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The Role of *luxS* in the Fire Blight Pathogen *Erwinia amylovora* Is Limited to Metabolism and Does Not Involve Quorum Sensing

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Erwinia amylovora **is a gram-negative phytopathogen that causes fire blight of pome fruit and related members of the family Rosaceae. We sequenced the putative autoinducer-2 (AI-2) synthase gene *luxS* from *E. amylovora.* Diversity analysis indicated that this gene is extremely conserved among *E. amylovora* strains. Quorum sensing mediated by LuxS has been implicated in coordinated gene expression, growth, and virulence in other enterobacteria; however, our evidence suggests this is not the function in *E. amylovora*. Mutational analysis pointed to a role in colonization of apple blossoms, the primary infection court for fire blight, although little if any role in virulence on apple shoots and pear fruit was observed. Expression of key virulence genes *hrpL* and *dspA/E* was reduced in mutants of two *E. amylovora* strains. Stronger effects on gene expression were observed for metabolic genes involved in the activated methyl cycle with mutants having greater levels of expression. No quorum-sensing effect was observed in coculture experiments with wild-type and mutant strains either in vitro or in apple blossoms. Known receptors essential for AI-2 quorum sensing, the LuxPQ sensor kinase or the Lsr ABC-transporter, are absent in *E. amylovora*, further suggesting a primarily metabolic role for *luxS* in this bacterium.**

Additional keywords: sulfur metabolism.

Fire blight, caused by the enterobacterium *Erwinia amylovora*, is one of the most devastating diseases of pome fruit and other rosaceous plants worldwide (Gordon Bonn and van der Zwet 2000). The most economically important phase of this disease is blossom blight originating from flower infections. The pathogen is spread during the flowering period from overwintering cankers or distant inoculum sources via pollinating insects (Thomson 2000) and, to a lesser extent, by physical dispersal. Under favorable environmental conditions (Thomson 2000) and in the presence of flower nectar, the pathogen population rapidly grows and enters the host via natural openings, nectarthodes. Once inside the host, the pathogen moves through the vascular system causing rapid death of shoots and, left unchecked, advances into the woody tissues, resulting in death of limbs and entire trees. Fire blight can spread rapidly, destroying entire orchards within a single season; however, even in infested areas, disease is unpredictable, with severe epidemics often followed by several years of low or absent severity. Although weather conditions play a major role in this phenomenon, many fundamentals of pathogen ecology (e.g., significance of survival on nonhost species) and interactions with host physiology (e.g., role of sorbitol flux in the host during vegetative growth) (Blachinsky et al. 2006) are still little understood.

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Although *E. amylovora* was the first phytopathogenic bacterium described, the major virulence factors have begun to be elucidated only relatively recently. A number of genes have been identified that contribute to virulence, particularly those involved in production of the extracellular polysaccharide amylovoran and the functionality of a type-III secretion system (T3SS) (Oh and Beer 2005). The T3SS is controlled by the alternative σ-factor HrpL and leads ultimately to the secretion of the effector DspA/E, which is believed to disrupt host cell function and may be part of a gene-for-gene interaction (Bogdanove et al. 1998). Current control measures beyond prevention and eradication are limited, with the most effective approaches, antibiotic applications during flowering and biological control, relying primarily on direct inhibition of pathogen growth or via substrate competition (Elgoorani and Hassanein 1991; Johnson and Stockwell 1998; McManus et al. 2002; Stockwell et al. 2002). Understanding virulence mechanisms can lead to novel control options, as demonstrated by the development of chemical inducers of plant defense responses (Messenger) based on *E. amylovora* harpin proteins secreted by the T3SS (Wei et al. 1992). Novel biocontrol strategies have been developed against related bacteria, based on the suppression of multiple virulence factors through quenching of quorum-sensing-mediated global regulatory systems. Thus far, this approach has been effective for pathogens such as *Pectobacterium carotovorum* (ex. *E. carotovora*) that rely on an *N*-homoserine lactone autoinducer-1 signal (AI-1) (Lee et al. 2002; Molina et al. 2003). AI-1 has been described recently in *E. amylovora* (Molina et al. 2005; Venturi et al. 2004); however, a global regulatory function has not been confirmed with mutational analysis, and chemical or biological approaches targeting autoinduction repression remain to be explored in this pathogen.

A second quorum-sensing system (QS-2) reliant on LuxS as the enzyme responsible for signal (AI-2) production is well described in diverse animal-associated bacteria (Vendeville et al. 2005). AI-2 has been implicated in virulence of several bacteria, such as *Vibrio cholerae* (Miller et al 2002), *Escherichia coli*, and *Salmonella typhimurium* (Surette and Bassler 1999), and it has been postulated to facilitate the transition to pathogenic existence inside the host (Surette and Bassler 1998, 1999). Recently, the presence of *luxS* was described in the pectolytic

enterobacterial phytopathogen *P. carotovorum* (Coulthurst et al. 2006; Laasik et al. 2006); however, the potential importance of AI-2 in plant–pathogen associations is still largely unknown. In members of *Vibrionaceae* (Bassler et al. 1994), the direct AI-2 precursor is (S)-4,5-dihydroxy-2,3-pentanedione (DPD), which is spontaneously transformed in the presence of borate (Winzer et al. 2002) into the actual AI-2 extracellular signal (furanosyl borate diester). This is the signal that then is detected by the two-component sensor kinase LuxPQ, and transduced inside the cell via the central signal relay protein LuxU and the terminal response regulator LuxO, which, together with σ^{54}, controls gene expression (Mok et al. 2003; Neiditch et al. 2005; Reading and Sperandio 2006). Enterobacteriaceae such as *E. coli* (Xavier and Bassler 2005) or *Salmonella* spp. (Surette and Bassler 1999) produce a different AI-2 signal that does not contain boron (i.e., [2R,4S]-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran [R-THMF]) (Miller at al. 2004), which is imported by the *luxS*-regulated (Lsr) ATP binding cassette (ABC)-transporter (Taga et al. 2003; Xavier and Bassler 2005). In the cell, LsrK phosporylates the AI-2 molecule, which then functions as an autoinducer by derepressing the Lsr operon (Wang et al. 2005). However, how the AI-2 signal is transduced to control pathogenicity-related genes remains unclear. A novel quorum-sensing regulator, MqsR, was detected in *E. coli* K12, where it stimulates biofilm formation by stimulating flagellar motion and motility (González Barrios et al. 2006) via the response regulator QseB and sensor kinase QseC (Sperandio et al. 2002). Another protein, TqsA, was shown to control the export of AI-2 in the same strain (Herzberg et al. 2006). In *Actinobacillus actinomycetemcomitans*, the ArcB sensor kinase is thought to contribute to the signal transduction cascade that directs the LuxS-dependent expression of iron acquisition genes under iron limitation (Fong et al. 2003). Thus, multiple components in addition to *luxS* are essential in a functioning QS-2 system.

Further complicating an assessment of the role of *luxS* is the fact that its function may not be limited to AI-2 synthesis. It is increasingly apparent that, in many bacteria, LuxS has a primary, or sole, role as a metabolic enzyme (*S*-ribosylhomocysteinase) in the activated methyl cycle (AMC) (Winzer et al. 2003). The AMC is responsible for the generation of the major methyl donor *S*-adenosylmethionine (SAM) and the recycling of methionine by detoxification of *S*-adenosyl-L-homocysteine (SAH). LuxS takes part in this cycle by salvaging the homocysteine moiety from the cycle intermediate *S*-ribosyl homocysteine (SRH). As a by-product of this reaction, DPD, the direct AI-2 precursor, is formed. Bacteria lacking an intact AMC lose the ability to recycle methionine and, thus, are dependent on its uptake or synthesis from environmental sulfur sources. In complex media, this amino acid, along with inorganic anionic sulfur species, is plentifully available and this handicap poses little or no problem to the mutant; however, when sulfur becomes a limiting resource, the metabolic charge required for de novo synthesis of methionine will negatively influence its growth (Doherty et al. 2006).

Despite the dual role of this gene, many recent reports have simply drawn a direct correlation between the mere occurrence of *luxS* and the presence and functionality of QS-2, sometimes supporting this observation with mutant analysis and additional phenotypic tests, such as the use of AI-2 reporter strain *V. harveyi* BB170 (Surette and Bassler 1999). However, relatively little effort typically is placed on identification of critical AI-2 receptor candidates. Moreover, the true functionality of QS-2 rarely is assessed by performing a chemical complementation of *luxS*-mutants using the pure AI-2 signal or supernatants of AI-2 producers; instead, assumptions are made based on the usual in trans genetic complementation, which also would restore the activity of the AMC (Vendeville et al. 2005). The aims of this study were, first, to characterize *luxS* in *Erwinia amylovora* and, then, use mutational analysis to investigate its role or roles in metabolism, quorum sensing, ecology, and virulence.

RESULTS

Sequence analysis of the *luxS* gene in *E. amylovora*.

Amplification of the *luxS* gene, using primers based on the complete genome sequence (Sanger Institute website), was obtained for all 21 *E. amylovora* strains analyzed. Representative examples of the complete sequence have been deposited in the National Center for Biotechnology Information (NCBI) database (accession numbers DQ457094 and DQ457095). Sequence information also was obtained for epiphytic *E. billingiae* LMG2613 (accession number DQ977724). Sequence analysis indicates that the 516 nucleotides of the *luxS* gene were identical in all but one *E. amylovora* strain analyzed (DQ457094, represented by *E. amylovora* CFBP1430), despite the fact that these were derived from genotypically diverse strains of worldwide origin which were isolated from different host plants. The only exception was *E. amylovora* FAW611 (DQ457095), which displayed two base-pair substitutions at positions 109 and 112 that resulted in a single substitution of aspartic acid with cognate acidic amino acid glutamic acid. Phylogenetic analysis indicates that the deduced amino acid sequence of LuxS of *E. amylovora* is most related to that of *E. tasmaniensis* (identity 98.2%) and *E. billingiae* (identity 90.1%), with sequences from other members of *Enterobacteriaceae*, such as *P. carotovorum*, *S. marcescens*, or *Yersinia* spp. (identity >85.4%), showing a more pronounced differentiation (Fig. 1).

Absence of known AI-2 receptor genes in *E. amylovora*.

