The *luxS* Gene of *Streptococcus pyogenes* Regulates Expression of Genes That Affect Internalization by Epithelial Cells

Mehran J. Marouni¹ and Shlomo Sela^{2*}

Department of Human Microbiology, Sackler School of Medicine, Tel-Aviv University, Tel-Aviv,¹ and Department of Food Sciences, Agricultural Research Organization (ARO), The Volcani Center, Beth-Dagan,² Israel

Received 9 December 2002/Returned for modification 28 April 2003/Accepted 14 July 2003

The gram-positive pathogen *Streptococcus pyogenes* was recently reported to possess a homologue of the *luxS* gene that is responsible for the production of autoinducer 2, which participates in quorum sensing of both gram-positive and gram-negative bacteria. To test the effect of LuxS on streptococcal internalization, a LuxS mutant was constructed in strain SP268, an invasive M3 serotype. Functional analysis of the mutant revealed that it was internalized by HEp-2 cells with higher efficiency than the wild type (wt). Several genes, including *hasA* (hyaluronic acid synthesis), *speB* (streptococcal pyrogenic exotoxin B), and *csrR* (capsule synthesis regulator), a part of a two-component regulatory system, are known to affect the internalization of strain SP268 (J. Jadoun, O. Eyal, and S. Sela, Infect. Immun. 70:462–469, 2002). Therefore, the expression of these genes in the mutant and in the wt was examined. LuxS mutation significantly reduced the mRNA level of *speB* and increased the mRNA level of *emm3*. No substantial effect was observed on transcription of *hasA* and *csrR*. Yet less hyaluronic acid capsule was expressed in the mutant. Further analysis revealed that *luxS* is under the regulation of the two-component global regulator CsrR. Our results indicate that LuxS activity in strain SP268 plays an important role in the expression of virulence factors associated with epithelial cell internalization.

Many species of bacteria regulate gene expression in response to cell population density through a mechanism called quorum sensing (14, 20). Intercellular communication controls gene expression in response to population density (10, 40). Response to environmental stress enables bacteria to acquire an adaptive response to provide significant benefits in the colonization of hosts, defense against competitors, adaptation to varying physical conditions, cellular differentiation, and species evolution (36). Cell-cell communication in the quorum-sensing process involves the synthesis, secretion, and detection of extracellular signaling molecules termed autoinducers. When these molecules reach a critical threshold concentration within a population, a signal transduction cascade is triggered, which forms the basis for alterations in gene expression (2).

In gram-negative bacteria, autoinducers are known to be derivatives of *N*-acyl homoserine lactones, which diffuse freely in and out of cells and interact directly with intracellular regulatory proteins (2). In gram-positive bacteria, autoinducers are generally secreted peptides that are processed from larger propeptides. These peptide autoinducers function as ligands for signal receptors such as two-component membrane-bound sensor with histidine protein kinase activity (2, 21). Recently, another quorum-sensing system has been identified that produces the signaling molecule autoinducer-2 (AI-2) in *Vibrio harveyi* (40). This system is highly conserved in both gram-positive and gram-negative bacteria and is likely to be used for interspecies communication (1). AI-2 is detected by a sensory histidine kinase located within the cytoplasmic membrane (32), and its chemical structure has recently been predicted as a

furanone (33). A gene termed *luxS* has been identified that is essential for the production of AI-2 (39, 40). This gene is highly conserved among numerous bacterial species, including both gram-positive and gram-negative bacteria (24). In pathogenic bacteria, the AI-2 signaling system plays an important role in the regulation of virulence factors, including the type III secretion system in enterohemorrhagic *Escherichia coli* (37). However, the role of the AI-2 system in the regulation of virulence genes is still unclear in many pathogenic bacteria.

Group A streptococcus (GAS) is a major human pathogen that causes a wide array of diseases ranging from mild pharyngotonsillitis and pyoderma to severe and life-threatening infections. Poststreptococcal sequelae of acute rheumatic fever and glomerulonephritis might also be caused by this pathogen (8, 12). The apparent increase in incidences of severe invasive infections during the last 2 decades has promoted intensive research on GAS pathogenesis (8, 34, 38). Much knowledge has now been accumulated regarding adherence to, and internalization of, GAS by epithelial cells. Numerous surface-associated and secreted components have been implicated in GAS internalization, including M protein (4, 11, 17), hyaluronic acid (HA) capsule (18, 35, 43), and streptococcal pyrogenic exotoxin B (SpeB) (5, 7, 42).

Recently, the presence of a *luxS* homolog in GAS was reported by Lyon et al., and the expression of AI-2 was demonstrated (23). Lyon et al. have generated a mutation in *luxS* and found the aberrant expression of several virulence properties that are regulated in response to growth phase, including enhanced hemolytic activity linked with an increase in expression of the hemolysin S-associated gene (*sagA*) and a dramatic reduction in the expression, but not transcription, of secreted proteolytic activity SpeB. These findings suggest that *luxS* has a prominent role in the regulation of GAS virulence gene factors (23). Here we report on the role of *luxS* gene on

* Corresponding author. Mailing address: Dept. of Food Sciences, ARO, The Volcani Center, P.O.B. 6, Beth-Dagan 50250, Israel. Phone: 972-3-9683750. Fax: 972-3-9683750. E-mail: shlomos@volcani.agri.gov.il.

expression of virulence factors in GAS and the effect upon internalization by HEP-2 epithelial cells.

