lungs, and blood after intranasal challenge. Ten mice were challenged with approximately 2×10^6 CFU of a 1:1 mixture of D39 and D39*luxS* organisms. Five mice were sacrificed after 24 or 48 h, and the number of bacteria in the various tissues was determined as described in Materials and Methods. Data are mean $\log_{10}$ CFU for the five mice, and the bars indicate the standard error. The asterisk denotes numbers significantly different from that of the wild type ($P < 0.001$) (unpaired Student's *t* test, two-tailed).

Determination of AI-2 activity in D39*luxS*. AI-2 produced by other bacterial species has been shown to induce bioluminescence in *V. harveyi* (3). We therefore examined the ability of both D39 and its isogenic *luxS* mutant to induce bioluminescence in the *V. harveyi* BB170 indicator strain, as described in Materials and Methods. Although the level of bioluminescence induced by the cell-free culture supernatant of D39 was markedly lower than that of the BB152 *V. harveyi* positive control strain, there was a significant difference in AI-2 activity between D39 and its *luxS* mutant. The D39*luxS* induced only 8 to 14% of the luminescence induced by D39.

Intranasal challenge studies. The abilities of D39*luxS* and wild-type D39 to colonize the mouse nasopharynx and to invade the lungs and blood were compared. Ten Swiss mice were inoculated intranasally with a 1:1 mixture of D39*luxS* and D39 (total inoculum, 2×10^6 CFU). Five mice were sacrificed after 24 and 48 h, and the relative numbers of D39 and D39*luxS* organisms in the nasopharynx, lung, and blood were determined as described in Materials and Methods (Fig. 1). There was no significant difference in the numbers of D39 and D39*luxS* organisms in the nasopharynx at either time point, and after 48 h, the output ratio for the wild-type and mutant strains remained similar to the input ratio. After 24 h, no bacteria were found in either the blood or the lungs. However, after 48 h, three out of the five mice sacrificed had significant levels of wild-type D39 in both the lungs and the blood (Fig. 1). Interestingly, D39*luxS* was not detected in either of these tissues, indicating that the mutant had a reduced capacity either to translocate from the nasopharynx and survive in the lung or

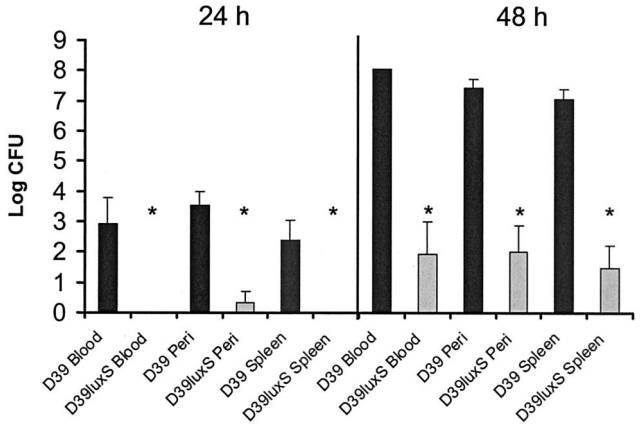

FIG. 2. Numbers of D39 and D39*luxS* organisms in peritoneal washout, blood, and spleen after intraperitoneal challenge. Ten mice were challenged with approximately 1×10^4 CFU of a mixture of D39 and D39*luxS* organisms at an input ratio of approximately 1:3. Five mice were sacrificed after 24 or 48 h, and the numbers of bacteria in the various tissues were determined as described in Materials and Methods. Data are mean $\log_{10}$ CFU for the five mice, and the bars indicate the standard error. The asterisks denote numbers significantly different from that of the wild type ($P < 0.001$) (unpaired Student's *t* test, two-tailed).

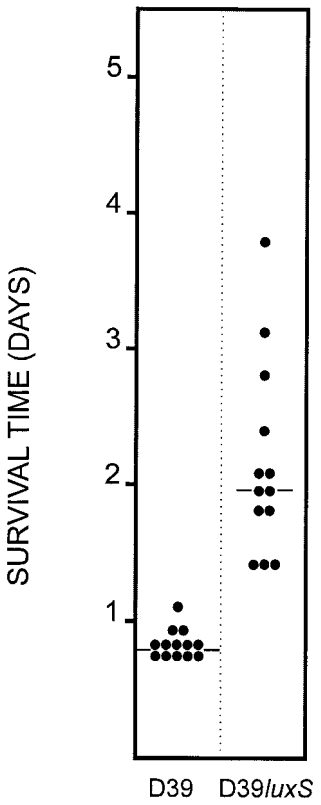

FIG. 3. Survival times of mice after intraperitoneal challenge. Groups of 13 BALB/c mice were challenged with approximately 6.5×10^4 CFU of either D39 or D39*luxS*. Survival time for each mouse was recorded. The two horizontal lines denote the median survival times for each group.

