*Oral Microbiology Immunology 2008: 23: 79–83*
*Printed in Singapore. All rights reserved*



ORAL MICROBIOLOGY
AND IMMUNOLOGY

Short communication

# LuxS and expression of virulence factors in *Streptococcus intermedius*

**D. Pecharki, F. C. Petersen, A. Aa. Scheie**
Department of Oral Biology, Faculty of Dentistry, University of Oslo, Oslo, Norway

*Pecharki D, Petersen FC, Scheie AAa. LuxS and expression of virulence factors in* Streptococcus intermedius.
*Oral Microbiol Immunol 2008: 23: 79–83.* © 2008 The Authors. Journal compilation © 2008 Blackwell Munksgaard.

**Background/aims:** Autoinducer-2 (AI-2) is used by several bacteria in quorum-sensing signaling and is a product of LuxS. The aim was to investigate the effect of LuxS mutation on expression of *Streptococcus intermedius* virulence factors.
**Methods:** *S. intermedius* mutants were constructed by insertion inactivation or gene deletion. Real time RT-PCR was used to assess transcription of *pas*, *ily* and *hyl*. Hyaluronidase and intermedilysin activities were measured biochemically.
**Results:** The results indicated that disruption of *luxS* in *S. intermedius* may affect hyaluronidase and intermedilysin gene expressions. No difference in antigen I/II expression was observed. Biochemical methods showed a five-fold decrease in hemolytic activity of the *luxS* mutant; however, secreted hyaluronidase activity was unaffected. The AI-2 precursor 4,5-dihydroxy-2,3-pentanedione complemented lack of AI-2 production by the mutant thus restoring hemolytic activity.
**Conclusions:** We suggest that AI-2 communication is involved in intermedilysin expression.

Key words: antigen I/II; autoinducer-2; hyaluronidase; intermedilysin; LuxS; *Streptococcus intermedius*

D. Pecharki, Postboks 1052 Blindern, 0316 Oslo, Norway
Tel.: +47 22840352;
fax: +47 22840302;
e-mail: ddasilva@odont.uio.no
Accepted for publication May 7, 2007

*Streptococcus intermedius* is not only a member of the commensal oral, gastrointestinal, and urinary floras, but may also be associated with deep-seated purulent infections, particularly in the brain and liver (3, 37). In the oral cavity, *S. intermedius* is mainly found in biofilms on teeth, but may be associated with periodontal disease and implantitis (33).

The autoinducer-2 (AI-2) signals produced by several gram-positive bacteria, including *S. intermedius* (20), are thought to mediate both intra- and inter-species communication (35, 39). DPD (4,5-dihydroxy-2,3-pentanedione), a product of LuxS, spontaneously cyclises to form AI-2 (26). The AI-2 molecules in different species are not necessarily identical, but are most likely derived from the same precursor, DPD (16). The structure of AI-2 produced by streptococci is presently unknown. AI-2 triggers gene regulatory cascades and may be involved in biofilm formation and virulence (35, 39). A number of proteins produced by *S. intermedius*, including antigen I/II (Ag I/II), hyaluronidase, and intermedilysin, have been implicated in its virulence and pathogenesis (8, 17, 31).

Ag I/II and hyaluronidase contain the LPXTG cell-wall anchoring motif. Both proteins are expressed extracellularly as well as on the cell surface. Ag I/II is a family of antigenically related proteins found in several streptococci (8); they have received a variety of names according to the species in which they were identified. In *S. intermedius* the protein is Pas with the coding gene *pas* (32). Ag I/II shows multifunctional activities, including binding to soluble extracellular matrix glycoproteins and host cell receptors, coaggregation with other bacteria, interactions with salivary glycoproteins, activation of monocytic cells, and biofilm formation (8, 19). In *Streptococcus mutans*, inactivation of *luxS* resulted in a mutant with reduced expression of the Ag I/II gene (15). Whether LuxS plays a role in Ag I/II gene expression in *Streptococcus intermedius* is unknown.

Hyaluronidase breaks down hyaluronan, a component found in virtually all tissues. Bacterial hyaluronidase acts as endo-*N*-acetylhexosaminidase by breaking the β-1-4 linkage of hyaluronan, with unsaturated disaccharides as the final products (12). Since hyaluronan is a major constituent of the ground substance of most connective tissues, its destruction facilitates the spread

of toxins from the primary infection site. Simultaneously, the disaccharides liberated may be transported and metabolized intracellularly by *S. intermedius* (6).

