CHAPTER IV

Functional and Phenotypic Characterization of *Histophilus somni*

***luxS* and Biofilm Formation**

ABSTRACT

Quorum sensing is a mechanism of interbacterial communication that has been shown to be involved in the regulation of several complex phenotypes and virulence in some pathogenic bacteria. In this chapter, the characterization of the *luxS* gene of *H. somni* as well as biofilm formation by different strains of *H. somni* are described. Based on the ability of the *luxS* gene from *H. somni* to complement autoinducer-2 biosynthesis in *Escherichia coli* DH5α, it is proposed that the gene encodes S- ribosylhomocysteinase. It is shown that culture supernatants from different members of the *Pasteurellaceae* differ significantly in their ability to induce luminescence in *V. harveyi* strain BB170. Based on this evidence and *in silico* analyses of LuxP proteins, it is proposed that AI-2 is not an 'universal signal'. It is also shown that several pathogenic isolates of *H. somni* form a prominent biofilm. A synthetic furanone has been shown to inhibit planktonic and biofilm growth of *H. somni*, raising the possibility that this molecule could potentially be used as an antibacterial agent. Preliminary results indicated that *luxS* as well as phosphorylcholine expression can influence biofilm formation by *H. somni*. However, further studies using *H. somni* mutants deficient in *luxS* and/or phosphorylcholine expression are required to verify these findings.

Introduction

Quorum sensing (QS), a mechanism used by some bacteria to sense and respond not only to members of their own species but also other bacterial species occupying the same ecological niche, is an evolutionarily conserved function (Fuqua and Greenberg, 1998; Federle and Bassler, 2003). Small molecules known as autoinducers, which are produced by these quorum sensing bacteria, have been shown to accumulate and regulate the expression of genes whose functions are more efficient at high cell densities.

Acylated homoserine lactone (AHL) molecules [also known as autoinducer-1 (AI-1) signals] mediate QS only in Gram-negative bacteria and small peptide molecules [also known as autoinducing peptides (AIPs)] mediate QS only in Gram-positive bacteria (Fuqua et al., 1994; Sturme et al., 2002). Due to the species-specificity of the AI-1 and AIP molecules, the QS mediated by AI-1 or AIP molecules is referred to as 'intraspecies' or 'species-specific' communication (Fuqua et al., 1994; Federle and Bassler, 2003).

A furanosyl borate diester autoinducer signal molecule has been shown to regulate QS-dependent bioluminescence in the Gram-negative marine bacterium *Vibrio harveyi* (Chen et al., 2002). Since an AI-1 molecule can also regulate QS dependent bioluminescence in this species, the furanosyl borate diester has been referred to as autoinducer-2 (AI-2). The product of *luxS* (S-ribosylhomocysteinase) has been shown to be an intermediary enzyme in the biosynthesis of AI-2 molecules. In *V. harveyi*, LuxP, which is a periplasmic protein, acts as a receptor for AI-2 molecules and transmits the signal to LuxQ, which is a sensor histidine kinase. A transcriptional regulator (LuxR) mediates gene expression in response to the AI-2 type QS signals (Chen et al., 2002; Federle and Bassler, 2003). Homologs of these and other genes involved in QS have been identified and characterized in several members of the genus *Vibrio*.

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In silico analyses revealed that the *luxS* gene, essential for synthesis of AI-2 in *V. harveyi*, was also present in numerous Gram-negative and Gram-positive bacteria. Using *V. harveyi* mutants as reporter strains that do not respond to the AHL (AI-1) signal molecules, it has been shown that culture supernatants from several *luxS* containing bacteria can induce luminescence via the AI-2 system (Federle and Bassler, 2003). These initial observations also lead to the proposal of the 'universal signal' hypothesis (Chen et al., 2002).

Many bacteria have been shown to sense the presence of their own species in high numbers using AI-1, AI-2, or the AIP signal molecules and accordingly regulate gene expression in a density-dependent fashion (Federle and Bassler, 2003). Quorum sensing has hitherto been shown to regulate genes involved in swarming motility, biofilm formation, exopolysaccharide production, and type III secretion (Keller and Surette, 2006; Kendall and Sperandio, 2007).

A biofilm is defined as "a community of microorganisms attached to a suitable surface" (O'Toole et al., 2000). A vast majority of bacteria in their original niche are thought to exist as structured biofilm communities (Hall-Stoodley et al., 2004). In nature, a bacterial biofilm is usually formed when cells transition from a planktonic state to a sessile state and adhere to a surface. An intricate matrix, usually comprised of polysaccharide material exuded by bacteria, provides the necessary stability and structure to the biofilm (Donlan and Costerton, 2002). A plethora of chronic infections of humans as well as animals involving several organs and prosthetic devices have been shown to be due to persistent biofilms (Trampuz and Widmer, 2006). In contrast to the planktonic state, bacterial biofilms are less susceptible to innate and acquired immune systems as well as to the action of antibiotics (Stewart and Costerton, 2001; Donlan and Costerton, 2002). In view of their complex nature, bacterial biofilms are thought to be regulated by several genes and environmental cues (Haase et al., 2006).

Bacterial biofilms are therefore the subject of intense research and are being studied from clinical as well as industrial perspectives. Medically important members of the *Pasteurellaceae* have also been shown to form biofilms *in vivo* and/or *in vitro* (Haas et al., 2006; Gallaher et al., 2006). For bacteria such as *H. somni* that thrive as commensals of cattle and sheep, the ability to adhere and form a film on the epithelial layers may provide a selective advantage over bacteria that cannot adhere. Preliminary studies have shown the ability of *H. somni* to attach to polyvinyl chloride (PVC) surfaces and form biofilm-like aggregations (Inzana, unpublished observations). However, a comparison of different *H. somni* strains to form biofilms under different growth conditions has not been performed previously. The genetic functions that regulate biofilm formation by *H. somni* are also unknown.

It has been reported that several bacteria related to *H. somni* possess a *luxS* gene and culture supernatants from these bacteria can induce AI-2 type signaling in *V. harveyi*. In *Actinobacillus actinomycetemcomitans*, AI-2 signaling increases leukotoxin and iron transport protein expression (Fong et al., 2001). Mutation of *luxS* in *A. actinomycetemcomitans* has also been shown to reduce growth under iron-limited aerobic conditions (Fong et al., 2003). However, compared to wildtype bacteria, a *Mannheimia haemolytica luxS* mutant has been observed to grow faster under iron-restricted conditions, synthesize more capsular polysaccharide, and adhere better to polarized bovine tracheal cells (van der Vinne et al., 2005). In *Haemophilus influenzae*, *luxS* mutants do form a biofilm and have been shown to be more invasive to human epithelial cells than wildtype bacteria (Daines et al., 2005). A homolog of the *V. harveyi luxS* gene has also been found in the genomes of *H. somni* strains 2336 and 129Pt. However, it is not known if this gene is functional and whether culture supernatants from *H. somni* can participate in interspecies signaling with *V. harveyi* reporter strains.

Halogenated furanones derived from the red marine alga *Delisea pulchra* have been shown to be very effective in inhibiting QS circuits in several Gram-negative bacteria without altering growth parameters (de Nys et al., 2006). The *Delisea pulchra* natural furanone, *(5Z)*-4-bromo-5-(bromomethylene)-3-butyl-2*(5H)*-furanone, has been shown to hinder AI-2 QS-mediated phenotypes in *V. harveyi* as well as *E. coli* (Ren et al., 2001). A synthetic furanone derivative (Fur-1) has also been shown to inhibit the growth of *Bacillus subtilis* (Ren et al., 2002). Therefore, these substances could potentially be used as therapeutic agents against bacterial infections. However, the effect of furanones on members of the *Pasteurellaceae* has not been examined previously, and there is a necessity to test their effect on different bacteria.

The objectives of the current study were to characterize the *luxS* gene of *H. somni* in the context of QS and biofilm formation, as well as to test the effect of furanones on *H. somni*. This chapter describes the *luxS* genes and their corresponding proteins from *H. somni* strains 2336 and 129Pt. The cloning and functional characterization of *luxS* from *H. somni* strain 2336 has shown that the gene encodes S-ribosylhomocysteinase that can complement AI-2 biosynthesis in *E. coli* DH5α. Using *V. harveyi* strain BB170 as a reporter, it has been shown that culture supernatants from different *luxS*-containing bacteria differ significantly in inducing bioluminescence.

The ability of different strains of *H. somni* to form biofilms under various growth conditions has also been described within this chapter and it has been shown that a synthetic furanone derivative (Fur-1) acts as a growth inhibitor of *H. somni* planktonic and sessile cells. Furthermore, *luxS* and phosphorylcholine expression have been shown to influence biofilm formation by *H. somni*. The chemical structure of the AI-2 molecule, the role of *luxS* in physiological regulation, and the factors that modulate biofilm growth remain to be further characterized within the context of *H. somni*.

Materials and Methods

Identification of *lux* homologs in *H. somni*

The genomes of *H. somni* strains 2336 and 129Pt were searched for homologs of genes involved in AI-2 QS (*luxS*, *luxR*, and *luxP*) using NCBI blastn and blastx programs. The putative genes, and their translated proteins, were compared with each other and their homologs from other species using blastp, clustalW, BioEdit, and BOXSHADE.

Cloning and expression of *H. somni luxS* in *E. coli*

Two primers [*luxS*f (5'-ACAATGTCATGACCTGCTCGAT-3') and *luxS*r (5'-CACAAGGAATGCCAAGGTTTTC-3')] were designed for PCR amplification of a 1,718 bp region containing the *luxS* gene from *H. somni*. Primers were purchased from Integrated DNA Technologies, Coralville, IA, and were used to amplify the *luxS* gene from *H. somni* strain 2336 genomic DNA utilizing PCR beads (Amersham Biosciences, Piscataway, NJ) as outlined below:

PCR mix	PCR protocol
*luxS*f primer 1 µl (10 pmol/µl)	96^0 C for 5 min
*luxS*r primer 1 µl (10 pmol/µl)	96^0 C for 1 min ⎫
Genomic DNA template 5 µl (60 ng/µl)	52^0 C for 1 min ⎬ 35 cycles
Sterile double distilled water QS 25 µl	72^0 C for 3 min ⎭
One PCR bead	72^0 C for 10 min

The PCR product was TOPO cloned into pCR2.1-TOPO (Invitrogen) to create plasmid pCR2.1TOPO*luxS* in *E. coli* DH5α and the insert was confirmed by DNA sequencing. *E. coli* DH5α containing pCR2.1TOPO vector was used as negative control in the AI-2 assays.

<u>Culture supernatant preparation</u>

H. influenzae, *Haemophilus parasuis*, *Actinobacillus pleuropneumoniae*, *M. haemolytica* A1, and *H. somni* were cultured in Brain Heart Infusion (BHI) [Becton Dickinson, Sparks, MD] broth containing 0.1% Trizma base (Sigma, St. Louis, MO) and 0.5% dextrose (Sigma). In addition, the broth was supplemented with 0.1% TMP (for *H. somni*), or 10 μg/ml NAD (for *A. pleuropneumoniae*), or 10 μg/ml NAD and 5 μg/ml Hemin (for *H. influenzae* and *H. parasuis*). *E. coli* DH5α containing pCR2.1TOPO*luxS* and *E. coli* DH5α containing pCR2.1TOPO were cultured in Luria Bertani (LB) [Becton Dickinson, Sparks, MD] broth containing 100 μg/ml ampicillin (Sigma).

The bacteria mentioned above were cultured at 37^0C with 200 rpm shaking until they reached mid-logarithmic phase of growth. The cultures were centrifuged at 5000 x g in a Sorvall RC5C centrifuge in the SS-34 rotor. The culture supernatants were filtered using 0.2 μm syringe filters (Nalgene, Rochester, NY). The filtrates (100 μl) were plated on agar plates to check sterility and purity. Sterile BHI medium with all the above supplements was used as the negative control in the AI-2 bioassay.

V. harveyi strain BB152, which has been engineered to synthesize only AI-2, was used as the positive control strain in the AI-2 bioassay (Surette and Bassler, 1998). Autoinducer Bioassay (AB) medium, which is a minimal medium containing casamino acids and salts, was prepared as reported by Greenberg et al. (1979). *V. harveyi* strain BB152 from an LB agar plate overnight culture was inoculated into AB medium and incubated at 30^0C with 175 rpm shaking for 16 hours and the culture supernatant, containing the AI-2 molecule, was prepared as described above. Sterile AB medium was used as a negative control. Filtered culture supernatants were stored at -20^0 C until further use.