MATERIALS AND METHODS

Bacterial strains, plasmids, and tissue culture cells. GAS strain SP268 serotype M3 was originally isolated from a patient with an invasive disease (28). The *hasA* and *speB* isogenic mutants of SP268 were previously described (19). Streptococcal strains were grown in Todd-Hewitt broth (Difco Laboratories, Detroit, Mich.) supplemented with 0.2% yeast extract (THY). Strains were grown in standing culture overnight (16 h) at 37°C. *E. coli* strain DH5 α MCR (Life Technologies, Paisley, United Kingdom) was used for cloning procedures. Antibiotics were used at the following concentrations: for GAS, erythromycin (1 μ g/ml); for *E. coli*, erythromycin (500 μ g/ml). The epithelial cell line employed in this study was HEP-2 (human larynx). The cells were maintained in Dulbecco's modified Eagle medium containing 2 mM L-glutamine and 10% fetal calf serum (Biological Industries, Kibbutz Bet-HaEmek, Israel) and supplemented with 200 μ g of streptomycin/ml and 200 U of penicillin/ml.

DNA techniques. Purification of GAS chromosomal DNA and plasmid electroporation were performed as previously described (6). Restriction enzymes, T4-DNA ligase, and *Taq* polymerase were used according to the manufacturer's instructions (Fermentas Inc., Hanover, Md.). Purifications of plasmids and PCR-amplified fragments were performed with the Plasmid Midi kit (Qiagen Inc., Santa Clarita, Calif.) and High Pure PCR product purification kit (Boehringer Mannheim, GmbH, Mannheim, Germany), respectively.

Site-specific mutagenesis of the *luxS* gene. An in-frame deletion in *luxS* was constructed, as depicted in Fig. 1. DNA primers of the *luxS* gene were designed based on the *Streptococcus pyogenes* genome sequence. Primer pairs Pse-104–Pse-105 and Pse-106–Pse-107 were used to amplify a 1,412-bp DNA fragment consisting of the 5' region of *luxS* and a 533-bp fragment consisting of the 3' end of the gene, respectively (Table 1). Amplification was performed in a PCR machine (PTC-150, MiniCycler MJ RESEARCH) using a 5-min hot start and the following reaction conditions: 94°C for 1 min, 58°C for 1 min, and 72°C for 1 min for 33 cycles. The 1,412-bp DNA fragment was digested with *Bam*HI and *Xba*I, and the product was ligated to an *Xba*I/*Hind*III digest of the 533-bp DNA fragment. The ligated fragment, which contained an in-frame deletion of 325 bp in the *luxS* gene, was ligated to a *Bam*HI/*Hind*III digest of pJRS233 (kindly provided by J. Scott, Emory University, Atlanta, Ga.). This plasmid is a temperature-sensitive *E. coli* gram-positive shuttle vector that replicates in gram-positive bacteria at 30°C but not at 37°C (30). The recombinant plasmid pJRS233/*luxS* was purified from *E. coli* DH5 α MCR and transformed into GAS strain SP268 by electroporation. Integration of the plasmid into the SP268 chromosome and allelic replacement was selected essentially as described previously (18). The presence of a *luxS* deletion in 20 clones was verified by PCR analysis using primer pair Pse-111–Pse-112 (Table 1; Fig. 1). One clone harboring a smaller PCR product than the wild-type (wt) strain was selected for further analysis. Sequencing data of the amplified chromosomal gene confirmed the presence of the specific in-frame deletion of 325 bp within the *luxS* gene.

Transcription analysis. Total RNA was isolated from GAS strains as previously described (19). Briefly, 3 ml of bacterial culture from mid-log or stationary phase (overnight) were centrifuged, and the pellet was resuspended in 90 μ l of TE (10 mM Tris-HCl [pH 8] and 1 mM EDTA [pH 8]). Five microliters of 10% sodium dodecyl sulfate and 5 μ l of Tween lysing solution (2 ml of Tween 20, 5 ml of 1 M Tris-HCl [pH 8.0], 10 ml of 0.5 M EDTA [pH 8.0] and 83 ml of double-distilled water) were added, and the suspension was mixed and incubated at 65°C for 15 min. Total RNA was purified following the addition of TRI reagent according to the manufacturer's instructions (Molecular Research Center, Inc., Cincinnati, Ohio). The pellet (RNA) was immediately frozen in dry ice-acetone solution and was kept at –70°C until used.