Another virulence factor of *S. intermedius* is the toxin called intermedilysin. Intermedilysin is a member of the cholesterol-dependent cytolysins, a large family of structurally related pore-forming toxins (23, 34). Structural analysis has indicated that intermedilysin binds to human CD59 (5), and that cholesterol is necessary for its insertion, but not necessarily at the stage of receptor binding (4). Intermedilysin is found extracellularly and bound to the bacterial surface, presumably because of the interaction of the strongly basic part with the negatively charged teichoic acid on the bacterial cell wall (28). Unlike other cytolysins, intermedilysin targets exclusively human cells (14, 17, 23). Intermedilysin from *S. intermedius* causes direct cell membrane damage in human erythrocytes and in cell lines from major organs (17, 23). Interestingly, cytolysin expression seems to be influenced by AI-2 communication in *Streptococcus pneumoniae* (9) and *Streptococcus pyogenes* (13).

The aim of this study was to investigate whether expression of *S. intermedius* virulence factors, including Ag I/II, hyaluronidase, and intermedilysin, might be regulated by LuxS. Analyses using real time reverse transcription-polymerase chain reaction (RT-PCR) demonstrated that disruption of *luxS* in *S. intermedius* resulted in a mutant with reduced transcription of the intermedilysin gene *ily* and the hyaluronidase gene *hyl*, whereas no significant differences were observed in the Ag I/II gene *pas*. Hyaluronidase activity, measured in the supernatants, was not affected in the *luxS* mutant. The ability of *S. intermedius* to lyse erythrocytes was reduced in the *luxS* mutant, consistent with the observed effect on *ily* expression. Synthetic AI-2 in the form of DPD restored the hemolytic activity in the *luxS* mutant.

*Table 1.* Strains and primer sequences used in this study

| Strain or primer | Relevant genotype or primer sequence (5′ to 3′)[1] | Reference or primer purpose |
|---|---|---|
| *S. intermedius* | | |
| 11324 | NCTC | NCTC |
| SI006 | NCTC 11324 *luxS′*::pSF151::*′luxS* | (20) |
| SI007 | NCTC 11324 Δ*luxS*::Pc Em | This study |
| SI008 | NCTC 11324 Δ*ily*::Pc Kan | This study |
| *V. harveyi* BB170 | luxN::Tn5, AI-1 sensor$^-$, AI-2 sensor$^+$, reporter strain | (2, 30) |
| Primers | | |
| SI007 construction | | |
| FP195 | CTCCGCTTGTCCACGTAAAT | Upstream flanker |
| FP196 | AGG/CGCGCCCCCAGCAGTTGGGATAGAAC | Upstream flanker |
| FP197 | AGGCCGG/CCCTGCCGGACTAGATTTCACA | Downstream flanker |
| AS016 | GGAGCATTTGATTATTTGATTCGTCCA | Downstream flanker |
| FP015 | GG/CGCGCCCCGGGCCCAAAATTTGTTTGAT | ermAM cassette |
| FP016 | GGCCGG/CCAGTCGGCAGCGACTCATAGAAT | ermAM cassette |
| FP017 | TTTTGTTCATGTAATCACTCCTTC | confirm insertion |
| FP018 | CACGCCAAAGTAAACAATTTAAG | confirm insertion |
| SI008 construction | | |
| FP189 | TCCATCTAACTCTTATCCCCAAA | Upstream flanker |
| FP190 | AGG/CGCGCCTGCAGCTTCAGAGTTGCTGT | Upstream flanker |
| FP191 | AGGCCGG/CCAGCCACTGGACTAGCTTGG | Downstream flanker |
| FP192 | GGGAGAACCCACAGGTCTTT | Downstream flanker |
| FP001 | AGG/CGCGCCGTTTGATTTTTAATG | Kan cassette |
| FP068 | AGGCCGG/CCTAGGTACTAAAACAATTCATCCAGTA | Kan cassette |
| FP037 | TCATTTTCTCCCACCAGCTT | confirm insertion |
| FP038 | GCGCCTACGAGGAATTTGTA | confirm insertion |
| Real-time RT-PCR | | |
| FP110 | TGCTCATGAGGCAGAAGTTG | *pas* amplification |
| FP111 | TGCTTTACTGGCAGCATTTG | *pas* amplification |
| FP112 | TGCTGAAAAAGTGCAACAGG | *hyl* amplification |
| FP113 | ATCAAGCCAAGCATTCCATC | *hyl* amplification |
| FP114 | TTAGCACTTGGGGAACAACC | *ily* amplification |
| FP115 | TGCGAAGATTCAAGGCTTCT | *ily* amplification |
| FP116 | TGAAGAAGGTTTTCGGATCG | 16S rRNA amplification |
| FP117 | CGCTCGGGACCTACGTATTA | 16S rRNA amplification |

