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Functional Quorum Sensing Systems Affect Biofilm Formation and Protein Expression in *Yersinia pestis*

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Abstract. Gram-negative bacteria predominantly use two types of quorum sensing (QS) systems - LuxI-LuxR, responsible for synthesis of N-acylhomoserine lactones (AHL or AI-1 signal molecule), and LuxS, which makes furanones (AI-2 signal molecule). We showed that LuxS and two LuxI-LuxR (YtbIR and YpsIR) systems are functional in *Y. pestis*. Four different AHL molecules were detected in *Y. pestis* extracts using TLC bioassays. Our data suggest that YtbIR is responsible for the production of long chain AHLs. Confocal laser scanning microscopy showed that biofilm formation is decreased in an *ytbIR ypsIR luxS* mutant. Two-dimensional gel electrophoresis revealed altered levels of protein expression in a *Y. pestis* triple QS mutant at 26°C and 37°C.

15.1 Introduction

In the phenomenon known as quorum sensing (QS), bacterial cells communicate with each other via small extracellular molecules that indicate population density. The N-acylhomoserine lactones (AHLs) of Gram-negative bacteria have been the most extensively studied signaling molecules in QS systems and are also referred to as AI-1. AHLs vary in length, side chain substitutions, and the degree of acyl saturation, making them relatively species specific - the AHL produced by one bacterial species generally does not serve as a density signal for broad range of bacteria. AHLs are synthesized by the LuxI family of synthase proteins. At sufficiently high concentrations, AHLs diffuse back into the bacterial cell and bind to a LuxR-type regulatory protein. The LuxR-AHL complex activates the expression of a variety of genes (Fuqua et al. 2001).

In animal and plant pathogens, such as *Agrobacterium tumefaciens, Aeromonas hydrophila, Erwinia chrysanthemi, Pseudomonas aeruginosa*, and *Vibrio anguillarum*, AHL systems control the expression of a number of exported products that are proven or putative virulence factors. One rationale for QS regulation is that the production of virulence factors by isolated bacteria may cause a neutralizing host response. However, coordinated expression by a large population may overwhelm

host defenses and lead to invasion of cells, tissues, and blood vessels, followed by dissemination.

An independent, non-AHL, QS autoinducer (termed AI-2) has been identified. In this system, the signaling molecules are furanones produced by LuxS (Surette et al. 1999; Chen et al. 2002). Unlike AI-1, the AI-2 autoinducer is not species specific; AI-2 from *Vibrio harveyi* serves as a signal for *Salmonella* and *Escherichia*. Homologues of *luxS* are also widespread among bacteria - examples include *Borrelia burgdorferi, Clostridium perfringens,* enterohemorrhagic and enteropathogenic *E. coli, Haemophilus influenzae, Klebsiella pneumoniae, Mycobacterium tuberculosis, Neisseria gonorrhoeae, Neisseria meningitidis, Salmonella typhi, Salmonella typhimurium, Staphylococcus aureus, Streptococcus pneumoniae, Vibrio cholerae, V. anguillarum, Yersinia enterocolitica*, and *Y. pestis* (Schauder and Bassler 2001). There is evidence that the AI-2 system regulates expression of virulence factors in *S. typhimurium* and *V. cholerae*. In enterohemorrhagic and enteropathogenic *E. coli*, LuxS regulates the expression of intimin, the intimin receptor, and the type III secretion system encoded within the locus of enterocyte effacement (LEE) pathogenicity island (Sperandio et al. 1999).

We have identified three QS loci in the genome of *Y. pestis*, two being LuxIR-like and one LuxS-like, and have investigated the role of these systems in the production of signal molecules as well as biofilm formation.

