

## Second Acyl Homoserine Lactone Production System in the Extreme Acidophile *Acidithiobacillus ferrooxidans*<sup>∇</sup>

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Received 19 December 2006/Accepted 26 February 2007

The acidophilic proteobacterium *Acidithiobacillus ferrooxidans* is involved in the industrial biorecovery of copper. It is found in acidic environments in biofilms and is important in the biogeochemical cycling of metals and nutrients. Its genome contains a cluster of four genes, *glyQ*, *glyS*, *gph*, and *act*, that are predicted to encode the  $\alpha$  and  $\beta$  subunits of glycine tRNA synthetase, a phosphatase, and an acyltransferase, respectively (GenBank accession no. DQ149607). *act*, cloned and expressed in *Escherichia coli*, produces acyl homoserine lactones (AHLs) principally of chain length C<sub>14</sub> according to gas chromatography and mass spectrometry measurements. The AHLs have biological activity as shown by *in vivo* studies using the reporter strain *Sinorhizobium meliloti* Rm41 SinI<sup>-</sup>. Reverse transcription-PCR (RT-PCR) experiments indicate that the four genes are expressed as a single transcript, demonstrating that they constitute an operon. According to semiquantitative RT-PCR results, *act* is expressed more highly when *A. ferrooxidans* is grown in medium containing iron than when it is grown in medium containing sulfur. Since AHLs are important intercellular signaling molecules used by many bacteria to monitor their population density in quorum-sensing control of gene expression, this result suggests that *A. ferrooxidans* has two quorum-sensing systems, one based on Act, as described herein, and the other based on a Lux-like quorum-sensing system, reported previously. The latter system was shown to be upregulated in *A. ferrooxidans* grown in sulfur medium, suggesting that the two quorum-sensing systems respond to different environmental signals that may be related to their abilities to colonize and use different solid sulfur- and iron-containing minerals.

Quorum sensing (QS) is a process by which bacteria communicate via the secretion and detection of chemical signaling molecules termed autoinducers. It is an important method for the regulation of population density-dependent cellular processes, such as the production of antibiotics and virulence factors, conjugation, transformation, swarming behavior, and biofilm formation (20). Several different QS systems have been discovered including the LuxIR paradigm, in which the signaling molecule is an acyl homoserine lactone (AHL), used principally by gram-negative bacteria (22, 39, 40); the furanosyl-borate diester signaling system (3); the peptide signaling systems used primarily by gram-positive bacteria (7); the LuxS/autoinducer-2 signaling used for interspecies communication; and the 3/epinephrine/norepinephrine interkingdom signaling system (36).

Although QS systems have been described in a wide variety of microorganisms (26), there are few reports of the presence of QS in extremophiles. AHL-based QS has been detected in the haloalkaliphilic archaeon *Natronococcus occultus* (21) and in the haloalkaliphilic *Halomonas* genus of bacteria (17). In

addition, peptide-based QS has been detected in the hyperthermophilic bacterium *Thermotoga maritima* (13). Little is known about QS systems in other extremophilic archaea, although a genome-wide survey indicated that a furanosyl-borate diester signaling system may be prevalent in archaea (38). The paucity of information regarding bacterial communication in extreme conditions, especially in acidic environments, prompted the present investigation.

Recently, a classic LuxIR system was described in the extreme acidophile *Acidithiobacillus ferrooxidans* (8, 27). *A. ferrooxidans* is a chemolithoautotrophic, mesophilic, facultative aerobe of the gammaproteobacterium group. It obtains energy and electrons by the oxidation of hydrogen and reduced sulfur compounds to sulfate and FeII to FeIII. It grows in extremely acidic conditions (<pH 2) and fixes CO<sub>2</sub> and N<sub>2</sub> to acquire cellular carbon and nitrogen. *A. ferrooxidans* is used for the industrial recovery of copper and gold in various parts of the world (25). It is also an important source of acid mine drainage and may play a significant role in the biogeochemical cycling of iron, sulfur, heavy metals, and nutrients in acidic environments (12). In an effort to broaden our understanding of QS in *A. ferrooxidans*, the complete genome was searched for candidate genes with similarity to all known genes involved in QS. We discovered an ortholog of *hdtS*, which encodes an AHL synthase in *Pseudomonas fluorescens* F113 (15), in the *A. ferrooxidans* genome, and we termed it *act* for acyl transfer function.