DNA probes derived from internal fragments of *hasA*, *speB*, *csrR*, *emm3*, and *luxS* genes were PCR amplified from SP268 chromosome using primer pairs PhasA-F–PhasA-R, PspeB-F–PspeB-R, JL2F–JL20R, Pse-75–Pse-76, and Pse-111–Pse-112, respectively (Table 1). A probe derived from the housekeeping gene *rpsL* served as an internal control to assess the amount of total mRNA in the various samples, as described previously (13). The *rpsL* probe was amplified using PrpsIF and PrpsIR primers (Table 1). The probes were labeled by digoxigenin (DIG)-dUTP, according to the manufacturer's instructions (Boehringer Mannheim). For Northern blot hybridization, 5 μ g of total RNA was loaded and run on a formaldehyde gel, as described previously (32). The labeled DNA fragments were used to detect the corresponding transcripts following hybridization, according to the manufacturer's instructions (Roche). Visualization of hybridized mRNA bands was performed by adding the chemiluminescent substrate CSPD (Boehringer Mannheim) and exposing the membrane to Kodak film

FIG. 1. Construction of the *LuxS* mutant. Chromosomal DNA regions surrounding an internal DNA fragment of *luxS* were amplified by PCR, digested, and ligated to each other to generate a fragment with a 325-bp deletion within *luxS*. The fragment was then ligated to a *Bam*HI/*Hind*III digest of pJRS233 to create plasmid pJRS233 Δ *luxS*. The recombinant plasmid was then introduced into SP268 strain by electroporation. Homologous recombination and allelic replacement resulted in an in-frame chromosomal deletion in *luxS*. Arrows indicate primers used in the study (see details in Table 1). B, *Bam*HI; X, *Xba*I; H, *Hind*III. *, designation according to the complete genome sequence of *S. pyogenes* MGAS315 (accession number NC_004070).

(X-omat AR). To quantify the amount of specific mRNA transcripts, the intensity of the hybridized bands was measured by densitometry using Kodak 1D image analysis software (Eastman Kodak Co., Rochester, N.Y.). Intensities of hybridizing bands were quantified by densitometry, and the relative abundance of each specific message was calculated as the ratio of the message of interest to that of the fluorescence intensity of the 23S rRNA band obtained for the identical sample. Intensities of the transcripts derived only from exponential growth cultures were also compared to the intensity of the *rpsL*-hybridized band in each lane. Both quantification methods yielded similar results.

Quantification of cysteine protease activity. Cysteine protease activity was measured by the azocasein assay, as described previously (42). Briefly, 200 μ l of overnight culture supernatant was added to 400 μ l of a prewarmed reaction mixture (2.7 mg of azocasein per ml in 50 mM Tris-HCl [pH 8.0]), and the mixture was incubated at 37°C for 20 min. The reaction was stopped by addition of 100 μ l of 15% ice-cold trichloroacetic acid and incubation on ice for 15 min. After centrifugation, an equal volume of 0.5 M NaOH was added to the supernatant, and the absorbance was measured at 450 nm. In control experiments, 200 μ l of sterile THY medium was used. To verify that the protease activity measured was indeed due to cysteine protease activity, the effect of the specific

TABLE 1. List of oligonucleotide primers used in this study

Primer	Sequence	Location	Source or reference
Pse-75	ACGCTGCGACCAATTAACAACCTAGAGGAG	3' region of <i>emm3</i>	31
Pse-76	AACTGCAGAGCTTAGTCTTCTCTTTGCG	5' region of <i>emm3</i>	31
Pse-104	CGGGATCCTTGCTACCAGCTTGGTCATT	Upstream of <i>luxS</i> gene	This study
Pse-105	GCTCTAGACTGCACTAGGCGAACATCAA	5' of <i>luxS</i> gene	This study
Pse-106	GCTCTAGATCCTTTTAGTCGCCATGTCA	3' of <i>luxS</i> gene	This study
Pse-107	CCCAAGCTTGGATCAAGCAGCTCAAGGAC	Downstream of <i>luxS</i> gene	This study
Pse-111	TCAGCTATTACCGGCTCTTCT	Upstream of <i>luxS</i> gene	This study
Pse-112	TCAAGGCTGATGCTAATTTTG	Downstream of <i>luxS</i> gene	This study
PpUC-F	GTAAACGACGGCCAGTG	Upstream to MCS of pJRS233	
LMB-3	CAGGAACAGCTATGAC	Downstream to MCS of pJRS233	
PhasA-F	ACGTTATCGTTTACCCTTCCC	5' region of <i>hasA</i>	9
PhasA-R	GGGGCTGCAGAGTGACCTTTTACGTGCCCC	3' region of <i>hasA</i>	9
PspeB-F	GATCAAACTTTGCTCGTAACG	Upstream region of <i>speB</i>	42
PspeB-R	CTAAGGTTTGATGCCTACAACAG	Downstream region of <i>speB</i>	42
JL2F	TTGCAAGGGTTGTTTGATG	5' region of <i>csrR</i>	19
JL20R	GGCAATCAGTGTAAGGCAG	3' region of <i>csrR</i>	19
PrpsIF	GAATGTAGATGCCTACAATTAACCA	5' region of <i>rpsL</i>	13
PrpsIR	TTTACGACTCAATTTCTCTTTATCCC	3' region of <i>rpsL</i>	13

cysteine-protease inhibitor, E64, on the protease activity was tested. It was found that E64 completely inhibited the protease activity (data not shown).