15.2 Materials and Methods

15.2.1 Bacterial Strains, Plasmids, Culture Conditions and Recombinant DNA Techniques

Y. pestis strains and newly constructed plasmids used in this study are listed in Table 1. *E. coli* cells were grown on Luria broth (LB). *Y. pestis* cells were grown on TBA plates and in Heart Infusion Broth and LB. Cell growth was monitored on a Spectronic Genesys5 spectrophotometer at 620 nm. Where required, ampicillin (Ap; 100 μg/ml), kanamycin (Km; 50 μg/ml), or streptomycin (Sm; 50 μg/ml) was added to cultures. Standard recombinant DNA methods (Ausubel et al. 1987) were used to isolate and construct the various plasmids. Genomic DNA was isolated by the CTAB method. The plasmids were transformed into *E. coli* and *Y. pestis* cells using a standard $CaCl_2$ procedure and electroporation, respectively. When necessary, plasmid DNA or PCR products were sequenced by Retrogen, Inc. Synthetic oligonucleotide primers were purchased from Integrated DNA Technologies. To construct a deletion in *ytb*IR, two fragments of 646 and 643 bp, respectively, were amplified by PCR from either end of the *ytb*IR locus using pWSKYtbIR and primer pairs, luxIR1/2 (GACTAGTTTTGGCATACATTTGTTCAGC and TTCACCTCCCAGTTAAGA CT) and luxIR3/4 (TGTCGATATAATCACGCAGTG and GCTCTAGAGGCGT GTTAGCATTATTTGTC). The PCR products were ligated together and the ligation reaction was used as a template for a second PCR reaction with primers luxIR1 and

Table 1. Bacterial strains and plasmids used in this study

Strains or plasmids	Relevant characteristics	Reference or source
Y. pestis strains		
KIM6+	Pgm^+ Pla^+ Yps^+ Ytb^+ $LuxS^+$	Fetherston et al. 1992
KIM6	Pgm^-(Δpgm^-) Pla^+ Yps^+ Ytb^+ $LuxS^+$	Fetherston et al. 1992
KIM10+	Pgm^+ Pla^- Yps^+ Ytb^+ $LuxS^+$	Fetherston et al. 1992
KIM6-2083+	Pgm^+ Pla^+ Yps^+ Ytb^+ $LuxS^-$ (*luxS::kan2083)*	This study
KIM6-2106+	Pgm^+ Pla^+ Yps^- (Δ*ypsIR2106)* Ytb^+ $LuxS^+$	This study
KIM6-2107+	Pgm^+ Pla^+ Yps^+ Ytb^- (Δ*ytbIR2107)* $LuxS^+$	This study
KIM6-2108+	Pgm^+ Pla^+ Yps^- Ytb^- $LuxS^+$	This study
KIM6-2109+	Pgm^+ Pla^+ Yps^- Ytb^- $LuxS^-$	This study
KIM5(pCD1Ap)+	Pgm^+ Pla^+ Yps^+ Ytb^+ $LuxS^+$ Lcr^+	
KIM5-2083 (pCD1Ap)+	Pgm^+ Pla^+ Yps^+ Ytb^+ $LuxS^-$ Lcr+	This study
Plasmids		
pCD1Ap	71.7 kb, pCD1 with *bla* cassette inserted into *'yadA* , Lcr^+; Ap^r	Gong et al. 2001
pWSKYps1	4.1-Kb *Cla*I-*Sac*I fragment from KIM10+ cloned into pWSK29 (Wang and Kushner, 1991); Km^r *ypsIR*$^+$	This study
pWSKYps2	3.7-Kb *Xho*I-*Eco*RV fragment from pLuxYsp-1 cloned into pWSK29; Ap^r	This study
pKNGYps3	715-bp *Sal*I-*Dra*I and 1550-bp *Dra*I-*Xba*I fragments from pLuxYps2 ligated into pKNG101; Sm^r *ΔypsIR2106*	This study
pWSKYtbIR	8.065-Kb *Bgl*II fragment from KIM10+ ligated into the *Bam*HI site of pWSK29; Km^r *ytbIR*$^+$	This study
pBSYtbIR	1.289-Kb PCR fragment cloned into the *Eco*RV site of pBluescript; Ap^r	This study
pKNGYtbIR	1.2-Kb *Bam*HI-*Xba*I fragment from pBSYtbIR ligated into the same sites of pKNG101; Sm^r *ΔytbIR2107*	This study
pLuxS1	0.82-Kb *Hind*III fragment from KIM10+ cloned into pBluescript II KS (Stratagene); Ap^r *luxS*$^+$	This study
pLuxS2	0.85-kb *Xma*I-*Sal*I from pLuxS1 cloned into pKNG101 (Kaniga et al., 1991); Sm^r	This study
pLuxS3	1.27-kb *Bam*HI fragment consisting of Km^r gene from pUC4K (Yanisch-Perron et al.1985) cloned in *Bam*HI of *luxS* gene in pLUXS2 ; *luxS::kan2083*	This study

luxIR4. The 1289 bp fragment, which removes 1203 bp from the *ytb*IR locus, was cloned into the suicide vector pKNG101, generating pKNGYbtIR. To construct a