to enter the bloodstream. The experiment was repeated with a higher dose of D39*luxS* relative to D39 (the input ratio was approximately 8:1). The ratios of D39*luxS*:D39 recovered from the nasopharynx after 24 and 48 h were again essentially unchanged from the input ratios (7.0:1 and 7.2:1, respectively). As in the previous experiment, neither strain was detected in lungs or blood after 24 h. However, after 48 h, two out of the five mice examined had significant levels of wild-type D39, but not D39*luxS*, in these tissues (result not presented). An additional experiment involving intranasal inoculation with D39 and D39*luxS* separately indicated that both strains were capable of colonizing the nasopharynx to a similar extent, suggesting that cross-complementation of D39*luxS* with AI-2 released from D39 was not essential for survival of the mutant in this niche (data not shown).

Intraperitoneal challenge studies. Ten BALB/c mice were injected intraperitoneally with approximately 1×10^4 CFU of a mixture of D39 and D39*luxS* at an input ratio of approximately 1:3. Five mice were sacrificed 24 and 48 h after challenge, and the relative numbers of organisms of the two strains were determined in peritoneal washout fluid as well as in the blood and in spleen homogenates (Fig. 2). After 24 h, no D39*luxS* bacteria were recovered from either the blood or spleen (the lower limit of detection was approximately 10^2 CFU/ml), and only one of the five mice had detectable levels of D39*luxS* in the peritoneal washout. However, high numbers of wild-type D39 organisms were present in the peritoneal washout of all five mice and in the blood and spleens of four. Although the D39:D39*luxS* input ratio was approximately 1:3, the output ratio was of the order of 1,000:1 for all sites examined. After 48 h, all five mice had high numbers of D39 organisms in all tissues examined. Interestingly, D39*luxS* was now detected in the peritoneal fluid, blood, and spleens of three of the five mice, albeit at levels approximately 10^5-fold lower than D39. Clearly D39*luxS* is less able than D39 to proliferate in vivo, but it is nevertheless capable of increasing its numbers between 24 and 48 h.

Comparative virulence of D39 and D39*luxS*. In view of the fact that D39*luxS* was able to increase in numbers between 24 and 48 h after intraperitoneal challenge, its capacity to kill mice was compared with that of D39. Two groups of 13 BALB/c mice were challenged by intraperitoneal inoculation with approximately 6.5×10^4 CFU of either D39*luxS* or D39, and their survival was followed (Fig. 3). All challenged mice succumbed, although the median survival time for the D39*luxS* group (44 h) was significantly longer than for the D39 group (19 h) ($P < 0.001$, Mann-Whitney U test). The appropriate *S. pneumoniae* strain was isolated from heart blood collected post mortem from each of the mice.

Adherence to A549 and HEp-2 cells. The nasopharyngeal challenge data suggesting that D39*luxS* and D39 could colonize the nasopharynx to similar extents but that only D39 could translocate to the lungs suggested the possibility of differences in their capacities to adhere to epithelial cells from the upper and lower respiratory tract. Accordingly, the abilities of these strains to adhere in vitro to cell lines derived from either the lung (A549) or the larynx (HEp-2) were compared. At an initial inoculum of 1.0×10^7 CFU per well, total adherence after 2 h to A549 cells was $(2.95 \pm 0.71) \times 10^4$ and $(2.94 \pm 0.53) \times 10^4$ CFU per well for D39 and D39*luxS*, respectively.