In silico analysis was performed with *E. amylovora* in order to find other AI-2 quorum-sensing-related genes. No homologues of the *luxPQ* genes coding for two-component AI-2 receptor of *V. harveyi* or the *luxOU* genes encoding the associated transduction pathway were found in the completed genome of *E. amylovora* Ea273 produced by the *Erwinia amylovora* Sequencing Group at the Sanger Institute in Cambridge, U.K. Although it is true that different proteins showed a moderate homology (≤51.3%) with the C-terminal domain (from position 1,225 to the end of the open reading frame) of *V. harveyi* LuxQ sensor kinase/phosphatase (accession number P54302), it must be noted that this region corresponds to the histidine kinase A, the histidine kinase-like ATPase, and the signal receiver domains of the LuxQ protein. In fact, these hits correspond merely to the cytoplasmic signal-transduction domain of other *E. amylovora* sensor kinases. Along the same line, no homologue of the Lsr operon, responsible for the transport and processing of AI-2 in *S. typhimurium* and other members of *Enterobacteriaceae*, or of the *Escherichia coli* quorum-sensing regulators MqsR, QseBC, and exporter TqsA, could be found in *Erwinia amylovora* Ea273. Finally, the *A. actinomycetemcomitans* ArcB sensor kinase did not yield any match in the *E. amylovora* genome and a search limited to *Erwinia* spp. of all NCBI databases with relevant functional terms associated with the second quorum-sensing system (e.g., AI-2, LuxS, LuxP, LuxQ, lsr operon, AI-2 receptor, and so on) yielded positive matches only for autoinducer-production protein LuxS, but not for any of the known receptors or AI-2-associated signal transduction proteins.

Construction and verification of *luxS* mutants and complementation of defective strains.

E. amylovora luxS mutants were constructed in two strains using polymerase chain reaction (PCR) to obtain a 2.1-kb

*Bam*HI assembly of the flanking regions of a *luxS* from strain CFBP1430. This was interrupted with an Ω cassette carrying a kanamycin resistance gene and cloned into the suicide vector pCAM-MCS (Burse et al. 2004). The resulting plasmid, named pCAM-AΩB, was mobilized into rifampicin-resistant strains CFBP1430 and FAW610 by biparental mating with *Escherichia coli* S17-1 λ-*pir*. Double homologue recombination in *Erwinia amylovora* was sought by overnight incubation at 28°C and isolation of the resulting colonies based on kanamycin and rifampicin resistance and tetracycline sensitivity on Chromocult coliform agar. Correct integration of the Ω cassette was verified in parallel PCRs using primers corBext1/agemO and gshAext/Omega, respectively (data not shown). Mutant strains CFBP1430ΔluxS and FAW610ΔluxS were complemented in trans by electroporation with the plasmid pMF8805 carrying the intact *luxS* gene of strain CFBP1430 controlled by its own promoter, yielding strains CFBP1430Δ+ and FAW610Δ+, respectively.

Complete knock-out of the synthesis of *luxS* mRNA in the two mutants and its restoration in the complemented strains was confirmed using reverse-transcription (RT)-PCR (Fig. 2). Similarly, a modest activation of the biosensor strain *V. harveyi* BB170 was confirmed by means of cross-feeding assays in wild-type and complemented strains, but not in the mutant (Table 1). However, this value, on average, was never higher than the value (i.e., 10% of the stimulation by *V. harveyi* BB120) which commonly is considered to be the threshold for a positive evaluation of the assay (Bassler et al. 1997).

Bacterial growth under sulfur-limiting conditions.

A straight approach to understand whether the *luxS*-deficient phenotype is to be ascribed to defective quorum sensing or is

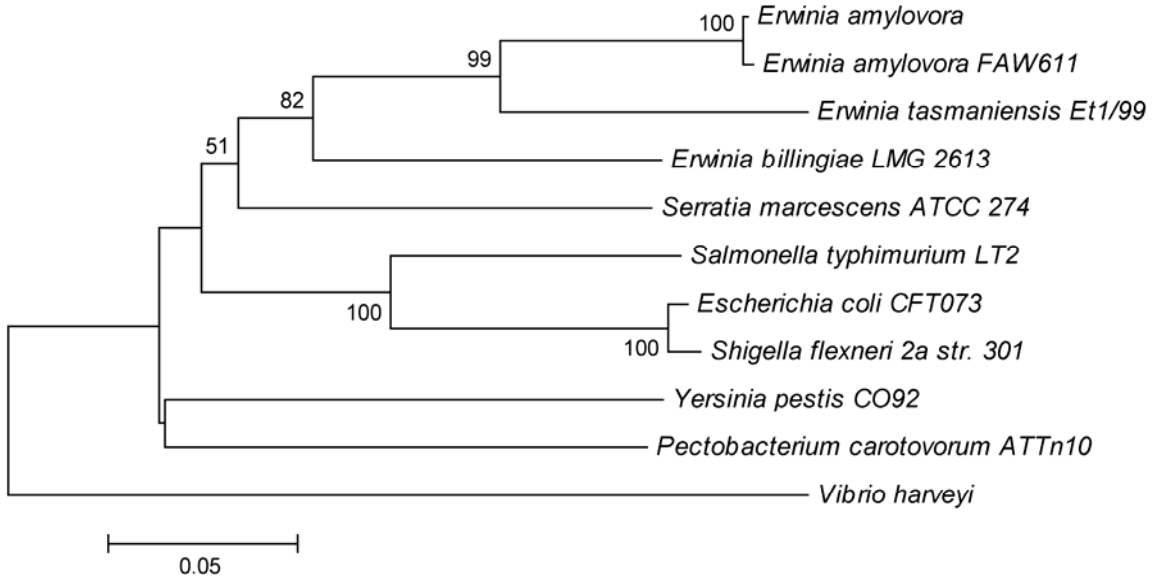

Fig. 1. Phylogenetic relationships on the basis of complete *luxS* sequences between different bacteria belonging to the family of *Enterobacteriaceae*. The distance tree was generated by the neighbor-joining method with the Jukes-Cantor formula, using *Vibrio harveyi* as outgroup. Nodal supports were assessed by 1,000 bootstrap replicates. Only bootstrap values greater than 50% are shown. The scale bar represents the number of substitutions per site. With the exception of *Erwinia amylovora* and *E. billingiae* LMG 2613 sequences, which were produced in this work, all *luxS* sequences were retrieved at the National Center for Biotechnology Information database or in published genomes projects: *E. tasmaniensis* Et1/99 (AM117930), *Serratia marcescens* ATCC 274 (AJ628150), *Salmonella typhimurium* LT2 (NC_003197), *Escherichia coli* CFT073 (AE014075), *Shigella flexneri* 2a str. 301 (NC_004337), *Yersinia pestis* CO92 (AL590842), *Pectobacterium carotovorum* ATTn10 (AJ628151), and *V. harveyi* (AF120098). *Erwinia amylovora* represents the ensemble of all *E. amylovora* strains examined in this work, which shared 100% consensus, with the exception of strain FAW611.

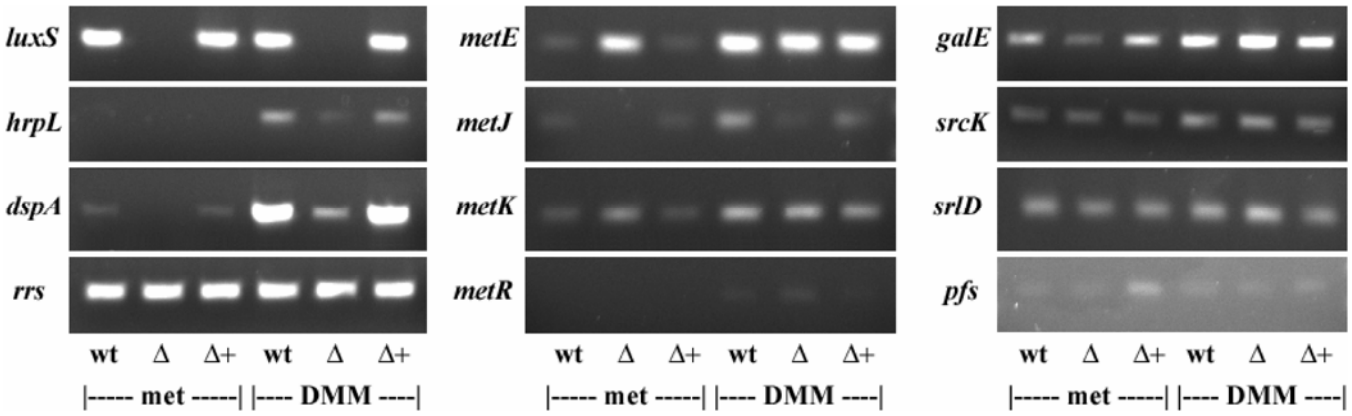

Fig. 2. Effect of the *luxS* mutation on the expression of several pathogenicity-related and metabolic genes of *Erwinia amylovora* FAW610. Genetic expression was assessed by semiquantitative reverse-transcription polymerase chain reaction in wild-type (wt), *luxS* mutant (Δ), and complemented strain (Δ+) in both Davis minimal medium (DMM) and modified DMM with 100 μM methionine as sole sulfur source (met). The picture shows the expression of all genes after 16 h of cultivation (middle of the exponential growth phase) except for *metJ*, *metR*, and *pfs,* which were below detection at that time and are shown after 26 h (end of the exponential growth phase). Expression of *rrs* (encoding 16S-rRNA) was confirmed to be constitutive throughout all growth phases and was validated as a control for sample normalization.

due merely to an interrupted metabolic process is to test the growth of the mutant with methionine (which is part of the AMC) or the other sulfur-containing amino acid (cysteine) as sole sulfur source, and to compare the result with bacteria grown in media where this chemical element is not a limiting factor. Cultivation experiments showed that the growth of *E. amylovora* FAW610 *luxS* mutant strains generally was unaffected compared with the corresponding wild-type or complemented strains in rich media such as Luria-Bertani (LB) (Fig. 3A) or in normal Davis minimal medium (DMM) (Fig. 3D), where the total SO_4^- concentration is as high as 8.4 mM. All strain derivatives reached absorbance values of up to 0.870 and 0.710, respectively, at the end of the exponential growth phase. Nevertheless, in DMM, unlike the wild-type or complemented strain, the *luxS* mutant showed a loss in turbidity (i.e., decrease in absorbance) after reaching the stationary phase at 40 h (Fig. 3D). In the modified sulfate-free DMM where methionine (met) was present at 100 μM-concentration as the sole sulfur source, the growth of the *luxS* mutant was considerably impaired, attaining an absorbance that was lower (0.235) than that attained by the wild-type (0.428) or the complemented strain (0.435) (Fig. 3B). Similar results were obtained at 50 μM (data not shown), whereas this gap was noticeably narrower when the concentration of methionine in the medium was only 10 μM (Fig. 3C). Conversely, if just cysteine (cys) was present as a sole sulfur source in the medium, no differences in bacterial growth could be observed at 100 μM (Fig. 3E) or 50 μM (data not shown), with the concentration of all three derivatives reaching absorbance values of up to 0.890 and 0.750, respectively. At 50 μM, however, the density of the mutant strain in stationary phase dropped slightly to an absorbance value of 0.621 after 72 h. Unlike results in media with 100 μM with methionine, impairment of mutant growth relative to the wild-type or complemented strain was observed only at low (10 μM) cysteine concentration (Fig. 3F).