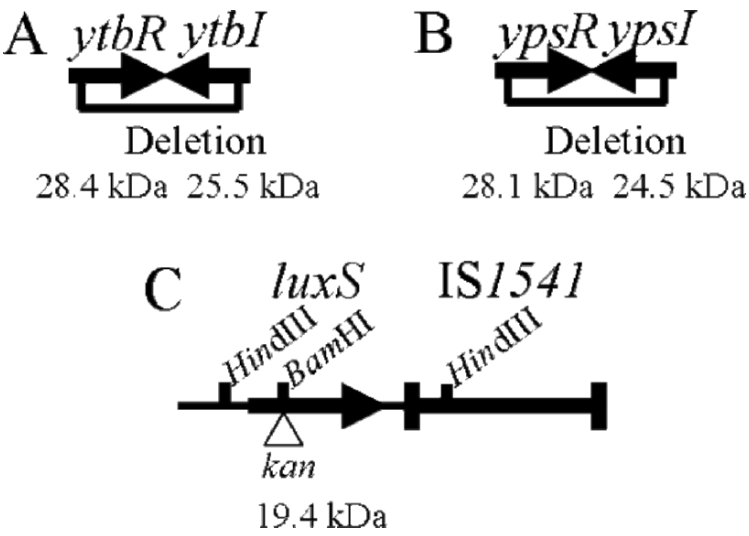

Fig. 1. Genetic organization of *Y. pestis ytbIR*, *ypsIR,* and *luxS*. The construction of mutations in *ytbIR* (A), *ypsIR*(B) and *luxS* (C) is shown.

deletion in *ypsIR*, a 1.5-kb *Dra*I fragment was eliminated from the *ypsIR* locus and cloned into pKNG101 yielding pKNGYps3. To construct a mutation in *luxS*, a 1.27-kb *Bam*HI fragment containing a Km^r gene from pUC4K was ligated into the *Bam*HI of a cloned *luxS* gene, generating the pLuxS3 suicide plasmid. Suicide plasmids were electroporated into KIM6+. Cells from Sm^r colonies were grown overnight without Sm and plated on TBA sucrose plates to isolate sucrose-resistant mutants that had completed allelic exchange. The triple QS mutant was constructed sequentially with the three suicide plasmids. All mutations were confirmed by PCR. Fig. 1 depicts the three QS loci and constructed mutations.

15.2.2 Assays

V. harveyi BB170, which does not respond to AI-1, was used in AI-2 autoinducer bioassays of cell-free culture media as previously described (Surette and Bassler 1998; Surette et al. 1999). Briefly, an overnight culture of *V. harveyi* BB170 was diluted 1:5,000 into fresh AB medium. Supernatants from late-exponential phase cultures grown in glucose-free LB were added to a final concentration of 10% (v/v) to early exponential phase BB170 cultures and light production was monitored for 5 hours using a TopCount luminescence counter with 96-well format (Packard).

E. coli MG4 (pKDT17) (Pearson et al. 1997) was used in liquid assays to measure AI-1 autoinducer levels in culture supernatants from early-stationary phase cultures of *Y. pestis* strains grown in LB with or without 50mM MOPS (pH 7.1) at 26°C or 37°C. The pKDT17 reporter plasmid contains a *lasB-lacZ* fusion that is induced by certain exogenous autoinducer molecules. *E. coli* (pKDT17) was grown in LB overnight at 30°C, back diluted to an OD620 of 0.1 and incubated with supernatants

for 5 hours at 30°C. The β-galactosidase activity was measured as described previously (Miller 1992).

15.2.3 Thin Layer Chromatography

Two different strains were used to detect AHL signal molecules in *Y. pestis*: *Agrobacterium tumefaciens* NTL4 (pCF218 + pCF372) which carries *traR* and a *traI-lacZ* fusion, and *Chromobacterium violaceum* strain CV026. The *Agrobacterium* strain produces a blue color in the presence of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) in response to AHLs (Fuqua and Winans 1996; Luo et al. 2001) while a purple pigment is produced by CV026 in response to AHLs (Throup et al. 1995).

The extraction of AHLs and thin layer chromatography was described by Shaw et al. (1997). *Y. pestis* strains were grown in LB with or without 50mM MOPS (pH 7.1) at 26°C or 37°C and AHLs were extracted three times with ethyl acetate. The extracts were concentrated using rotary evaporation. AHL extracts and synthetic standards were applied to a C_{18} TLC plate (Macherey-Nagel) and developed in 60:40 (v/v) methanol/water until the front reached the top. The TLC plate was dried and overlaid with 1% LB or 1% ABT agar containing *C. violaceum* CV026 or *A. tumefaciens* NTL4 (pCF218 + pCF372), respectively, and incubated at room temperature until AHL spots were detected.