[1]Restriction sites are underlined: *Asc*I (GG/CGCGCC), *Fsc*I (GGCCGG/CC).

## Bacterial strains and growth conditions

The *S. intermedius* strains used in this study were the type strain NCTC 11324 and its derivatives (Table 1). The sequences of the oligonucleotide primers used are listed in Table 1.

Mutants with disruption of *luxS* were constructed by insertion–inactivation as previously described for *Streptococcus anginosus* (20). The PCR ligation mutagenesis strategy, in which two PCR amplified flanking sequences of the gene are ligated to a resistance cassette, was used for gene deletion (10). Briefly, *Asc*I or *Fse*I restriction sites were incorporated into the oligonucleotide primers used to generate the flanking DNA fragments and the resistance cassette (Table 1). The erythromycin cassette (ermAM) for *luxS* disruption was amplified from PcEm (11) with the primer pair FP015-FP016. The kanamycin-resistance cassette (Kan cassette) used for *ily* disruption was PCR amplified from plasmid pR410 (29) with the primer pair FP001-FP068 (PcEm and pR410 were a kind gift from D. A. Morrison). The primer pairs FP195-FP196 and FP197-AS016 were used to amplify the upstream and downstream flanking regions of *luxS*, respectively. The *ily* flanking regions were amplified by the primer pairs FP189-FP190 and FP191-192. After restriction enzyme digestion with *Asc*I and *Fse*I, the upstream or downstream amplicons were ligated to the respective cassettes in two separate reactions. The two ligation products were then mixed and PCR-amplified with the distal primers FP195-AS016 for *luxS* and FP189-FP192 for *ily.* The resultant amplicons were used to transform *S. intermedius* NCTC 11324, as described below. The gene deletion in each mutant was confirmed by PCR with distal primers and internal primers for the Kan cassette or ermAM. The primer pairs FP195-FP018 and FP017-AS016 were used for confirmation of the *luxS* deletion, and the primer pairs FP189-FP038 and FP037-FP192 were used for confirmation of the *ily* deletion. For gene disruption, the cells were grown in Todd-Hewitt broth (THB) with 2.5% horse serum, and transformation was induced by the competence-stimulating peptide CSP 11325 as previously described (21). Transformants were selected by growth on THB agar plates containing the appropriate selective antibiotics.

The strains were stored at −70°C in brain-heart infusion broth (Difco Laboratories, Detroit, MI) supplemented with 15%

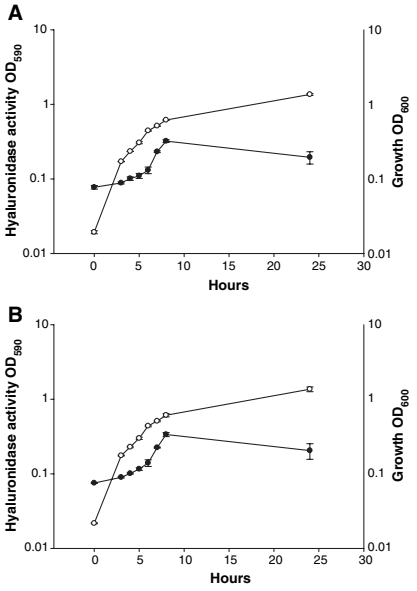


*Fig. 1. Streptococcus intermedius* wild type (A) and *luxS* mutant (B) hyaluronidase activity and growth followed during 24 h. Hyaluronidase activity was determined as the concentration of reducing *N*-acetyl-D-glucosamine ends generated from hyaluronan hydrolysis by measuring $OD_{590}$ (●). Growth was monitorated by measuring $OD_{600}$ (○). The results represent mean values and standard errors of three independent experiments with three parallels each.