Table 1. Luminescence induction of the AI-2 biosensor *Vibrio harveyi* BB170 by cell-free supernatants of *Erwinia amylovora* CFBP1430 and FAW610 strain derivatives[a]

Strain[b]	AB	LB	Davis	hrp
V. harveyi BB120	100	nd	nd	nd
CFBP1430	6.5 (±1.0)	7.5 (±3.4)	8.5 (±2.7)	9.5 (±1.0)
CFBP1430ΔluxS	bd	0.3 (±0.3)	bd	0.7 (±0.6)
CFBP1430Δ+	7.0 (±2.7)	6.5 (±0.9)	8.0 (±2.0)	10 (±2.7)
FAW610	4.0 (±1.5)	6.5 (±1.7)	6.5 (±1.7)	9.0 (±1.8)
FAW610ΔluxS	bd	0.3 (±0.3)	bd	0.7 (±0.6)
FAW610Δ+	5.0 (±2.7)	7.5 (±1.0)	6.5 (±0.0)	9.5 (±3.1)

[a] *E. amylovora* was grown for 14 h at 28°C in four different culture media and spent supernatants were added (1:10 vol/vol) to newly inoculated cultures of *Vibrio harveyi* BB170 Luminescence was measured after 4 h of incubation at 30°C.

[a] Values represent percent induction with respect to the response of the biosensor when grown in autoinducer assay broth (AB) medium (1:10 vol/vol) supplemented with spent *V. harveyi* BB120 supernatant (± standard deviation). LB = Luria-Bertani medium, nd = not determined, and bd = below detection.

Bacterial growth in coinoculated cultures.

In order to understand whether the *luxS*-deficient phenotype really involves quorum sensing, we tested whether the wild-type or the complemented strain were able to relieve the growth deficit of the *luxS* mutant by the means of extracellular factors in cocultivation experiments under sulfur-limiting conditions. In DMM, all strain derivatives reached concentrations of up to 3×10^9 CFU ml^{-1} at the end of the exponential growth

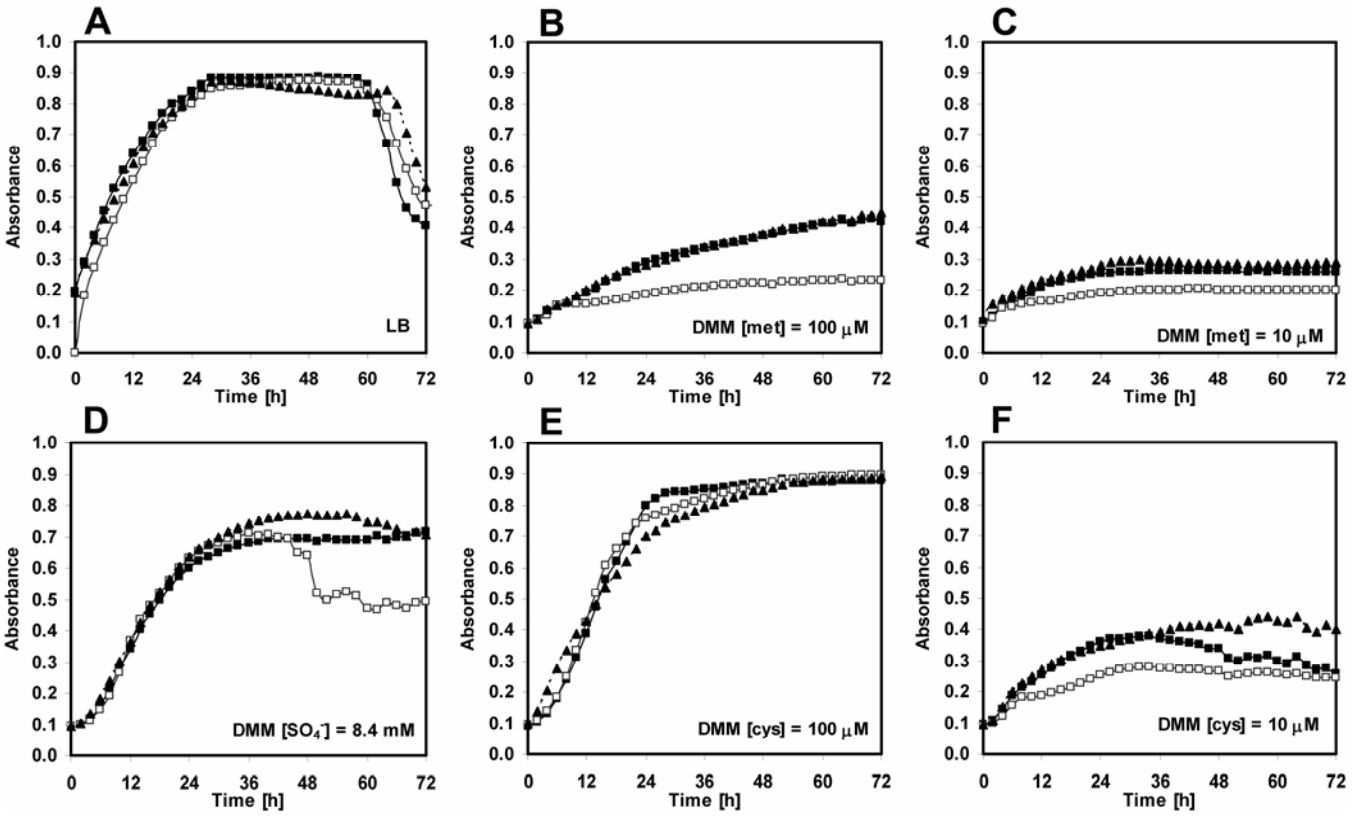

Fig. 3. Effect of sulfur limitation on the growth of *Erwinia amylovora* FAW610 derivatives. The bacteria were cultivated in 200-μl microcultures at 28°C in sulfur-rich media using **A,** Luria-Bertani medium or **D,** Davis minimal medium (DMM [SO_4^-] = 8.4 mM) or under sulfur limitation in modified DMM with **B,** 100 μM or **C,** 10 μM methionine (DMM [met] and **E,** 100 μM or **F,** 10 μM cysteine (DMM [cys] as sole sulfur sources. The wild type is represented by black squares (■), the *luxS* mutant by empty squares (□), and the complemented strain by black triangles (▲).

phase whereas, in the modified sulfate-free DMM where methionine (met) was present at a concentration of 100 μM as the sole sulfur source, the growth of the *luxS* mutant was considerably impaired, attaining a cell number that was lower (3.4×10^8 CFU ml^{-1}) than that of the wild-type (9.4×10^8 CFU ml^{-1}) or the complemented strain (8.7×10^8 CFU ml^{-1}) (Fig. 4A). This phenotype was not relieved under coculture conditions, when wild-type and isogenic mutant strains (Fig. 4B) where coinoculated at the same concentration in one flask. The growth of the coinoculated strains was again comparable in normal DMM, reaching cell densities of 1.7×10^9 CFU ml^{-1}, but not in the modified sulfate-free DMM, where the mutant growth was only approximately one-fifth (1.7×10^8 CFU ml^{-1}) compared with the wild type (7.2×10^8 CFU ml^{-1}). Similar results where obtained when the coinoculation experiment was performed using the mutant and complemented strain (Fig. 4C). In normal DMM, both strains reached comparable cell densities (1.8×10^9 and 1.9×10^9 CFU ml^{-1}, respectively); however, under sulfur-limiting conditions, the complemented strain (6.7×10^8 CFU ml^{-1}) was more competitive than the *luxS* mutant (2.4×10^8 CFU ml^{-1}).

Genetic expression in *luxS* mutants.

Semiquantitative RT-PCR confirmed the complete knockout of the *luxS* gene in the corresponding mutant in all strain derivatives and throughout all growth phases, and the constitutive expression of the *rrs* gene which, therefore, was validated as internal control for sample normalization (Fig. 2). The expression of carbon metabolism genes generally was not affected appreciably in the *luxS* mutant, and was modulated only by the growth phase of the bacterial culture or the growth medium used. For *galE*, this stands in contrast with a previous report that suggested that this gene (coding for UDP-galactose 4-epimerase) is expressed constitutively by *E. amylovora* (Metzger et al. 1994). In fact, of all carbon metabolism genes analyzed, only the transcription of *galE* showed a general decline in the *luxS* mutant, especially after reaching the end of the exponential growth phase (Table 2).

Conversely, the *luxS* mutation had an unequivocal impact on the expression of genes that are related to the AMC. Expression of *metE*, *metK*, and *metR* (activator) was upregulated, whereas *pfs* and especially *metJ* (repressor) were downregulated in the *luxS* mutants, particularly when bacteria where cultured in modified DMM with methionine as sole sulfur source (Fig. 2). The expression of the regulatory gene *hrpL*, encoding the T3SS-specific σ factor, which was evident only in DMM, was downregulated in the *luxS* mutant throughout the entire exponential growth phase. The expression of the pathogenicity-related gene *dspA*, encoding for a T3SS-secreted effector, also was impaired in the *luxS*-deficient derivatives, possibly due to the reduced transcription of the positive regulatory gene. Again, this effect on *dspA* was more evident under sulfur limitation in modified DMM (Fig. 2).

Motility and biofilm assays.

Swimming and swarming were assessed by measuring the distance covered by the bacteria from the original spotting point after 48 h of growth at 23°C on semiliquid agar. The spreading pattern of the colony was similar for both strains and in all agar concentration tested (0.3, 0.5, and 0.8%), with the development of three circular concentric zones (chemotactic rings) of apparently different cell densities. The diameter of each zone was measured, but no significant difference in swimming or swarming was detected between the wild-type strains and their derivatives in media with a range of 0.3 to 0.8% agarose. Illustrative results for the 0.3% agar concentration (swimming) are displayed in Table 3. Strains FAW610 and CFBP1430 were equally able to form a biofilm in rich medium (LB, SOBG [20 g of tryptone, 5 g of yeast extract, 0.5 g of NaCl, 2.4 g of $MgSO_4$, 0.186 g of KCl, and 2% glycerol per liter], or SOCG [SOBG supplemented with 20 mM glucose]) at both temperatures tested (24 or 30°C). No difference between wild-type, mutant, and complemented strains was observed in biofilm development in stationary cultures of *E. amylovora* grown for 2 weeks in the above media.

Colonization of detached apple blossoms.

