

A Lux-like Quorum Sensing System in the Extreme Acidophile *Acidithiobacillus ferrooxidans*

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ABSTRACT

The genome of the acidophilic, proteobacterium *Acidithiobacillus ferrooxidans*, contains linked but divergently oriented genes, termed *afeI* and *afeR*, whose predicted protein products are significantly similar to the LuxI and LuxR families of proteins. A possible promoter and Lux box are predicted upstream of *afeI*. A cloned copy of *afeI*, expressed in *E. coli*, encodes an enzyme that catalyzes the production of a diffusible compound identified by gas chromatography and mass spectrometry as an unsubstituted *N*-acyl homoserine lactone (AHL) of chain length C₁₄. This AHL can be detected by a reporter strain of *Sinorhizobium meliloti* Rm41 suggesting that it is biologically active. The reporter strain also responds to extracts of the supernatant of *A. ferrooxidans* grown to early stationary phase in sulfur medium indicating that a diffusible AHL is produced by this microorganism. Semi-quantitative RT-PCR experiments indicate that *afeI* and *afeR* are expressed maximally in early stationary phase and are more expressed when *A. ferrooxidans* is grown in sulfur- rather than iron-containing medium. Given the predicted amino acid sequence and functional properties of AfeI and AfeR it is proposed that *A. ferrooxidans* has a quorum sensing system similar to the LuxI-LuxR paradigm.

Key terms: *A. ferrooxidans*, quorum-sensing, LuxI and LuxR, Lux box, homoserine lactone.

INTRODUCTION

Quorum sensing is a mechanism for regulating gene expression in response to changes in cell density in bacterial populations. One type of quorum sensing mechanism, widely used by proteobacteria, involves the production of an *N*-acyl homoserine lactone (AHL) autoinducer. AHL is synthesized from S-adenosylmethionine (SAM) (Hanzelka and Greenberg, 1997) via the activity of LuxI-type acyl synthase. AHL molecules diffuse through the bacterial membrane and when a critical extra-cellular threshold concentration is reached, they are detected by intracellular LuxR-type proteins that can regulate not only the expression of *luxI* but also other target genes involved in a variety of behavioral responses (reviewed in

Pappas *et al.*, 2004). In many instances it has been shown that LuxR regulates gene expression by binding to upstream consensus sequences, termed Lux-boxes, upregulating gene transcription. The types of responses regulated by the quorum sensing genetic circuits include bioluminescence (Nealson, 1999), the horizontal transfer of DNA (Winans *et al.*, 1999; Whitehead *et al.*, 2001), the formation of biofilms (Conway *et al.*, 2002) and the production of pathogenetic factors, antibiotics and other secondary metabolites (Pesci and Iglewski, 1999; reviewed in Miller and Bassler, 2001)

Acidithiobacillus ferrooxidans, formerly called *Thiobacillus ferrooxidans* (Kelly and Wood, 2000) is a chemolithoautotrophic, γ -proteobacterium that obtains energy and electrons by the oxidation of reduced sulfur

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compounds to sulfate or FeII to FeIII. It is a mesophilic, facultative aerobe that fixes atmospheric CO₂ and N₂ to provide cellular C and N. It thrives in extremely acidic conditions (pH 1-2) and is often confronted with high concentrations of metals. These multiple challenges make it an excellent choice for understanding microbial physiology in extreme environments.

A. ferrooxidans is a member of a consortium of microorganisms found in bioleaching operations and other naturally low pH environments (reviewed in Rawlings, 2002) and is known to occur in biofilms (Gehrke *et al.*, 1998, Schippers and Sand, 1999). A knowledge of its role in the development and maintenance of biofilms is important for generating a comprehensive description of its role in mineral leaching and environmentally associated processes. Quorum sensing processes have been invoked in the establishment and maintenance of biofilms in a number of environmental bacteria (Bollinger *et al.*, 2001; Conway *et al.*, 2002; Huber, *et al.*, 2001), but only two preliminary reports of the presence of potential quorum sensing genes in *A. ferrooxidans* have been published (Barreto

et al., 2003; Farrah *et al.*, 2004). This lack of information provoked the present study.

METHODS

Bacterial strains, plasmids and media

Acidithiobacillus ferrooxidans ATCC 23270 was grown in 9K salts medium (pH 2.4) supplemented with elemental sulfur or iron (FeSO₄) as described by Yates and Holmes (1988). *Escherichia coli* JM109 was grown in Luria-Bertani (LB) medium. *Agrobacterium tumefaciens* NT1 was grown in LB medium containing kanamycin 50 µgml⁻¹ at 30° C. *Sinorhizobium meliloti* Rm41 was grown in LB at 30° C and *S. meliloti* Rm41 SinI⁻ was grown in LB or MMgly medium (11 g of Na₂HPO₄, 3 g of KH₂PO₄, 0.5 g NaCl, 1g of NH₄Cl, 5 ml of glycerol, 1 mg of biotin, 27.8 mg of CaCl₂, and 246 mg of MgSO₄ per liter) at 30° C supplemented with 1% (w/v) L-arabinose. Antibiotics were added where appropriate at the following final concentrations neomycin 200 mg µl⁻¹, gentamycin 50 mg µl⁻¹. Details of the phenotypes and sources of bacteria are shown in Table I.