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<sup>∇</sup> Published ahead of print on 9 March 2007.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype/phenotype	Reference or source
<b>Strains</b>		
<i>Acidithiobacillus ferrooxidans</i> ATCC 23270	Type strain	ATCC
<i>Escherichia coli</i> JM109	F <sup>+</sup> <i>traD36 lacI<sup>q</sup>(lacZ)M15 proA<sup>+</sup>B<sup>+</sup>/e14(mcrA) (lac-proA) thi gyrA96(Nal<sup>r</sup>) endA hsdR17(r<sub>K</sub> m<sub>K</sub><sup>+</sup>) relA1 supE44 recA traR traG::lacZ</i> ; biosensor reporter; Km <sup>r</sup>	Promega
<i>Agrobacterium tumefaciens</i> NT1		34
<i>Sinorhizobium meliloti</i> Rm41	Type strain; wild-type AHL overproducer	18
Rm41 SinI <sup>-</sup>	<i>sinR sinI::lacZ</i> ; biosensor reporter; Gm <sup>r</sup> Neo <sup>r</sup>	18
<b>Plasmids</b>		
pKK223-3	Expression plasmid vector; Amp <sup>r</sup>	Pharmacia
pAf-act	<i>act</i> of <i>A. ferrooxidans</i> expressed from pKK223-3 <i>tac</i> promoter; Amp <sup>r</sup>	This study

This paper provides evidence for the role of Act in the formation of AHLs in *A. ferrooxidans*.

#### MATERIALS AND 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 (FeSO<sub>4</sub>) as described previously (44). *Escherichia coli* JM109 was grown in Luria-Bertani (LB) medium. *Agrobacterium tumefaciens* NT1 was grown in LB medium containing 50 µg ml<sup>-1</sup> kanamycin at 30°C. *Sinorhizobium meliloti* Rm41 was grown in LB medium at 30°C, and *S. meliloti* Rm41 SinI<sup>-</sup> was grown in LB medium or MMgly medium (11 g of Na<sub>2</sub>HPO<sub>4</sub>, 3 g of KH<sub>2</sub>PO<sub>4</sub>, 0.5 g of NaCl, 1 g of NH<sub>4</sub>Cl, 5 ml of glycerol, 1 mg of biotin, 27.8 mg of CaCl<sub>2</sub>, and 246 mg of MgSO<sub>4</sub> per liter) at 30°C supplemented with 1% (wt/vol) L-arabinose. Antibiotics were added where appropriate at the following final concentrations: 200 µg ml<sup>-1</sup> neomycin and 50 µg ml<sup>-1</sup> gentamicin. Details of the phenotypes and sources of bacteria are shown in Table 1.

**Bioinformatic analyses.** Candidate protein coding genes were identified in the genome sequence of *A. ferrooxidans* ATCC 23270, made available by The Institute for Genome Research (<http://www.tigr.org>) using Glimmer (<http://www.tigr.org>), Critica (<http://www.ttatus.com>), and BlastX (<http://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 (<http://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 (<http://www.ncbi.nlm.nih.gov>), the suite of protein characterization programs available in InterproScan (<http://www.ebi.ac.uk/interpro>), Blocks (<http://www.blocks.fhrc.org>), and ClustalW (<http://www.ebi.ac.uk/ClustalW>). Conserved motifs in the ClustalW alignments were visualized using Logos (4).