(volume/volume; V/V) glycerol. Incubations were at 37°C in a 5% $CO_2$ aerobic atmosphere. Before each experiment the cells were grown on THB agar plates for 48 h. *S. intermedius* colonies were then transferred to trypticase soy broth (TSB; Difco Laboratories) and incubated for 24 h. For the *luxS* mutant SI006, kanamycin was used at a final concentration of 500 μg/ml in the first overnight cultures, whereas no antibiotic was used for the deletion mutants SI007 and SI008. *S. intermedius* wild type and *luxS* mutants from first overnight cultures were diluted 1 : 200 in fresh TSB and grown once more overnight without antibiotics. The cells were then diluted 1 : 100 in TSB and supernatant and cells were collected at various time-points as specified below. Inactivation of the *luxS* gene in *S. intermedius* had no effect on growth compared with the wild type (Fig. 1A,B).

To assess the AI-2 activity over time, supernatants were collected at various time-points and assayed for bioluminescence as described by Surette and Bassler (30), except that frozen *Vibrio harveyi* BB170 aliquots were used. The highest AI-2 activity for *S. intermedius* in this study was found in supernatants collected between 4 and 6 h (Fig. 2).

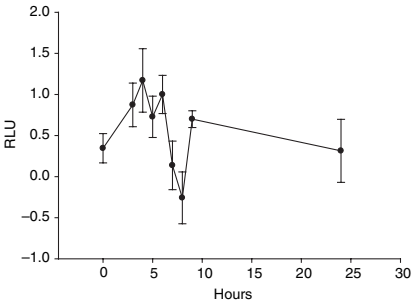


*Fig. 2.* Detection of streptococcal AI-2 production by measuring the level of bioluminescence induced in *Vibrio harveyi* BB170. Relative change in bioluminescence by the *Streptococcus intermedius* wild type was calculated by subtracting the luminescence values obtained with *S. intermedius* wild type and *luxS* mutant during 24 h of growth. The AI-2 activity at 6 h was normalized to 1. Results are mean values and standard errors of two parallels from three independent experiments.

## Transcription of intermedilysin and hyaluronidase, but not Ag I/II gene, is downregulated in the *luxS* mutant

It has been shown that transcription of virulence factors may be regulated by *luxS* in several bacteria, including streptococci (9, 13, 27, 36). To assess the impact of *S. intermedius* LuxS on *hyl*, *pas*, and *ily* expression, we compared the expression profiles of a wild-type strain and its *luxS* isogenic mutant SI006 by real-time RT-PCR. Total RNA from *S. intermedius* wild type and the *luxS* mutant SI006 was extracted at early-growth-phase (approximately 4 h) with values between 0.2 and 0.3 for optical density measured at 600 nm ($OD_{600}$). The High pure RNA isolation kit (Roche Diagnostics GmbH, Mannheim, Germany) was used according to the manufacturer's recommendation, except that the cells were incubated at 37°C for 30 min in 100 μl lysis buffer containing 20 mg/ml lysozyme and 100 U mutanolysin. RNA concentration was adjusted to 100 ng/μl, and samples were stored at −70°C until use. Complementary DNA templates were created from 50 ng RNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche) following the manufacturer's protocol.

Real-time RT-PCR was carried out in an MX4000® multiplex detection system (Stratagene, La Jolla, CA) using qPCR Mastermix for SYBR Green I (Eurogentec, Seraing, Belgium). Gene-specific primers were designed to amplify the *hyl*, *pas* and *ily* genes (Table 1). To normalize the data, primers pairs were designed to amplify a sequence in the 16S rRNA genes and gyrase A gene as housekeeping control (15). The gradient thermocycling program was set for 40 cycles at 95°C for 15 s, 58°C for 30 s, and 72°C for 30 s, with an initial cycle at 95°C for 10 min. During each cycle, the accumulation of PCR products was detected by monitoring the increase in fluorescence of the reporter dye from double-stranded-DNA-binding SYBR green. Dissociation curves were run immediately after the last PCR. To exclude DNA contamination of the RNA samples, replicate control assays were performed in which reverse transcriptase was omitted. Data were collected and analyzed using the software and graphics program MX4000 v 4.00 (Stratagene). Standard curves were obtained for the *hyl*, *pas*, *ily*, and 16S rRNA genes. The relative differences in expression were analyzed with the Relative expression Software Tool (v.1.9.12) (22).