<u>Autoinducer bioassay</u>

V. harveyi reporter strain BB170, which responds only to the AI-2 molecule (Surette and Bassler, 1998), was used in the AI-2 bioassay. Briefly, the reporter strain from an LB agar plate overnight culture was inoculated into AB medium and incubated at 30^0 C with 175 rpm shaking for 16 hours. The culture was diluted 1:5000 in AB medium and 90 μl was added to each well. Culture supernatants from different bacteria were added (10 μl/well) to test the presence of the AI-2 signal. The principle of AI-2 bioassay is outlined in fig. 4.01.

The AI-2 assay was performed in black, 96-well plates (PerkinElmer Life and Analytical Sciences, Inc. Wellesley, MA) in quadruplicate and luminescence in each well was measured immediately after the addition of the culture supernatants using a TopCount-NXT microplate scintillation/luminescence counter (Fig. 4.02) [Packard Instrument Co., Meriden, CT]. The plate was incubated at 30^0 C and luminescence induction was recorded every hour thereafter for 6-8 hours. Luminescence in each well was counted for 5 seconds, the average count of four wells for each sample was calculated, and this value was used in assessing the response of the reporter strain. Each culture supernatant was tested at least 3 times in assays performed on different days.
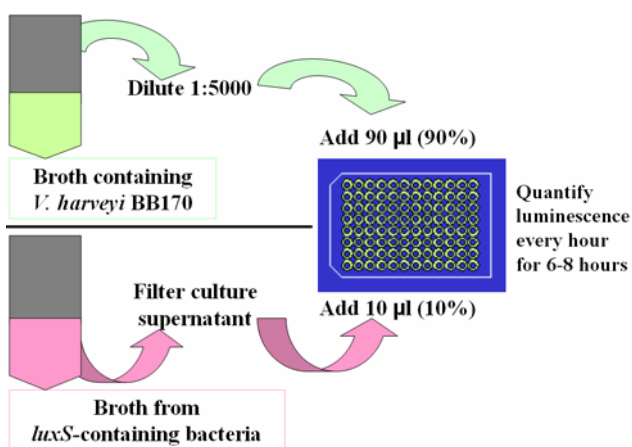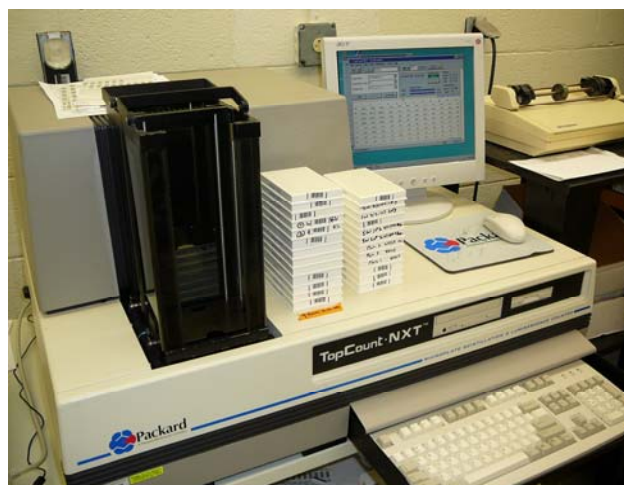

Fig. 4.01: Principle of AI-2 bioassay   Fig. 4.02: Packard luminescence counter

Biofilm formation, staining, and visualization

 H. somni was cultured on Columbia blood agar (Becton Dickinson, Sparks, MD) plates containing 5% sheep blood in a candle jar for 24-36 hours at 37^0C. A loopful of fresh bacteria from agar plates was resuspended in BHI broth containing 0.1% Trizma base and 0.1% TMP. For assaying in 96-well PVC plates, 150 μl of the bacterial suspension was added to each well. For assaying in 50-ml plastic conical tubes, 3 ml of the bacterial suspension was added to each tube. The 96-well PVC plates and tubes were incubated at 37^0C for 72 hours. Bacterial adherence was tested by staining with 0.001% crystal violet for 10 minutes at room temperature (O'Toole et al., 1999). The plates were washed once with tap water to remove unbound stain. Stained biofilms in 50-ml tubes were not washed to prevent loss of the slimy layer. Crystal violet stained biofilms were photographed using a handheld digital camera. Biofilm formation by *E. coli* was assayed as above, except that the incubation period was reduced to 24 hours.

 For assaying biofilm formation on glass surfaces, 1 ml of the bacterial suspension was inoculated into 50 ml broth in 150-ml glass containers and a sterile glass slide was placed inside. The cultures were incubated at 37^0C for up to five days. Biofilms on glass slides were stained with 150 μg of Concanavalin A-Alexa Fluor® 488 conjugate (CAAF) [Molecular Probes, Eugene, OR] dissolved in phosphate buffered saline (5 mg/ml). The staining was performed at 37°C for one hour. Imaging of *H. somni* biofilms cultured on glass slides and stained with CAAF was performed using an inverted Axiovert 100 M equipped Zeiss LSM 510 confocal laser scanning microscope (Carl Zeiss, Jena, Germany). Sugar (glucose and/or mannose) residues in the bacterial polysaccharide matrix are bound by CAAF, which fluoresces green at an excitation wavelength of 495 nm with an emission maxima of 519 nm. Acquisition and analyses of images were performed as described by Kuhn et al. (2002).

<u>Effect of furanone on *H. somni*</u>

The synthesis and characterization of the furanone have been described previously (Ren et al., 2002). The furanone was dissolved in 95% ethanol to a final concentration of 14.9 mg/ml. To test the effect of furanone on biofilm formation, *H. somni* was cultured in plastic tubes and on glass slides as described before after adding the desired concentrations of the furanone. Ethanol (95%) was added as a control at quantities that equaled the volume of the solvent containing the highest concentration of the furanone used in a particular experiment. Biofilm formation was examined by crystal violet staining and confocal laser microscopy as described above.

To test the effect of furanone on planktonic growth, 50 ml of BHI broth was inoculated with *H. somni* strain 2336 in klett flasks and various quantities of the furanone were added at the beginning of the experiment. Control flasks contained *H. somni* strain 2336 and 95% ethanol or *H. somni* strain 2336 only. The cultures were incubated for six hours at 37^0 C and 150 rpm shaking. Growth was assessed by recording the density of each of the inoculations using a Klett-Summerson colorimeter every hour. The growth rate of *H. somni* strain 2336 was compared to the growth rates of *H. somni* strain 2336 cultured with furanone or 95% ethanol.

<u>Cloning of *luxS* into plasmids pHS649SS and pLSKS</u>

Construction of the shuttle vector pHS649SS has been described in chapter III. Plasmid pLSKS, which is a derivative of the broad host range shuttle vector pLS88, has been described by Wood et al. (1999). *H. somni luxS* from pCR2.1TOPO*luxS* was subcloned by standard procedures into pHS649SS and pLSKS to create plasmids pHS649SS*luxS* and pLSKS*luxS*, respectively. *H. somni* strain 129Pt was transformed with these plasmids by electroporation as described by Sanders et al. (1997). Biofilm formation by these transformants was tested as described above.

Results

Characterization of *luxS* from *H. somni*

In *H. somni*, LuxS consists of 168 amino acids and is encoded by the 507 bp putative *luxS* open reading frame (ORF). In *H. somni* strain 2336, *luxS* is located on the plus strand in the region from 1,672,823 bp to 1,673,329 bp of the 2,263,870 bp chromosome. In *H. somni* strain 129Pt, *luxS* is located on the minus strand in the region from 583,282 bp to 583,788 bp of the 2,008,359 bp chromosome. In both the strains, *luxS* is flanked by *purT* and *tktA*, which encode enzymes involved in nucleotide and carbohydrate metabolism and transport, respectively (Fig. 4.03). Within the two *H. somni* strains, the *luxS* ORFs are 90% identical, whereas the LuxS proteins are 90% identical and 95% similar (Figs. 4.04 and 4.05). Some features of *luxS* and LuxS are presented in table 4.01. LuxS appears to be well conserved across different members of the *Pasteurellaceae* and *Neisseriae* and the closest homologs of *H. somni* LuxS occur in strains of *H. influenzae* (Table 4.02).

Table 4.01: Comparison of *luxS* and LuxS from *H. somni* strains

Features	*H. somni* strain 2336	*H. somni* strain 129Pt
luxS G+C content	35.11%	33.73%
luxS A+T content	64.89 %	66.27%
LuxS predicted molecular weight	18981.5801 Da	19081.6309 Da

Cloning of *luxS* from *H. somni* strain 2336

In *H. somni* strains 2336 and 129Pt, *luxS* appears to form an independent transcriptional unit with its own promoter. Since *H. somni* strain 2336 is pathogenic in cattle, it was decided to characterize the *luxS* gene from this strain. A detailed map of the 1,718 bp region containing the *luxS* gene from *H. somni* strain 2336 is shown in fig. 4.06. The product of the *luxS* PCR amplification is shown in fig. 4.07.
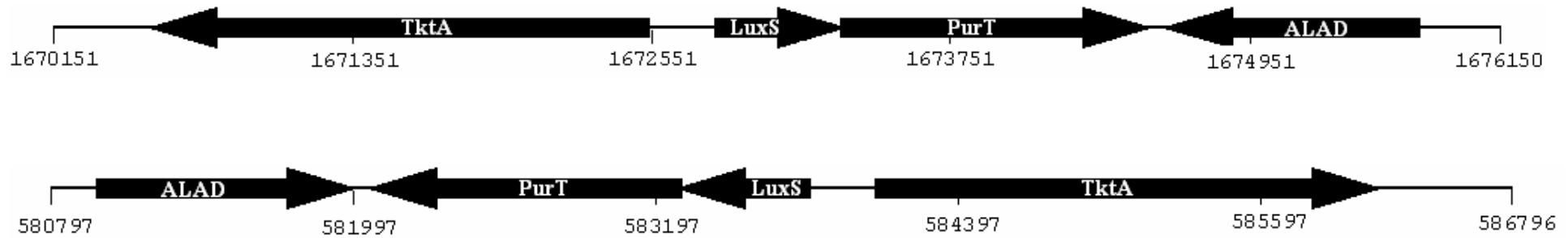
Fig. 4.03: Map of the *H. somni* chromosomal region containing the *luxS* gene

Top; A 6,000 bp region of *H. somni* strain 2336 chromosome from 1,670,151 bp to 1,676,150 bp containing four genes.

Bottom; A 6,000 bp region of *H. somni* strain 129Pt chromosome from 580,797 bp to 586,796 bp containing four genes.

ALAD; Delta-aminolevulinic acid dehydratase, PurT; Formate-dependent phosphoribosylglycinamide formyltransferase (GAR transformylase; nucleotide transport and metabolism), LuxS; S-ribosylhomocysteinase (autoinducer-2 production protein), TktA; Transketolase (carbohydrate transport and metabolism).

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2336    ATGCCACTACTTGACAGTTTTAAAGTTGATCATACGAGAATGAATGCCCCTGCTGTGCGT GTTGCAAAAACAATGCGAACACCAAAAGGCGACAATATCACTGTTTTTGATCTTCGTTTT 120
129Pt   ATGCCACTACTTGACAGTTTTAAAGTTGATCATACGAGAATGAATGCCCCTGCCGTACGT ATAGCAAAAACAATGCAAACACCGAAAGGCGACAATATCACCGTTTTTGATCTTCGTTTT 120
        *********************************************** ** *** *  * ************* ****** ******************* ******************
2336    TGTATTCCTAATAAAGAAATCCTCTCTCCTAAAGGCATTCACACTTTAGAACACTTATTC GCAGGATTTATGCGTGATCATTTAAATAGTGAACAAATTGAAATTATTGATATTTCGCCC 240
129Pt   TGTATTCCAAATAAAGAGATCCTCTCATCTAAAGGAATTCATACCTTAGAACATCTATTT GCAGGATTTATGCGTGATCATTTAAATAATGAACAAGTTGAAATTATTGATATTTCGCCT 240
        ******** ******** ******** ******** ***** ** ******** * *** ***************************** ****** *********************
2336    ATGGGCTGTCGAACCGGATTTTATATGTCATTAATTGGCATGCCGAATGAACAACAAGTT GCAAACGCTTGGTCAGCTTCAATGCAAGATATTTTAAACGTCAAAAATCAAGCCGAAATT 360
129Pt   ATGGGATGCCGAACCGGATTTTATATGTCATTAATTGGCACACCAAATGAACAACAAGTT GCTGAAGCTTGGCTGATTTCTATGCAAGATATTTTAAACATTAAAAATCAAGACGAAATT 360
        ***** ** ***************************** ** ** ************** ** * ****** *** ***************** * ********* * ******* *******
2336    CCTGAACTAAACGAATATCAATGTGGAACCTATACTGAACATTCATTGGAAGATGCACAT AATATTGCACGGAATATTCTTAATCGAGGCGTAGGTATTAATAAAAATGAAGATCTACTT 480
129Pt   CCTGAACTAAACGAATATCAATGTGGAACTTATACTGAGCATTCGTTGGAAGAAGCACAA AATATTGCACAAAATATCCTTCATCGAGGCGTAGGTATTAATAAAAATGAAGATCTACTT 480
        ***************************** ******** ***** ******** *****  ********* ***** *** *************************************
2336    TTAGATGATAATTTACTCAATTCTTAA 507
129Pt   TTAGATGATAATTTACTCAATTCTTAA 507
        ***************************
```

Fig. 4.04: ClustalW alignment of *luxS* ORFs from *H. somni* strains 2336 and 129Pt

The two genes are highly homologous and have 461 identical nucleotides (marked by asterisks) but differ at 46 nucleotides (marked by

blank spaces).