Population sizes of *E. amylovora* FAW610 strain derivatives were assessed after artificial inoculation in the hypanthium of detached apple blossoms in order to determine the role of *luxS* in bacterial colonization ability. Each flower originally was inoculated with a population of $\approx 10^2$ CFU/flower of the respective strain derivative, which increased after 2 days of incubation to 5.3×10^8 and 6.3×10^8 CFU/flower in the wild-type and the

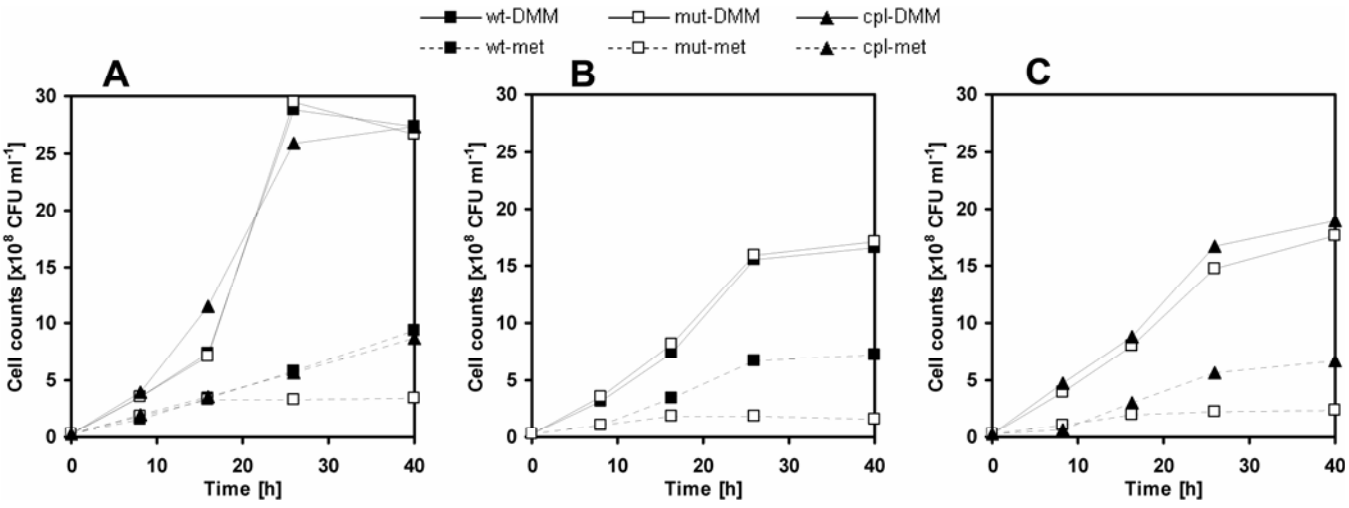

Fig. 4. Competition experiments between *luxS*-positive and *luxS*-negative *Erwinia amylovora* FAW610 derivatives. The failure of *luxS*-positive strains to complement *E. amylovora* FAW610Δ*luxS* in co-cultivation assay is indicative of the absence of an extracellular signal. The bacteria were cultivated at 28°C in Davis minimal medium (DMM [SO_4^-] = 8.4 mM) (solid line) or under sulfur-limiting conditions in modified DMM with methionine [met] = 100 μM as sole sulfur source (dotted line) in either **A,** separate cultures or **B,** a 1:1 ratio of wild-type/mutant and **C,** mutant/complemented strain at a starting optical density at 600 nm = 0.018. Bacteria were enumerated by colony counts on Luria-Bertani supplemented with the appropriate antibiotics. The wild type is represented by black squares (■), the *luxS* mutant by empty squares (□), and the complemented strain by black triangles (▲).

Table 2. Expression of metabolic and pathogenicity related genes in *Erwinia amylovora* FAW610 strain derivatives during growth in Davis minimal medium (DMM) or modified DMM with 100 μM methionine (met) as sole sulfur source[a]

	met			DMM		
Gene, *t* (h)	**wt**	**Δ**	**Δ+**	**wt**	**Δ**	**Δ+**
luxS						
16	1.18	bd	1.15	1.09	bd	1.05
26	1.2	bd	1.36	1.27	bd	1.08
40	1.47	bd	1.44	1.25	bd	1.12
hrpL						
16	bd	bd	bd	0.4	0.08	0.26
26	bd	bd	bd	0.27	0.02	0.18
40	bd	bd	bd	bd	bd	bd
dspA						
16	0.08	bd	0.08	1.18	0.44	1.2
26	0.83	bd	0.82	1.26	1.05	1.13
40	1.15	bd	1.08	1.43	1.33	1.31
metE						
16	0.12	0.95	0.07	1.12	1.16	1.01
26	0.06	0.66	bd	1.1	1.24	0.98
40	bd	bd	bd	0.95	1.33	0.89
metJ						
16	0.01	0.01	0.02	0.03	0.01	0.01
26	0.11	bd	0.06	0.49	0.02	0.17
40	0.15	bd	0.25	1.31	0.17	0.52
metK						
16	0.15	0.34	0.1	0.46	0.4	0.32
26	0.05	0.49	0.12	0.53	0.63	0.7
40	0.29	0.94	0.43	0.95	0.66	0.93
metR						
16	bd	bd	bd	bd	bd	bd
26	bd	bd	0.01	0.04	0.06	0.01
40	0.02	0.25	0.01	0.39	0.82	0.12
galE						
16	0.55	0.21	0.69	0.88	1.08	0.75
26	0.45	0.05	0.56	0.79	0.07	0.46
40	0.55	0.13	0.54	0.53	0.05	0.52
srcK						
16	0.14	0.15	0.13	0.39	0.41	0.31
26	0.32	0.53	0.66	0.8	0.39	0.55
40	0.89	0.6	0.61	0.67	0.92	0.78
srlD						
16	0.44	0.3	0.36	0.41	0.49	0.3
26	0.7	0.54	0.72	0.56	0.6	0.58
40	0.64	0.58	1	1.65	1.08	1.01
pfs						
16	bd	bd	bd	bd	bd	bd
26	0.03	0.03	0.27	0.09	0.04	0.07
40	0.04	bd	0.04	0.04	0.05	0.08
rrs[b]						
16	*1,823*	*1,986*	*1,879*	*2,027*	*1,856*	*2,103*
26	*1,947*	*1,909*	*1,711*	*1,702*	*1,890*	*1,909*
40	*1,703*	*1,791*	*1,651*	*1,439*	*1,705*	*1,677*

[a] The expression of different genes was measured as the intensity of densitometric signals obtained after semiquantitative reverse-transcription polymerase chain reaction (RT-PCR). All values were calculated relative to the absolute value (shown in italics) obtained for *rrs* (constitutively expressed control) and are comparable only within the same gene; bd = below detection, *t* = time in hours, and wt = wild type; Δ = mutant, Δ+ = complemented mutant.

[b] Absolute densitometric intensity obtained after 25 cycles of RT-PCR using *rrs*-primers (used for normalization).

complemented strain, respectively. The colonization ability of the *luxS* mutant was noticeably impaired, being approximately a log-value lower (6.1×10^7 CFU/flower) (Fig. 5).

In order to observe its competitiveness in apple blossoms and to evaluate whether its hypothetical loss of AI-2 production could be complemented by the same compound produced by the intact gene product, the *luxS* mutant also was coinoculated on the flowers in a 1:1 mix with either the wild-type or the complemented strain derivative. The results obtained were comparable with those achieved when the bacteria were inoculated individually. In both cases, the population size of the *luxS* mutant was approximately an order of magnitude lower (3.3×10^7 respectively 2.1×10^7 CFU/flower) relative to those obtained for the wild-type (5.4×10^8 CFU/flower) and the complemented (5.2×10^8 CFU/flower) strain.

Virulence assay on young apple shoots.

The role of *luxS* in virulence was assessed on 1-year-old 'Golden Delicious' grafted plants inoculated with a range of bacterial concentrations (10^3 to 10^9 CFU ml^{-1}). The level of disease measured as percent lesion length increased with higher inoculum concentrations (Fig. 6). However, no differences in virulence were observed when the mutant, complemented mutant, and wild-type strain were compared at any inoculum concentration (Fig. 6). No infection was evident with any of the three strains at the lowest inoculum concentration (10^3 CFU ml^{-1}). The formation of characteristic bacterial ooze was observed in most plants inoculated with 10^5 CFU ml^{-1} or higher, regardless of the presence of a functioning *luxS*. Similar results showing a lack of difference in strains at any inoculum concentration were obtained in a second trial; however, the overall level of disease was lower, preventing pooling of the data. Similarly, no difference in disease severity was obtained in a preliminary trial performed using 10^9 CFU ml^{-1} suspensions of individual strain derivatives from *E. amylovora* FAW610 and CFBP1430 on 6-week-old apple seedlings grown from Golden Delicious fruit (data not shown).

Virulence assay on immature pear.

Regardless of the starting concentration of the inoculum, no significant difference was observed in the diameter of the lesion formed on the surface of immature pear fruit inoculated with the *luxS* mutants of FAW610 compared with the wild-type or complemented strains. The wild-type and complemented strains also were not more efficient in colonizing the fruit tissue than the corresponding mutant strain at any inoculum concentration (Fig. 7). The final concentration of bacteria in the pear tissues was similar in all cases, even if the inoculum concentration varied by four orders of magnitude. A comparison between the virulence and colonization of *luxS* derivatives was performed with similar results also for strain CFBP1430 using an inoculum containing 10^7 CFU ml^{-1} (data not shown).

DISCUSSION

The *luxS* gene product first was described as the enzyme responsible the production of AI-2, the extracellular signal molecule regulating the expression of luminescence by the means

Table 3. Effect of *luxS* mutation on motility of *Erwinia amylovora* CFBP1430 and FAW610 strain derivatives

Strain[a]	CFBP	CFBPΔ*luxS*	CFBPΔ+	FAW610	FAW610Δ*luxS*	FAW610Δ+
Zone 1	0.30 ± 0.06	0.32 ± 0.04	0.27 ± 0.05	0.30 ± 0.06	0.33 ± 0.05	0.32 ± 0.08
Zone 2	1.65 ± 0.05	1.72 ± 0.04	1.58 ± 0.12	1.60 ± 0.09	1.80 ± 0.13	1.68 ± 0.15
Zone 3	3.28 ± 0.25	3.38 ± 0.31	3.52 ± 0.16	3.45 ± 0.29	3.35 ± 0.41	2.90 ± 0.43

[a] Swimming behavior was assessed after 48 h of incubation at 24°C in 0.3% Luria-Bertani agar. Values represent the diameter of the different zones (chemotactic rings) expressed in centimeters (± standard deviation).

of the second quorum-sensing system in *V. harveyi* (Bassler et al. 1993). A crucial role of LuxS in the AMC and in sulfur metabolism subsequently has become evident in certain bacteria (Winzer et al. 2002). Many studies, focused on different bacterial strains, have assumed that the presence of a functional *luxS* gene was a sufficient condition for the existence of an AI-2-based quorum-sensing system in the corresponding bacteria (Winzer et al. 2003).