15.2.4 Confocal Laser Scanning Microscopy

Samples were processed for confocal microscopy as previously described (Lawrence and Neu 1999). Briefly *Y. pestis* strains, grown overnight on TBA slants, were used to inoculate the defined medium, TMH, to an optical density at 620 nm of 0.1. Glass coverslips were placed in the cultures which were incubated overnight at 30°C. The coverslips were washed twice, incubated with FITC-conjugated wheat germ agglutinin (WGA) for 20 minutes, rinsed with distilled water to remove unbound WGA, and examined by confocal laser scanning microscopy (CLSM).

15.2.5 Two-Dimensional Gel Electrophoresis

Y. pestis strains were cultured in PMH2 with 10mM $FeCl_3$ at either 26 or 37°C to early stationary phase. Proteins were radiolabeled for 2 hours with ^{35}S-amino acids (DuPont NEN Research Products). Samples were prepared as described in the Bio-Rad manual "2-D electrophoresis for proteomics". Supernatants were subjected to isoelectric focusing at between pH 3 to 10 by using precast Immobiline™ DryStrips (Amersham Biosciences), followed by reducing SDS-PAGE in precast Excel 8-18% acrylamide gradient gels (Amersham Biosciences) or 10% polyacrylamide gels. Labeled proteins were detected by autoradiography.

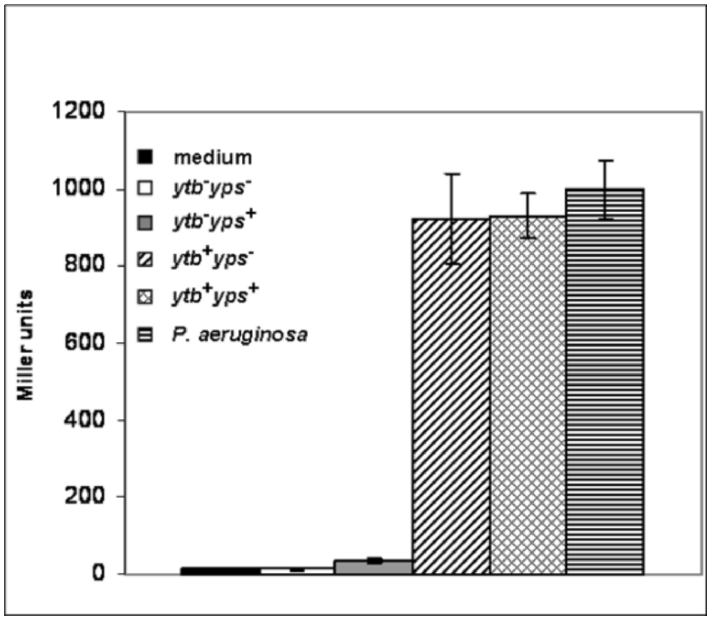

Fig. 2. Activation of *lasB::lacZ* reporter by *Y. pestis* supernatants. *E. coli* cells carrying pKDT17 (encoding the *lasB::lacZ* reporter) were exposed to cell-free culture supernatants from *Y. pestis* strains, *E. coli* (negative control), or *P. aeruginosa* (positive control) and samples were collected after 5 h of incubation.

15.3 Results and Discussion

15.3.1 QS Loci in *Y. pestis*

The *Y. pestis* KIM genome contains two *luxIR* systems and a *luxS* homolog. The two *luxIR* homologs are nearly identical (99%) to the *Yersinia pseudotuberculosis* genes and were designated *ytbIR* and *ypsIR* in keeping with the *Y. pseudotuberculosis* nomenclature (Deng et al. 2002; Atkinson et al. 1999). The deduced amino acid sequence of *Y. pestis* LuxS shows 87-89% identity to LuxS proteins from *E. coli, S. typhimurium, Shigella flexneri* and several other bacteria (Blattner et al. 1997; McClelland et al. 2001; Wei et al., 2003). The *ytbIR, ypsIR,* and *luxS* loci were cloned from *Y. pestis* KIM6+ genomic DNA. *Y. pestis* strains with single, double, and triple mutations in the QS systems were constructed.