TABLE I
Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype/phenotype	Reference or source
Strains		
<i>Acidithiobacillus ferrooxidans</i> ATCC23270	Type strain	ATCC
<i>Escherichia coli</i> JM109	[F ⁺ TraD36lacI ^q (lacZ)M15proA ⁺ B ⁺ /e14(McrA)(lac-proA)thiyrA96(NaI ^r)endAhsdR17(rkmk ⁺)relA1supE44recA]	Promega
<i>Agrobacterium tumefaciens</i> NT1	<i>traR</i> , <i>traG::lacZ</i> , biosensor reporter, Km ^r	Shaw <i>et al.</i> , 1997
<i>Sinorhizobium meliloti</i> Rm41	Type strain, wild type AHL overproducer	Llamas <i>et al.</i> , 2004
Rm41 SinI ⁻	<i>sinR</i> , <i>sinI::lacZ</i> , biosensor reporter, Gm ^r Neo ^r	Llamas <i>et al.</i> , 2004
Plasmids		
pGEMT-Easy	plasmid vector, Amp ^r	Promega
PGEX-2t	GST gene fusion vector, Amp ^r	Pharmacia
pT7blue3-3	plasmid vector, Amp ^r	Novagen
pAfeI	<i>afeI</i> expressed from pGEX-2t tac promoter, Amp ^r	This study
pAfeR-I	<i>afeR::Lux box:afeI</i> promoter: <i>afeI::lacZ</i> , Amp ^r	This study
pAfeΔR-I	Lux box: <i>afeI</i> promoter: <i>afeI::lacZ</i> , Amp ^r	This study

Bioinformatic Analyses

Candidate protein coding genes were identified in the partial genome sequence of *A. ferrooxidans* ATCC 23270, made available by The Institute for Genome Research (TIGR, www.tigr.org) using Glimmer (www.tigr.org), Critica (www.ttaxis.com) and BlastX (www.ncbi.nlm.nih.gov), followed by manual curation of the predicted genes to correct errors in start site prediction and identify missing candidate genes. The annotated genome was displayed in the interactive format of Artemis (www.sanger.ac.uk/Software/Artemis). The following bioinformatic programs were used to further characterize candidate genes and their predicted protein products: BlastP and PsiBlast (www.ncbi.nlm.nih.gov), the suite of protein characterization programs available in InterproScan (www.ebi.ac.uk/interpro), Blocks (www.blocks.fhcrc.org) and ClustalW (www.ebi.ac.uk/ClustalW). Additional protein motif finding programs were used to detect potential helix-turn-helix (npsa-pbil.ibcp.fr/cgi-bin/primanal_hth.pl) and signal peptide motifs (www.psport.nibb.ac.jp). A candidate LuxR transcription factor binding sites (Lux box) was identified by using a motif model of the LuxR binding site in DNA (McGuire *et al.*, 2000). The putative target was subjected to a cross-species comparison (phylogenetic footprinting) to increase confidence in the prediction using a Gibbs sampling-based motif-detection procedure (McCue *et al.*, 2001; Frazer *et al.*, 2003). Candidate sigma-70 promoters were detected using a neural network program for prokaryotic sigma-70 promoters (www.fruitfly.org/seq_tools/promoter.html).

Purification and identification of AHL

A. ferrooxidans and recombinant *E. coli* pAfeI early stationary-phase cultures (including cells and supernatants) were extracted with dichloromethane (DCM) at a ratio of 70:30 (culture:DCM) as described (McClean *et al.*, 1997). DCM was removed by rotary evaporation and the residue

reconstituted in 100 μ l DCM for fractionation by sep-pack C₁₈ preparative columns. Fractions were eluted with 1 ml in a gradient of methanol in water (20-40-60-75-95 %, v/v). Five fractions (F1-F5) were collected, concentrated to 10 ml and assayed for activity using the AHL assays described above. Samples (1 μ l) were also injected in the splitless mode into a gas chromatography-mass spectrometry (GC/MS) system consisting of a Autosystem XL gas chromatograph (Perkin-Elmer, Boston, MA, USA) with a MDN-5 column (Supelco, Bellefonte, PA, USA), coupled to a Perkin-Elmer Turbo Mass mass spectrometer. Helium served as carrier gas. The mass spectrometer was operated in the electron impact ionization mode at 70 eV as described (Seeger *et al.*, 2001, 2003). The following AHL standards were purchased from Fluka: *N*-hexanoyl-DL-homoserine lactone (AHL-C₆), *N*-octanoyl-DL-homoserine lactone (AHL-C₈) and *N*-tetradecanoyl-DL-homoserine lactone (AHL-C₁₄).

Isolation of DNA, recombinant DNA techniques, DNA sequencing

DNA was isolated from *A. ferrooxidans* as described (Barreto *et al.*, 2005). The following standard recombinant DNA techniques: digestion of DNA with restriction enzymes, agarose gel electrophoresis, purification of DNA fragments, PCR amplification of DNA, DNA ligation, plasmid preparation and transformation of *E. coli*, were performed as described (Sambrook *et al.*, 1989). DNA sequencing was carried out by the Sanger dideoxynucleotide method (Sambrook *et al.*, 1989). The nucleotide sequence of the *afeR-I* locus has been assigned the GenBank accession number AY758559.

Construction of recombinant plasmids and analysis of gene expression

AfeI was amplified with Elongase mix (Invitrogen) using genomic DNA as a template with the primers AfeI-4 and AfeI-5 (Table II). The resulting amplified DNA was cloned into pGEMT-easy as described

by the supplier (Promega). *AfeI* was subcloned pGEMT-easy into pGEX-2t (Amershan Pharmacia Biotech) as described by the suppliers. The resulting plasmid was termed pAfeI. pGEX-2t and pAfeI were separately transformed into *E. coli* JM109 by electroporation and the transformants were grown on LB plates containing ampicillin 100 $\mu\text{g ml}^{-1}$.

Genomic DNA corresponding to the entire predicted *afeR*, the first 120 bps of *afeI* and the whole of the intergenic region between these two genes (Fig. 1) was amplified by PCR using the primers AfeR-3 and AfeI-3 (Table II). The resulting amplified DNA was cloned in pT7-Blue3 (Novagen) and subsequently subcloned into pQF50 (Farinha and Kropinski, 1990) in an orientation that places the expression of *lacZ* in pQF50 under the control of the putative promoter of *afeI*. The resulting plasmid was termed pAfeR-I and was introduced into *E. coli* JM109 by electroporation and the transformants were grown in LB plates containing ampicillin 100 $\text{mg } \mu\text{l}^{-1}$. In addition, a plasmid similar to pAfeR-I but lacking *AfeR* was constructed and termed pAfe Δ R-I.