E. amylovora strains were collected on three different continents and on different host plants and represent the wide genotypic diversity of this pathogen (Jock et al. 2002). Nevertheless, all *E. amylovora* strains had the *luxS* gene, with 20 of 21 having 100% homology, and one (FAW611) differing in just 2 bp, resulting in one substitution between two cognate acidic amino acids. This result strongly suggests that this gene is of great importance for the biology of the fire blight pathogen. However, the fact that no homologues of known AI-2 receptors, exporters, or QS-2 regulators could be identified, either in the finished genome assembly of the *E. amylovora* Ea273 or through BLAST searching of other published *E. amylovora* sequences, casts substantial doubts on the existence of an AI-2-based quorum-sensing system in the fire blight pathogen and on the possible role of *luxS* as a key enzyme for the production of an AI-2 signal in this pathogen. Although it was not unexpected that no significant homology could be found to the LuxPQ two-component sensor kinase (a receptor that, thus far, was found only in the *Vibrionaceae* family) or the MqsR quorum-sensing regulator (which has been reported in association with QS-2 exclusively in *Escherichia coli*), the absence from the *Erwinia amylovora* genome of an Lsr receptor-complex (similar to the one found in *Escherichia coli, Salmonella typhimurium*, and other members of *Enterobacteriaceae*) was more surprising. The same is true also for the TqsA exporter or the QseBC and ArcB sensor kinases, which are also widespread in the *Enterobacteriaceae* family, even in species that do not display the familiar Lsr receptor (*unpublished data*). The absence of QseC homologs suggests that *Erwinia amylovora* also does not possess the critical components of a third quorum-sensing system recently described in *Escherichia coli* O157:H7. In this strain, LuxS is not directly involved in synthesis of a QS-3 signal molecule; however, *luxS*

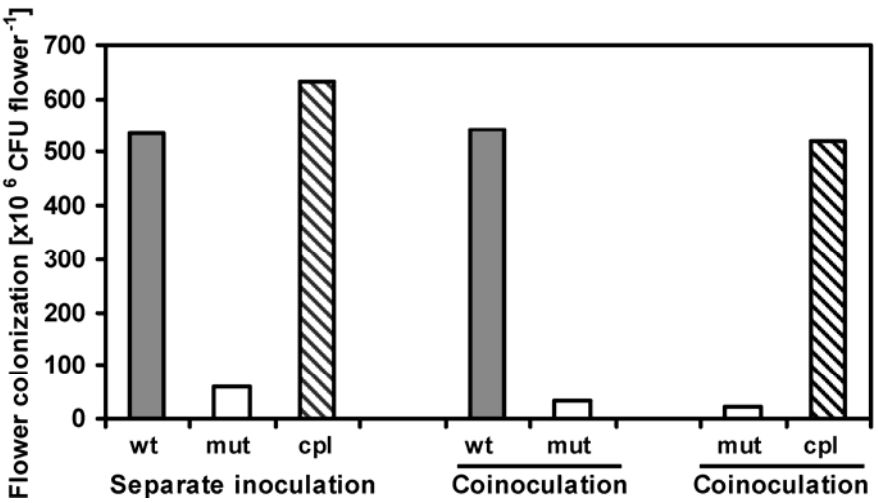

Fig. 5. *Erwinia amylovora* FAW610 colonization of detached 'Golden Delicious' apple flowers. Each flower was inoculated in the stigma or hypanthium with a suspension containing approximately 10^2 CFU of a single strain derivative (separate inoculation) or 1:1 mixes of wild-type/mutant and mutant/complemented strain, both at 0.5×10^2 CFU each (co-inoculation). CFU were enumerated after 2 days of incubation of the flowers at 20°C and 100% relative humidity. Values are the mean of five replicates containing two flowers each from one experiment. The experiment was repeated once with similar results.

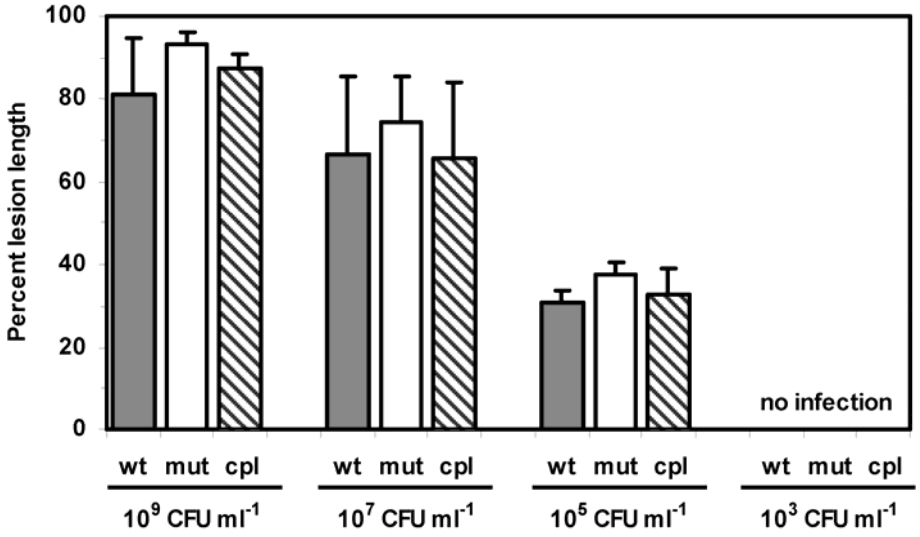

Fig. 6. Role of *luxS* in virulence of *Erwinia amylovora* FAW610 on 1-year-old grafted 'Golden Delicious' plants. Shoots were inoculated with bacterial suspensions containing approximately 10^9, 10^7, 10^5, or 10^3 CFU ml^{-1} of a single strain derivative. Disease progress was measured after 14 days. Values for percent lesion length represent the means of three replicates with standard error bars.

mutation reduces signal production due to pleiotrophic effects that result in the augmented use of the oxaloacetate pathway to compensate for the inability of the bacteria to generate SAM via the (AMC) (Sperandio et al. 2003).

Undeniably, cross-feeding assays using *V. harveyi* BB170 showed that both the wild type and the *luxS*-complemented derivative of either CFBP1430 and FAW610 were able to trigger a faint but clear response in the AI-2 reporter strain, and that this function was suppressed in the mutants (Table 1). These results can be explained by the fact that a background level of DPD (the natural AI-2 precursor) is produced as by-product of the LuxS-mediated reaction that leads to detoxification of SAH by rescuing the homocysteine (HC) moiety from the AMC-cycle intermediate SRH. DPD is an unstable transitional molecule thought to be spontaneously converted into the active AI-2 molecule (i.e., furanosyl borate diester in Vibrionales) (Winzer et al. 2002) or *R*-THMF in Enterobacteriales (Miller at al. 2004). A recent report by Turovskiy and Chikindas (2006) demonstrates that the traditional BB170-based autoinducer-2 bioassay is a qualitative, not quantitative, method that may be influenced by the composition of the culture broth (e.g., by low or high glucose levels in the medium). Therefore, the apparent weak positive results obtained in the BB170 bioassay with wild-type and complemented strains, and its loss in the mutants, may simply reflect the functionality of LuxS in the AMC, and may not be indicative of the presence of a working quorum-sensing system so much as a metabolic phenomenon.

This hypothesis was confirmed by the means-of-cultivation experiments in which strain derivatives were grown in rich medium, in minimal medium with sufficient sulfur supplies, or in minimal medium under sulfur limitation. The resulting growth curves showed that *luxS* mutants were not impaired in LB or in DMM containing 8.4 mM SO_4^-, but displayed significantly reduced growth when methionine was used as sole sulfur source already at a concentration as low as 100 μM. In order to obtain a comparable effect with cysteine, the concentration of the amino acid must be lowered to 10 μM, showing that the mutation in the *luxS* gene directly affects methionine metabolism. The decrease in absorbance displayed in the stationary phase by the mutant in DMM and in modified DMM with 50 μM cysteine as sole sulfur source was consistent with a toxic effect generated by the accumulation of SAH (Pei and Zhu 2004) that results from the interruption of the AMC through by the *luxS* mutation. It is noteworthy that phenotypes displaying reduced growth under sulfur-limiting conditions were not relieved when the *luxS* mutant was cultured together with a strain with unaffected *luxS* activity (i.e., the wild type or the complemented derivative). This suggests that the strains with an intact *luxS* gene were unable to complement the *luxS* mutation by the means of a purported AI-2 production and secretion. Thus, the observed growth pattern was not caused by an extracellular signal, but was likely of a metabolic, intracellular nature. This conclusion is strengthened by analogous results recently obtained using a similar approach in other *luxS*-positive bacteria, such as *Staphylococcus aureus* or *Serratia plymuthica* (Doherty et al. 2006; Van Houdt et al. 2006). An additional indication that, in *Erwinia amylovora*, *luxS* is involved in sulfur and amino acid metabolism is the position of the *luxS* open reading frame in the *E. amylovora* genome. Specifically, the gene is contiguous to and arranged in tandem with *gshA*, a γ-glutamylcystein synthetase that is involved in the metabolism of the other sulfur-containing amino acid, cysteine.

Further support for the metabolic role of *luxS* in *E. amylovora* was provided by the RNA expression profiling of selected AMC-related genes during growth in sulfur-limiting media. Upregulation of *metE* (HC methyltransferase), *metK* (SAM synthetase), and *metR* (activator of the AMC) was observed in *luxS* mutants under sulfur limitation, while *pfs* (MTA/SAHase) and *metJ* (repressor of the AMC) were downregulated. This is indicative of a disturbance of the normal methionine pool in the cell caused by the interruption of the AMC following the inactivation of *luxS*. Similarly, the slight decrease in environmental fitness observed in the mutant—specifically, reduced ability to colonize apple flowers—most probably was due to this metabolic imbalance, rather than to a disturbed quorum-sensing system. An efficient methionine biosynthetic pathway previously was shown to be required for epiphytic fitness of plant-pathogenic bacteria (Andersen et al. 1998). The metabolic origin of this dysfunction, and the absence of an extracellular signal, is further suggested by the fact that, when the *luxS* mutant was coinoculated in apple flowers with a strain with *luxS* activity (i.e., the wild-type or the isogenic complemented strain), the mutant did not recover normal colonization ability. On the contrary, results obtained were even slightly more exaggerated when mutants were coinoculated with either the wild-type or complemented strains compared with the individ-