**Purification and identification of AHL.** Recombinant *E. coli* pAf-act early-stationary-phase cultures (including cells and supernatants) were extracted with dichloromethane (DCM) at a ratio of 70:30 (culture to DCM) as described previously (19). DCM was removed by rotary evaporation, and the residue was reconstituted in 100 µl DCM for fractionation by Sep-Pack C<sub>18</sub> preparative columns. Fractions were eluted with 1 ml in a gradient of methanol in water (20, 40, 60, 75, and 95%, vol/vol). Five fractions (F1 to F5) were collected, concentrated to 10 µl, and assayed for activity using the AHL assays described above. Samples (1 µl) were also injected in the splitless mode into a gas chromatography-mass spectrometry (GC/MS) system consisting of an Autosystem XL gas chromatograph (Perkin-Elmer, Boston, MA) with an MDN-5 column (Supelco, Bellefonte, PA) 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 previously (32, 33). The following AHL standards were purchased from Fluka: *N*-hexanoyl-DL-homoserine lactone (AHL-C<sub>6</sub>), *N*-octanoyl-DL-homoserine lactone (AHL-C<sub>8</sub>), and *N*-tetradecanoyl-DL-homoserine lactone (AHL-C<sub>14</sub>).

**Isolation of DNA, recombinant DNA techniques, and DNA sequencing.** DNA was isolated from *A. ferrooxidans* as previously described (1). The following standard recombinant DNA techniques were performed as previously described: 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* (29). DNA sequencing was carried out by the Sanger dideoxynucleotide method (29).

**Construction of recombinant plasmids and analysis of gene expression.** *act* was amplified by PCR with Elongase mix as described by the manufacturer (Invitrogen) using genomic DNA as a template with the primers P8 and P9 (Table 2). Amplified *act* was cloned into pKK223-3 as described by the suppliers, and the resulting recombinant plasmid was termed pAf-act. pAf-act was transformed into *E. coli* JM109 by electroporation, and the transformants were grown on LB plates containing ampicillin (100 µg · ml<sup>-1</sup>) at 37°C.

**AHL reporter plate assays.** *E. coli* JM109 (pAf-act) was cross streaked onto X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; 80 µg ml<sup>-1</sup>)-IPTG (isopropyl-β-D-thiogalactopyranoside; 40 µg ml<sup>-1</sup>)-LB medium reporter plates in the presence of *A. tumefaciens* NT1 (34) and *S. meliloti* Rm41 SinI<sup>-</sup> (18) as described by Latifi et al. (14) and Swift et al. (39). Plates were incubated overnight at 30°C. Activation of *lacZ* in these reporter strains was detected by the production of blue dye. As a negative control, *E. coli* JM109 lacking *act* but containing the plasmid vector pKK223-3 was also cross streaked on the same plates. As a positive control, the AHL-producing strain *S. meliloti* Rm41 was also cross streaked.

**Gene cotranscription analysis.** Five micrograms of total RNA was isolated from late-log/early-stationary-phase cells of *A. ferrooxidans* and was reverse transcribed by reverse transcription PCR (RT-PCR) as described previously (10) using the DNA primers listed in Table 2. Appropriate negative and positive controls were included in each RT-PCR experiment as described previously (10).

**Semiquantitative 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 (10) using the following DNA primers: P8 and P9 to amplify DNA corresponding to *act* and *recA1* and *recA2* to amplify DNA corresponding to *recA* (Table 2). Semiquantitative PCR was carried out for one cycle of incubation at 94°C for 1 min, followed by 15, 25, and 30 cycles of 90°C for 30 s each, 64°C for 1 min, and 72°C for 1 min. PCR products were visualized by agarose gel electrophoresis as previously described (10). Densitometry measurements of DNA were quantitated using Scion Image for Windows software. Appropriate negative and positive controls were included in each

TABLE 2. PCR and RT-PCR primers used in this study

Gene	Primer	Sequence (5' to 3')
<i>hyp</i>	P1	AAGCTTGGGCTGGTCTTTCT
	P2	ATGCCGGTAGGTGCCGGA
<i>glyQ</i>	P3	GTCCCCATAACGCGCTCCGTG
	P4	TTCCCGCGCGGCAACTC
<i>glyS</i>	P5	CTCGGGCTGCTCGCAGGC
	P6	GCCACTCTGCGGGCGATTGAT
<i>gph</i>	P7	CGACCCATGCTGGTACTGACGG
	P8	ATGATACGCAGCATCCGC
<i>act</i>	P9	TCAAAGCGCTTTTGCGCAGG
	P10	GCCGCTCGCGGGCGATG
<i>rimK</i>	recA1	CCGCCAACATTTCCCGGACC
	recA2	ACGCCGAGGTCCACCAGTTC

RT-PCR experiment as described previously (10). PCR was performed with up to 100-fold dilutions of template to ensure that assays were carried out in the linear range of template concentrations. 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.