HA determination. Bacteria were grown in THY broth (10 ml) to early logarithmic phase and centrifuged, and the pellet was resuspended in 0.5 ml of DDW. Cell-associated HA was extracted once with chloroform (1 ml) at room temperature for 1 h. Samples were then centrifuged, and the aqueous layer was taken for the determination of HA, as described previously (35). HA from *Streptococcus zooepidemicus* (General Biotechnology, Rehovot, Israel) was used as a standard.

Growth curves. One milliliter of an overnight culture of each strain was used to inoculate 50 ml of THY broth at 37°C. Samples (1 ml) were removed at hourly intervals, and the absorbance at 600 nm was determined by spectrophotometry (Pye Unicam, SP8-400).

Internalization assays. Internalization of GAS strains into epithelial cells was determined as described previously (17). Briefly, streptococci (10^6 CFU) suspended in RPMI-1640 devoid of serum were incubated with epithelial cells for 2 h at 37°C. Nonadherent bacteria were removed by washing with 1 ml of warm RPMI-1640 devoid of serum, and then extracellular adherent bacteria were killed by adding 1 ml of fresh RPMI-1640 supplemented with gentamicin (100 µg/ml) and penicillin (5 µg/ml) for 2 h. Control experiments using bacteria alone in RPMI-1640 or RPMI-1640 containing cell lysates verified that the indicated antibiotic concentrations killed 10^8 CFU/ml under the assay conditions. The infected monolayer was washed once with RPMI-1640 and lysed with ice-cold DDW (15 min at 4°C). Viable streptococci that survived antibiotic treatment were enumerated by plating serial dilutions (in duplicate) of cell lysate on Trypticase soy agar plates.

Statistical analysis. All assays were performed in triplicate and repeated at least three times on different days. Statistical analysis was performed by using Student's *t* test. *P* values were considered significant if *P* < 0.05.

RESULTS

Generation and characterization of an isogenic *luxS*-deficient mutant. A deletion mutant of strain SP268 was constructed by replacing the wt *luxS* allele with a disrupted allele containing a 325-bp in-frame deletion, as depicted in Fig. 1. The mutant colonies appeared to be smaller than the wt colonies on Trypticase soy agar plates (data not shown). Therefore, the growth rates of the wt and the mutant strains were studied. No difference in the growth rates was observed in THY broth medium (Fig. 2).

Uptake of *S. pyogenes* wt and LuxS mutant by epithelial cells. Antibiotic protection assays were used to compare the internalization capacity of the wt and isogenic LuxS mutant strains in a HEp-2 cell model. The results showed that the LuxS

mutant displayed significantly higher (about fivefold) internalization efficiency compared to the wt (Fig. 3).

Interactions between *luxS* and various *S. pyogenes* virulence genes. Since several streptococcal virulence genes have been previously implicated in internalization and a mutant deficient in LuxS expression had aberrant expression of several virulence properties (23), we have tested a possible effect of *luxS* mutation upon the transcription of some virulence genes previously associated with GAS internalization. Northern blot analysis showed that the *luxS* mutation had no effect on the transcription of *hasA* and resulted in a slight increase of *csrR* transcription (Fig. 4). However, the mutant transcribed significantly higher levels of *emm3* compared to the wt. In addition, the mutant transcribed significantly less (more than 10-fold) *speB* mRNA compared to the wt. It has been shown that CsrR affects the transcription of several virulence factors of *S. pyogenes*, including capsule and SpeB (13, 16, 19, 22). To test whether CsrR also regulates *luxS*, we have examined the transcription of the *luxS* gene in a CsrR-deficient mutant. Northern

FIG. 2. Growth curve of the LuxS mutant. Bacterial growth of wt (●) and mutant (◆) strains in THY broth was measured by recording the absorbance of the culture at 600 nm. The results of a single representative experiment are presented.

FIG. 3. Internalization of strain SP268 and its LuxS mutant. Bacteria were incubated for 2 h with the indicated epithelial cells, washed, and treated with antibiotics for another 2 h. Data are presented as the mean number of intracellular bacteria (CFU) per well that survived antibiotic treatment. The results are the averages of three independent experiments ($\pm$ standard errors of the mean). *, $P < 0.05$.

blot analysis of wt, LuxS⁻, and CsrR⁻ strains revealed that transcription of the *luxS* gene was much elevated in the CsrR mutant (Fig. 5), suggesting that *luxS* is repressed by CsrR. Interestingly, transcription of the truncated *luxS* was found to be much reduced in the LuxS mutant (Fig. 5).