Promoter activity of pAfeR-I.

Overnight *E. coli* JM109 (pAfeR-I) cultures were normalized to an optical

density at 600 nm of 0.1 in a volume of 5 ml of LB containing the desired AHL at the desired concentration. Cultures were grown with agitation at 37° C for 6 h, and β -galactosidase activities were determined by the Miller assay as previously described (Quatrini *et al.*, 2005).

AHL reporter plate assays

E. coli JM109 (pAfeI) was cross-streaked onto X-gal 80 $\text{mg } \mu\text{l}^{-1}$, IPTG 40 $\mu\text{g ml}^{-1}$ LB medium reporter plates in the presence *A. tumefaciens* NT1 (Shaw *et al.*, 1997) and *S. meliloti* Rm41 SinI- (Llamas *et al.*, 2004) as described by Latifi *et al.* (1995) and Swift *et al.* (1997). Plates were incubated overnight at 30° C. Activation of the *lacZ* gene in these reporter strains was detected visibly by the production of blue dye. As a negative control, *E. coli* JM109 lacking *afeI* but containing the plasmid vector pGEX-2t was also cross-streaked on the same plates. As a positive control, the AHL-producing strain *S. meliloti* Rm41 was also cross-streaked. The presence of AHL was also detected on solid media by growing *E. coli* (pAfeR-I) on X-Gal medium supplemented with ampicillin (100 $\mu\text{g ml}^{-1}$) in close proximity to the tester strain. The presence of AHL was observed when *E. coli* (pAfeR-I) turned blue.

TABLE II

PCR and RT-PCR primers used in this study

Gene	Primer name	Sequence (5' to 3') ^a
<i>afeI</i>	afeI-1	5'CAGGTTATAACCGGGCCAGCTG
	afeI-2	5'ATCAGCCGTTTTGCCCCGTG
	afeI-3	5' <u>AAGCTT</u> ATCCC GTTCTCCGTGTGCA
	afeI-4	5' <u>CTCGAG</u> CGCATGCAGGTTATAACC
	afeI-5	5' <u>GAGCTC</u> GCGGTCCAGATCTATCCA
<i>afeR</i>	afeR-1	5'GAGCATCGCCGCCTGCAATA
	afeR-1	5'AGGCCATCGATCCCACGGTA
	afeR-1	5' <u>GGTACCC</u> GACAGCAACCCGAGCATC
<i>afe1016</i>	afe1016-1	5'ATGACAGTGCAGAACGGAAT
	afe1016-1	5'AACATATCTATCCATGATAT
<i>recA</i>	recA-1	5'CCGCCAACATTTCCCGGACC
	recA-1	5'ACGCCGAGGTCCACCAGTTC

^a Underlined are added restriction enzyme sites.

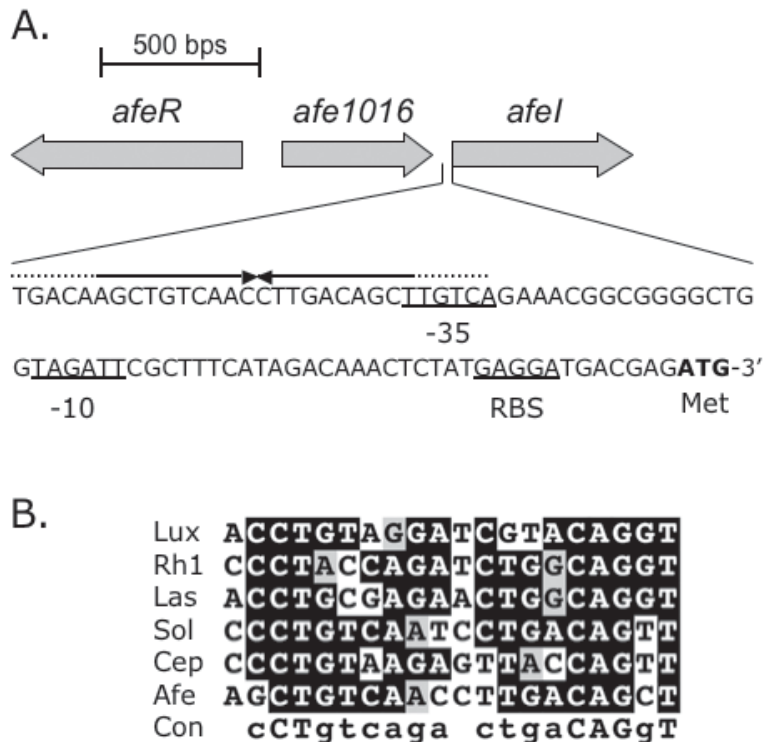


Figure 1. (A) Organization of the predicted genes of the Lux-like quorum sensing locus of *A. ferrooxidans*. Arrows indicate the proposed direction of transcription. The inwardly directed arrows indicate the predicted Lux box and the dotted lines demonstrate the potential extension of the conserved Lux box another 5 bps upstream and downstream. Underlined are predicted -35 and -10 regions of a sigma 70-like promoter and a ribosome binding site (RBS). The predicted initial ATG codon of *afeI* is indicated. (B) Comparison of the predicted Lux box of *A. ferrooxidans* (Afe) with other known Lux boxes, shaded nucleotides are the most conserved. Lux = Lux box of *Vibrio fischeri* (Devine *et al.*, 1988), Rhl = Rhl box, Las = Las box of *Pseudomonas aeruginosa* (Latifi *et al.*, 1995), Sol = Sol box of *Ralstonia solanacearum* (Flavier *et al.*, 1997), Cep = Cep box of *Burkholderia cepacia* (Lewenza *et al.*, 1999). Con = consensus sequence of the Lux boxes derived from the alignment of the six Lux boxes.