```
2336    MPLLDSFKVDHTRMNAPAVRVAKTMRTPKGDNITVFDLRFCIPNKEILSPKGIHTLEHLF AGFMRDHLNSEQIEIIDISPMGCRTGFYMSLIGMPNEQQVANAWSASMQDILNVKNQAEI 120
129Pt   MPLLDSFKVDHTRMNAPAVRIAKTMQTPKGDNITVFDLRFCIPNKEILSSKGIHTLEHLF AGFMRDHLNNEQVEIIDISPMGCRTGFYMSLIGTPNEQQVAEAWLISMQDILNIKNQDEI 120
        ********************:****:************************:********** *********.********:********************.**:******************* *******:** *******:*** **
2336    PELNEYQCGTYTEHSLEDAHNIARNILNRGVGINKNEDLLLDDNLLNS 168
129Pt   PELNEYQCGTYTEHSLEEAQNIAQNILHRGVGINKNEDLLLDDNLLNS 168
        *****************:*:***:***:********************
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Fig. 4.05: ClustalW alignment of LuxS proteins from *H. somni* strains 2336 and 129Pt

The two proteins are highly homologous and have 153 identical amino acids (marked by asterisks), 9 conserved amino acid substitutions

(marked by colons), 2 semiconserved amino acid substitutions (marked by periods), and 4 unrelated amino acid substitutions (marked by

blank spaces).

*luxS*f <u>acaatgtcatgacctgctcgat</u>taaattgtgcggctaacgttttttctgcaatcgccatgcctacagcgttagtaatgccttgacctaatggacccgttgtggtttcaatacctggtgcata

accgtattcagggtgccccggagtcttggaatgtaattgacgaaattgttttaaatcttcgatagaaagatcgtaaccggtgagatgcagtaaactataaattaacattgagccatgaccgt

tagataacacaaaacgatcacgatttgcccattgaggattagtcggattatgatttaaaaaatcacgccataatacttccgcaatatccgccattccatcggagctcccggatgcccagat

tttgctttttgtaccgcatccatacttaaaaaacggattgcatttgccaattccctacgagtta<u>cat</u>gattattctcctgcgaagttatgttgaattattcgatcggctattctaccctat
_{TktA}

ttttataaaaaaaagacgaattaatttgataggataagaaaaagtataaagctcaataaaataaagaaaatctacataaaatttaaaattatcgacaataaattaaaaatcatgacctatat

cataaaaaatcaggtttttgttatagcaatacgttacaatacaatttctaggtaaatacaataccggtcaataatctattttaatgctctctaaaggaaaaatt

_{LuxS}
<u>atg</u>ccactacttgacagttttaaagttgatcatacgagaatgaatgcccctgctgtgcgtgtt
M   P   L   L   D   S   F   K   V   D   H   T   R   M   N   A   P   A   V   R   V
gcaaaaacaatgcgaacaccaaaaggcgacaatatcactgtttttgatcttcgtttttgtatt
A   K   T   M   R   T   P   K   G   D   N   I   T   V   F   D   L   R   F   C   I
cctaataaagaaatcctctctcctaaaggcattcacactttagaacacttattcgcaggattt
P   N   K   E   I   L   S   P   K   G   I   H   T   L   E   H   L   F   A   G   F
atgcgtgatcatttaaatagtgaacaaattgaaattattgatatttcgcccatgggctgtcga
M   R   D   H   L   N   S   E   Q   I   E   I   I   D   I   S   P   M   G   C   R
accggattttatatgtcattaattggcatgccgaatgaacaacaagttgcaaacgcttggtca
T   G   F   Y   M   S   L   I   G   M   P   N   E   Q   Q   V   A   N   A   W   S
gcttcaatgcaagatattttaaacgtcaaaaatcaagccgaaattcctgaactaaacgaatat
A   S   M   Q   D   I   L   N   V   K   N   Q   A   E   I   P   E   L   N   E   Y
caatgtggaacctatactgaacattcattggaagatgcacataatattgcacggaatattctt
Q   C   G   T   Y   T   E   H   S   L   E   D   A   H   N   I   A   R   N   I   L
aatcgaggcgtaggtattaataaaaatgaagatctacttttagatgataatttactcaattct
N   R   G   V   G   I   N   K   N   E   D   L   L   L   D   D   N   L   L   N   S

taa atcttattttaataaaaaacacaatgtaaaaggacaaaaaa<u>atg</u>acaatattaggaacagctttaacacctaaagcaacaaaagtcatgcttcttggttcaggtgaactaggcaaagaagtcg
● _{PurT}
ttatagaattacaacgtcttggtgtagaagtgattgcggtagatcgctatgaaaatgcaccggcacaacaagttgcacaccgctcttcacactatttcaatgttagatggagatgctttgaaa

gcattaattgaaaaagaaaaaccggattatatcgtgccggaagtcgaagcaatcgcaacctcaacactagttgaattggagcaagccggctttaatgttgttccaactgctaaagctacaca

attaacgatgaaccgtgaaggtattcgtcgccttgcagcagaaaaattaaagttgccaacatcaaattatcaatttgtagataattttgatgactttca[aagtgcggt]cgaaaaccttggca
_{USS}
<u>ttccttgtg</u> *luxS*r

Fig. 4.06: Map of the cloned region containing *luxS* from *H. somni* strain 2336

DNA and protein sequences are shown in lower and upper cases, respectively. Sequences of the two primers, *luxS*f (5'-ACAATGTCATGACCTGCTCGAT-3') and *luxS*r (5'-CACAAGGAATGCCAA GGTTTTC-3'), have been underlined (dark lines). Start codons of LuxS, TktA, and PurT have been marked and underlined (red lines). The stop codon of LuxS is shown with a red dot. The amino acid encoded by each codon is marked below the first base of the codon. A 9 bp consensus *Haemophilus* DNA uptake signal sequence (USS) is shown in red and is boxed.
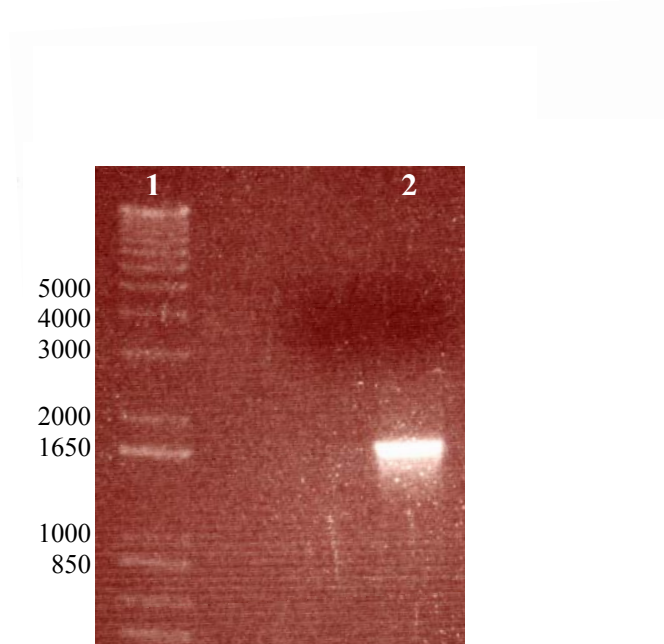
Fig. 4.07: PCR amplification of DNA containing *luxS* from *H. somni* strain 2336

Lane 1; kb plus DNA ladder (only bands from 850 bp to 5,000 bp are marked)

Lane 2; 1,718 bp PCR product containing *luxS*

Table 4.02: Comparison of *H. somni* strain 2336 LuxS to LuxS proteins from other bacteria

Bacterial Species	% Identity	% Similarity
H. influenzae Rd KW20	83	92
Neisseria meningitidis	79	88
Neisseria gonorrhoeae FA 1090	79	88
Pasteurella multocida subsp. *multocida* str. Pm70	83	91
Mannheimia succiniciproducens MBEL55E	80	90
Actinobacillus succinogenes 130Z	78	92
A. pleuropneumoniae serovar 1 str. 4074	75	87
Haemophilus ducreyi 35000HP	75	85
Vibrio harveyi	74	87
Campylobacter coli RM2228	72	87
Salmonella typhimurium LT2	73	84
E. coli K12	73	85
Clostridium perfringens ATCC 13124	48	65
Staphylococcus aureus RF122	43	63
Bacillus anthracis str. Ames	37	58
Streptococcus agalactiae str. COH1	36	56

Functional characterization of *luxS* gene from *H. somni* strain 2336

      To test whether *luxS* from *H. somni* strain 2336 is functional, *E. coli* DH5α cells were transformed with pCR2.1TOPO*luxS*. Due to a mutation in its native *luxS*, *E. coli* DH5α cannot synthesize the AI-2 signaling molecules and it has been shown previously that expression of *luxS* from other bacteria can be used to complement this deficiency (Surette et al., 1999). Culture supernatants from *E. coli* DH5α containing pCR2.1TOPO*luxS* were found to induce light production in *V. harveyi* strain BB170 (Fig. 4.08). This confirmed that *luxS* from *H. somni* strain 2336 is functional and encodes S-ribosylhomocysteinase.
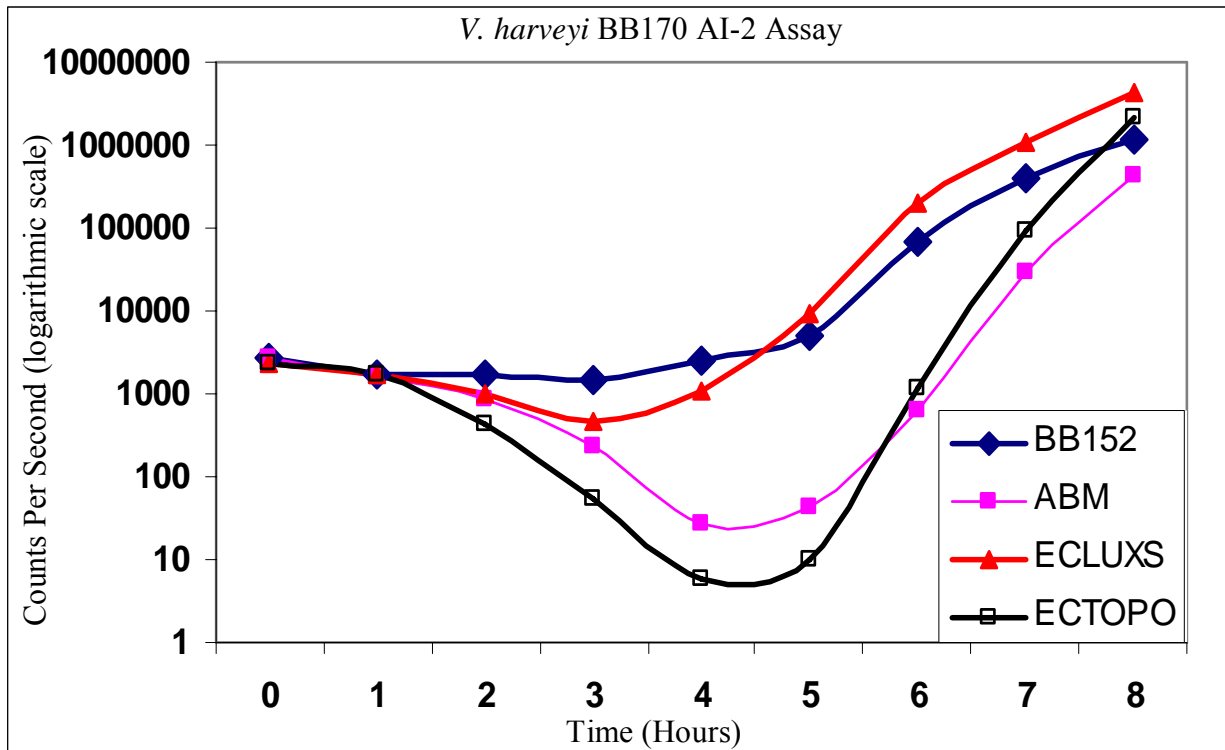
Fig. 4.08: Luminescence induction in the *V. harveyi* BB170 reporter strain by culture supernatants from *V. harveyi* and *E. coli*. BB152; *V. harveyi* strain BB152 (positive control), ABM; Autoinducer Bioassay Medium (negative control), ECLUXS; *E. coli* DH5α containing pCR2.1TOPO*luxS*, ECTOPO; *E. coli* DH5α carrying the TOPO vector only (negative control).