**Nucleotide sequence accession numbers.** The nucleotide sequence of the *act* locus has been assigned the GenBank accession number DQ149607. The sequence of *act* was determined and deposited in GenBank under accession number AAZ78229.

## RESULTS

The genome of *A. ferrooxidans* was searched for genes with similarity to all known genes involved in QS, and apart from the previously reported *luxIR* gene pair (8, 27) a significant hit was found only to *hdtS* (gi.11120074) of *P. fluorescens* F113 (15). *hdtS* has been shown to encode an AHL synthase responsible for the production of AHLs of various chain lengths. It has no significant similarity to the LuxI family of AHL synthases (15). The ortholog of *hdtS* in *A. ferrooxidans* was termed *act* (acyl transfer function). Predicted characteristics, including the identification of domains, motifs, and patterns of Act, are shown in Table 3.

A ClustalW alignment of Act with the top 10 BlastP hits in GenBank permitted the identification of the amino acid sequence conservation of this group of proteins; in particular it highlighted the NHQS and PEGTR motifs characteristic of the family of lysophosphatidic acid (LPA) acyltransferases previously identified (42) (data not shown). The alignment showed that the first of these motifs can now be better described as KHQSAWET and that both motifs are flanked by conserved hydrophobic amino acids. Given the overall amino acid sequence conservation and taking into account the conservation of key motifs, it is predicted that Act is an acyltransferase of the family of 1-acyl-*sn*-glycerol 3-phosphate *O*-acyltransferases or so-called LPA acyltransferases (EC 2.3.1.51). This family includes members that catalyze the conversion of LPA to phosphatidic acid. Phosphatidic acid is a crucial phospholipid intermediate in cell membrane biosynthesis.

**Organization and expression of a gene cluster containing *act*.** Analysis of the genome of *A. ferrooxidans* revealed the presence of a cluster of three genes, named *glyQ*, *glyS*, and *gph*, associated with *act* (Fig. 1). GlyQ and GlyS are predicted to correspond to the conserved  $\alpha$  and  $\beta$  subunits of glycyl-tRNA synthetase (class II, heterodimer family, EC 6.1.1.14), respectively. Gph (general function phosphatase) shares some sequence similarity to the HisB family of histidinol-phosphate phosphatases (EC 3.1.3.15); however, it lacks the diagnostic two-domain structure of this group, and we have assigned it only a general phosphatase function at the present time.

Analysis of the *act* gene cluster reveals that there is an overlap of 15 bp between *glyQ* and *glyS*, a space of 3 bp between *glyS* and *gph*, and an overlap of 3 bp between *gph* and *act*. This intergenic spacing suggests that the cluster is an operon (Fig. 1A). The *act* operon is preceded upstream by a hypothetical conserved gene of unknown function and is followed downstream by *rimK*, oriented in the opposite direction from the *act* locus. *rimK* is predicted to encode a ribosomal S6 protein modification enzyme. A possible ribosome binding site with the sequence GAGA was detected 5 nucleotides upstream of the predicted ATG start site of *glyQ* (Fig. 1A) that shows

TABLE 3. Predicted properties of Act

Property	Value or characteristic
Gene name (GenBank accession no.)	<i>act</i> (AAZ78229)
Predicted function	Family of 1-acyl- <i>sn</i> -glycerol 3-phosphate <i>O</i> -acyltransferases
Best BlastP hit	<i>Nitrosococcus oceani</i>
% Similarity	59
E value	4e-47
Score	190
Domain, motif, pattern	PF01553 SM00563 PS50239 PTHR10434 IPR002123

sequence similarity to the *A. ferrooxidans* consensus GAGGA (unpublished results). A predicted sigma-70-like promoter was found 26 nucleotides upstream of the ATG start site with the sequence TTCACA (−35 box)−18 bp−CATAAT (−10 box) with sequence similarity to the *A. ferrooxidans* consensus sigma-70-like promoter (TTGACA [−35 box]−18 bp−TATAAT) (unpublished results). The intergenic spacing and the presence of a predicted promoter are consistent with the idea that the *act* locus is an operon. In order to evaluate this possibility, RT-PCR experiments were undertaken using primers designed for each of the predicted genes. The results indicate that *glyQ*, *glyS*, *gph*, and *act* are cotranscribed as a single message whereas *hyp* and *rimK* are not transcribed as part of the same message, indicating that the *act* locus is an operon (Fig. 1B).