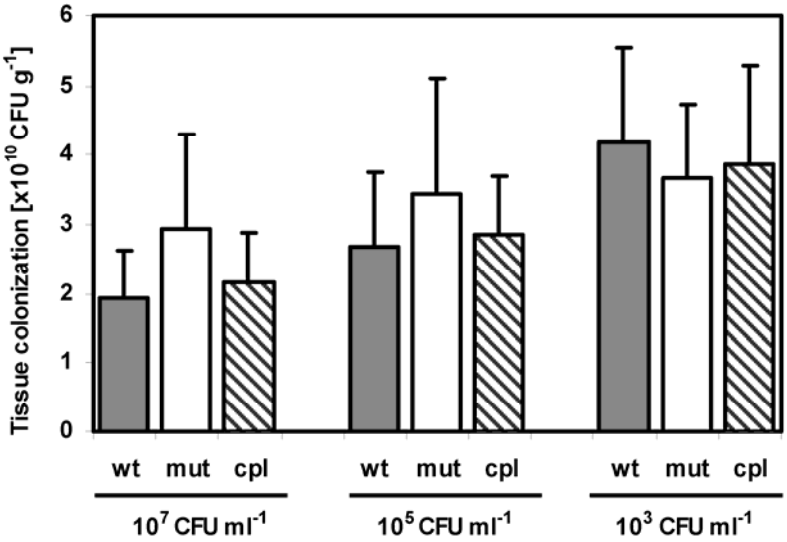

Fig. 7. *Erwinia amylovora* FAW610 colonization of immature pear tissue. Each fruit was inoculated in an artificial 1-mm-wide wound with 5 μl of a suspension containing approximately 10^7, 10^5, or 10^3 CFU ml^{-1} of a single strain derivative. CFU were enumerated after 7 days of incubation of the infected fruit at 24°C in a humidified chamber. Values are the mean of three replicates and error bars represent standard deviations.

ual inoculation of the strains, indicating an *luxS* role in competitiveness. Regardless of the molecular basis for reduced competitiveness, this observation points to *luxS* as a potential target for developing novel control approaches to reduce the ability of *E. amylovora* to establish in the primary fire blight infection court (Thomson 2000). Although we observed competition effects in flowers, which were inoculated with a relatively low concentration of bacteria in order to observe shifts in relative population size during growth, we have no indications that the role of *luxS* is dependent on bacterial concentration. In pear fruit colonization and virulence assays on apple plants, no difference was observed in the behavior of a *luxS* mutant and the wild type at any concentration between 10^3 and 10^9 CFU ml^{-1} (Figs. 6 and 7).

Pathogenicity on apple plants, immature pear fruit, and other functions that normally are associated with AI-2-related quorum sensing in animal pathogens, such as swarming and motility, were essentially unaffected in the *E. amylovora luxS* mutants. A recent shot-gun screening approach to identify key virulence genes in *E. amylovora* also failed to identify *luxS* as a major determinant of virulence on pear fruit (Zhao et al. 2005). However, we found that two important virulence genes, *hrpL* and *dspA*, were downregulated in *luxS* mutants, suggesting a possible role for *luxS* at some stage in disease development. In *Xanthomonas campestris* pv. *vesicatoria*, *hrp* induction requires the presence of sulfur-containing amino acids (Schulte and Bonas 1992; Wengelnik and Bonas 1996); thus, efficient pathogenicity may depend on an unaltered sulfur metabolism. Although we observed no clear role on highly susceptible apple shoots, recent preliminary data suggests that host physiological status may affect the importance of *luxS* in virulence on apple and further studies are planned to examine the influence of environmental stimuli on *luxS*. Both *hrpL* and *dspA* are responsive to bacterial growth phase and environmental stimuli (Wei et al. 2000); thus, a role of *luxS* in virulence via metabolism under certain conditions cannot be discounted entirely.

In summary, the lack of known AI-2 receptors in the *E. amylovora* genome, together with the failure of wild-type strains to chemically complement isogenic mutants in cocultivation experiments, strongly suggest that the role of *luxS* in the fire blight pathogen is of an intracellular metabolic nature and is not primarily related to AI-2 production or quorum sensing. Of course, the possibility that *E. amylovora* produces AI-2 to interfere with signaling of other bacteria or that the *E. amylovora* LuxS product is inadvertently utilized by other bacteria as an AI-2 signal cannot be ruled out completely.

MATERIALS AND METHODS

Bacterial strains and culture media.

Bacterial strains and plasmids used in this work are listed in Table 4. LB medium was used routinely for culturing *E. amylovora*, while autoinducer assay broth (AB) (Bassler et al. 1993) normally was used for *V. harveyi* bacterial growth. When necessary, the following antibiotics were added to the medium: ampicillin at 100 μg ml^{-1}, kanamycin at 50 μg ml^{-1} (Km^{50}), rifampicin at 100 μg ml^{-1} (Rif^{100}), and tetracycline at 50 μg ml^{-1} (Tc^{50}). *E. amylovora* AI-2 activity was assessed in LB, AB medium, *hrp*-inducing medium (50 mM potassium phosphate buffer, 7.6 mM $[NH_4]_2SO_4$, 1.7 mM $MgCl_2$, 1.7 mM NaCl, and 10 mM glucose, pH 5.7) (Huynh et al. 1989) or DMM (55 mM potassium phosphate buffer, 7.6 mM $[NH_4]_2SO_4$, 0.83 mM $MgSO_4$, 1.7 mM sodium citrate, and 6 mM glucose, pH 7.0). A sulfur-free modification of the latter (sf-DMM) was used in cultivation and cocultivation experiments and was prepared by replacing $MgSO_4$ and $(NH_4)_2SO_4$ with equal molar amounts of the corresponding chloride salts.

PCR conditions.

All PCRs described herein were performed either on DNA purified with the Promega Wizard DNA Extraction Kit (Promega Corp., Madison, WI, U.S.A.) or directly on bacterial lysates which were prepared as described by Rezzonico and associates (2003). Briefly, 5 μl of overnight LB culture was mixed with 95 μl of lysis buffer (i.e., 50 mM KCl; 10 mM Tris-HCl, pH 8.3; and 0.1% Tween 20) and heated for 10 min at 99°C in a Techne TC-412 thermal cycler (Midsci, St. Louis). PCR amplification was carried out in 10-μl reaction mixtures using 1 μl of cell lysate or 1 μl of purified DNA (1 ng/μl) and 0.4 mM each primer in a final concentration of 1× master mix of the HotStarTaq MasterMix Kit (Qiagen, Basel, Switzerland). Cycling conditions were substantially the same throughout this work, with an initial denaturation and activation of the HotStarTaq enzyme for 15 min at 95°C; followed by 35 cycles of 45 s of denaturation at 95°C, 45 s of annealing at 55°C, plus 30 s of elongation for every 500 bp of expected amplicon size; and a final elongation for 10 min at 72°C. Primers used for PCR and RT-PCR were designed based on the genome sequence of *E. amylovora* Ea273 using a Web-based primer-picking program *Primer3* (Rozen and Skaletsky 2000) and are listed in Table 5.

Sequence analysis of *luxS* and related genes in *E. amylovora* strains.

Primers corBtoluxS and gshAtoluxS were used as described above to amplify an 843-bp fragment spanning from *corB* to *gshA* in a collection of *E. amylovora* strains of worldwide origin (Table 4). PCR amplicons were purified from PCR mix by washing twice with 100 μl of double-distilled water (ddH_2O) on a MultiScreen PCR Plate (Millipore, Molsheim, France), resuspended in 30 μl of ddH_2O, and visually quantified in agarose gel. The cycle-sequencing reaction was performed with 20 to 40 ng of purified PCR product using the ABI PRISM BigDye Terminators v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, U.S.A.) according to the manufacturer's instructions using primers corBtoluxS and gshAtoluxS, each at a final concentration of 0.2 μM. Cycle-sequencing products were cleaned through water-swelled Sephadex G-50 columns (Amersham Biosciences, Uppsala, Sweden) on MultiScreen HV plates (Millipore) and sequenced on an ABI PRISM 3100 Genetic Analyzer. Obtained sequences were assembled using the Sequencher software (version 4.0.5; Gene Codes Corporation, Ann Arbor, MI, U.S.A.). For phylogenetic inference, representative *luxS* sequences of *E. amylovora* were aligned with *luxS* sequences of other gram-negative and gram-positive bacteria using the web-based program ClustalW (Thompson at al. 1994). Sites presenting alignment gaps were excluded from analysis. The Molecular Evolutionary Genetics Analysis program, version 2.1 (Kumar et al. 2001), was used to calculate evolutionary distances and to infer trees based on the neighbor-joining method with the Jukes-Cantor formula. Nodal robustness of the inferred trees was assessed by 1,000 bootstrap replicates. The presence of known AI-2 receptor genes was verified by comparing their published DNA sequences using BLAST with the translation of the completed genome of *E. amylovora* Ea273. Sequences used were *luxPQ* of *V. harveyi* BB7 (accession number U07069), *lsr*-operon genes of *Salmonella enterica* subsp. *enterica* serovar Typhi Ty2 (AE014613), and *mqsR* of *Escherichia coli* K12 (U00096).

Construction of plasmid pCAM-AΩB2 carrying *luxS* interrupted by an Ω cassette.

The flanking regions of *luxS* were amplified using primer pairs EcorEB-fw and BluxS-rD, yielding a 1,069-bp amplicon (fragment B2) containing the C-terminal regions of *luxS* and *corB*, and BluxS-fD and EgshA-rev, yielding a 1,058-bp am-

plicon (fragment A) containing the N-terminal region of *luxS* and the C-terminal region of *gshA* plus the intergenic region bearing the *luxS* promoter. Both fragments were digested with restriction enzyme *Bam*HI and ligated together using T4 ligase. The 2,119-bp band corresponding to the AB2 ligation was isolated on agarose, reamplified with EcorEB-fw and EgshA-rev, and digested with *Eco*RI. Plasmid pCAM-MCS (Burse et al. 2004) was linearized with *Eco*RI, dephosphorylated with shrimp alkaline phosphatase (SAP), and then ligated with the *Eco*RI-digested AB fragment. Ligation was transformed into chemically competent *E. coli* S17-1 λpir cells using standard transformation procedures (Sambrook et al. 1989) and transformants were selected using Tc^{50} selection. The presence of the AB2 fragment was verified by PCR amplification of a 408-bp amplicon using primers corBtoluxS and gshAtoluxS. The resulting plasmid, named pCAM-AB2, was extracted from *E. coli* using the QIAGEN Miniprep kit, linearized with *Bam*HI, and dephosphorylated with SAP. The Ω cassette, containing a kanamycin-resistance determinant, was amplified from plasmid pHP45ΩKm (Prentki and Krisch 1984) using primers BΩ-fw and BΩ-rev. The PCR product was digested with *Bam*HI and ligated with the linearized pCAM-AB2 plasmid using T4 ligase. Ligation was transformed into competent *E. coli* S17-1 λpir cells as described above and transformants containing the Ω cassette were selected on LB-Km^{50}. The correct insertion of the cassette was verified by PCR amplification of the 2,377-bp AΩB2-amplicon using primers corBtoluxS and gshAtoluxS, and the resulting plasmid was named pCAM-AΩB2.

luxS gene disruption by allelic exchange in *Erwinia amylovora* strains CFBP1430 and FAW610.