<u>Characterization of luminescence induction by culture supernatants from different bacteria</u>

It was found that culture supernatants from five different members of the *Pasteurellaceae* induce varying levels of light production in the reporter *V. harveyi* strain BB170 (Fig. 4.09). Least luminescence induction was observed with culture supernatants from *H. somni*. Altering *H. somni* culture parameters (aerobic and anaerobic growth with or without boric acid addition) had no effect on light induction characteristics. Similar results were obtained when the assay was repeated by adding culture supernatants at 20% (data not shown). This suggests that the responses in fig. 4.09 are not due to varying quantities of AI-2 being present in the culture supernatants, but are probably because of different structures of the AI-2 from various species.
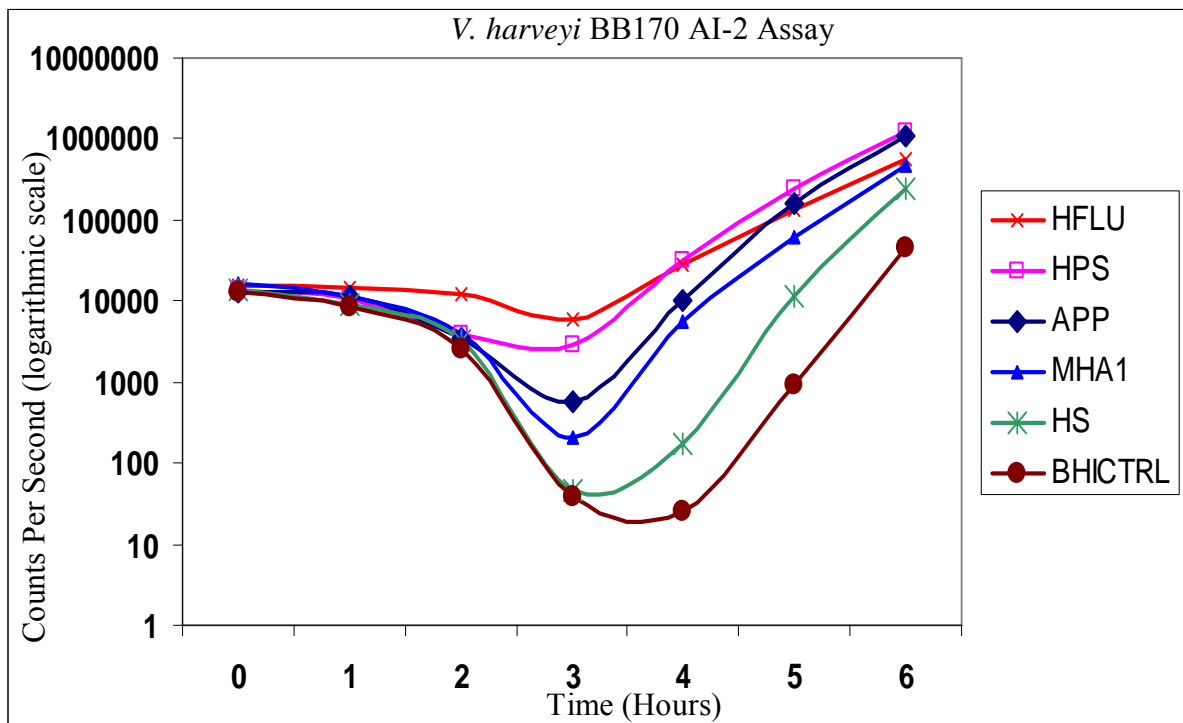
Fig. 4.09: Luminescence induction in the *V. harveyi* BB170 reporter strain by culture supernatants from different bacteria. HFLU; *Haemophilus influenzae*, HPS; *Haemophilus parasuis*, APP; *Actinobacillus pleuropneumoniae*, MHA1; *Mannheimia haemolytica* A1, HS; *Histophilus somni*.

<u>An *in silico* test of the 'universal signal' hypothesis and comparison of LuxP proteins</u>

In *V. harveyi*, LuxP is a periplasmic protein that acts as a receptor for signaling molecules and is a key protein involved in AI-2 QS. Chen et al. (2002) have shown that in *V. harveyi* LuxP, nine different amino acids are involved in forming the motif that binds the AI-2 molecule. This protein is also thought to bind to AI-2 molecules from other bacteria in the bioassay used to test the presence of these molecules. For the purpose of this analysis, it was hypothesized that if the AI-2 was a 'universal signal' as proposed, then the AI-2 binding domains and motifs would be universally identical/similar in the LuxP homologs. To test this hypothesis, a blastp search was performed to identify the extent to which LuxP is conserved among different bacteria. Only 23 full length homologs of LuxP from different species of bacteria were found in the GenBank non-redundant database. Partial homologs, annotated as "ABC-type sugar transport system, periplasmic component", were found in scores of other bacteria, including *H. somni*.

To further characterize these proteins, 19 relevant LuxP homologs were selected and a phylogenetic distance tree was constructed using the blast pairwise alignments (Fig. 4.10). This method is based on the principle of identification of local regions of similarity between a given pair of sequences. The 'sequence distance function' was derived based on identification of a set of high-scoring segment pairs between each pair of sequences and the tree was constructed by the neighbor-joining method, as described by Auch et al. (2006). The maximum sequence difference was set at 0.75. The tree in fig. 4.10 indicates that the LuxP homologs have a high degree of similarity. The protein sequences from fig. 4.10 were aligned using ClustalW-BOXSHADE to identify homologous AI-2 binding domains and motifs (Fig. 4.11). It was found that out of the nine amino acids that interact with the AI-2, six have unrelated substitutions within this group of proteins (Fig. 4.11), suggesting that the corresponding AI-2 molecules may be structurally different.
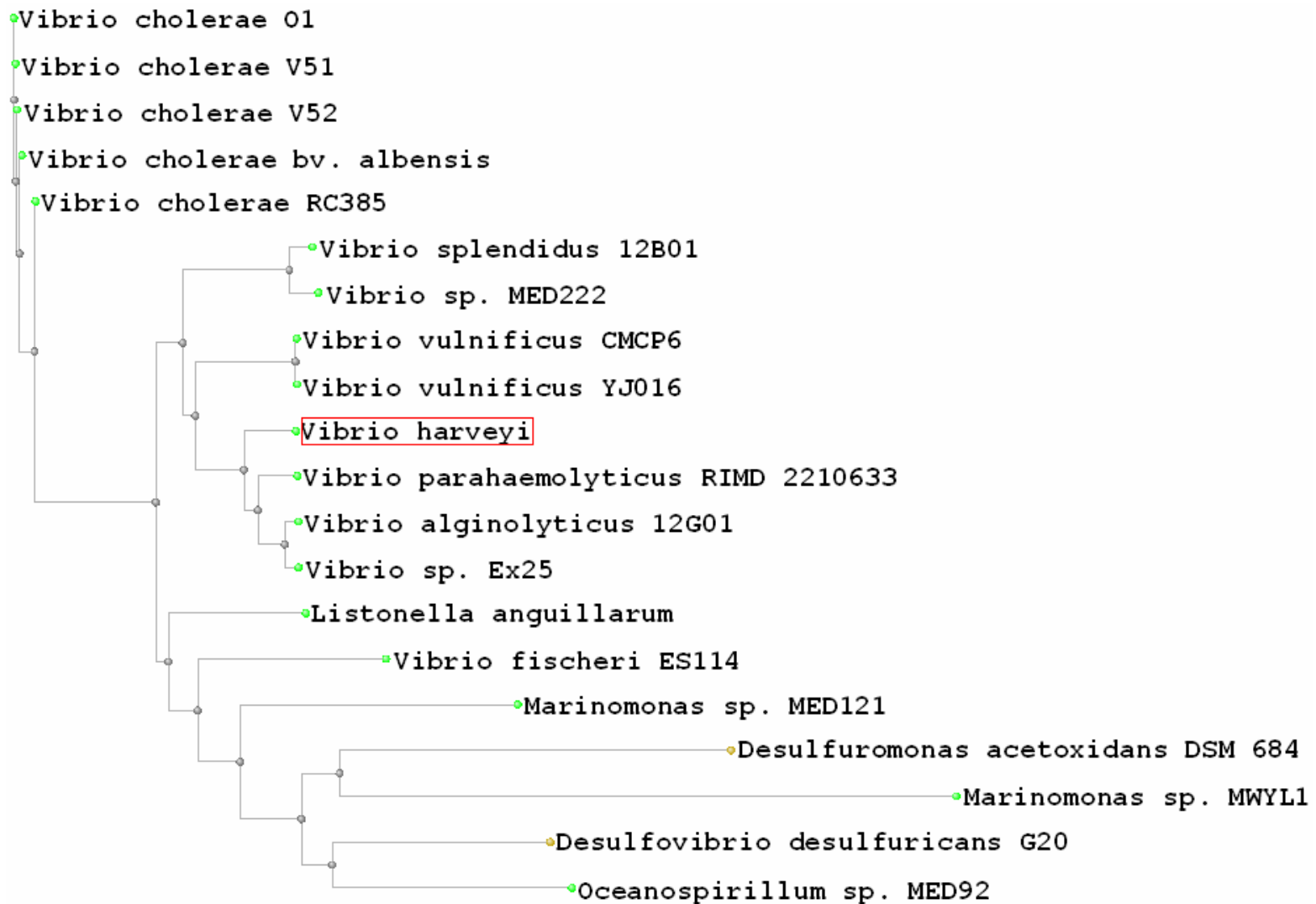
Fig. 4.10: Phylogenetic distance tree of LuxP homologs from different γ- proteobacteria (green nodes) and δ-proteobacteria (yellow nodes) constructed using the BLAST pairwise alignments. The blastp query sequence (*V. harveyi* LuxP) is shown with a red box.
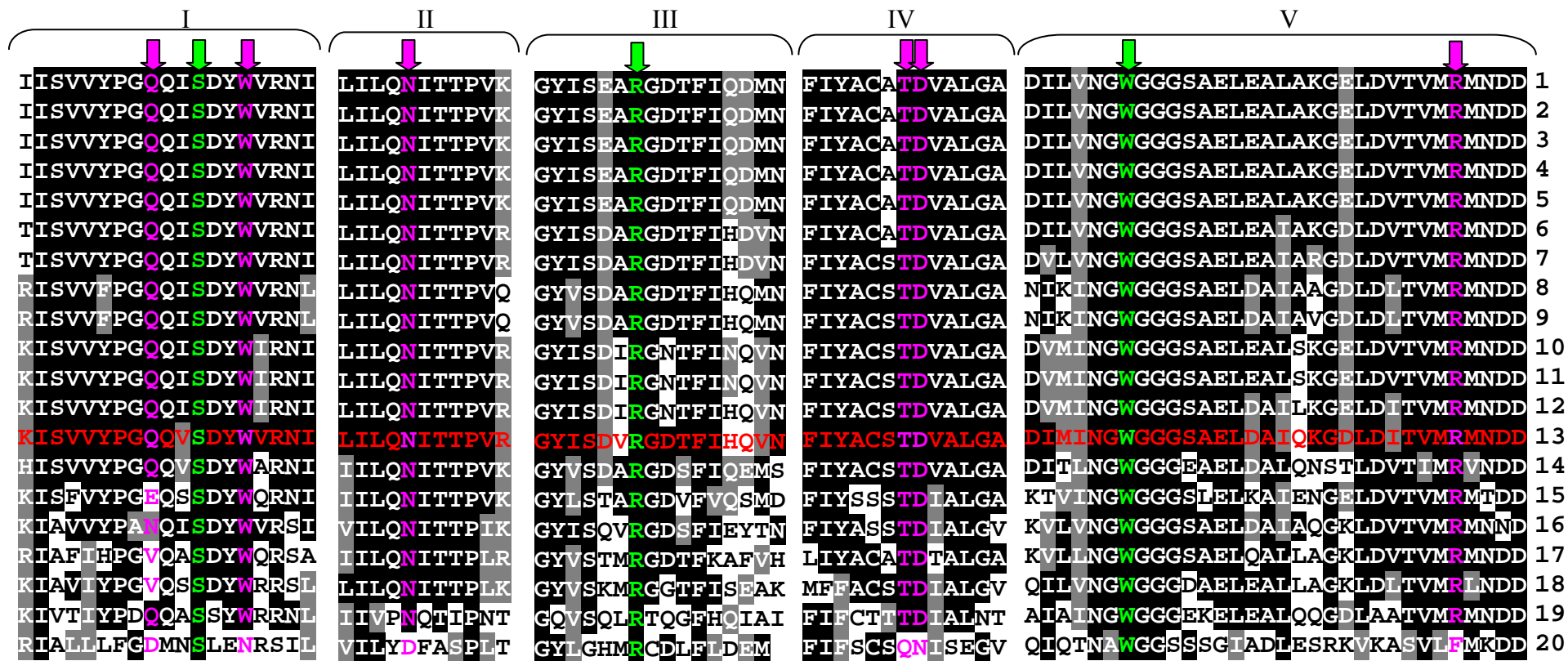
Fig. 4.11: ClustalW-BOXSHADE multiple sequence alignment of LuxP homologs from different bacteria

I, II, III, IV, and V show five conserved regions of the alignment that possibly form AI-2 binding domains. *V. harveyi* LuxP is shown in red. The arrows point to the nine different amino acids of *V. harveyi* LuxP that are involved in forming the motif that binds AI-2. Green arrows indicate amino acids that are identical (shown in green) in all the twenty proteins. Pink arrows indicate amino acids that differ in at least one species (shown in pink). 1; *Vibrio cholerae* O1, 2; *V. cholerae* V51, 3; *V. cholerae* V52, 4; *V. cholerae* bv. Albensis, 5; *V. cholerae* RC385, 6; *V. splendidus* 12B01, 7; *Vibrio* sp. MED222, 8; *V. vulnificus* CMCP6, 9; *V. vulnificus YJ016*, 10; *V. alginolyticus* 12G01, 11; *Vibrio* sp. Ex25, 12; *V. parahaemolyticus* RIMD 2210633, 13; *V. harveyi*, 14; *Listonella anguillarum*, 15; *V. fischeri* ES114, 16; *Marinomonas* sp. MED121, 17; *Desulfovibrio desulfuricans* G20, 18; *Oceanospirillum* sp. MED92, 19; *Desulfuromonas acetoxidans* DSM 684, 20; *Marinomonas* sp. MWYL1.