Table 2. Activation of *lasB::lacZ* reporter depends on pH and temperature

conditions	OD_{620} ~ 1.0		OD_{620} ~ 1.7		OD_{620} ~ 2.6-2.8		OD_{620} ~ 2.1-2.3	
	pH	MU[a]	pH	MU	pH	MU	pH	MU
37°C	6.92	543	7.37	683	-	-	7.85	459
37°C MOPS pH 7.1	6.94	673	7.06	824	-	-	7.15	937
26°C	6.85	1315	6.92	1297	7.54	1567	-	-
26°C MOPS pH 7.1	6.9	1146	6.92	1450	7.09	1582	-	-

[a] Miller units of β-galactosidase activity from *lasB::lacZ* reporter

15.3.2 Detection and Characterization of AHLs Produced by *Y. pestis*

We used a *P. aeruginosa lasB::lacZ* reporter to detect AI-1 signal molecules in *Y. pestis* supernatants. This reporter responds to autoinducers with a 12-carbon acyl group but will recognize autoinducers with 8-14 carbon acyl groups. *Y. pestis* produced AI-1 molecules that activated the reporter. Supernatants from cells grown at 26°C gave higher values than those from 37°C grown cells (Fig. 2). In *Y. pseudotuberculosis*, the AHL levels were almost undetectable when the cells were grown at 37°C. Yates et al. (2002) showed that during the growth of *Y. pseudotuberculosis* at 37°C in LB, the medium becomes alkaline when the cells reach stationary phase. The combination of temperature and pH leads to lactonolysis, i.e. ring opening of the AHLs. However, lactonolysis could be reduced using LB buffered with MOPS (pH 7.1) (Yates et al. 2002). Using LB and buffered LB at 26°C, we found no difference between the activating ability of supernatants obtained from stationary phase cultures despite some differences in pH (Table 2). The activity of the reporter upon addition of supernatant from stationary phase cultures grown at 37°C was significantly lower. When we added supernatants from cultures grown in buffered medium at 37°C, a two-fold increase in *lasB::lacZ* reporter activity was observed. Therefore our results are in agreement with the previous data for *Y. pseudotuberculosis* (Yates et al. 2002), suggesting that pH and temperature dependent lactonolysis may also occur in *Y. pestis*. Comparison of the various *luxIR* mutants showed that the Ytb system plays a critical role in the activation of the *lasB* reporter. While a mutation in the *yps* operon gave a slight reduction in the activating ability of *Y. pestis* supernatants towards *lasB::lacZ*, deletion of *ybtRI* almost completely abolished the stimulatory activity of supernatants from this mutant (Fig. 2).

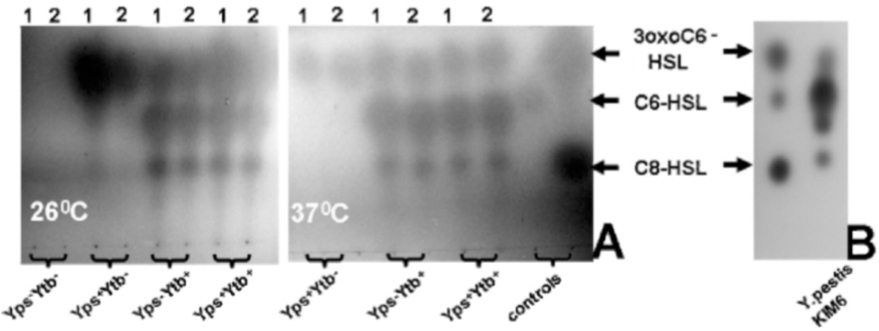

Fig. 3. TLC chromatograms of AHLs extracted from *Y. pestis* strains. A and B AHLs detected using *A. tumefaciens* NTL4 (pCF218 + pCF372) and *C. violaceum* CV026, respectively. 1 and 2 HSLs extracted from strains grown in LB medium and LB medium buffered with MOPS, respectively.