Plasmid pCAM-AΩB2 is a suicide vector for members of *Enterobacteriaceae* because it bears the R6K origin of replication and can be maintained only in host strains, such as *Escherichia coli* S17-1 λpir, producing the R6K-specified protein π. Furthermore, *E. coli* S17-1 λpir contains the λpir gene. This strain has chromosomally integrated conjugal transfer functions (RP4 transfer functions); therefore, when it is used as a spe-

Table 4. Bacterial strains and plasmids used in this work

Strain or plasmid	Geographic origin (year)	Plant origin, relevant characteristics[a]	Reference[b]
Erwinia amylovora strains			
CFBP1232	United Kingdom (1959)	*Pyrus communis*	Jock et al. 2002
CFBP1430	Lille, France (1972)	*Crataegus* sp.	Jock et al. 2002
BPIC847	Arcadia, Greece (1984)	*P. communis*	Zhang et al. 1998
JL1185	Washington, U.S.A. (1988)	*P. communis*	Loper et al. 1991
01SFR-BO	Ravenna, Italy (1991)	*Sorbus* sp.	Jock et al. 2002
Ea02	Vollèges, Switzerland (2002)	*Cotoneaster* sp.	Molina et al. 2005
Ea153	Oregon, U.S.A. (1989)	*Malus domestica*	Johnson et al. 1993
Ea1/79	Germany (1979)	*Cotoneaster* sp.	Jock et al. 2002
Ea263	Baden-Wüttenberg, Germany (1996)	*M. domestica*	Jock et al. 2002
Ea273	New York, U.S.A. (1971)	*M. domestica*	Beer et al. 1991
Ea321 [CFBP1367]	Angers, France (unknown)	*Crataegus* sp.	Beer et al. 1991
OMP-BO 379	Emilia Romagna, Italy (2001)	*P. communis*	A. Calzolari (PC)
Ea4/82	Egypt (1982)	*P. communis*	Jock et al. 2002
FAW610 [ACW24849]	Appenzell, Switzerland (2001)	*M. domestica*	J. Vogelsanger (PC)
FAW611 [ACW26599]	Switzerland (2001)	*Cydonia* sp. (Quince)	J. Vogelsanger (PC)
OPM-BO 691.2	Emilia Romagna, Italy (1995)	*P. communis*	Zhang et al. 1998
IPV-BO 1077/7	Emilia Romagna, Italy (1994)	*P. communis*	Babini and Mazzucchi 2000
IPV-BO 5010	Lombardia, Italy (2003)	*P. communis*	Venturi et al. 2004
IPV-BO 5011	Lombardia, Italy (2003)	*P. communis*	Venturi et al. 2004
IPV-BO 5039	Friuli Venezia Giulia, Italy (2003)	*Crataegus* sp.	Venturi et al. 2004
IPV-BO 5357	Piemonte, Italy (2003)	*Cotoneaster* sp.	Venturi et al. 2004
Other *Erwinia* strains			
E. billingiae LMG 2613	United Kingdom (1959)	*P. communis*	Mergaert et al. 1999
E. amylovora derivatives			
CFBP1430Δ*luxS*	…	Km^r, *luxS* interrupted by the Ω cassette of pHP45ΩKm	This work
CFBP1430Δ+	…	Km^r, Tc^r, CFBP1430Δ*luxS* complemented by pMF8805	This work
FAW610Δ*luxS*	…	Km^r, *luxS* interrupted by the Ω cassette of pHP45ΩKm	This work
FAW610Δ+	…	Km^r, Tc^r, FAW610Δ*luxS* complemented by pMF8805	This work
Escherichia coli strains			
S17-1 λpir	…	*recA thi pro hsdR* [res^- mod^+][RP4::2-Tc::Mu-Km::Tn*7*] λ *pir* phage lysogen	Simon et al. 1983
TOP10	…	Chemical competent; F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*araleu*) 7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G	Invitrogen
Vibrio harveyi strains			
BB120/ATCC BBA-1116	…	Wild type, AI-1^+ AI-2^+, sensor-1^+ sensor-2^+	Bassler et al. 1997
BB170/ATCC BBA-1117	…	AI-1^+ AI-2^+, sensor-1^- sensor-2^+	Bassler et al. 1993
Plasmids			
pHP45ΩKm	…	Amp^r, Km^r, pHP45 containing the Km^r cassette	Prentki and Krisch, 1984
pCAM-MCS	…	Amp^r, cloning vector	Burse et al. 2004
pCAM-AB2	…	Amp^r, 2.1-kb fragment containing the flanking regions of *luxS* cloned into *Eco*RI site of pCAM-MCS	This work
pCAM-AΩB2	…	Amp^r, Km^r, Ω cassette from pHP45Ω cloned into *Bam*HI site of pCAM-AB2	This work
pME3088	…	Tc^r, cloning vector	Voisard et al. 1994
pMF8805	…	Tc^r, 1.1-kb fragment containing luxS cloned into *Eco*RI site of pME3088	This work

[a] Km^r, Tc^r, Amp^r, and Str^r = resistant to kanamycin, tetracycline, ampicillin, and streptomycin, respectively.
[b] PC = personal communication and Invitrogen, Basel, Switzerland.

cific host strain into which the transposon vector DNA is transformed, the transfer occurs by means of a biparental mating and a helper strain is not necessary. Overnight cultures (5 ml) of *E. coli* S17-1 λpir/pCAM-AΩB2 in LB-Km^{50} and 3-day-old, late-stationary-phase cultures of rifampicin-resistant *Erwinia amylovora* in LB-Rif^{100} were pelleted by centrifugation, washed twice with fresh LB, mixed together in a 3:1 ratio, and then resuspended in 20 µl of LB before being finally spotted in the center of LB plates. Plates were incubated at 37°C for 2, 5, and 7.5 h; then, the cells were eluted with 300 µl of LB medium and plated on LB agar supplemented with rifampicin (100 µg ml^{-1}) and kanamycin (50 µg ml^{-1}) to select for transformed *E. amylovora*. Plates were incubated overnight at 28°C and the resulting colonies were analyzed. To exclude mutants that resulted from single crossover events (i.e., plasmid integration in the chromosome), kanamycin-resistant colonies were transferred in parallel onto Chromocult coliform agar (Merck, Dietikon, Switzerland) supplemented with kanamycin (50 µg ml^{-1}) and onto LB agar plates supplemented with ampicillin (100 µg ml^{-1}). Kanamycin-resistant and ampicillin-sensitive colonies with the distinctive *E. amylovora* pink coloration on Chromocult agar were selected and allelic exchange was verified by PCR using primer-pair combinations corBext1/agemO and gshAext/Omega.

Complementation of *E. amylovora* mutant strains CFBP1430Δ*luxS* and FAW610Δ*luxS*.

Primers EcorB-fw and EgshA-rD were used to amplify a 1,124-bp fragment containing the *luxS* gene and its promoter plus the C-terminal regions of the flanking genes *corB* and *gshA* ("Fragment bSa"). Plasmid pME3088 (Voisard et al. 1994), bearing a tetracycline-resistance determinant, was linearized with *Eco*RI, dephosphorylated with SAP, and then ligated with the *Eco*RI-digested bSa-fragment. Ligation was trans-

Table 5. Primers used in this work

Primer[a]	Sequence 5′–3′[b]	Utilization, target gene function	Reference
Mutant			
EcorEB-fw	CCAC**GAATTC**AGTACTGCCGAAAACCGTTG	Amplification of fragment B2	This work
BluxS-rD	CCAC**GGATCC**CACTGCCAAAAGAGAAGCTG	Amplification of fragment B2	This work
BluxS-fD	CCAC**GGATCC**TGGTGTGATCGACGGTAAAG	Amplification of fragment A	This work
EgshA-rev	CCAC**GGATCC**GCGATCTTGGCTACACCAAT	Amplification of fragment A	This work
EcorB-fw	CCAC**GAATTC**GCCTGGTCACCATTGAAGAT	Amplification of fragment bSa	This work
EgshA-rD	CCAC**GAATTC**CGAAGCCTTTTTACAAAGCAA	Amplification of fragment bSa	This work
BΩ-fw	CCAC**GGATCC**CAATTCCCCTGCTCGCGCAGG	Amplification of Ω-cassette	This work
BΩ-rev	CCAC**GGATCC**CAGCTTAGTAAAGCCCTCGCT	Amplification of Ω-cassette	This work
corBtoluxS	GATTAAGCAGGTGCGCATTA	Verification of fragments AB2 and AΩB2	This work
gshAtoluxS	AAGTGATACGCTTAGCTTCG	Verification of fragments AB2 and AΩB2	This work
corBext1	TTACAAGCTGCGTCATCAGG	Verification of allelic exchange	This work
agemO	ACTGTGGGTCAAGGATCTGG	Verification of allelic exchange	This work
gshAext	GGTTGGGTGATCCCTTACCT	Verification of allelic exchange	This work
Omega	CACCAAGGTAGTCGGCAAAT	Verification of allelic exchange	This work
luxSic1	GGCATTCACACGCTGGA	Detection of *luxS*	T. Paternoster
luxSic2	CATCGCCGCRTTCCACGC	Detection of *luxS*	T. Paternoster
RT-PCR			
RTluxS-fw	CTTTACCGTCGATCACACCA	Amplification of 420-bp *luxS* amplicon, methionine metabolism/AI-2 signal	This work
RTluxS-rev	ATATGACGGGCGATATCCTG		This work
RT16S-fw	CAGAAGAAGCACCGGCTAAC	Amplification of 342-bp *rrs* amplicon, 16S rDNA synthesis	This work
RT16S-rev	AAACGATGTCGACTTGGAGG		This work
RThrpL-fw	CGATGGGCTACCGCTTAATA	Amplification of 431-bp *hrpL* amplicon, σ-factor, control of *hrp* gene expression	This work
RThrpL-rev	TCAAATGTGCTGCAATAGCC		This work
RTdspA-fw	AAAACCGCACTGACCAAATC	Amplification of 451-bp *dspA* amplicon, virulence effector	This work
RTdspA-rev	CTGGCACCTTCTTGCTAAGG		This work
RTgalE-fw	CGATGACGTGGTGATACTGG	Amplification of 495-bp *galE* amplicon, UDP-glucose 4-epimerase	This work
RTgalE-rev	ATCTGACCGGATTCATGAGC		This work
RTmetE-fw	TCAGATTGATGAGCCTGCAC	Amplification of 304-bp *metE* amplicon, homocysteine transmethylase	This work
RTmetE-rev	ACGATATCCACTCGCCAAAC		This work
RTmetJ-fw	CTGAATGGAACGGCGAATA	Amplification of 275-bp *metJ* amplicon, transcriptional repressor of *met* genes	This work
RTmetJ-rev	AAGAGGCAAAAGCGATCATG		This work
RTmetK-fw	GCTTTGATGCCAATTCCTGT	Amplification of 326-bp *metK* amplicon, *S*-adenosylmethionine synthetase	This work
RTmetK-rev	TGTCTACTCAACACGCCGAG		This work
RTmetR-fw	TATTGCCGCAGATACAGCAG	Amplification of 339-bp *metR* amplicon, transcriptional activator of *met* genes	This work
RTmetR-rev	TATCTCACCCCAGGATCTCG		This work
RTpfs-fw	GAAGTTGCCCTGCTGAAATC	Amplification of 338-bp *pfs* amplicon, MTA/SAH nucleosidase	This work
RTpfs-rev	GGTGATGCCTTTATCAACGG		This work
RTscrK-fw	ACGCCACTTCACCTTTATGG	Amplification of 271-bp *scrK* amplicon, fructokinase	This work
RTscrK-rev	GATGATGTCTGCCAGCTCAA		This work
RTsrlD-fw	TGGCAGCAAGCATAACTCTG	Amplification of 284-bp *srlD* amplicon, sorbitol-6-phosphate dehydrogenase	This work
RTsrlD-rev	TGTCACTCGCGTAAAACAGC		This work