In vitro assays of β -galactosidase production.

A. tumefaciens NT1 and *S. meliloti* Rm41 SinI⁻ were grown to 0.5 OD₆₀₀ in 2 ml of LB/MC broth with 1% (w/v) L-arabinose at 30° C. The cultures were diluted 1:10 ml in Z buffer (0.06 M Na₂HPO₄, 0.04 M KCl, 0.001 M MgSO₄, 0.05 M β -mercaptoethanol) and assayed by the Miller procedure (Miller, 1972) as previously described (Quatrini *et al.*, 2005) to determine Miller units of activity, using o-nitrophenyl- β -D-galactopyranoside as the substrate. Each sample was assayed in triplicate, and each experiment was

repeated at least three times. AHL crude extracts from Rm41 or *A. ferrooxidans* or recombinant *E. coli* pAfeI or the different synthetic and commercial short and long-chain AHLs (see above) were added to the medium at the time of inoculation.

Semi-quantitative RT-PCR measurements of gene expression

Five micrograms of total RNA was isolated from *A. ferrooxidans* and was reverse transcribed by PCR (RT-PCR) as described previously (Guacucano *et al.*, 2000) using the following DNA primers: *afeI*-1 and *afeI*-2 to amplify DNA corresponding to the

predicted *afeI*, *afeR-1* and *afeR-2* corresponding to *afeR* and *recA-1*, *recA-2* to amplify DNA corresponding to *recA* (control gene to evaluate constitutive expression) (Table II). Semi-quantitative PCR was carried out by one cycle of incubation at 94⁰ C for 1 min, followed by 15, 20, 25 and 30 cycles of 90⁰ C for 30 s each, 64⁰ C for 1 min, and 72⁰ C for 1 min. PCR products were visualized by agarose gel electrophoresis as previously described (Guacucano *et al.*, 2000). Densitometry measurements of DNA were quantitated using Scion Image for Windows software. Appropriate negative and positive controls were included in each RT-PCR experiment as described previously (Guacucano *et al.*, 2000). PCR was performed with up to 100-fold dilutions of template to ensure that assays were carried out in the linear range of template concentration. Reproducibility was assessed by performing at least two

independent RT reactions for each time point and at least three PCRs using each of these templates.

RESULTS

Identification and organization of the afeI-afeR locus in the genome of A. ferrooxidans

Putative genes, termed *afeI* and *afeR* (formerly *traI* and *traR*, Barreto *et al.*, 2003), have been detected in the genome of *A. ferrooxidans* using bioinformatic procedures. The predicted AfeI and AfeR protein products exhibit significant amino acid sequence similarity and conservation of motifs, domains and patterns with the LuxI and LuxR family of proteins, respectively, involved in quorum sensing in many Gram-negative bacteria (Table III) (Pappas *et al.*, 2004).

TABLE III

Predicted characteristics of the genes and protein products of the Lux-like quorum sensing locus of *A. ferrooxidans* (Gen Bank acc. o AY 758559)

Gene name	Function assigned	Best BlastP hit	% similarity	E value	Score	Motif, pattern
<i>afeI</i>	Autoinducer lactone synthase	<i>Burkholderia pseudomallei</i>	69	1e-47	190	PD002752 PR01549 PF00765 SSF55729 IPR001690
<i>afeI016</i>	unknown	<i>Burkholderia pseudomallei</i>	51	1e-14	81	
<i>afeR</i>	Autoinducer-binding transcriptional regulator	<i>Burkholderia mallei</i>	62	7e-55	215	PD000307 PR00038 PF001968 SM00421 PS50043 IPR000729 IPR005143

% similarity = similarity of amino acid sequence. E value and scores were derived from BlastP. PD = Prodom, PR = Prosite, PF = Pfam, SSF = SuperFamily, SM = Smart, PS = Profile Scan and IPR = InterproScan.

AfeI and *afeR* are organized in a divergent fashion, as has been observed in several other bacteria (Stevens and Greenberg, 1997; Lewenza *et al.*, 1999; reviewed in Miller and Bassler, 2001) (Fig. 1A). A conserved hypothetical gene (*afe1016*, Fig. 1A) of unknown function (Table III) is located in the intergenic region between *afeI-afeR* oriented in the same direction as *afeI*. A similar organization of *afeI-hypothetical gene-afeR* has been observed in *Burkholderia pseudomallei* (Lewenza *et al.*, 1999; reviewed in Gray and Garey, 2001). The function of the conserved hypothetical gene remains unknown. A candidate sigma 70-like promoter, exhibiting potential -35 and -10 consensus regions, was detected 34 bps upstream of the proposed ATG start codon of *afeI*. Immediately upstream of the -35 consensus region is a predicted Lux box of 20 base pairs that exhibits pseudo-palindromy and conservation of nucleotide sequence with other characterized Lux boxes (Fig. 1B) (Fuqua and Winans, 1996; Whiteley and Greenberg, 2001; Schuster *et al.*, 2004). The pseudo-palindrome can be extended for another 5 bps on either side of the predicted Lux box, making the palindrome a total of 30 bps and superimposing it over the possible -35 promoter region (Fig. 1A).

AfeI catalyzes the formation of an unsaturated *N*-acylhomoserine lactone (AHL) of chain length C_{14}

In many characterized Gram-negative bacterial systems, the product of *afeI* is a homoserine lactone synthase that catalyzes the production of an *N*-acylhomoserine lactone (AHL) from S-adenosylmethionine (SAM) (Hanzelka and Greenberg, 1997). In order to evaluate whether the putative *afeI* of *A. ferrooxidans* also catalyzed the production of AHL, *afeI* was amplified by PCR and cloned into the *E. coli* expression vector pGEX-2t under the control of a *tac* promoter for inducible, high level expression.