Activity of SpeB and expression of capsule in the LuxS mutant. Augmentation of the internalization rate in GAS was previously shown to be associated with inactivation of *speB* (7, 19). Since the LuxS mutant transcribed significantly less *speB* mRNA, the elevated internalization rate of the mutant might be related to the lack of *speB* expression. Therefore, the SpeB cysteine protease activity of the wt and the LuxS mutant was examined. The cysteine-protease activity in the mutant was significantly lower than that of the wt and comparable to that of a SpeB-deficient mutant (Table 2).

FIG. 4. Transcription analysis of *hasA*, *emm3*, *speB*, and *csrR* genes. Shown are Northern blots containing 5 μ g of total RNA derived from the indicated strains, hybridized with DIG-labeled DNA probes specific for internal parts of the tested genes. Numbers represent relative abundance of the indicated message of the mutant strains and reflect the ratio of the transcript of interest relative to that of the 23S rRNA. The ratios for the mutant strains are shown compared with the wt, which has been assigned a value of 1.0.

FIG. 5. Transcription analysis of the *luxS* gene. A Northern blot containing 5 μ g of total RNA of the indicated strains was hybridized with DIG-labeled DNA probes specific for the *luxS* gene. Numbers represent relative abundance of the indicated message of the mutant strains and reflect the ratio of the transcript of interest relative to that of the 23S rRNA. The ratios for the mutant strains are shown compared with the wt strain, which has been assigned a value of 1.0.

It has been demonstrated that GAS internalization is hindered by HA capsule (18, 19, 35). As shown in Table 2, the LuxS mutant expressed about 50% less cell-associated HA than the wt. This may suggest that capsule expression is controlled at a posttranscription level by LuxS and that a lower amount of capsule in the mutant has contributed to the higher internalization rate of the mutant.

DISCUSSION

Conserved *luxS* homologs exist in over 30 species of both gram-negative and gram-positive bacteria, and it has been proposed that the AI-2 system plays an important role in interspecies communication (2, 40). Recently, Lyon et al. have shown that AI-2 is also expressed in *S. pyogenes* HSC5, an M6 serotype (23). An insertion mutation in *luxS* was reported to reduce the secretion and protease activity (but not gene transcription) of SpeB and to increase expression of the hemolysin S-associated gene *sagA* (23). In this study we have examined the role of LuxS in the internalization of an M3 invasive serotype (SP268) by HEp-2 cells. We have constructed an isogenic mutant harboring an in-frame deletion within the *luxS* gene. The mutant had a growth rate similar to that of the wt; however, transcription of the *emm3* gene was greatly increased. In contrast, transcription of the *speB* gene and the truncated *luxS* was much reduced. The effect of the mutation on the transcription of the truncated gene could be attributed simply to insta-

TABLE 2. Relative amounts of cell-associated HA capsule and cysteine protease activity of SP268 and its isogenic mutants

Strain	Amt of HA ^a	Cysteine protease activity ^a
SP268 (wt)	100 $\pm$ 14.3	100 $\pm$ 5.1
LuxS ⁻	52.3 $\pm$ 10.9 ^b	16 $\pm$ 2.7 ^b
SpeB ⁻	ND ^c	3.2 $\pm$ 1.9 ^b
HasA ⁻	2.7 $\pm$ 0.45 ^b	ND

^a The amounts of cell-associated capsule or cysteine protease activity were measured as described in Materials and Methods. Data are presented as the percent of the cell-associated capsule (fg/CFU) or cysteine protease activity of the control (SP268) $\pm$ standard error of the mean.

^b Statistically significant difference compared to SP268 ($P < 0.05$).

^c ND, not determined.

FIG. 6. A schematic model showing the interactions between *luxS*, CsrR, SpeB, capsule, and M3 protein. Positive and negative effect are indicated by + and –, respectively. Solid lines represent effect on gene expression, and lines with dashes indicate effect on internalization. CsrR down-regulates the expression of capsule and transcription of *luxS*. In contrast, it activates expression of SpeB. AI-2, the product of the LuxS pathway, activates the expression of SpeB and capsule. On the other hand, AI-2 negatively regulates the expression of M3 protein. Both capsule and SpeB negatively affect GAS internalization (5, 19, 35, 42), while expression of M3 protein enhances uptake by epithelial cells (4). The outlined interactions between CsrR, capsule, and SpeB are based on previous reports (13, 16, 18, 19, 22).

bility of the mutated gene. Alternatively, this finding might reflect an autoregulation by the LuxS product.