[a] Mutant = mutant construction and verification and RT-PCR = reverse-transcription polymerase chain reaction.
[b] Sequences in bold are the recognition sites for the restriction enzymes.

formed into competent TOP10 *Escherichia coli* cells (Invitrogen) according to the manufacturer's instructions. The insertion of the bSa-fragment was verified in the transformed colonies (Tc^{50} selection) by PCR, again using EcorB-fw and EgshA-rD. The plasmid bearing the intact *luxS* gene, named pMF8805, was extracted from *E. coli* using the QIAGEN Miniprep kit and transformed by electroporation (12.5 V/cm, 25 μF, 200 Ω) into competent *Erwinia amylovora* CFBP1430 and FAW610 Δ*luxS* mutant strains. Verification of *luxS* expression in CFBP1430 and FAW610 wild-type, mutant, and complemented strains was assessed by RT-PCR using the primers RTluxS-fw and RTluxS-rev and PCR conditions described below, but using an annealing temperature of 60°C and 40 cycles of PCR.

Detection of AI-2 production in *E. amylovora* using *V. harveyi* reporter strain BB170.

Stimulation of light production in *V. harveyi* reporter strain BB170 (sensor 1^- sensor 2^+) by *E. amylovora* was assessed as described by Surette and Bassler (1998). BB170 was grown overnight at 30°C in AB medium (Greenberg et al. 1979) to an optical density at 600 nm (OD_{600}) of approximately 1.0. The culture was diluted 1:5000 in fresh medium and cell-free culture fluids from *E. amylovora* were added at 10% final concentration. The resulting light production was monitored with a Fluoroskan Ascent FL luminometer (Thermo Labsystem, Helsinki, Finland). The maximal stimulation of light production occurred at 4 h after dilution and addition of the culture fluids. All experiments were performed at least three times. The stimulation of light production obtained from BB120 wild-type strain cell culture fluid in AB medium was considered to be 100% and the results were considered positive when the stimulation of light was, in all three experiments, higher than 10% of the result obtained for BB120 (Bassler et al. 1997).

Growth under sulfur-limiting conditions in vitro.

The growth ability of *E. amylovora* under sulfur-limiting conditions was studied using 200-μl microcultures in 100-well honeycomb plates (well volume 400 μl) at 28°C with regular shaking at 15-min intervals. FAW610 wild type, the mutant (FAW610Δ*luxS*), and the complemented strain (FAW610Δ*luxS*/pMF8805) were grown overnight in separate cultures on DMM, collected by centrifugation, and washed twice with sterile 0.9% NaCl. All strains were inoculated at an initial OD_{600} = 0.018 on LB, DMM (total SO_4 molarity = 8.4 mM), or modified sf-DMM either with methionine or cysteine (10, 50, or 100 μM) as sole sulfur sources. Growth curves were measured in quadruplicate by periodically quantifying the absorbance through a 420- to 580-nm wide band filter using a Bioscreen C MBR system (Growth Curves Oy, Helsinki, Finland).

Competition assay under sulfur-limiting conditions.

For competition experiments, FAW610 and FAW610Δ*luxS* or FAW610 and FAW610Δ*luxS*/pMF8805 were coinoculated in a 1:1 ratio at the same total OD_{600} = 0.018 for each individual culture (corresponding to approximately 2×10^7 CFU ml^{-1}) in 10 ml of DMM or modified sf-DMM with 100 μM methionine. Separate cultures of single-strain derivatives were used as controls. The bacteria were sampled after 7, 22, 31, and 53 h of growth at 28°C and 220 rpm, and viable counts were performed by serial dilutions on selective (LB-Km^{50} and LB-Tc^{50} for mutant and complemented strain, respectively) and nonselective LB agar (total bacteria), enabling subtractive determination of the wild-type population.

Genetic expression in *luxS* mutants.

Genetic expression in *E. amylovora* strains was evaluated by monitoring the production of gene-specific mRNAs by semi-quantitative RT-PCR in DMM and in modified DMM with 100 μM methionine as sole sulfur source. Primers used for RT-PCR are listed in Table 5. The expression of following genes was monitored: *luxS* (methionine metabolism/putative AI-2 signal production); pathogenicity-related genes *hrpL* (alternative σ-factor for type III secretion system genes) and *dspA* (secreted avirulence protein); sorbitol metabolism gene *srlD* (sorbitol-6-phosphate dehydrogenase); galactose metabolism gene *galE* (UDP-glucose 4-epimerase); fructose metabolism gene *scrK* (fructokinase); and AMC genes *metE* (homocysteine transmethylase), *metK* (*S*-adenosylmethionine synthetase), *metJ* (transcriptional repressor of *met* genes), *metR* (transcriptional activator of *met* genes), and *pfs* (5′-methylthioadenosine/*S*-adenosylhomocysteine nucleosidase). RNA extraction was performed from *E. amylovora* cells using the RNeasy mini kit (Qiagen) at three different sampling times: 16 h (middle of exponential growth phase), 26 h (end of exponential growth phase), and 40 h (stationary phase). Reverse transcription was conducted using the QuantiTect Reverse Transcription kit (Qiagen) and 400 ng of total RNA per reaction. After the reverse transcription reaction, cDNA was purified through QIAquick PCR purification columns (Qiagen), and the cDNA quantity was measured spectrophotometrically and adjusted in each sample to a final concentration of 0.8 ng μl^{-1} to be used in PCR amplification. A constitutive *rrs* gene encoding for 16S rRNA was used as internal control for sample normalization. To ensure proper semiquantitative results, the PCR on cDNA was performed as described above, but the number of PCR cycles was adjusted individually for each primer pair in order to fall in the exponential phase of the amplification reaction (Walther et al. 1998); therefore, the relative expression values are comparable only within each individual gene, not among the different genes.

Motility and biofilm assays.

Motility assays were performed with *E. amylovora* CFBP1430 and FAW610 strains and derivatives on LB plates containing decreasing (0.8, 0.5, and 0.3%), concentration of agar as described (Dixit et al. 2002). Briefly, for every strain, 1 μl of LB overnight cultures of wild-type, mutant, and complemented strains was spotted on the same plate and grown for 48 h at 23°C. The diameters of the three visible chemotactic rings were measured. Average values and standard deviations ($n = 6$) were calculated. Both strains were further tested for biofilm formation in rich medium as described by Yap and associates (2005) using LB, SOBG or SOCG medium. A 10-μl aliquot of an overnight bacterial culture was suspended in 10 ml of liquid medium and cultures were incubated at 24 or 30°C in stationary reaction tubes for up to 2 weeks. The formation of a biofilm around the edge of the culture tube was evaluated qualitatively by estimating the thickness of the bacterial layer at the air-liquid surface interface.

Colonization of detached apple blossoms.

Detached flower assays were performed following Pusey (2000) using newly opened Golden Delicious apple flowers of similar age collected in an orchard of the ACW in Wädenswil. Each detached flower was maintained with the cut peduncle submerged in 10% sterile sucrose solution in isolated indentations in sterile trays. Overnight bacterial cultures in LB were washed twice in 0.9% NaCl solution and diluted in phosphate-buffered saline (PBS) to a final cell concentration of 10^4 CFU ml^{-1}. Application of bacteria to flowers was achieved by touching each hypanthium with a 10-μl droplet of the suspension. Flowers were enclosed in plastic boxes with moist paper towels to maintain near 100% relative humidity and then incubated for 2 days at 20°C. After incubation, 10 flowers per treatment

were sampled to determine bacterial population sizes. Two stigmas and a portion of the supporting style were placed in sterile microcentrifuge tubes containing 900 µl of sterile PBS. Tubes were vortexed briefly and placed in a sonication bath for 30 s. Samples were vortexed again and serial dilutions in 10-fold dilution steps were spread on nutrient sucrose agar (NSA)-Rif. Bacterial colonies were counted after 2 days of incubation at 28°C. To evaluate competitiveness in apple blossoms, wild-type and mutant as well as mutant and complemented strains were applied on the flowers in a 1:1 mix. Each strain was used at half concentration with respect to the experiment featuring the single strains. The experiment was repeated once.

Virulence assay on young apple trees.

Virulence on young apple trees was evaluated using the standard assay of internal bacterial movement (Momol et al. 1998) and fire blight resistance (Khan et al. 2006), with slight modifications. Shoots of 15 to 30 cm in length of potted 1-year-old grafted Golden Delicious plants were inoculated in a quarantine greenhouse with bacterial suspensions adjusted to cell densities of 10^9, 10^7, 10^5, and 10^3 CFU ml^{-1}. Shoot tips of trees were inoculated by inserting a 0.46-mm-diameter (26-gauge) hypodermic needle through the stem just above the youngest opened leaf. Sufficient inoculum was introduced to fill the wound, leaving drops at each end of the wounds when the needle was removed. After 14 days, disease progress was measured as the percentage of total shoot length with observable lesion formation starting from the inoculation point, following Momol and associates (1998). The experiment was repeated once with three replicate plants in each trial.

Virulence assay on immature pear fruit.

Virulence on immature pear fruit was evaluated by inoculating the fruit with 5 µl of bacterial suspensions adjusted to cell densities of 10^7, 10^5, and 10^3 CFU ml^{-1} through an artificial lesion produced using a 1-mm sterile steel tip (Zhao et al. 2005) after surface sterilization with 10% bleach solution. Four randomly chosen, immature pear fruit (*Pyrus communis*) of similar size were inoculated for each treatment using bacterial strain FAW610 and derivatives. After 1 week of incubation in a humidified chamber at 24°C, the progress of the disease was recorded. The virulence of the strain derivatives was analyzed by measuring the diameter of the lesion on the fruit. For bacterial population studies, the inoculation site was excised using a no. 4 cork borer and homogenized in 0.5 ml of 1× PBS. Serial dilutions in 10-fold dilution steps were spread on NSA-Rif. After 2 days of incubation at 28°C, bacterial colonies in the appropriate dilution were counted and the number of cultivable cells per gram of pear tissue was calculated. The experiment was repeated once.

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AUTHOR-RECOMMENDED INTERNET RESOURCE

Wellcome Trust Sanger Institute *Erwinia amylovora* webpage: www.sanger.ac.uk/Projects/E_amylovora