After growth in the wells of PVC plates for 72 hours and staining with crystal violet, *H. somni* formed a visible biofilm, comparable to that of *Pseudomonas aeruginosa*. However, different *H. somni* strains exhibited a marked variability in their abilities to form biofilms in PVC wells (Fig. 4.12). Generally, pathogenic isolates formed a significant biofilm whereas preputial isolates formed a weak biofilm when tested in this assay. Laboratory strains of *E. coli* used as controls in the assay were unable to form biofilms (data not shown).
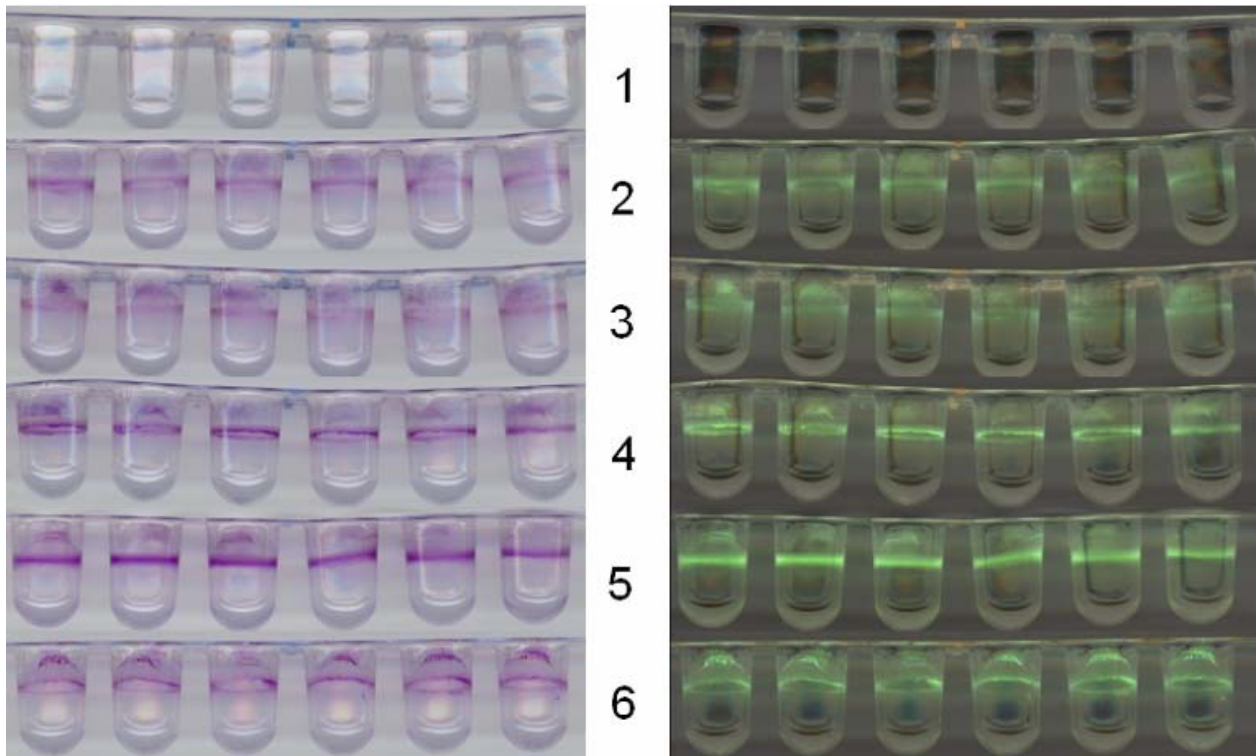
Fig. 4.12: Biofilm formation by *H. somni* strains in the wells of a PVC plate (side view) 1; BHI medium, 2; strain 2336, 3; strain 129Pt, 4; strain 8025, 5; strain 738, 6; strain 649. Left image: bacterial attachment to the PVC surface was stained with crystal violet and photographed. Right image: same as in left but the image has been 'inverted' to render crystal violet fluoresce green.

H. somni strains also exhibited a slimy biofilm growth in plastic conical tubes after 72 hours of incubation followed by staining with crystal violet (Fig. 4.13).
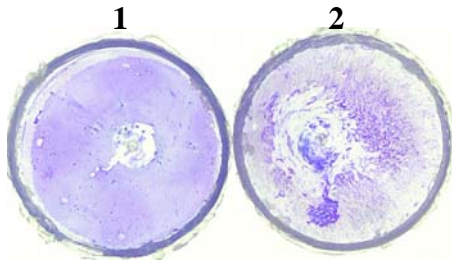

Fig. 4.13: Biofilm formation by *H. somni* in 50-ml plastic conical tubes (top view) 1; strain 2336, 2; strain 129Pt.

H. somni was also observed to form biofilms on glass slides and coverslips. Confocal laser microscopy of *H. somni* cultured on glass slides using BHI medium followed by staining with CAAF revealed that a mature biofilm-like growth occurs between 3 and 5 days of incubation (Fig. 4.14).
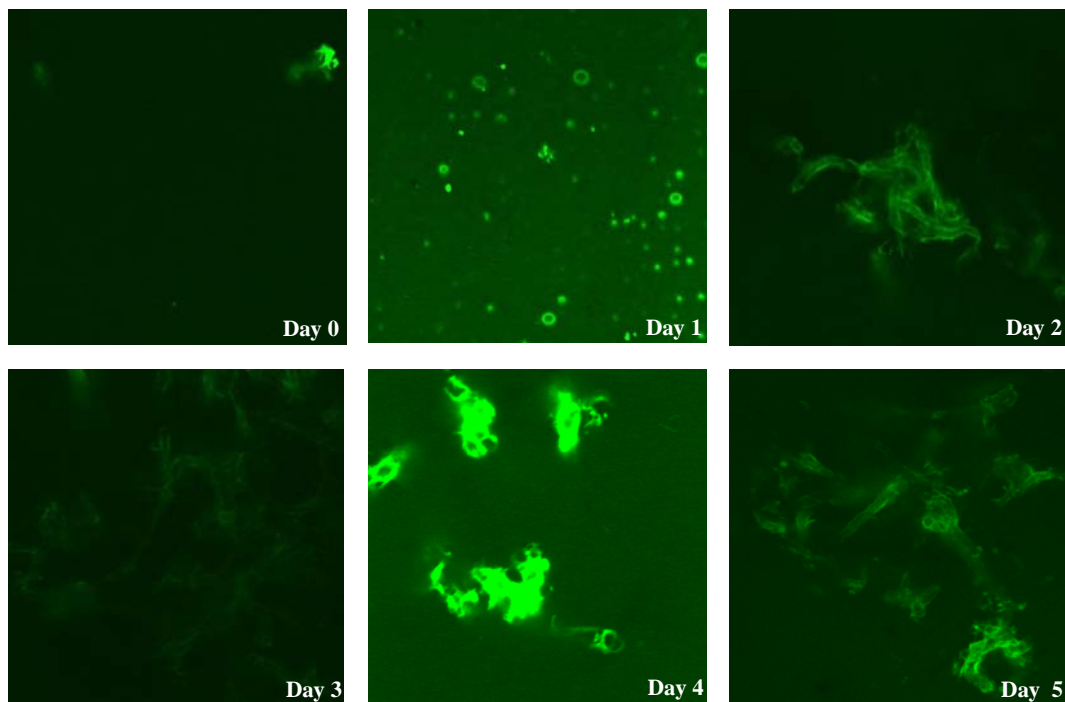

Fig. 4.14: Confocal laser microscopy of *H. somni* strain 738 cultured on glass slides Day 0; bacterial seeding, Day 1; bacterial attachment, Days 2 and 3; biofilm development, Day 4; biofilm maturation, Day 5; biofilm degeneration.

Biofilm formation by phosphorylcholine variants of *H. somni* pneumonia strain 93

As outlined in chapter I, the phase-variable addition of phosphorylcholine (ChoP) to cell surface components can facilitate attachment and survival of bacterial commensals and pathogens associated with mucosal surfaces. Phosphorylcholine has previously been shown to promote the establishment of stable biofilms of nontypeable *H. influenzae* (Hong et al., 2006). Variants of *H. somni* pneumonia strain 93 were selected for either high or low expression of ChoP by colony immunoblotting with an anti-phosphorylcholine antibody. The ChoP-positive as well as ChoP-negative variants were further enriched through selective passage in culture (Elswaifi, 2006). The ability of these variants to form biofilms *in vitro* was tested. In four different experiments, it was found that ChoP-negative variants of *H. somni* strain 93 form considerably less biofilm compared to ChoP-positive variants of strain 93 (Fig. 4.15). This indicated that ChoP expression may influence *H. somni* biofilm formation *in vivo*.

Biofilm formation by *H. somni* strain 129Pt and *E. coli* DH5α overexpressing *luxS*

To test the phenotypic consequences of expression of *luxS* on multicopy plasmids, *luxS* was cloned into pHS649SS as well as pLSKS, and *H. somni* strain 129Pt was transformed with *luxS*-containing plasmids. The ability of the transformants to form biofilms was tested by culturing in 50-ml plastic conical tubes. It was found that strains containing multiple copies of the *luxS* gene formed slightly thicker biofilms compared to strains containing a single copy of the *luxS* gene (Fig. 4.16).

Furthermore, in a similar assay, *E. coli* DH5α containing pCR2.1TOPO*luxS* formed slightly more biofilm compared to *E. coli* DH5α containing pCR2.1TOPO (Fig. 4.16). This indicates that *luxS* expression may influence *H. somni* biofilm formation *in vivo*.