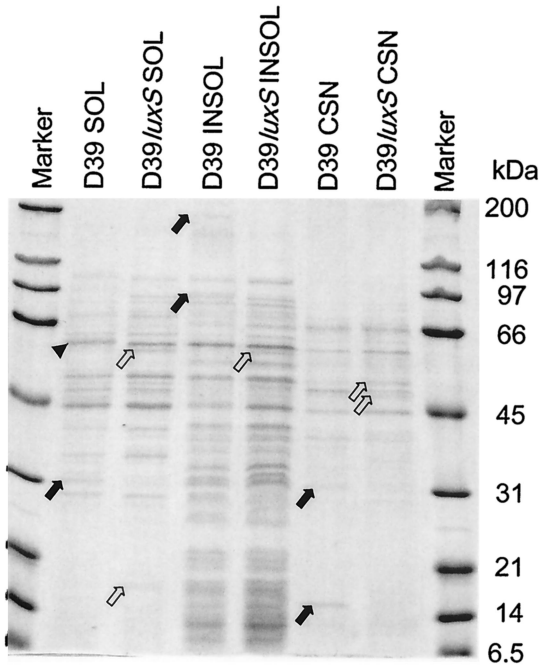

FIG. 4. Protein profiles of subcellular fractions of D39 and D39*luxS*. Supernatants (100,000 × *g*) (SOL) and pellets (INSOL) of French pressure cell lysates of *S. pneumoniae* cells, as well as trichloroacetic acid-precipitated culture supernatant from the original THY cultures (CSN), were separated by SDS-PAGE (12% gel) and stained with Coomassie blue, as described in Materials and Methods. Solid arrows indicate protein species present in D39 but absent in D39*luxS*. Open arrows indicate protein species present in D39*luxS* but absent in D39. The arrowhead indicates a protein band with altered mobility between D39 and D39*luxS*. Molecular masses of size markers are indicated on the right of the figure.

Under the same conditions, total adherence to HEp-2 cells was $(8.88 \pm 0.97) \times 10^4$ and $(1.05 \pm 0.13) \times 10^5$ CFU per well for D39 and D39*luxS*, respectively. Interestingly, although there was no significant difference between the adherences of the two strains to either cell line, both D39 and D39*luxS* adhered more efficiently to HEp-2 cells than to A549 cells ($P < 0.05$).

Comparative protein expression in D39 and D39*luxS*. The protein expression profiles of D39 and D39*luxS* were then compared. The strains were grown in THY broth to an A_{600} of approximately 0.5, and the cells were fractionated as described in Materials and Methods. Fractions of the soluble (cytoplasmic) and insoluble (membrane and cell wall) extracts, as well as the culture supernatant, were analyzed by SDS-PAGE stained with Coomassie blue (Fig. 4). At least 10 changes were detected in the protein expression profiles for the various cell fractions between D39 and D39*luxS*, indicating that the mutation has pleiotropic effects. Both strains were in the opaque phase, and so these changes in expression pattern were not due to phase variation. Furthermore, both increases and decreases in staining intensity for particular protein bands, as well as one example of an apparent shift in mobility, were observed between fractions from the mutant and its parent. The apparent molecular mass of the proteins affected ranged from approximately 180 to 12 kDa. Similar differences in protein expression profile between D39 and D39*luxS* fractions were also seen when lower-cell-density cultures were tested (A_{600} of 0.1 rather than 0.5) (result not shown).

Levels of expression of previously characterized virulence-related proteins (29, 30) were also compared by Western immunoblot analysis. D39 and D39*luxS* were grown in THY broth, and whole-cell extracts were separated by SDS-PAGE. Proteins were then transferred to nitrocellulose, and filters were probed with polyclonal antisera specific for pneumolysin (Ply), choline binding protein A (CbpA), neuraminidase (NanB), autolysin (LytA), pneumococcal surface protein A (PspA), and pneumococcal surface antigen A (PsaA). No difference in labeling intensity was observed between the mutant and parent strains for any of the virulence proteins tested (result not shown).

DISCUSSION

At least three major classes of quorum-sensing molecules involved in cell density-dependent gene regulation have been described in bacteria. The first to be studied were the small peptide pheromones associated with development of genetic competence in gram-positive bacteria, most notably *S. pneumoniae* and *Bacillus subtilis* (37, 38, 39, 40). In the late 1970s, various acylhomoserinelactones were shown to be involved in quorum-sensing circuits in gram-negative bacteria, and studies by Bassler and colleagues elucidated the mechanism whereby these so-called class I autoinducers (AI-1) regulated luminescence in certain *Vibrio* spp. (3, 7, 16, 24, 25). Both the peptide pheromones and acylhomoserinelactones exhibit species specificity as far as cognate receptor interactions are concerned, and they are viewed as mechanisms whereby bacteria sense and respond to the presence of their own species in an environmental niche (12). More recently, an additional broader-spectrum quorum-sensing molecule, AI-2, has been described. AI-2 has been implicated in regulation of virulence gene expression in several pathogens on the basis of studies involving mutagenesis of *luxS*, a gene required for AI-2 biosynthesis. The presence of *luxS* homologues in both gram-positive and gram-negative bacteria implied that AI-2-mediated quorum-sensing circuits might play regulatory roles in many species, including virulence gene regulation in important pathogens. Thus, AI-2 antagonists capable of interfering with virulence gene expression might represent a novel class of antimicrobial agents. The significantly reduced ability of D39*luxS* culture supernatant relative to that of wild-type D39 to elicit bioluminescence in a *V. harveyi* AI-2 reporter strain clearly shows that *luxS* is important for the production of AI-2-like activity in *S. pneumoniae*.