The results indicated that the *S. intermedius luxS* mutant, defective in AI-2 production, expressed less *hyl* and *ily* than the wild type, with expression ratios of 0.640 and 0.316, respectively. No difference in *pas* expression was observed, (expression ratio 1.078). The results presented were also normalized by gyrase A amplification, giving essentially the same results. We used bacterial samples from exponential growth phase because that is when maximum AI-2 activity is observed in *S. intermedius* supernatants (20). Also, in several other bacteria, including *S. pneumoniae*, disruption of *luxS* has the most pronounced effects on gene expression during exponential growth, when cell density is rapidly increasing (9). Based on the results from real-time RT-PCR we decided to assess whether these differences in the transcription of *hyl* and *ily* resulted in differences in phenotypes. We thus compared the hyaluronidase activity and the capacity of lysis of human erythrocytes in the wild type and *luxS* mutant.

## Hyaluronidase activity is similar in the wild type and *luxS* mutant

Measurement of hyaluronidase activity was based on the colorimetric method described by Reissig et al. (24), slightly modified by Asteriou et al. (1). It determines the concentration of reducing *N*-acetyl-D-glucosamine ends generated from hyaluronan hydrolysis; *N*-acetyl-D-glucosamine (Sigma A 8625) was used as a standard. Briefly, bacteria from the second overnight culture were transferred to 1.5 ml TSB. The supernatants were collected at different time-points and fro-

zen. Prewarmed hyaluronan solution (200 µl of 1 mg/ml solution) containing 5 mM ammonium acetate at pH 5 was incubated with 100 µl culture supernatant at 37°C for 1 h. The reaction was stopped by adding 50 µl borate solution, followed by vortexing and incubation in a boiling water bath for 3 min. After cooling, 1.5 ml of a 10-fold diluted *p*-dimethylaminobenzaldehyde (DMAB) solution was added to each tube. In this method, the *N*-acetyl-D-glucosamine generated from degradation of hyaluronic acid is converted to a furan derivative, which reacts with the DMAB to form a red complex. The color was determined spectrophotometrically at 590 nm after 20 min of incubation at 37°C. Each assay was performed in triplicate in three independent experiments.

The hyaluronidase accumulates during growth up to the early stationary phase. The level dropped at 24 h being lower than at 9 h. *S. intermedius* wild type and *luxS* mutant SI006 showed similar hyaluronidase activity in all phases (Fig. 1). The fact that no hyaluronidase activity was observed in a *S. intermedius hyl* mutant strain (data not shown) proves that just one copy of the hyaluronidase gene exists.

### Lysis of human erythrocytes is reduced in the *luxS* mutant

To evaluate whether hemolysin activity is affected in the *luxS* mutant we used a hemolytic assay as previously described (18), with slight modification. Briefly, human erythrocytes were washed three times in phosphate-buffered saline (PBS) by centrifugation. Hemolytic reactions were carried out in a total volume of 500 µl. The reaction mixture contained 410 µl PBS, 15 µl of 50% (V/V) erythrocyte/PBS suspension and 75 µl of culture supernatants from the wild-type or the *luxS* mutant (SI006-SI007) collected at time 0, after 3 or 6 h growth in TSB. After 1 h in 37°C, the reaction mixtures were centrifuged at 7000 ***g*** for 5 min. Three hundred microliters of each supernatant were dispensed into a 96-well microtiter plate (Nunc, Copenhagen, Denmark), and the hemoglobin released from the erythrocytes into the supernatant was measured at 530 nm in a Synergy HT Multi-Detection Microplate Reader (Biotek Instruments, Winooski, VT). Each assay was performed in triplicate in three independent experiments. At time zero and at 3 h the hemolytic activity was low and no differences were observed between the wild type and the *luxS* mutant. The *S. intermedius* wild type showed, however five times more hemolytic activity at 6 h ($OD_{600}$ 0.450) of growth than the *luxS* deletion mutant (Fig. 3). The results are consistent with those from real-time RT-PCR, in which expression of *ily* in the *luxS* mutant was reduced.