We used TLC to examine the spectrum of AHLs produced by *Y. pestis.* Three compounds migrating similar to 3-oxo-C6-HSL, C6-HSL and C8-HSL (Fig. 3A) were detected using *A. tumefaciens* NTL4 (pCF218 + pCF372). The same compounds were identified in *Y. pseudoberculosis* (Atkinson *et al*. 1999). A fourth compound migrating between C6-HSL and C8-HSL, similar to N-3-oxo-octanoyl homoserine lactone (3-oxo-C8-HSL) produced by *P. aeruginosa* PAO1 (data not shown), was detected using *C. violaceum* CV026 (Fig. 3B). Yates et al. (2002) identified a similarly migrating compound from *Y. pseudoberculosis* supernatant as C7-HSL. The amount of all AHLs, especially 3-oxo-C6-HSL, was reduced at 37°C, likely due to lactonolysis. The AHL profile of the *yps* mutant did not differ from that of the wild type strain in the levels of C6-HSL and C8-HSL but did have a 2-3 fold decrease in the amount of 3-oxo-C6-HSL. In supernatants from the *ytb* mutant, only 3-oxo-C6-HSL and traces of C6-HSL were detected. Our data suggests that the Ytb system is responsible for the synthesis of 3-oxo-C6-HSL, C6-HSL and C8-HSL and the Yps system produces 3-oxo-C6-HSL. These results are in agreement with recently published data on the AHL profiles in *Y. pseudotuberculosis ytbI* and *ypsI* mutants (Ortori et al. 2006) and those of Kirwan et al. (2006) where expression of YtbI (termed YspI in strain CO92 nomenclature) resulted in production of 3-oxo-C6-HSL and 3-oxo-C8-HSL.

15.3.3 Functionality of the *Y. pestis* LuxS System

We used a bioassay to demonstrate that the *Y. pestis* produces a functional AI-2 signal molecule. Supernatants obtained from KIM6+, a *luxS* mutant and a complemented *luxS* mutant were tested in luminescence bioassays using *V. harveyi* strain BB170 that recognizes only the AI-2 molecule. Activity of the culture supernatants

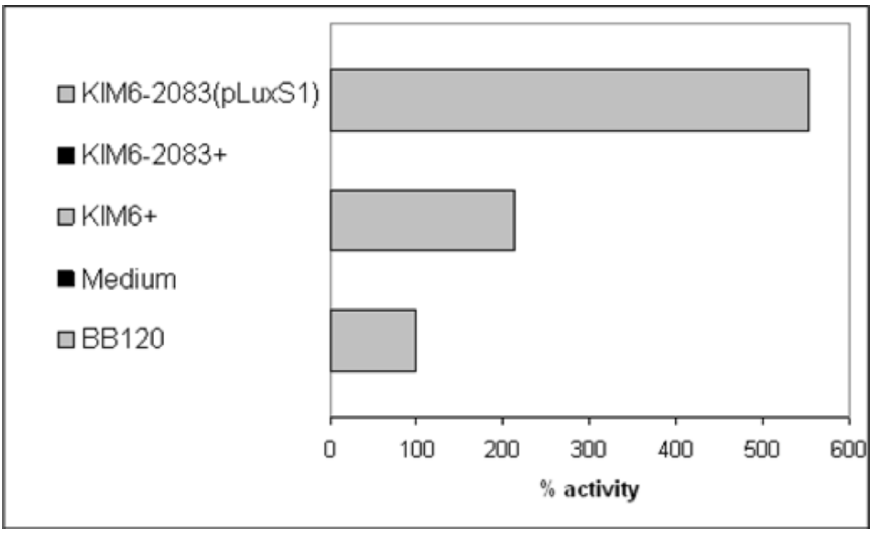

Fig. 4. LuxS activity in *Y. pestis* strains. Cell-free culture supernatants from *Y. pestis* cultures were tested, in a bioassay, for the presence of AI-2 signal molecule produced by LuxS. Activity present was compared to that produced by *V. harveyi* BB120 which was normalized to 100%.

from *Y. pestis* strains at 5 h of incubation was compared to the AI-2- producing *V. harveyi* BB120 strain which was normalized to 100% (Fig. 4). *Y. pestis* KIM6+ showed a high level of activity while the *luxS::kan* mutant, KIM6-2083+, lost all activity. Complementing this mutant with the *luxS*$^{+}$ recombinant plasmid, pLuxS2, restored high-level production of the AI-2 molecule. Uninoculated medium had no stimulatory activity (Fig. 4). 2-D gel electrophoresis of *Y. pestis* proteins identified only modest differences in protein expression between the parental strain and the *luxS::kan* mutant (data not shown).

To determine the role of LuxS in the pathogenesis of plague, mice were infected subcutaneously with KIM5(pCD1Ap)+ or KIM5-2083(pCD1Ap)+ cells grown at 26^{o}C to approximate conditions of a flea bite. LD_{50} analysis did not show a loss of virulence in the *luxS::kan* mutant strain. Further studies will be necessary to assess whether this mutation has more modest affects on the disease course and/or organ specificities.