S. pneumoniae is one of the foremost human pathogens, responsible for millions of deaths each year, and increasing rates of resistance to conventional antibiotics are a major concern. The *S. pneumoniae* genome contains a *luxS* homologue, and in the present study, we have examined the involvement of this gene in pathogenesis of disease in a mouse model with a view to assessing the potential of AI-2 antagonists for treatment of pneumococcal infections. The genetic organization of the *luxS* region of the pneumococcal chromosome is such that insertion-duplication mutagenesis of this gene is unlikely to result in polar effects on neighboring genes. Comparison of in vitro growth rates at differing starting densities and in either

serum broth or THY also did not reveal any differences between wild-type *S. pneumoniae* D39 and D39*luxS*, unlike the situation in *S. pyogenes* (20). Moreover, when administered intranasally, both the wild type and the mutant were capable of establishing colonization of the nasopharynx, and neither strain outcompeted the other in this niche. These findings indicate that mutation of *luxS* does not appear to have any detrimental effect on basic cellular metabolic processes required for growth in vitro or in the nasopharynx. However, unlike D39, D39*luxS* was not found in either the lungs or the blood of mice after intranasal challenge with the mixture of strains. This may be a consequence of inability to translocate from the nasopharyngeal niche or an inability to resist host defenses (e.g., polymorphonuclear leukocytes and alveolar macrophages) in the deeper tissues. Whether this defect is a direct consequence of inability to adjust gene expression in response to AI-2 is uncertain. Given that the wild type and mutant strains cocolonized the nasopharynx at similar densities, D39*luxS* may have been able to respond to exogenous AI-2 released from D39. However, when the strains were administered individually, the numbers of D39*luxS* and D39 organisms recovered from the nasopharynx were not significantly different.

The higher fitness of D39 relative to D39*luxS* was also evident when the two strains were coadministered intraperitoneally. The wild-type strain readily outcompeted the mutant, and after 24 h, only D39 could be found in the blood or spleen. Nevertheless, the numbers of D39*luxS* organisms in peritoneal washout, blood, and spleen increased by 48 h, although the numbers of D39 organisms were at least 10^5-fold higher at all three sites. The capacity of D39*luxS* to survive and proliferate after intraperitoneal challenge was not dependent on coadministration of D39, because all mice challenged with D39*luxS* alone died within 4 days. However, all mice challenged with the same dose of D39 succumbed within 28 h, indicating that the wild-type strain is significantly more virulent.

This is the first report that mutagenesis of *luxS* directly affects virulence of a gram-positive pathogen. A recent study by Lyon et al. (20) reported both positive and negative effects on expression of virulence-related genes in a *luxS* mutant of *S. pyogenes*, but whether these have a net impact on virulence in animal models is not known. In the present study, we did not observe differences in expression of any of six previously characterized virulence-related proteins, but both increases and decreases in levels of expression of at least 10 unidentified proteins in various cell fractions were evident from SDS-PAGE analysis of D39 and D39*luxS*. Some of these changes in protein expression may account for the altered virulence phenotype of D39*luxS*. Additional changes may also be detectable using higher-resolution separation protocols (e.g., two-dimensional gels) and more sensitive detection techniques such as silver staining. It also seems likely that proteomic analysis of the two strains will identify additional pneumococcal proteins directly involved in pathogenesis of disease. Notwithstanding the clear contribution of *luxS* to pneumococcal virulence, it is evident from this study that disruption of the gene does not completely attenuate *S. pneumoniae*. This unequivocal finding argues against the likely efficacy of AI-2 antagonists for treatment of pneumococcal disease.

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