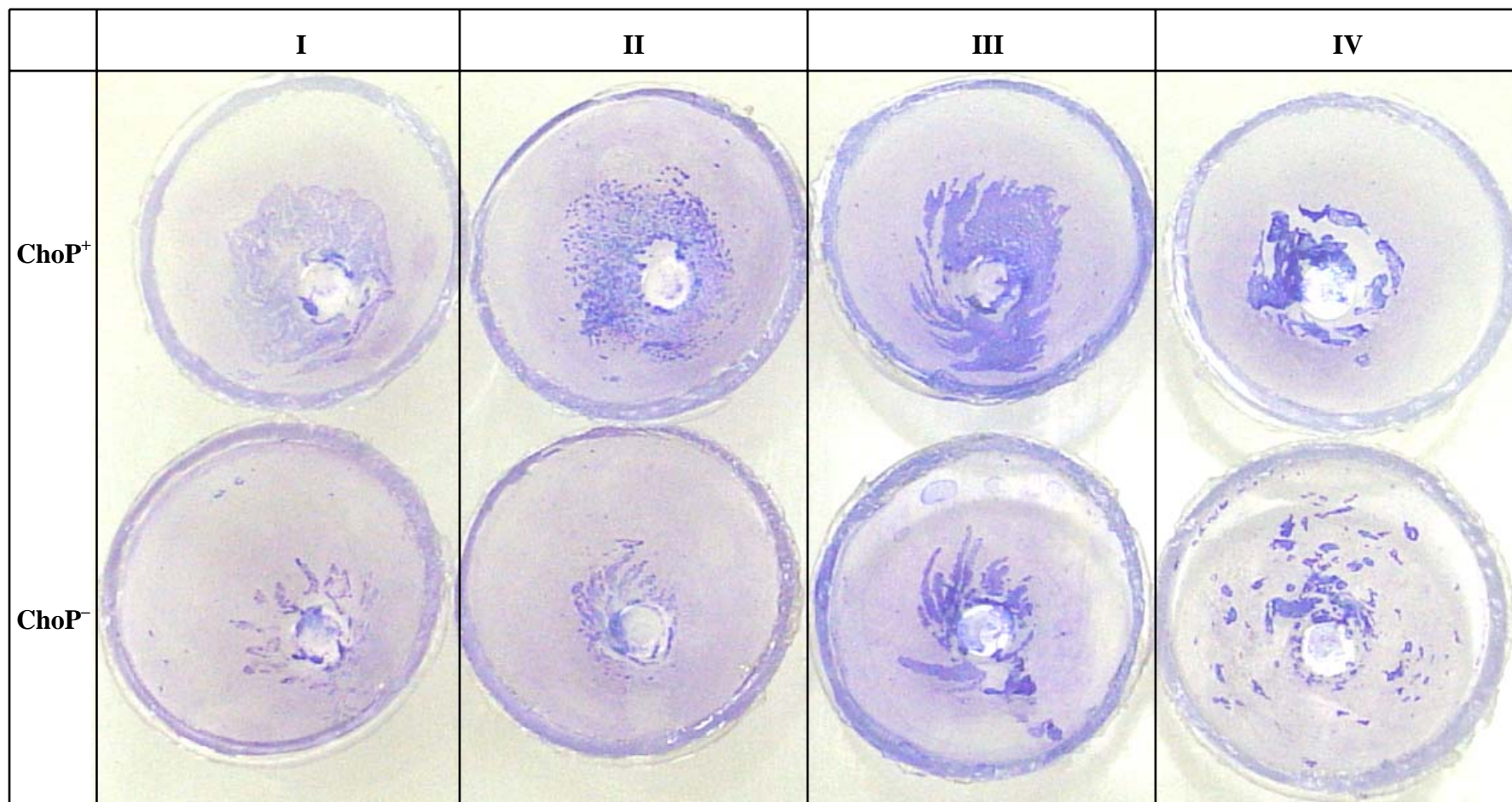Fig. 4.15: Biofilm formation by phosphorylcholine variant strains of *H. somni*

ChoP$^+$; strain 93 ChoP-positive variants, ChoP$^-$; strain 93 ChoP-negative variants.

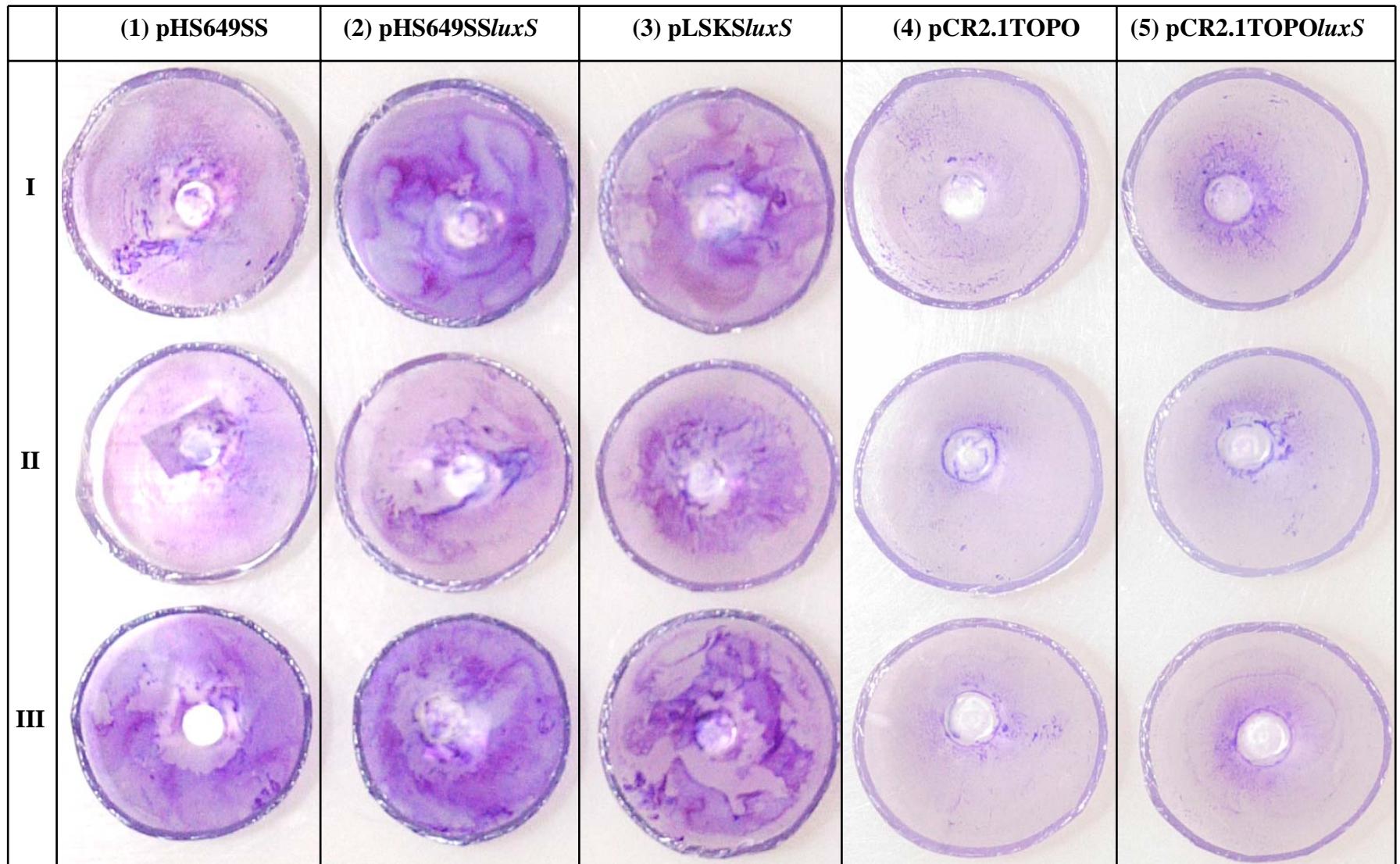I, II, III, and IV indicate assays performed using same strains on four different days.

Fig. 4.16: Biofilm formation by *H. somni* strain 129Pt and *E. coli* DH5α overexpressing *luxS*

1, 2, 3; *H. somni* strain 129Pt containing pHS649SS, pHS649SS*luxS*, and pLSKS*luxS*, respectively. 4, 5; *E. coli* DH5α containing pCR2.1TOPO and pCR2.1TOPO*luxS*, respectively. I, II, and III indicate assays performed using same strains on three different days.

Effect of furanone on biofilm growth of *H. somni* strain 738

        To test the effect of a synthetic derivative of the *Delisea pulchra* furanone, *(5Z)*-4-bromo-5-(bromomethylene)-3-butyl-2*(5H)*-furanone [Fur-1], on biofilm formation by *H. somni*, strain 738 was cultured on glass slides using BHI medium for up to five days with various concentrations of furanone. Biofilm development was analyzed by confocal laser microscopy as outlined previously. The furanone was found to inhibit *H. somni* biofilm growth in a dose-dependent manner, with no inhibition observed at 5 and 10 μg/ml of furanone, and complete inhibition at 20 and 40 μg/ml of furanone (Fig. 4.17).
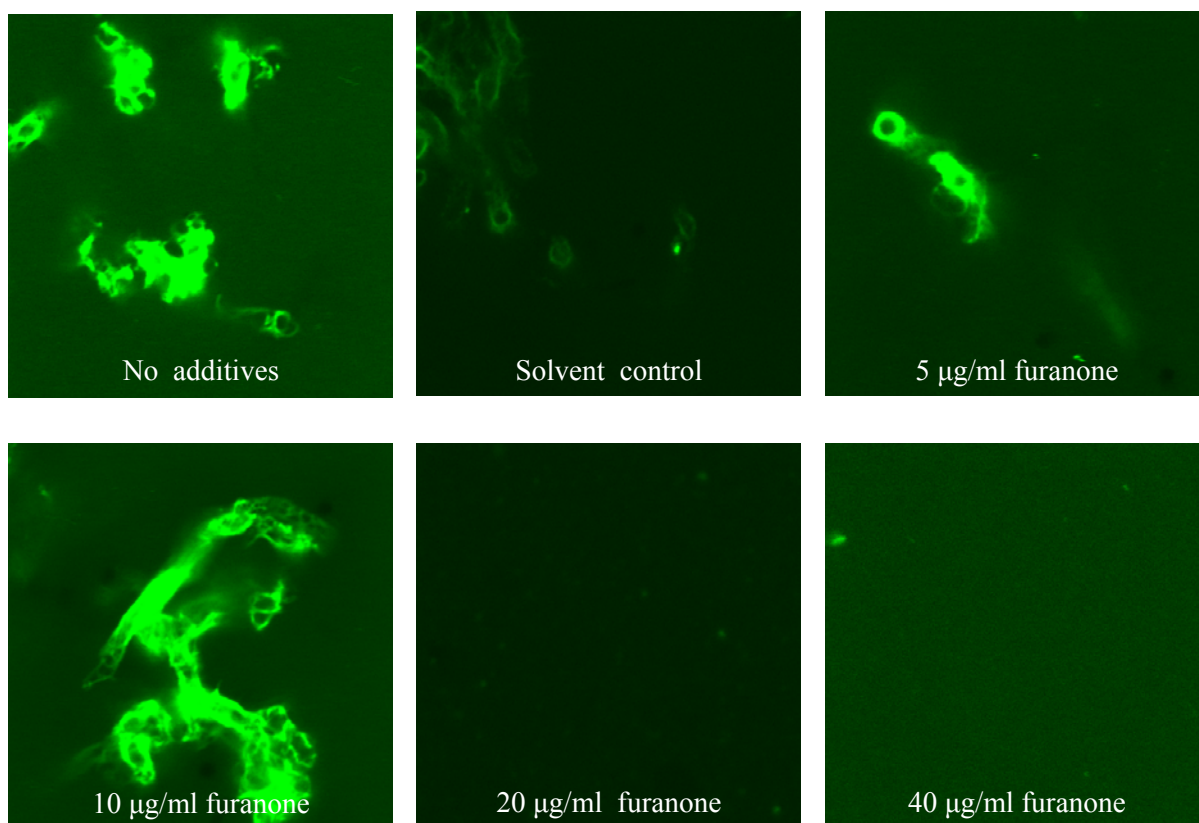

Fig. 4.17: Effect of furanone on biofilm growth of *H. somni* strain 738 cultured on glass slides for 4 days. Concentrations of furanone added are shown on each image. Solvent control refers to the addition of 135 μl of 95% ethanol.