Our findings contradict those of Lyon et al. in two points. First, these authors have reported that a LuxS mutation resulted in a 30% reduction in growth rate in the same medium as we have used (THY), while we did not observe such a difference. Interestingly, Lyon et al. found no such difference when the strains were grown in C medium, a peptide-rich, carbohydrate-poor medium. It is unlikely that the nature of the *luxS* mutation in the two studies affected these results, since we have generated an in-frame deletion within the *luxS* gene, while Lyon et al. have constructed an insertion mutation. However, a control mutant that harbored insertion downstream to *luxS* had a phenotype similar to the wt, negating the possibility of a polar effect (23). Therefore, the dissimilar results might be strain specific. Second, a defect in SpeB expression was re-

ported that was not associated with impaired *speB* transcription (23), while we found that reduction in SpeB expression was related to decreased transcription of the gene (Fig. 4). This disparity might be due to the growth medium used to cultivate the bacterium for growing the GAS prior to the preparation of RNA for Northern blot analysis or for SpeB determination. We have employed THY medium, while C medium, an optimal medium to support high-level expression of the GAS cysteine protease, was used by Lyon et al. Furthermore, in the study of Lyon et al., mRNA for transcription analysis of *SpeB* was isolated from GAS following growth to early stationary phase (4.5 h), while we isolated the mRNA at late stationary phase (overnight 16 h) of GAS. Taken together, it is possible that the growth phase and culture environment utilized in the two studies have differently affected *speB* regulation.

Although transcription of the *hasA* gene was not affected in the mutant, it expresses about 50% of the amount of capsule expressed in the wt. The latter observation might indicate that LuxS is also responsible for posttranscriptional control of capsule expression, which might explain the small colony phenotype observed in the mutant.

It might be hypothesized that the effect of LuxS on the expression of SpeB, M3, and capsule is responsible for the increased internalization rate observed in the mutant. Still other, yet-unknown *luxS*-regulated genes might contribute to this effect. One might also suggest that the LuxS mutant replicates at a higher growth rate than the wt during the internalization assay, which may have a significant impact on interpretation of the internalization data. However, it was previously found that strain SP268 could not replicate in cell culture medium (RPMI-1640) devoid of serum, thus negating this possibility (24). Still, it has recently been reported that GAS could replicate within HEp-2 cells (27). Although we have no indication for such a phenomenon in the SP268 strain, we cannot rule out the possibility that the *luxS* mutation has affected intracellular replication.

Both capsule SpeB and M3 proteins were previously implicated in GAS internalization (4, 5, 18, 19, 35, 42). Capsule and SpeB impede bacterial uptake (5, 18, 19, 35, 42), while expression of M3 protein promotes internalization (4). SpeB is a cysteine protease that could cleave host cell molecules, such as fibronectin (8), and release several bacterial surface proteins, including C5a peptidase, and M1 protein (3). Previous studies have demonstrated that isogenic *speB* mutants were internalized better than their wt strains by both endothelial and epithelial cells (5, 19). Since both fibronectin (17, 24, 29, 41) and M3 protein (4) were shown to promote GAS internalization, it is possible that the lack of active SpeB in the LuxS mutant enables intact surface molecules, such as M3 protein and fibronectin-binding molecules, to mediate internalization. Thus, it might be concluded that expression of LuxS directly or indirectly affects the expression of a variety of surface determinants that are also involved in GAS internalization. Unlike other strains, SP268 does not express the Fn-binding protein, PrtF1/SfbI (28), previously associated with GAS internalization (17, 26), and the nature of its fibronectin-binding molecules remains unknown.

In order to adapt to and survive at various niches in the human host, GAS must be able to finely regulate gene expression. It has recently been reported that inactivation of a re-

sponse regulator of a two-component regulatory system, CsrRS (recently renamed CovRS, for control of virulence) (13), represses transcription of HA capsule (16, 19, 22) and expression of SpeB (16, 19), which are two principle virulence factors that also affect SP268 internalization (19). A recent genome scale analysis revealed that CsrR (CovR) controls, directly and indirectly, the expression of as much as 15% of all GAS chromosomal genes (15). However, the sequence corresponding to the *luxS* gene was not found among them (15). This might be explained by difference in times where mRNA was extracted (16 h in our study, compared to 10 h). In addition, a low abundance of the *luxS* transcript in the wt strain and a higher sensitivity of the Northern blot analysis might have influenced these results.

Finally, our results suggest that CsrR also affects the transcription of *luxS*; therefore, even though this finding has not been reported by Graham et al., some of the CsrR-regulated genes in fact might be controlled at least partially by the AI-2 quorum-sensing system. The majority of bacterial functions controlled by LuxS in pathogenic and nonpathogenic species have not yet been identified (2). Our results provide further insight regarding the role of LuxS in the global network regulatory system of GAS, which regulates, among other things, the expression of major virulence factors and epithelial cell internalization. The current knowledge regarding the interactions between *luxS*, CsrR, SpeB, capsule, and M3 and their effects upon GAS internalization is summarized in Fig. 6.

ACKNOWLEDGMENTS

We thank J. Scott (Emory University, Atlanta, Ga.) for providing pJRS233 plasmid.

This study was supported in part by a grant from the Chief Scientist's Office of the Ministry of Health, Israel, and by a grant from the Israel Science Foundation awarded to S. Sela.