Cultures of *E. coli* JM109 containing pAfeI, were extracted with dichloromethane and the extract was subjected to preparative fractionation with sep pack C18 as described in Methods. Five fractions were recovered

(F1–F5) and independently subjected to GC/MS analysis. The retention times and mass spectra were compared to the retention times and mass spectra from standards of *N*-tetradecanoyl-DL-homoserine lactone (AHL- C_{14}), *N*-hexanoyl-DL-homoserine lactone (AHL- C_6) and *N*-octanoyl-DL-homoserine lactone (AHL- C_8). Fraction F5 contained as main product a compound identified by retention time and mass spectrum obtained from GC/MS analysis as AHL- C_{14} (Fig. 2) suggesting that this is the major AHL product of AfeI. However, the production of minor amounts of other classes of AHL cannot be discounted. The other fractions (F1-F4) did not yield products with mass spectra of any of the standard AHLs checked (data not shown). Supernatant extracts derived from *E. coli* JM109 containing the cloning vector pGEX-2t without *afeI* did not yield a product with mass spectrum related to AHL (data not shown) indicating that the presence of *afeI* is required for the production of AHL.

AHL encoded by afeI has biological activity

In order to evaluate whether the AHL derived from the expression of *afeI* has biological activity, *E. coli* JM109 pAfeI was cross-streaked on X-gal indicator agar plates with reporter strains of *S. meliloti* Rm41 SinI- (responds to unsubstituted AHL- C_{10-16}) and *A. tumefaciens* NT1 (responds to AHL- C_{4-14}). AHL was detected by *S. meliloti* Rm41 SinI- (dark stain, streak b, Fig. 3A) but only barely by *A. tumefaciens* NT1 (streak b, Fig. 3B) indicating that the AHL produced by AfeI is biologically active in the heterologous strain *S. meliloti* Rm41 SinI-. Further, this activity is most likely to result from the production of an unsubstituted AHL of chain length C_{10-16} , consistent with the results from the GC/MS experiment reported above that suggest the AHL contains a chain length of C_{14} .

In control experiments, neither of the reporter strains respond to *E. coli* JM109 containing plasmid pGEX-2t (*lacking afeI*) showing that *afeI* is required (streak a, Fig. 3A and B). As a positive control, it can be seen that both indicator strains respond to *S. meliloti* Rm41 that produces AHLs of chain lengths C_{10-16} (streak c, Fig. 3A and B).

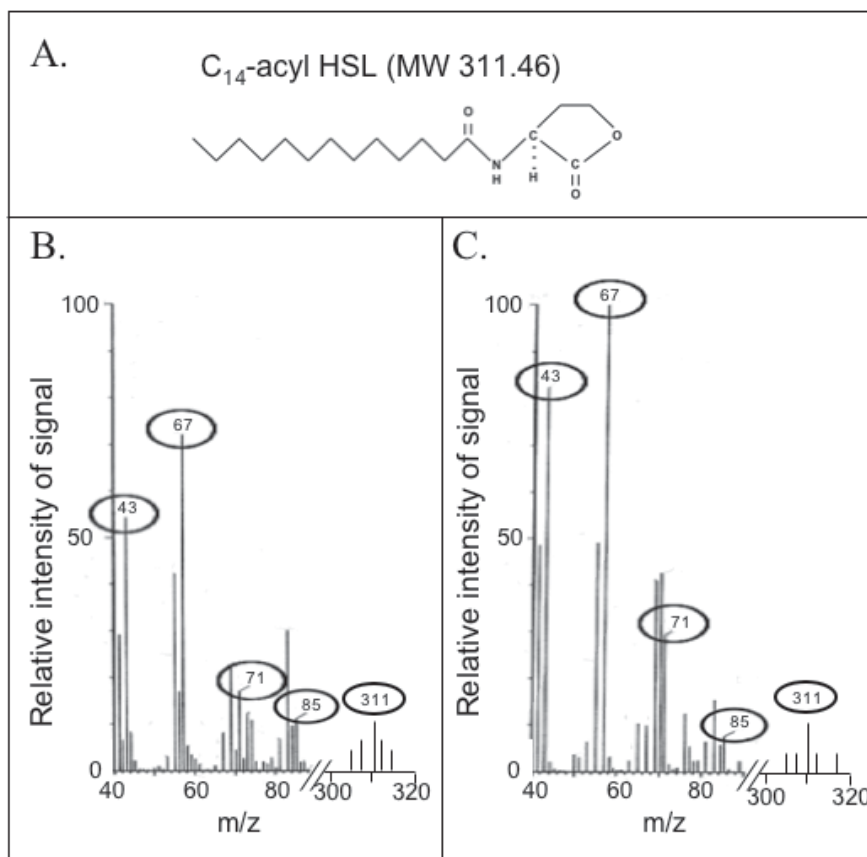


Figure 2. Identification of an acyl-homoserine lactone (AHL) whose principal chain length is C_{14} in the extracellular supernatant derived from a culture of *E. coli* JM109 expressing *afeI*. (A) Structure of a standard unsubstituted AHL of chain length C_{14} . Mass spectra of (B) a supernatant extract from *E. coli* strain pAfeI and (C) a synthetic unsubstituted C_{14} -AHL (the scale to the right of the line break in the x axis has been amplified 5 times to show the m/z at 311).

In order to determine if the AHL produced directly by *A. ferrooxidans* is biologically active, an extract of an early stationary phase whole culture of *A. ferrooxidans* was isolated by sep-pack C_{18} preparative column fractionation and fraction F5 (see Methods) was applied to the reporter strain *S. meliloti* SinI⁻ and the induction of β -galactosidase was monitored by the Miller assay. A significant induction of β -galactosidase activity was detected (Fig. 3C, e), demonstrating that fraction F5 contains an active AHL- C_{10-16} , consistent with the results from GC/MS. As controls, it is shown that a non-substituted AHL- C_8 standard does not induce β -galactosidase activity (Fig. 3, b), whereas both a non-substituted AHL- C_{14} standard (Fig. 3C, c)

and a crude extract of a culture of *S. meliloti* Rm41 (Fig. 3C, d) induce β -galactosidase activity. The addition of a blank without AHL resulted in an almost undetectable response (Fig. 3C, a). It is concluded that fraction F5 from an extract of a culture of *A. ferrooxidans* contains an active AHL identified by GC/MS analysis as AHL- C_{14} .