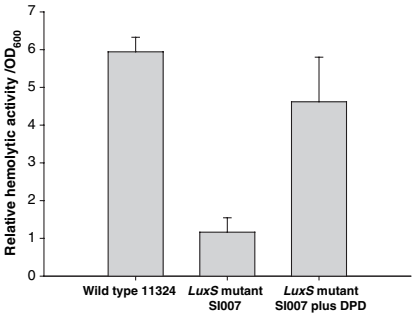


*Fig. 3. Streptococcus intermedius* wild type and *luxS* mutant (plus and minus DPD) hemolytic activity measured after 6 h of growth ($OD_{530}$). Hemolytic activity was calculated according to cell density. The data correspond to mean values and standard errors from three independent experiments. The results from the *luxS* mutant plus DPD were from the highest hemolytic activity of each experiment with concentrations of DPD varying between 0.4 and 32 nM.

To ascertain that the reduction in hemolytic activity was in fact the result of intermedilysin activity, we constructed an *S. intermedius ily* mutant strain (SI008). No hemolytic activity by the *ily* mutant was observed (data not shown).

### *S. intermedius luxS* mutant regained the hemolytic activity by chemical complementation with DPD

To verify that the reduction in lysis of erythrocytes was the result of a lack of AI-2-mediated signaling, concentrations varying from 0.4 to 32 nM of DPD (Omm Scientific, Dallas, TX) were added to the *luxS* mutant SI007 growth medium. Supernatants of the *luxS* mutant were collected after 3, 5, and 6 h. DPD had no effect on the growth of the *luxS* deletion mutant (data not shown). Hemolytic activity was partially restored by growth of the *luxS* mutant in the presence of DPD (Fig. 3). The optimum DPD concentration for complementation varied between 0.4 and 32 nM in the three experiments. This concentration range was similar to that described in DPD complementation studies investigating biofilm formation by a *Streptococcus oralis luxS* mutant and *Actinomyces naeslundii* (25).

AI-2-mediated communication is involved in the regulation of several bacterial processes, including the expression of toxins, protease activity, biofilm, motility, cell division, and cell internalization (35, 39). In this study we investigated whether *S. intermedius pas*, *hyl*, and *ily* genes, encoding putative virulence and colonization factors, were affected in the *luxS* mutant. No significant differences in *pas* expression were observed. In *S. mutans*, both downregulation (15) and no effect (36) in expression of the Ag I/II gene have been reported in a *luxS* mutant.

The *S. intermedius luxS* mutant showed downregulation of *hyl* expression, although the hyaluronic activity was unaffected. A study using microarray in *S. pneumoniae* showed no difference in *hyl* expression in a *luxS* mutant (9).

Our results showed that inactivation of *luxS* in *S. intermedius* resulted in a mutant with reduced intermedilysin gene expression and hemolytic activity against human erythrocytes. Disruption of *luxS* in *S. pneumoniae* has also been shown to reduce the pneumolysin gene expression required for virulence in animals (9), whereas in *S. pyogenes*, disruption of *luxS* enhances streptolysin activity (13). Yamaguchi et al. (40) suggested that other toxins may be involved in hemolytic activity. In *S. intermedius* NCTC 11324 we found that inactivation of *ily* abolished hemolytic activity, thus indicating that *ily* may be the sole hemolytic toxin in *S. intermedius.*

Surface-bound intermedilysin in *S. intermedius* has been shown to be an important factor for invasion into host cells, and secretion of the cytolysin is thought to be essential for subsequent cell death (28). Reduced *ily* expression in the exponential phase by the *S. intermedius luxS* mutant may reduce its colonization and invasion significantly, thus allowing host defense mechanisms to gradually eliminate the bacteria.

LuxS also functions as an integral component of the activated methyl cycle and so has an alternative role in the cell (38). Complementation of the *luxS* mutant with DPD restored, almost completely, the hemolytic activity, thus indicating that the differences observed between the wild type and the *luxS* mutant were in fact a result of AI-2. Consequently, virulence of *S. intermedius* could possibly be reduced through interference with AI-2 communication.

*S. intermedius* is found in the oral cavity and in the gastrointestinal tract, niches known to harbor a variety of bacterial species. Human hemolytic strains of *S. intermedius* are isolated most frequently among infection- and abscess-related strains (7). The ability to orchestrate the

expression of virulence factors via AI-2 communication may contribute to the adaptation and survival of *S. intermedius* in complex niches. In this study, we showed that *ily* expression and subsequent hemolytic activity, among the major virulence factors in *S. intermedius*, are decreased in the *luxS* mutant. Targeting AI-2 quorum-sensing signaling may represent a novel strategy to reduce virulence expression and fight diseases. Further studies are, however, necessary to address the mechanisms involved in this regulation.