Effect of furanone on biofilm growth of *H. somni* strain 2336

To further test the effect of furanone on biofilm formation, *H. somni* strain 2336 was cultured in 50-ml plastic conical tubes for 72 hours after addition of various concentrations of furanone. The furanone was again found to inhibit *H. somni* biofilm growth in a dose-dependent manner, with no inhibition observed at 12 and 14 µg/ml and complete inhibition at 16 and 18 µg/ml (Fig. 4.18). Therefore, furanone appears to affect the growth of *H. somni* under culture conditions designed to favor sessile growth.
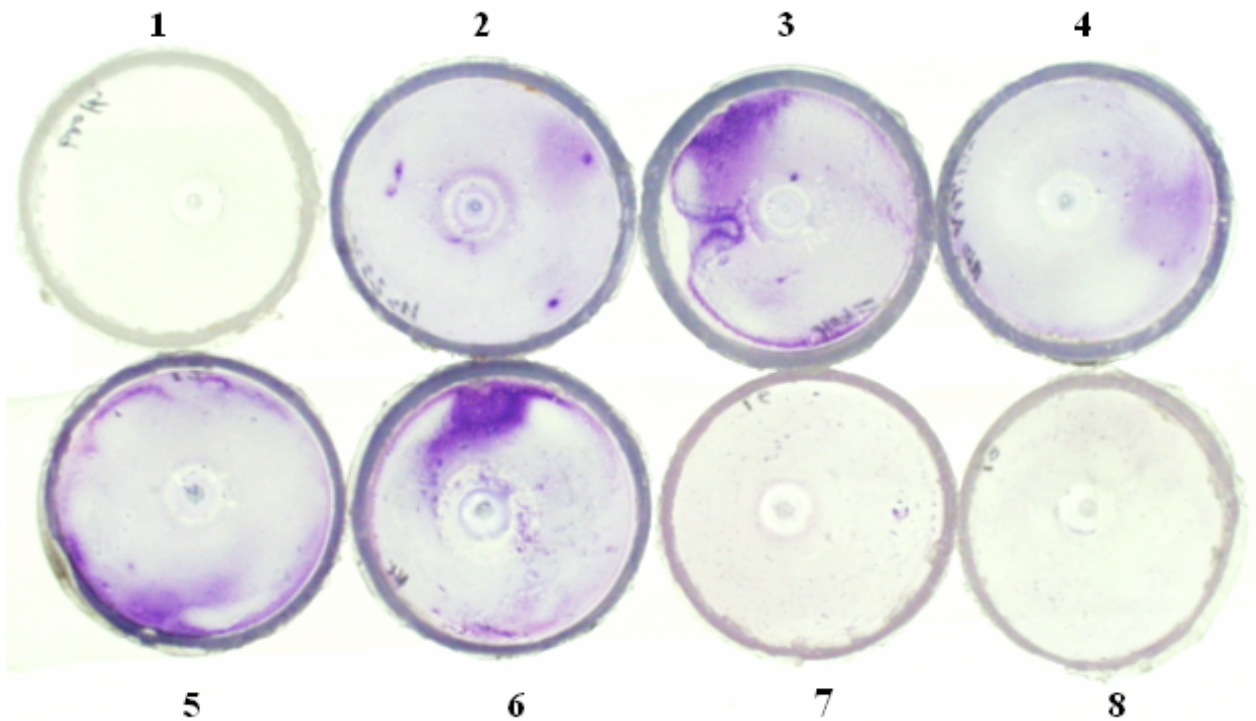

Fig. 4.18: Effect of furanone on biofilm growth of *H. somni* strain 2336 cultured in 50-ml plastic conical tubes for 72 hours. 1; BHI medium, 2; bacteria with no additives, 3; bacteria with 6 µl of 95% ethanol (solvent control), 4; bacteria with no additives, 5; bacteria with 12 µg/ml of furanone, 6; bacteria with 14 µg/ml of furanone, 7; bacteria with 16 µg/ml of furanone, 8; bacteria with 18 µg/ml of furanone.

Effect of furanone on planktonic growth of *H. somni* strain 2336

       To test if furanone inhibits planktonic growth of *H. somni* when cultured in BHI medium with shaking, bacteria were incubated with various concentrations of furanone. Furanone was found to inhibit the growth of *H. somni* strain 2336 in a dose-dependent manner during a 6 hour culture period, with complete inhibition observed at 16 μg/ml (Fig. 4.19). This confirms that furanone affects the growth of *H. somni* under culture conditions designed to favor planktonic growth.
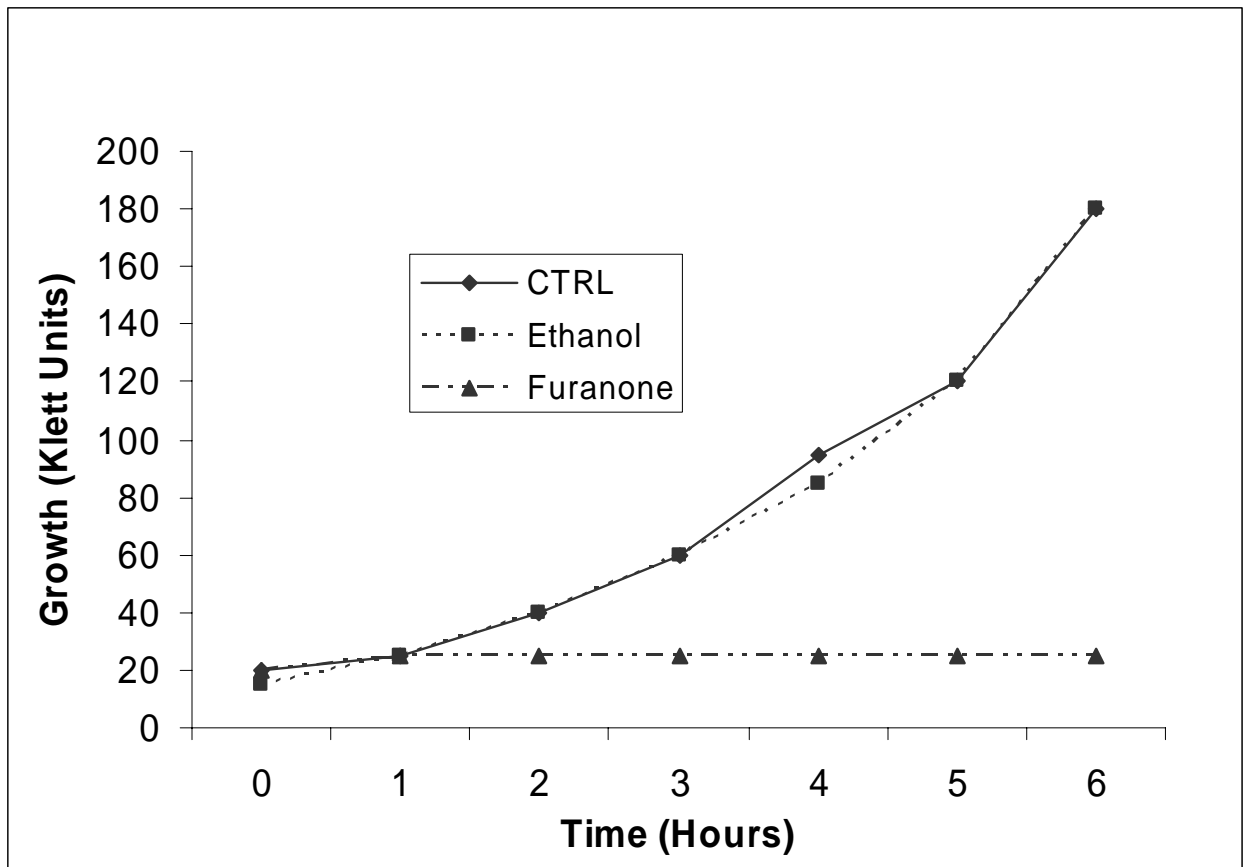

Fig. 4.19: Effect of furanone on planktonic growth of *H. somni* strain 2336 cultured in BHI medium with shaking. CTRL; bacteria cultured with no additives, Ethanol; bacteria cultured with 55 μl of 95% ethanol (solvent control), Furanone; bacteria cultured with 16 μg/ml furanone.

Discussion

In this study, the positioning of *luxS* ORFs on the chromosomes of *H. somni* strains 2336 and 129Pt were compared using genome sequence data. Although the *luxS* ORFs are highly homologous and are flanked by similar genes in both strains of *H. somni*, they are located on different regions of the chromosomes and occur in opposite orientations, indicating extensive genome rearrangement during evolution. Functional characterization using a complemented *E. coli* strain revealed that *H. somni* strain 2336 *luxS* encodes S- ribosylhomocysteinase that participates in AI-2 biosynthesis.

During the biosynthesis of AI-2, the product of *luxS* catalyzes the conversion of S-ribosylhomocysteine to 4,5-dihidroxy-2,3-pentanedione (DPD). The final steps in the biochemical conversion of DPD to the functional AI-2 signaling molecules remain poorly understood. Nevertheless, the location of *luxS* among a cluster of genes involved in metabolism and transport indicated that LuxS plays other roles in cell physiology and metabolism besides being an intermediary enzyme in the biosynthesis of AI-2. Recent studies have shown that deletion of *Lactobacillus rhamnosus luxS* results in pleiotropic physiological effects and further emphasize the multiple roles played by LuxS (Lebeer et al., 2007). In few other bacteria, *luxS* mutants have been shown to be growth deficient under certain conditions (Blehert et al., 2003; Fong et al., 2005). Therefore, it is very likely that the phenotypes due to direct effects of a lack of AI-2 production overlap with the phenotypes due to indirect effects of growth deficiency in such bacteria. In this study, several attempts were made to delete *luxS* in *H. somni* by homologous recombination, but in vain. In view of this, it is possible that *luxS* is indispensable to metabolism as well as regulation in some bacteria and mutations may have a lethal effect. Future attempts to create and characterize *luxS* mutants of *H. somni* will need to consider the above factors.

Considerable variability in luminescence induction by culture supernatants from different bacteria was observed during efforts to test the reactivity of *H. somni* AI-2 molecules with *V. harveyi* strain BB170. These results point to the possibility that the AI-2 molecules from different bacteria are, like the AI-1 molecules, a group of related entities rather than the unique, single, universal signal as originally proposed. The degree of relatedness of these molecules to the *V. harveyi* AI-2 is probably what determines the outcome of the reporter assay. When the AI-2 molecules of a certain *luxS* containing bacterium are structurally different from those of *V. harveyi*, the affinity and specificity of ligand (AI-2) and receptor (*V. harveyi* LuxP) interactions may be altered. This non-specific interaction could lead to structural modifications of LuxP, ineffective signal transduction, and very little luminescence induction in the reporter strain (Stock, 2006).

Furthermore, addition of boric acid has been reported to increase the production of AI-2 molecules as well as luminescence induction in *V. harveyi* (Chen et al., 2002). In the current report, addition of boric acid (Sigma) at 0.1 mg/ml had no effect on luminescence induction by *H. somni* culture supernatants. However, culture supernatants from *V. harveyi* BB152 grown in the presence of boric acid were twice as effective in luminescence induction compared to those from *V. harveyi* BB152 grown in the absence of boric acid (data not shown). These results indicate that AI-2 molecules from bacteria other than *V. harveyi* may not contain boron.

If the universal signal hypothesis was true, then the LuxP protein, which binds the AI-2 signal, would be expected to be very similar or to have similar AI-2 binding domains in all *luxS*-containing bacteria that were examined. This crucial point has often been overlooked in the past in the light of the universal signal hypothesis. However, detailed analyses of LuxP, LuxR, and LuxS homologs from different bacteria revealed that the protein components of the AI-2

biosynthetic pathway are strikingly similar whereas the protein components of the AI-2 response pathway, notably LuxP, are different.

Similar results have been reported previously, when culture supernatants from several bacterial species induced a range of responses in the *V. harveyi* luminescence system (Greenberg et al., 1979; Bassler et al., 1997; Forsyth and Cover, 2000). It has also been shown that the AI-2 molecule from *Salmonella typhimurium* is structurally different from that of *V. harveyi* (Miller et al., 2004). Thus, the results of the current study support the hypothesis that AI-2 molecules from different bacteria have different structures. A simple model is presented fig. 4.20 to reflect these findings.
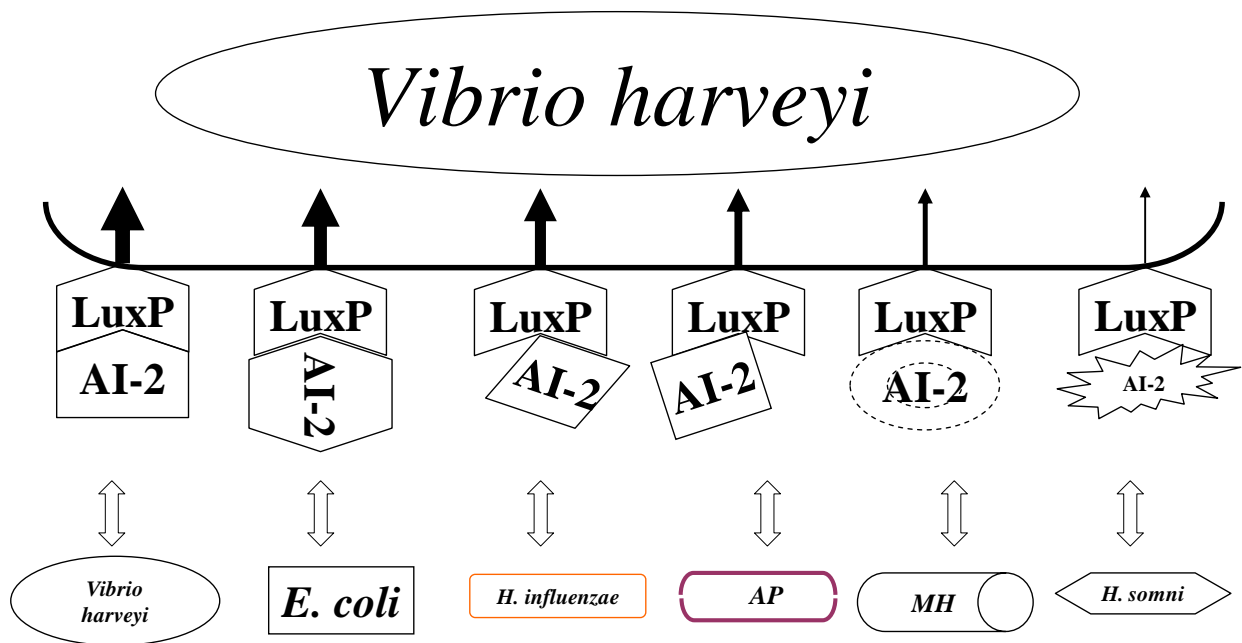

Fig. 4.20: Hypothetical diversity of AI-2 from different bacteria. When AI-2 molecules from various bacteria have different structures, luminescence induction varies as a function of their interaction with *V. harveyi* LuxP. The bidirectional arrows represent the production and release of AI-2 by different bacteria. The dark arrows represent relative differences in light induction by culture supernatants from respective bacteria. AP; *A. pleuropneumoniae*, MH; *M. haemolytica*.