AfeI expression is induced by AHL

The question arises as to whether the predicted Lux box-promoter region of *A. ferrooxidans* can positively regulate the expression of *afeI*. A reporter plasmid, pAfeRI was constructed that fuses the first 120 bps of *afeI* to the promoterless *lacZ* of plasmid pQF50. Included in the construction

is the complete predicted *afeR*, together with the entire intergenic region between *afeR* and *afeI* (Fig. 4A). This region is predicted to contain the promoter and Lux box of *afeI*. pAfeR-I was cloned into *E. coli* JM109 and the resulting cells were cross-streaked on X-Gal indicator plates in close proximity to *S. meliloti* Rm41 producing AHLs-C₆₋₁₆. The blue color that developed at the junction of the cross streaking indicates the detection of AHL by *E. coli* containing pAfeR-I (streak a, Fig. 4C). A control plasmid, termed pAfeΔR-I, was constructed

that is similar to pAfeR-I but lacks the *afeR* gene (Fig. 4B). *E. coli* JM109 containing pAfeΔR-I does not respond to exogenous AHL (streak b, Fig. 4C) indicating that the presence of *afeR* is required for the response. This is interpreted to mean that AHLs-C₁₀₋₁₆ are capable of inducing transcription of the *lacZ* reporter gene from the *afeI* promoter via the activation of this promoter by LuxR, as has been demonstrated in other Gram-negative bacteria. It is also consistent with the idea that a Lux box that responds to LuxR is present in pAfeR-I.

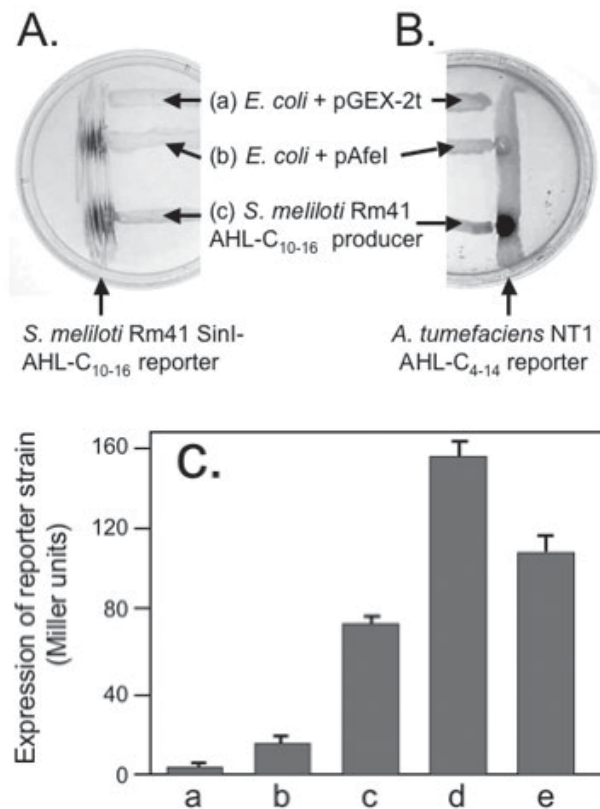


Figure 3. Biological activity of *A. ferrooxidans afeI*. (A) Demonstration that *E. coli* JM109 containing *afeI* is capable of inducing the expression of β -galactosidase (dark spot in streak b) in Reporter strains of (A) *S. meliloti* SinI- and (B) *A. tumefaciens* NT1 streaked with *E. coli* JM109 containing plasmid pGEX-2T lacking *afeI* (streak a), plasmid pAfeI containing *afeI* (streak b) or *S. meliloti* Rm41 that produces AHLs of various chain lengths from C₁₀ to C₁₆. Plates contain X-gal that produces a blue color (appears as a dark stains in Fig. 3) in the presence of β -galactosidase activity. (C) Measurement of β -galactosidase activity in Miller units (see Methods). *S. meliloti* Rm41 SinI- AHL reporter strain was grown for 6 h in the presence of: (a) no added AHL; (b) Unsubstituted AHL-C₈; (c) Unsubstituted AHL-C₁₄; (d) crude extract from a culture of *S. meliloti* strain Rm41 producing AHL₁₀₋₁₆, and (e) fraction F5 from sep-pack C₁₈ preparative column fractionation of an extract from a culture of *A. ferrooxidans*. The means and standard deviations of triplicate experiments are shown.

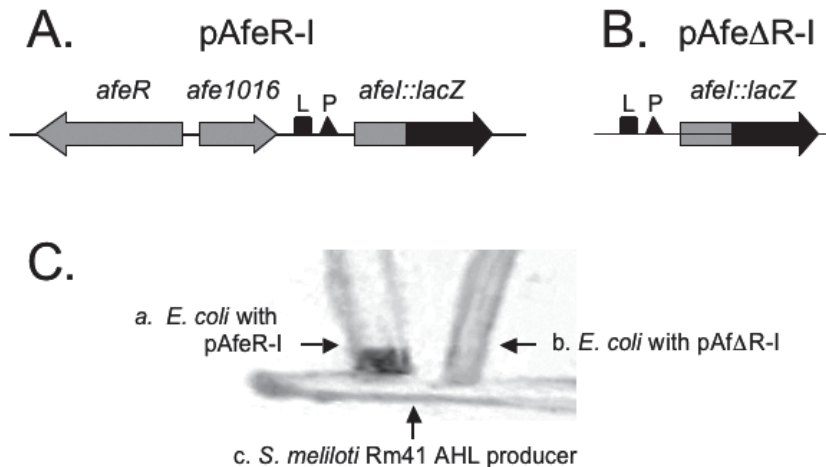


Figure 4. Demonstration that *afeI* expression can be activated by AHL. (A) Illustration of pAfeR-I showing the construction used for reporting the presence of exogenous AHL. L and P show the location of the predicted Lux box and putative promoter, respectively, of *afeI*. (B) Illustration of pAfeΔR-I (C) *E. coli* JM109 containing either (a) pAfeR-I or (b) pAfeΔR-I crossed streaked on X-Gal indicator plates with (c) *S. meliloti* Rm41 that produces AHL C₁₀₋₁₆. The dark stain on streak (a) indicates the presence of β-galactosidase activity.