## References


1. Asteriou T, Deschrevel B, Delpech B et al. An improved assay for the *N*-acetyl-D-glucosamine reducing ends of polysaccharides in the presence of proteins. Anal Biochem 2001: **293**: 53–59.
2. Bassler BL, Greenberg EP, Stevens AM. Cross-species induction of luminescence in the quorum-sensing bacterium *Vibrio harveyi*. J Bacteriol 1997: **179**: 4043–4045.
3. Claridge JE 3rd, Attorri S, Musher DM et al. *Streptococcus intermedius*, *Streptococcus constellatus*, and *Streptococcus anginosus* (''*Streptococcus milleri* group'') are of different clinical importance and are not equally associated with abscess. Clin Infect Dis 2001: **32**: 1511–1515.
4. Giddings KS, Johnson AE, Tweten RK. Redefining cholesterol's: role in the mechanism of the cholesterol-dependent cytolysins. Proc Natl Acad Sci USA 2003: **100**: 11315–11320.
5. Giddings KS, Zhao J, Sims PJ, Tweten RK. Human CD59 is a receptor for the cholesterol-dependent cytolysin intermedilysin. Nat Struct Mol Biol 2004: **11**: 1173–1178.
6. Homer K, Shain H, Beighton D. The role of hyaluronidase in growth of *Streptococcus intermedius* on hyaluronate. Adv Exp Med Biol 1997: **418**: 681–683.
7. Jacobs JA, Schot CS, Schouls LM. Haemolytic activity of the '*Streptococcus milleri* group' and relationship between haemolysis restricted to human red blood cells and pathogenicity in *S. intermedius*. J Med Microbiol 2000: **49**: 55–62.
8. Jenkinson HF, Demuth DR. Structure, function and immunogenicity of streptococcal antigen I/II polypeptides. Mol Microbiol 1997: **23**: 183–190.
9. Joyce EA, Kawale A, Censini S et al. LuxS is required for persistent pneumococcal carriage and expression of virulence and biosynthesis genes. Infect Immun 2004: **72**: 2964–2975.
10. Lau PC, Sung CK, Lee JH et al. PCR ligation mutagenesis in transformable streptococci: application and efficiency. J Microbiol Methods 2002: **49**: 193–205.
11. Lee MS, Morrison DA. Identification of a new regulator in *Streptococcus pneumoniae* linking quorum sensing to competence for genetic transformation. J Bacteriol 1999: **181**: 5004–5016.
12. Li S, Kelly SJ, Lamani E et al. Structural basis of hyaluronan degradation by *Streptococcus pneumoniae* hyaluronate lyase. EMBO J 2000: **19**: 1228–1240.
13. Lyon WR, Madden JC, Levin JC et al. Mutation of *luxS* affects growth and virulence factor expression in *Streptococcus pyogenes*. Mol Microbiol 2001: **42**: 145–157.
14. Macey MG, Whiley RA, Miller L, Nagamune H. Effect on polymorphonuclear cell function of a human-specific cytotoxin, intermedilysin, expressed by *Streptococcus intermedius*. Infect Immun 2001: **69**: 6102–6109.
15. Merritt J, Kreth J, Shi W, Qi F. LuxS controls bacteriocin production in *Streptococcus mutans* through a novel regulatory component. Mol Microbiol 2005: **57**: 960–969.
16. Miller ST, Xavier KB, Campagna SR et al. *Salmonella typhimurium* recognizes a chemically distinct form of the bacterial quorum-sensing signal AI-2. Mol Cell 2004: **15**: 677–687.
17. Nagamune H, Ohnishi C, Katsuura A et al. Intermedilysin, a novel cytotoxin specific for human cells secreted by *Streptococcus intermedius* UNS46 isolated from a human liver abscess. Infect Immun 1996: **64**: 3093–3100.
18. Nagamune H, Whiley RA, Goto T et al. Distribution of the intermedilysin gene among the anginosus group streptococci and correlation between intermedilysin production and deep-seated infection with *Streptococcus intermedius*. J Clin Microbiol 2000: **38**: 220–226.
19. Pecharki D, Petersen FC, Assev S, Scheie AA. Involvement of antigen I/II surface proteins in *Streptococcus mutans* and *Streptococcus intermedius* biofilm formation. Oral Microbiol Immunol 2005: **20**: 366–371.