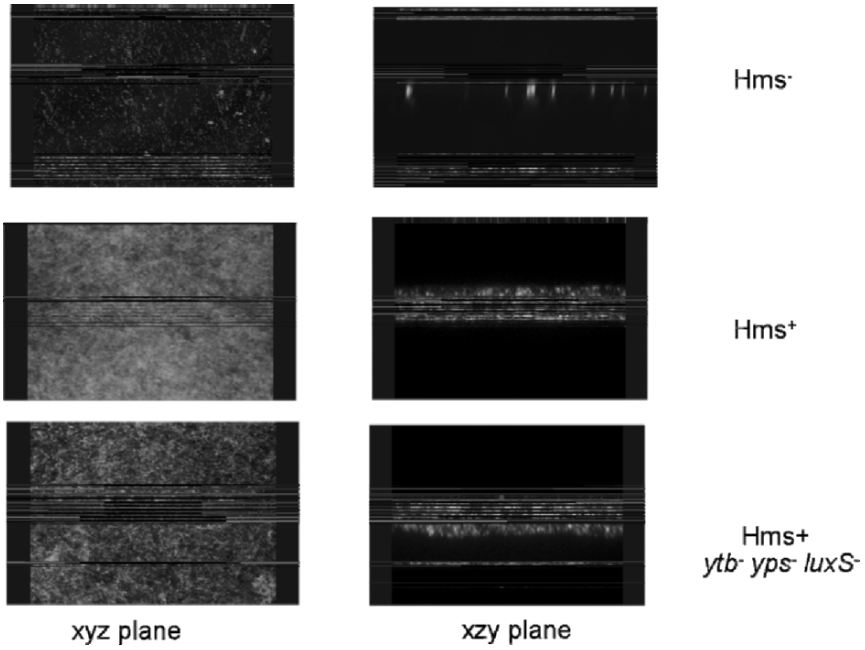

Fig. 5. CLSM images of *Y. pestis* biofilms stained with FITC-WGA.

15.3.4. Effect of QS Systems on Biofilm Formation and Protein Synthesis in *Y. pestis*

In a number of other bacteria, regulation of biofilm formation by quorum sensing systems has been shown (Parsek and Greenberg 2005). In many cases, quorum sensing systems are required for the early stages of biofilm development. Biofilm formation has been shown to be critical for transmission of plague to mammals by some fleas (Hinnebusch et al. 1996; Jarrett et al. 2004). In vitro biofilm formation in *Y. pestis* has been demonstrated using crystal violet staining and Congo red binding assays as well as CLSM (Kirillina et al. 2004). We could not detect any difference between the parent strain and the triple QS mutant by Congo red binding and crystal violet staining assays. However, using CLSM we consistently observed a mild defect in biofilm development in the triple QS mutant (Fig. 5). Similar results were obtained using cells expressing the green fluorescent protein (data not shown). However, the two AHL systems did not appear to play any role in the blockage of *Xenopsylla cheopis* and transmission of plague to mammals (Jarrett et al. 2004). Whether quorum sensing systems are important for transmission of plague by some other flea species or unblocked fleas (Eisen et al. 2006) remains to be elucidated.

The total protein profile of KIM6+ and the triple QS mutant (KIM6-2109) was analyzed by 2-D gel electrophoresis. For *Y. pestis* grown at 26^{o}C, at least seven proteins that were highly expressed in KIM6+ were missing or reduced in KIM6-