REFERENCES

- Bassler, B. L., E. P. Greenberg, and A. M. Stevens. 1997. Cross-species induction of luminescence in the quorum-sensing bacterium *Vibrio harveyi*. *J. Bacteriol.* **179**:4043–4045.
- Bassler, B. L. 1999. How bacteria talk to each other: regulation of gene expression by quorum sensing. *Curr. Opin. Microbiol.* **2**:582–587.
- Berge, A., and L. Björck. 1995. Streptococcal cysteine proteinase releases biologically active fragments of streptococcal surface proteins. *J. Biol. Chem.* **270**:9862–9867.
- Berkower, C., M. Ravins, A. E. Moses, and E. Hanski. 1999. Expression of different group A streptococcal M proteins in an isogenic background demonstrates diversity in adherence to and invasion of eukaryotic cells. *Mol. Microbiol.* **31**:1463–1475.
- Burns, E. H., Jr., S. Lukowski, J. Rurangirwa, A. Podbielski, and J. M. Musser. 1998. Genetic inactivation of the extracellular cysteine protease enhances in-vitro internalization of group A streptococci by human epithelial and endothelial cells. *Microb. Pathog.* **24**:333–339.
- Caparon, M. G., and J. R. Scott. 1991. Genetic manipulation of pathogenic streptococci. *Methods Enzymol.* **204**:556–586.
- Chaussee, M. S., R. L. Cole, and J. P. Van-Putten. 2000. Streptococcal erythrogenic toxin B abrogates fibronectin-dependent internalization of *Streptococcus pyogenes* by cultured mammalian cells. *Infect. Immun.* **68**:3226–3232.
- Cunningham, M. W. 2000. Pathogenesis of group A streptococcal infections. *Clin. Microbiol. Rev.* **13**:470–511.
- DeAngelis, P. L., J. Papaconstantinou, and P. H. Weigerl. 1993. Molecular cloning, identification, and sequence of the hyaluronan synthase gene from group A *Streptococcus pyogenes*. *J. Biol. Chem.* **268**:19181–19184.
- De Kievit, T. R., and B. H. Iglewski. 2000. Bacterial quorum sensing in pathogenic relationships. *Infect. Immun.* **68**:4839–4849.
- Dombeck, P. E., D. Cue, J. Sedgewick, H. Lam, S. Ruschkowski, B. Finlay, and P. P. Cleary. 1999. High-frequency intracellular invasion of epithelial cells by serotype M1 group A streptococci: M1 protein mediated invasion and cytoskeletal rearrangements. *Mol. Microbiol.* **31**:859–870.
- Efstathiou, A. 2000. Group A streptococci in the 1990s. *J. Antimicrob. Chemother.* **45**:S3–S12.
- Federle, M. J., K. S. McIver, and J. R. Scott. 1999. A response regulator that represses transcription of several virulence operons in the group A streptococcus. *J. Bacteriol.* **181**:3649–3657.
- Fuqua, W. C., S. C. Winans, and E. P. Greenberg. 1994. Quorum sensing in bacteria: the LuxR-LuxI family of cell density-responsive transcriptional regulators. *J. Bacteriol.* **176**:269–275.
- Graham, M. R., L. M. Smoot, C. A. Lux, Migliaccio, K. Virtaneva, D. E. Sturdevant, S. F. Porcella, M. J. Federle, G. J. Scott, and J. M. Musser. 2002. Virulence control in group A streptococci by a two-component gene regulatory system: global expression profiling and *in vivo* infection modeling. *Proc. Natl. Acad. Sci. USA* **99**:13855–13860.
- Heath, A., V. J. DiRita, N. L. Barg, and N. C. Engleberg. 1999. A two-component regulatory system, CsrR-CsrS, represses expression of three *Streptococcus pyogenes* virulence factors, hyaluronic acid capsule, streptolysin S, and pyrogenic exotoxin B. *Infect. Immun.* **67**:5298–5305.
- Jadoun, J., V. Ozeri, E. Burstein, E. Skutelsky, E. Hanski, and S. Sela. 1998. Protein F1 is required for efficient entry of *Streptococcus pyogenes* into epithelial cells. *J. Infect. Dis.* **178**:147–158.
- Jadoun, J., and S. Sela. 2000. Mutation in CsrR global regulator reduces *Streptococcus pyogenes* internalization. *Microb. Pathog.* **29**:311–317.
- Jadoun, J., O. Eyal, and S. Sela. 2002. Role of CsrR, hyaluronic acid, and SpeB in the internalization of *Streptococcus pyogenes* M type 3 by epithelial cells. *Infect. Immun.* **70**:462–469.
- Kleerebezem, M., L. E. Quadri, O. P. Kuipers, and W. M. De Vos. 1997. Quorum sensing by peptide pheromones and two-component signal-transduction systems in gram-positive bacteria. *Mol. Microbiol.* **24**:895–904.
- Lazazzera, B. A., and A. D. Grossman. 1998. The ins and outs of peptide signaling. *Trends Microbiol.* **6**:288–294.
- Levin, J. C., and M. R. Wessels. 1998. Identification of *csrR/csrS*, a genetic locus that regulates hyaluronic acid capsule synthesis in group A streptococci. *Mol. Microbiol.* **30**:209–219.
- Lyon, W. R., J. C. Madden, J. Stein, and M. G. Caparon. 2001. Mutation of *luxS* affects growth and virulence factors expression in *Streptococcus pyogenes*. *Mol. Microbiol.* **42**:145–157.
- Marouni, M. J., A. Barzilai, N. Keller, E. Rubinstein, and S. Sela. Intracellular survival of persistent group A streptococci in cultured epithelial cells. *Int. J. Med. Microbiol.*, in press.
- Miller, M. B., and B. L. Bassler. 2001. Quorum sensing in bacteria. *Annu. Rev. Microbiol.* **55**:165–199.
- Molinari, G., S. R. Talay, P. Valentin-Weigand, M. Rohde, and G. S. Chhatwal. 1997. The fibronectin-binding protein of *Streptococcus pyogenes*, SfbI, is involved in the internalization of group A streptococci by epithelial cells. *Infect. Immun.* **65**:1357–1363.
- Molinari, G., M. Rohde, C. A. Guzman, and G. S. Chhatwal. 2000. Two distinct pathways for the invasion of *Streptococcus pyogenes* in non-phagocytic cells. *Cell. Microbiol.* **2**:145–154.
- Natanson, S., S. Sela, A. E. Moses, J. M. Musser, M. G. Caparon, and E. Hanski. 1995. Distribution of fibronectin-binding proteins among group A streptococci of different M types. *J. Infect. Dis.* **171**:871–878.
- Okada, N., I. Tatsuno, E. Hanski, M. Caparon, and C. Sasakawa. 1998. *Streptococcus pyogenes* protein F promotes invasion of HeLa cells. *Microbiology* **144**:3079–3086.
- Perez-Casal, J., J. A. Price, E. Maguin, and J. R. Scott. 1993. An M protein with a single C repeat prevents phagocytes of *Streptococcus pyogenes*: use of a temperature-sensitive shuttle vector to deliver homologous sequences to the chromosome of *S. pyogenes*. *Mol. Microbiol.* **8**:809–819.
- Podbielski, A., B. Melzaer, and R. Luttkicken. 1991. Application of the polymerase chain reaction to study the M protein (-like) gene family in beta-hemolytic streptococci. *Med. Microbiol. Immunol.* **180**:213–227.
- Sambrook, J., E. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
- Schauder, S., K. Shokat, M. G. Surette, and B. Bassler. 2001. The LuxS family of bacterial autoinducers: biosynthesis of a novel quorum-sensing signal molecule. *Mol. Microbiol.* **41**:463–476.
- Schlievert, P. M., A. P. Assimacopoulos, and P. P. Cleary. 1996. Severe invasive group A streptococcal disease: clinical description and mechanisms of pathogenesis. *J. Lab. Clin. Med.* **127**:13–22.
- Schrager, H. M., J. G. Rheinwald, and M. R. Wessels. 1996. Hyaluronic acid capsule and the role of streptococcal entry into keratinocytes in invasive skin infection. *J. Clin. Invest.* **98**:1954–1958.
- Shapiro, J. A. 1998. Thinking about bacterial populations as multicellular organisms. *Annu. Rev. Microbiol.* **52**:81–104.
- Sperandio, V., J. L. Mellies, W. Nguyen, S. Shin, and J. B. Kaper. 1999. Quorum sensing controls expression of the type III secretion gene transcription and protein secretion in enterohemorrhagic and enteropathogenic *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **96**:15196–15201.
- Stevens, D. L. 1999. The flesh eating bacterium: what's next? *J. Infect. Dis.* **179**:S366–S374.