***act* shows increased expression when *A. ferrooxidans* is grown in iron-containing medium rather than sulfur-containing medium.** RNA was isolated from cultures of *A. ferrooxidans* grown in either 9K medium supplemented with sulfur or the same medium supplemented with iron at pH 2. The RNA was amplified by RT-PCR using primers specific for *act* and using different numbers of cycles of amplification. The RT-PCR products were visualized by agarose gel electrophoresis (Fig. 1C). As a control, RT-PCR amplification was simultaneously carried out using primers specifically designed for *recA*. *recA* expression is independent of the growth medium of *A. ferrooxidans* (16). There is more *act*-specific RT-PCR product after 25 and 30 cycles of PCR amplification using RNA from cells grown in medium supplemented with iron than after the same procedure using RNA from cells grown in medium supplemented with sulfur, suggesting that there are more transcripts of *act* in cells grown in iron.

**In vivo gene reporter assay for AHL production.** Since HdtS has been shown elsewhere to exhibit AHL synthase activity (15), it was decided to test Act for similar function. Unique primers were designed for *act*, and PCR was used to amplify a DNA fragment corresponding to the predicted coding region. This DNA fragment was cloned into the expression vector pKK223-3, and the resulting recombinant plasmid was termed pAf-act. The sequence of *act* was determined and deposited in GenBank under accession number AAZ78229.

*E. coli* JM109 containing pAf-act was streaked onto X-Gal indicator agar plates in juxtaposition to two sensitive bacterial reporter strains of AHLs, *Sinorhizobium meliloti* Rm41 SinI<sup>−</sup> (18) and *Agrobacterium tumefaciens* NT1 (34). These two reporter strains have been constructed to produce  $\beta$ -galactosidase in response to the presence of unsubstituted AHLs of C<sub>12</sub>