Biofilm formation appears to be a recurrent theme among bacteria of diverse taxa. As a sessile mode of growth characterized by the attachment of bacteria to inanimate and animate surfaces, a biofilm may represent a complex community wherein bacteria manifest sophisticated phenotypes that could help them to survive and reproduce better than in the planktonic state (Hall-Stoodley et al., 2004). In the current study, it is not surprising that different strains of *H. somni* exhibited marked variation in their ability to form biofilms. These differences may reflect the underlying genetic differences and the evolutionary dissimilarities between the strains, which have only now begun to be understood better with the availability of complete genome sequences. The reduced ability of *H. somni* strain 129Pt to form biofilms may also be due to slow growth rates that have been observed during the planktonic culture of this strain.

The decoration of cell surface structures with ChoP appears to be common in bacteria that colonize the mucosal surfaces. It has been demonstrated that ChoP expression is important in the pathogenesis of *H. influenzae* (Weiser et al., 1998). It is also known that *H. influenzae* biofilms contain a higher percentage of ChoP-positive variants (Hong et al., 2007). In view of this, it is interesting to note that ChoP-positive variants of *H. somni* strain 93 formed more biofilm compared to ChoP-negative variants of strain 93 in the current study. It is also noteworthy that the *licA* gene, essential for the biosynthesis of ChoP, is truncated in *H. somni* strain 129Pt (Elswaifi, 2006), and this strain forms less biofilm compared to *H. somni* strain 2336 (Fig. 4.13).

Although overexpression of *luxS* appeared to influence the biofilm phenotype in *H. somni* and *E. coli*, these observations need to be further confirmed using the 'flow cell' method of biofilm quantification. It is possible that in *H. somni*, as in other bacteria, biofilm formation is regulated by several genes. The roles of *luxS* and ChoP expression in biofilm formation by

H. somni will need to be investigated in detail by construction of isogenic mutants that lack one or both of these functions. The pathogenic role of *H. somni* biofilm formation will also need to be examined in the bovine host using biofilm-deficient mutants.

Natural and synthetic furanones are gaining importance as novel antibacterial agents. In the current study, a synthetic furanone derivative (Fur-1) was found to inhibit biofilm as well as planktonic growth of *H. somni*. Although furanones have been shown previously to inhibit QS-mediated phenotypes in a few Gram-negative bacteria without affecting their growth (de Nys et al., 2006), such an effect on *H. somni* could not be confirmed using the biofilm assay performed in 96-well PVC plates or 50-ml plastic conical tubes. Furanones have also been found to inhibit the growth of Gram-positive bacteria at concentrations above 10 μg/ml (Ren et al., 2002; Jones et al., 2005), and the growth of Gram-negative bacteria at concentrations above 30 μg/ml (Ren et al., 2001). In view of this, it is possible that furanones have variable effects on different bacteria under different growth conditions. Nevertheless, the current study shows the antibiotic effect of furanone on *H. somni*.

In conclusion, this study has shown that *H. somni* LuxS is a S- ribosylhomocysteinase that is possibly involved in AI-2 biosynthesis. Comparative analyses also support the concept that AI-2 is not a universal QS signal, as originally proposed. A novel method to test biofilm formation by bacteria using conical tubes has also been developed. Using 50-ml plastic conical tubes and the traditional 96-well PVC plates, it has been demonstrated that most pathogenic strains of *H. somni* form a prominent biofilm. A physiological role for *luxS* and ChoP expression during sessile growth has been proposed based on their effect on biofilm formation *in vitro*. The antibacterial effect of furanone indicated that the substance has potential clinical applications. Isogenic mutants of *H. somni* and sophisticated experimental approaches are required to verify these findings.

References

Auch AF, Henz SR, Holland BR, Goker M (2006). Genome BLAST distance phylogenies inferred from whole plastid and whole mitochondrion genome sequences. *BMC Bioinformatics* **7**: 350.

Bassler BL, Greenberg EP, Stevens AM (1997). Cross-species induction of luminescence in the quorum-sensing bacterium *Vibrio harveyi*. *Journal of Bacteriology* **179**: 4043–4045.

Blehert DS, Palmer RJ Jr, Xavier JB, Almeida JS, Kolenbrander PE (2003). Autoinducer 2 production by *Streptococcus gordonii* DL1 and the biofilm phenotype of a *luxS* mutant are influenced by nutritional conditions. *Journal of Bacteriology* **185**: 4851-4860.

Chen X, Schauder S, Potier N, Van Dorsselaer A, Pelczer I, Bassler BL, Hughson FM (2002). Structural identification of a bacterial quorum-sensing signal containing boron. *Nature* **415**: 545-549.

Daines DA, Bothwell M, Furrer J, Unrath W, Nelson K, Jarisch J, Melrose N, Greiner L, Apicella M, Smith AL (2005). *Haemophilus influenzae luxS* mutants form a biofilm and have increased virulence. *Microbial Pathogenesis* **39**: 87-96.

de Nys R, Givskov M, Kumar N, Kjelleberg S, Steinberg PD (2006). Furanones. *Progress in Molecular and Subcellular Biology* **42**: 55-86.

Donlan RM and Costerton JW (2002). Biofilms: survival mechanisms of clinically relevant microorganisms. *Clinical Microbiology Reviews* **15**: 167-193.

Elswaifi S (2006). The molecular characterization of phosphorylcholine (ChoP) on *Histophilus somni* lipooligosaccharide: contribution of ChoP to bacterial virulence and pathogenesis. *PhD dissertation*, Virginia Polytechnic Institute and State University, Blacksburg, VA.

Federle MJ and Bassler BL (2003). Interspecies communication in bacteria. *Journal of Clinical Investigation* **112**: 1291-1299.

Fong KP, Chung WO, Lamont RJ, Demuth DR (2001). Intra- and interspecies regulation of gene expression by *Actinobacillus actinomycetemcomitans* LuxS. *Infection and Immunity* **69**: 7625-7634.

Fong KP, Gao L, Demuth DR (2003). *luxS* and *arcB* control aerobic growth of *Actinobacillus actinomycetemcomitans* under iron limitation. *Infection and Immunity* **71**: 298-308.

Forsyth MH and Cover TL (2000). Intercellular communication in *Helicobacter pylori*: *luxS* is essential for the production of an extracellular signaling molecule. *Infection and Immunity* **68**: 3193-3199.

Fuqua WC, Winans SC, Greenberg EP (1994). Quorum sensing in bacteria: the LuxR-LuxI family of cell density-responsive transcriptional regulators. *Journal of Bacteriology* **176**: 269–275.

Fuqua WC and Greenberg EP (1998). Self perception in bacteria. *Current Opinion in Microbiology* **1**: 183–189.

Gallaher TK, Wu S, Webster P, Aguilera R (2006). Identification of biofilm proteins in non-typeable *Haemophilus Influenzae*. *BMC Microbiology* **6**: 65.

Greenberg EP, Hastings JW, Ulitzer S (1979). Induction of luciferase synthesis in *Beneckea harveyi* by other marine bacteria. *Archives of Microbiology* **120:** 87–91.

Haase EM, Bonstein T, Palmer RJ Jr, Scannapieco FA (2006). Environmental influences on *Actinobacillus actinomycetemcomitans* biofilm formation. *Archives of Oral Biology* **51**: 299-314.

Hall-Stoodley L, Costerton JW, Stoodley P (2004). Bacterial biofilms: from the natural environment to infectious diseases. *Nature Reviews Microbiology* **2**: 95-108.

Hong W, Mason K, Juriscek J, Novotny L, Bakaletz LO, Swords WE (2007). Phosphorylcholine decreases early inflammation and promotes establishment of stable biofilm communities of nontypeable *Haemophilus influenzae* strain 86-028 NP in a chinchilla model of otitis media. *Infection and Immunity* **75**: 958-965.

Jones MB, Jani R, Ren D, Wood TK, Blaser MJ (2005). Inhibition of *Bacillus anthracis* growth and virulence-gene expression by inhibitors of quorum-sensing. *Journal of Infectious Diseases* **191**: 1881-1888.

Keller L and Surette MG (2006). Communication in bacteria: an ecological and evolutionary perspective. *Nature Reviews Microbiology* **4**: 249-258.

Kendall MM and Sperandio V (2007). Quorum sensing by enteric pathogens. *Current Opinion in Gastroenterology* **23**: 10-15.

Kuhn DM, George T, Chandra J, Mukherjee PK, Ghannoum MA (2002). Antifungal susceptibility of Candida biofilms: unique efficacy of amphotericin B lipid formulations and echinocandins. *Antimicrobial Agents and Chemotherapy* **46**: 1773-1780.

Lebeer S, De Keersmaecker SC, Verhoeven TL, Fadda AA, Marchal K, Vanderleyden J (2007). Functional analysis of *luxS* in the probiotic strain *Lactobacillus rhamnosus* GG reveals a central metabolic role important for growth and biofilm formation. *Journal of Bacteriology* **189**: 860-871.

Miller ST, Xavier KB, Campagna SR, Taga ME, Semmelhack MF, Bassler BL, Hughson FM (2004). *Salmonella typhimurium* recognizes a chemically distinct form of the bacterial quorum-sensing signal AI-2. *Molecular Cell* **15**: 677-687.

O'Toole GA, Pratt LA, Watnick PI, Newman DK, Weaver VB, Kolter R (1999). Genetic approaches to study of biofilms. *Methods in Enzymology* **310**: 91-109.

O'Toole G, Kaplan HB, Kolter R (2000). Biofilm formation as microbial development. *Annual Review of Microbiology* **54**: 49-79.

Ren D, Sims JJ, Wood TK (2001). Inhibition of biofilm formation and swarming of *Escherichia coli by* (5Z)-4-bromo-5-(bromomethylene)-3-butyl-2(5H)-furanone. *Environmental Microbiology* **3**: 731-736.

Ren D, Sims JJ, Wood TK (2002). Inhibition of biofilm formation and swarming of *Bacillus subtilis* by (5Z)-4-bromo-5-(bromomethylene)-3-butyl-2(5H)-furanone. *Letters in Applied Microbiology* **34**: 293-299.

Sanders JD, Tagawa Y, Briggs RE, Corbeil LB (1997). Transformation of a virulence associated gene of *Haemophilus somnus* into a strain lacking the gene. *FEMS Microbiology Letters* **154**: 251-258.

Stewart PS and Costerton JW (2001). Antibiotic resistance of bacteria in biofilms. *Lancet* **358**: 135-138.

Stock AM (2006). Transmembrane signaling by asymmetry. *Nature Structural & Molecular Biology* **13**: 862-863.

Sturme MH, Kleerebezem M, Nakayama J, Akkermans AD, Vaugha EE, de Vos WM (2002). Cell to cell communication by autoinducing peptides in gram-positive bacteria. *Antonie van Leeuwenhoek* **81**: 233-43.

Surette MG and Bassler BL (1998). Quorum sensing in *Escherichia coli* and *Salmonella typhimurium*. *Proceedings of the National Academy of Sciences of the U.S.A.* **95**: 7046-7050.

Surette MG, Miller MB, Bassler BL (1999). Quorum sensing in *Escherichia coli*, *Salmonella typhimurium*, and *Vibrio harveyi*: a new family of genes responsible for autoinducer production. *Proceedings of the National Academy of Sciences of the U.S.A.* **96**: 1639-1644.

Trampuz A and Widmer AF (2006). Infections associated with orthopedic implants. *Current Opinion in Infectious Diseases* **19**: 349-356.

van der Vinne AN, Lo RY, Shewen PE (2005). Construction and analysis of a *Mannheimia haemolytica* A1 *luxS* mutant. *Veterinary Microbiology* **110**: 53-66.

Weiser JN, N Pan, McGowan KL, Musher D, Martin A, Richards J (1998). Phosphorylcholine on the lipopolysaccharide of *Haemophilus influenzae* contributes to persistence in the respiratory tract and sensitivity to serum killing mediated by C-reactive protein. *Journal of Experimental Medicine* **187**: 631-640.

Wood GE, Dutro SM, Totten PA (1999). Target cell range of *Haemophilus ducreyi* hemolysin and its involvement in invasion of human epithelial cells. *Infection and Immunity* **67**: 3740-3749.