39. Surette, M. G., and B. Bassler. 1999. Regulation of autoinducer production in *Salmonella typhimurium*. *Mol. Microbiol.* **31**:585–595.
40. Surette, M. G., M. B. Miller, and B. Bassler. 1999. Quorum sensing in *Escherichia coli*, *Salmonella typhimurium*, and *Vibrio harveyi*: a new family of genes responsible for autoinducer production. *Proc. Natl. Acad. Sci. USA* **96**:1639–1644.
41. Terao, Y., S. Kawabata, E. Kunitomo, J. Murakami, I. Nakagawa, and S. Hamada. 2001. Fba, a novel fibronectin-binding protein from *Streptococcus pyogenes*, promotes bacterial entry into epithelial cells, and the *fba* gene is positively transcribed under the Mga regulator. *Mol. Microbiol.* **42**:75–86.
42. Tsai, P. J., C. F. Kuo, K. Y. Lin, Y. S. Lin, H. Y. Lei, F. F. Chen, J. R. Wang, and J. J. Wu. 1998. Effect of group A streptococcal cysteine protease on invasion of epithelial cells. *Infect. Immun.* **66**:1460–1466.
43. Wessels, M. R., J. B. Goldberg, and A. E. Moses. 1994. Effects on virulence of mutations in a locus essential for hyaluronic acid capsule expression in group A streptococci. *Infect. Immun.* **62**:433–441.

Editor: V. J. DiRita