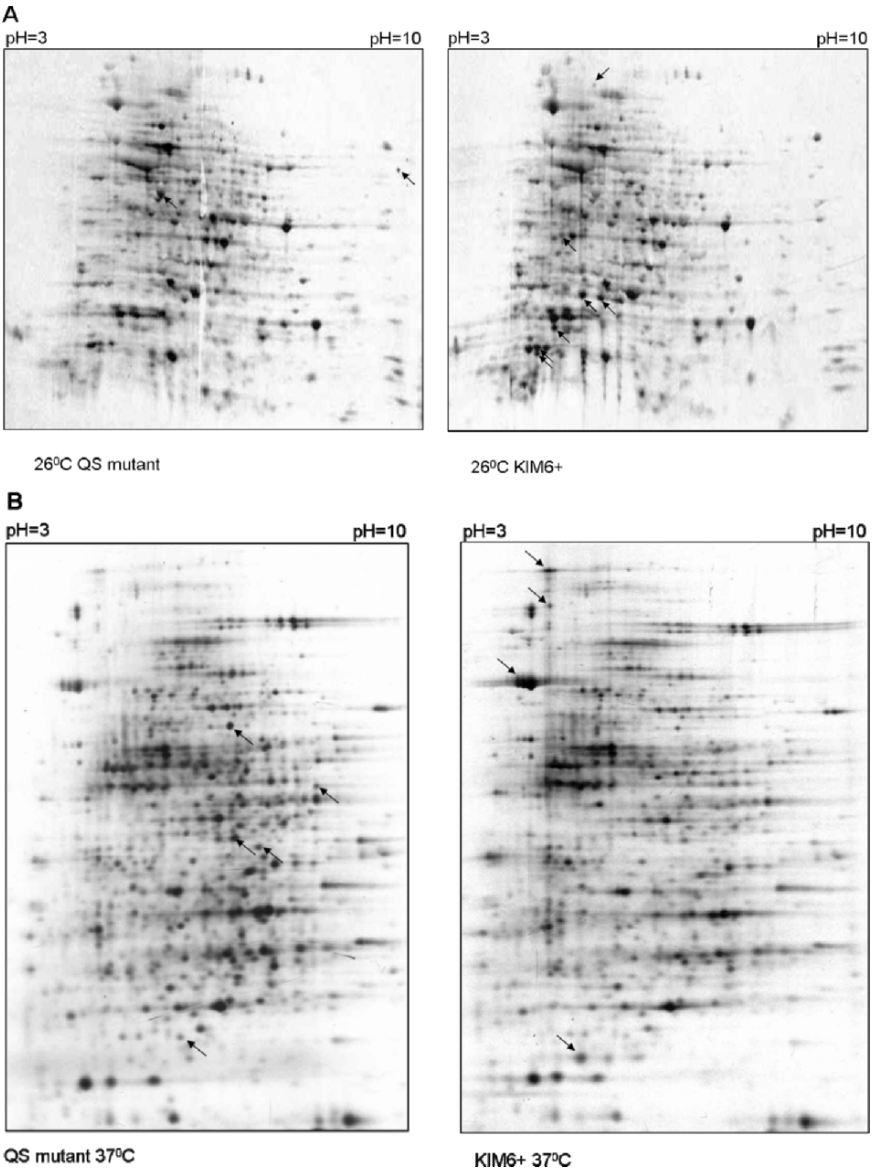

Fig. 6. Effect of QS on *Y. pestis* protein expression. Arrowheads indicate representative proteins whose expression was altered.

2109 while there were two proteins in the QS mutant that appear to be missing in KIM6+ (Fig. 6A). This differential expression might explain the defect in biofilm formation of the *Y. pestis* triple QS mutant at ambient temperature. At least eleven

proteins were differentially expressed in KIM6+ compared to the triple QS mutant when the bacteria were grown at 37°C (Fig. 6B). Moreover, a different set of the proteins was affected at 37°C compared to 26°C indicating that QS regulation in *Y. pestis* is temperature-dependent. Using protein microarrays, Chen et al. (2006) have suggested that expression of a number of virulence-associated proteins including F1, LcrV, KatY and pH6 were decreased in an *ypeRI/yspRI* mutant [*ytb* and *yps* were annotated as *ysp* and *ype*, respectively in CO-92 genome (Parkhill et al. 2001)]. Swift et al. (1999) noted a slight delay in time of death from infection with a *ypeR* mutant compared to the wild-type strain of *Y. pestis*. This suggests that quorum sensing may play a role in the pathogenesis of plague. During a mammalian infection *Y. pestis* exists in acidic conditions as a result of inflammation, thus breakdown of the AHLs as a result of lactonolysis at 37°C might be reduced.

15.4 Conclusions

Using several bioassays we demonstrated that *Y. pestis* has functional LuxI-LuxR and LuxS systems. Four compounds which are likely 3-oxo-C6-HSL, C6-HSL, C8-HSL and 3-oxo-C8-HSL were detected in *Y. pestis* supernatants. Our data suggest that YtbIR system was responsible for synthesis 3-oxo-C6-HSL, C6-HSL and C8-HSL. In contrast YpsIR system failed to produce long chain molecules. CLSM demonstrated that biofilm formation in vitro was reduced in a triple QS mutant suggesting that development of plague biofilm in fleas and transmission of bubonic plague to mammals can be affected by QS. Differential expression of proteins in the QS mutant and wild type strain at different temperatures suggests that QS can regulate protein expression in *Y. pestis* during infection of fleas, at ambient temperature, as well as during infection of mammals at 37°C.

15.5 Acknowledgements

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