AfeI and *afeR* expression is maximal in early stationary phase when *A. ferrooxidans* is grown in sulfur

Semi-quantitative RT-PCR was used to determine the expression of *afeI* and *afeR* at three different stages, mid-log, early stationary and late stationary phases, during the culture of *A. ferrooxidans* in 9K medium supplemented with sulfur. As an internal standard, *recA* RNA, isolated during the three growth stages, was also amplified by semi-quantitative PCR. Expression of *recA* does not change during growth of *A. ferrooxidans* (Liu *et al.*, 2000). The cDNA products resulting from RT-PCR amplification of RNA from *afeI*, *afeR* and *recA* at non-saturating cycles of RT-PCR were visualized by agarose gel electrophoresis and the resulting ethidium bromide stained bands were quantitated by densitometry and image analysis. The amount of product derived from *afeI* and *afeR* was plotted as a percentage relative to the amount of product derived from *recA* (Fig. 5A). Maximal expression of both *afeI* and *afeR* occurred in early stationary phase.

RNA was isolated from early stationary phase cultures of *A. ferrooxidans* grown in

either 9K medium supplemented with sulfur or iron was amplified by RT-PCR using different numbers of cycles of amplification and the products visualized by agarose gel electrophoresis (Fig. 5B). RT-PCR amplification was simultaneously carried out on *recA* RNA (Fig. 5C). Whereas the amount of RT-PCR amplified product from *recA* does not vary when RNA is isolated from cells grown on 9K supplemented with sulfur or iron, there is more product from both *afeI* and *afeR* from cells grown on 9K supplemented with sulfur compared to iron.

DISCUSSION

Closely linked, but divergently oriented, genes (*afeI* and *afeR*) were found in the genome of the acidophilic proteobacterium, *Acidithiobacillus ferrooxidans* using bioinformatic procedures (Fig. 1). The predicted protein products of these genes are significantly similar to the LuxI and LuxR families of proteins, respectively, including conserved characteristic motifs, domains and patterns (Table III). A potential gene (*afe1016*) of unknown function was detected in the intergenic

region between *afeI* and *afeR*. A potential gene (gi: 53721910) with similarity to *afe1016* and located between genes of the *luxI* and *luxR* families was detected in *Burkholderia pseudomallei* K96243. This suggests the possibility that the function of *afe1016* might be quorum sensing related.

A candidate sigma 70-like promoter and a possible Lux box were detected in the intergenic space between *afeI* and *afeR* (Fig. 1). The putative Lux box of *A. ferrooxidans* is predicted to be a palindrome of 20 bps and is located immediately adjacent to, and upstream of, the candidate -35 consensus of the predicted *afeI* promoter. A similar juxtaposition of Lux boxes and promoters of other *luxI*-like genes has been observed (Schuster *et al.*, 2004; Whiteley and Greenberg, 2001; Whiteley *et al.*, 1999; Fuqua and Winans, 1996; Wagner *et al.*, 2003). However, unlike these characterized

Lux boxes, the candidate *A. ferrooxidans* Lux box, exhibits an additional 10 bps of palindromic sequence of which 5 bps overlap the -35 consensus sequence. The significance of this observation remains to be determined.

When *afeI* is cloned and expressed in *E. coli* JM109 an AHL can be isolated from the culture supernatant identified as an unsubstituted AHL of chain length C₁₄ by GC/MS analysis (Fig. 2), although the presence of minor amounts of other AHLs cannot be ruled out. In cross-streaking assays (Fig. 3A,B) with the AHL reporter strains *S. meliloti* SinI⁻ and *A. tumefaciens* NT1, that respond respectively to unsubstituted AHL-C₁₀₋₁₆ or substituted AHL-C₄₋₁₄, only the former reacts significantly to the AHL of *A. ferrooxidans* consistent with the idea that the major product of AfeI is an unsubstituted AHL of longer chain length.

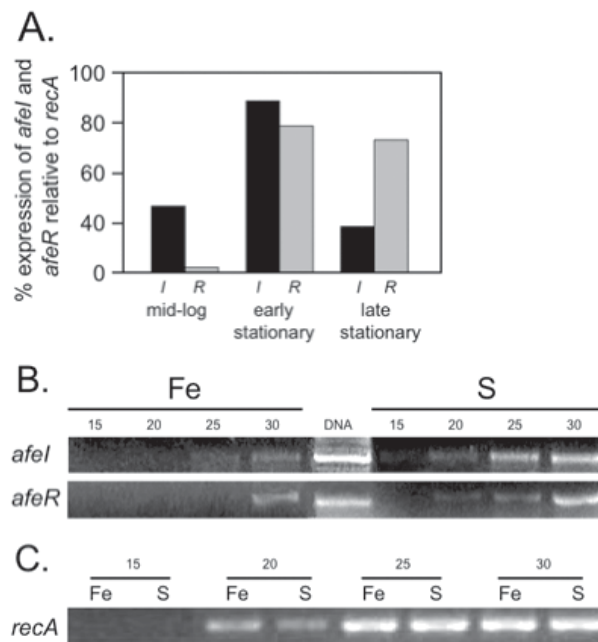


Figure 5. Determination of expression of *afeI* and *afeR* by semiquantitative RT-PCR during different phases of growth of *A. ferrooxidans* and from cultures grown in either 9K medium supplemented with sulfur or iron. (A) I = *afeI* expression and R = *afeR* expression. Mid-log = 72hrs, early stationary phase = 120 hrs and late stationary phase = 240 hrs. (B) RT-PCR amplification of *afeI* and *afeR* RNA from cells grown in either 9K medium supplemented with iron (Fe) or sulfur (S). (C) RT-PCR amplification of *recA* RNA. (B) and (C) Numbers refer to the number of cycles of PCR amplification carried out. DNA = PCR amplification of DNA using *afeI* or *afeR* specific primers to determine the specificity of the chosen primers in the PCR reaction.