20. Petersen FC, Ahmed NA, Naemi A, Scheie AA. LuxS-mediated signalling in *Streptococcus anginosus* and its role in biofilm formation. Antonie Van Leeuwenhoek 2006: **90**: 109–121.
21. Petersen FC, Pecharki D, Scheie AA. Biofilm mode of growth of *Streptococcus intermedius* favored by a competence-stimulating signaling peptide. J Bacteriol 2004: **186**: 6327–6331.
22. Pfaffl MW, Horgan GW, Dempfle L. Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res 2002: **30**: e36.
23. Polekhina G, Giddings KS, Tweten RK, Parker MW. Insights into the action of the superfamily of cholesterol-dependent cytolysins from studies of intermedilysin. Proc Natl Acad Sci USA 2005: **102**: 600–605.
24. Reissig JL, Storminger JL, Leloir LF. A modified colorimetric method for the estimation of *N*-acetylamino sugars. J Biol Chem 1955: **217**: 959–966.
25. Rickard AH, Palmer RJ Jr, Blehert DS et al. Autoinducer 2: a concentration-dependent signal for mutualistic bacterial biofilm growth. Mol Microbiol 2006: **60**: 1446–1456.
26. Semmelhack MF, Campagna SR, Federle MJ, Bassler BL. An expeditious synthesis of DPD and boron binding studies. Org Lett 2005: **7**: 569–572.
27. Stroeher UH, Paton AW, Ogunniyi AD, Paton JC. Mutation of *luxS* of *Streptococcus pneumoniae* affects virulence in a mouse model. Infect Immun 2003: **71**: 3206–3212.
28. Sukeno A, Nagamune H, Whiley RA et al. Intermedilysin is essential for the invasion of hepatoma HepG2 cells by *Streptococcus intermedius*. Microbiol Immunol 2005: **49**: 681–694.
29. Sung CK, Li H, Claverys JP, Morrison DA. An rpsL cassette, janus, for gene replacement through negative selection in *Streptococcus pneumoniae*. Appl Environ Microbiol 2001: **67**: 5190–5196.
30. Surette MG, Bassler BL. Quorum sensing in *Escherichia coli* and *Salmonella typhimurium*. Proc Natl Acad Sci USA 1998: **95**: 7046–7050.
31. Takao A, Nagashima H, Usui H et al. Hyaluronidase activity in human pus from which *Streptococcus intermedius* was isolated. Microbiol Immunol 1997: **41**: 795–798.
32. Tamura H, Kikuchi T, Shirato R, Kato H. Cloning and DNA sequencing of the surface protein antigen I/II (PAa) of *Streptococcus cricetus*. FEMS Microbiol Lett 2001: **196**: 251–256.
33. Tanner A, Maiden MF, Lee K et al. Dental implant infections. Clin Infect Dis 1997: **25** (suppl 2): S213–217.
34. Tilley SJ, Saibil HR. The mechanism of pore formation by bacterial toxins. Curr Opin Struct Biol 2006: **16**: 230–236.
35. Vendeville A, Winzer K, Heurlier K et al. Making 'sense' of metabolism: autoinducer-2, LuxS and pathogenic bacteria. Nat Rev Microbiol 2005: **3**: 383–396.
36. Wen ZT, Burne RA. LuxS-mediated signaling in *Streptococcus mutans* is involved in regulation of acid and oxidative stress tolerance and biofilm formation. J Bacteriol 2004: **186**: 2682–2691.
37. Whiley RA, Beighton D, Winstanley TG et al. *Streptococcus intermedius*, *Streptococcus constellatus*, and *Streptococcus anginosus* (the Streptococcus milleri group): association with different body sites and clinical infections. J Clin Microbiol 1992: **30**: 243–244.
38. Winzer K, Hardie KR, Williams P. LuxS and autoinducer-2: their contribution to quorum sensing and metabolism in bacteria. Adv Appl Microbiol 2003: **53**: 291–396.
39. Xavier KB, Bassler BL. LuxS quorum sensing: more than just a numbers game. Curr Opin Microbiol 2003: **6**: 191–197.
40. Yamaguchi T, Koreeda H. Distribution and characterization of hemolytic activity by an oral anaerobe from the *Streptococcus milleri* group. Oral Microbiol Immunol 2004: **19**: 132–135.