More than ten different types of AHLs have been identified in different organisms in which an acyl chain of 4 to 18 carbon residues is attached to conserved homoserine lactone group. Additional variation occurs via substitution at the third carbon with hydroxy or oxo moieties and by the presence of double bonds in the acyl chain. Long chain AHLs have been identified in several bacteria including *Paracoccus denitrificans* (AHL-C₁₆), *Rhodobacter capsulatus* (AHL-C₁₄ and AHL-C₁₆), *Rhodobacter sphaeroides* (Schaefer *et al.*, 2002), *Rhizobium leguminosarum* (AHL-oxo-C₁₄) (Gray *et al.*, 1996) and *Sinorhizobium meliloti* (AHL-C₁₂, -oxo-C₁₄, -oxo-C_{16:1}, -C_{16:1} and -C₁₈) (Marketon *et al.*, 2002). The detection of long chain AHLs is complicated and their final identification usually requires radioactive assays. In the present study a combination of experiments involving GC/MS and cross-streaking in the presence of reporter strains of bacteria allowed the identification of an unsaturated AHL- C₁₄.

According to the results of the cross-streaking assays (Fig. 3A, B), *A. ferrooxidans* AHL is capable of crossing the *E. coli* membrane. This suggests that no additional *A. ferrooxidans* gene products are absolutely required for the export of *A. ferrooxidans* AHL from *E. coli*. However, it remains to be determined if this is also true for its export from native *A. ferrooxidans* cells. In some organisms quorum sensing lactones cross the membrane via multidrug pumps such as MexAB and OprD (Evans *et al.*, 1998; Kohler *et al.*, 2001). Since active AHL can be isolated from *A. ferrooxidans* cultures, it indicates that it is stable to the low pH (pH 3.6) of the medium. Acid stability of homoserine lactones has been previously reported (Yates *et al.*, 2002).

The upregulation of a reporter β -galactosidase gene by exogenous AHL occurred only if the reporter gene was linked to the predicted promoter and Lux box of *afeI* and only if *luxR* was present (Fig. 4). This is consistent with the well characterized model of the positive regulation of *luxI* by LuxR that has been described in many Gram-negative bacteria

(reviewed in Pappas *et al.*, 2004). It suggests that AfeR might mediate its regulatory role by binding to the predicted Lux box upstream of *luxI* as has been described in many Lux quorum sensing systems (Fuqua and Greenberg, 1998). This hypothesis has been recently experimentally validated (Barreto, M., Lefimil, C., Rivas, M., Holmes, D. and E. Jedlicki, submitted, 2005). The predicted gene *af1016*, of unknown function, is located in the intergenic region between *luxI* and *luxR*. However, it is not known if it plays a role in quorum sensing.

Evidence from semi-quantitative real time PCR (Fig. 5A) demonstrates that both *afeI* and *afeR* are preferentially expressed in early stationary phase as has been reported for several other Gram negative bacteria (Whiteley *et al.*, 1999). The expression of *A. ferrooxidans luxI* is higher than that of *luxR* in mid-log whereas the converse is true in late stationary phase (Fig. 5A). An interpretation of these results is that LuxR serves as a repressor of *luxI* in *A. ferrooxidans*. An alternative hypothesis is that the relative levels of *luxI* and *luxR* do not necessarily reflect the cellular concentrations of their respective protein products. Post-transcriptional control of LuxI levels has been demonstrated in *Erwinia carotovora* where it has been shown that RsmA negatively regulates synthesis of 3-oxo-C6-AHL by destabilizing the transcript encoding LuxI (Cui *et al.*, 1995). CsrA, a member of the RsmA family of regulators, also destabilizes specific mRNAs and RsmA family members are widespread in bacteria (White *et al.*, 1996). Regulated stability of AHL synthase transcripts may therefore be relatively common.

Both *luxR* and *luxI* exhibit increased expression in sulfur- versus iron medium (Fig. 5B). This could reflect the fact that in early stationary phase there is a higher cell population density in sulfur grown medium and this increased density might be driving the increased gene expression. Alternatively, the expression *luxR* and *luxI* might be modulated by genetic signals resulting from sensing differences in the two media.

Given the significant amino acid similarity and conservation of protein domains, motifs and patterns of AfeI and AfeR with the LuxI and LuxR family of quorum sensing proteins and given their observed similarity of function, it is proposed that *afeI* and *afeR* are orthologs of *luxI* and *luxR*. Since the genomic organization of *luxI* and *luxR* and the predicted location of a Lux box are also conserved and since *luxI* expression can be induced by AHL, it is further proposed, as a testable hypothesis, that *A. ferrooxidans* contains a quorum sensing system that works according to the LuxI-LuxR paradigm. It will be important in the future to determine the genetic targets of LuxR. Initial progress in this direction has been made (Barreto, M., Lefimil, C., Rivas, M., Holmes, D. and E. Jedlicki, submitted, 2005). In addition, a number of alternate quorum sensing systems have been described in bacteria (Pappas et al., 2004) and recent evidence suggests that *A. ferrooxidans* contains a second quorum sensing system based on the HtdS paradigm (Rivas, M., Holmes, D. and E. Jedlicki, submitted, 2005). A future challenge will be to build models of the cellular behavior(s) specified by these two quorum sensing systems and to determine their activating signals and the integration of their genetic responses.

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