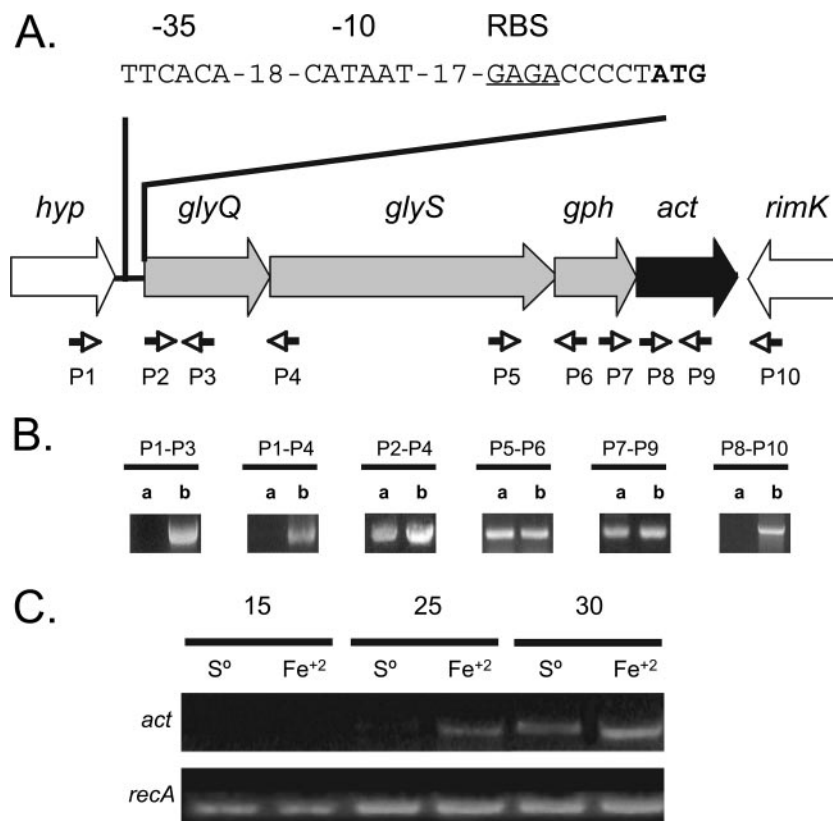


FIG. 1. Organization and coexpression of the *act* locus of *A. ferrooxidans* and differential expression of *act*. (A) Organization of the *act* locus. Shaded arrows indicate the direction of transcription. Small black arrows indicate the position and orientation (5' to 3') of primers used in RT-PCR or PCR studies. A predicted sigma-70-like promoter (-35 and -10 regions separated by 18 bp), a possible ribosome binding site (RBS; underlined), and the proposed ATG initiation codon (bold) are indicated. *hyp*, conserved hypothetical gene of unknown function; *rimK*, predicted ribosomal protein S6 modification protein. (B) Coexpression of the *act* locus. Results of RT-PCR (using RNA as a substrate [columns a]) and PCR experiments (using genomic DNA as a substrate [columns b]) are shown. The nucleotide sequence of the *act* locus has been deposited in GenBank under accession number DQ149607. (C) Differential expression of *act* when cells were grown in either iron-containing (Fe<sup>2+</sup>) or sulfur-containing (S<sup>0</sup>) medium as determined by semiquantitative RT-PCR. The numbers refer to the numbers of PCR cycles performed. The expression of the constitutively expressed *recA* is included as a control.

to C<sub>16</sub> in chain length or substituted AHLs of C<sub>4</sub> to C<sub>14</sub> in chain length, respectively. A positive response to AHL can be observed as a blue stain on appropriate indicator plates. An AHL produced by *E. coli* JM109 carrying pAf-act was detected strongly by *S. meliloti* Rm41 SinI<sup>-</sup> (dark stain, streak b, Fig. 2A) but only weakly by *A. tumefaciens* NT1 (barely visible stain, streak b, Fig. 2B). Neither of the reporter strains responded to *E. coli* JM109 containing plasmid pKK223-3 (lacking *act*), demonstrating that *act* is required for the activity (streak a, Fig. 2A and B). As a positive control, both reporter strains respond to *S. meliloti* Rm41, which produces AHLs of C<sub>6</sub> to C<sub>16</sub> in chain length (streak c, Fig. 2A and B). This suggests that the *act* from *A. ferrooxidans* encodes an enzyme with homoserine lactone synthase function that produces AHLs enriched in chain lengths of C<sub>12</sub> to C<sub>16</sub> when expressed in the heterologous host *E. coli* JM109.

**Identification by GC/MS of the AHL product of Act.** Given the evidence from the bacterial reporter strains for the production of AHLs enriched in chain lengths of C<sub>12</sub> to C<sub>16</sub> encoded by *act*, further characterization of the putative AHLs was proposed. *E. coli* JM109 pAf-act was grown to early stationary phase, and both cells and the supernatant were extracted

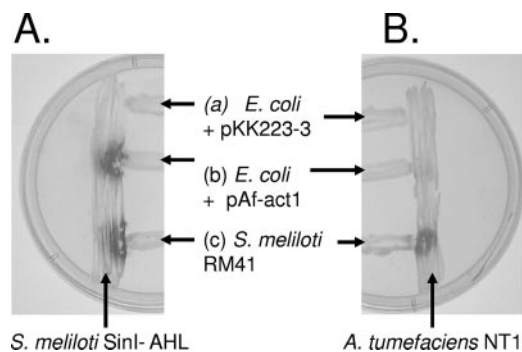


FIG. 2. Demonstration of the biological activity of *A. ferrooxidans* Act. (A) *E. coli* containing pAf-act is capable of inducing the expression of  $\beta$ -galactosidase (dark spot in streak b) in a reporter strain of *S. meliloti*, SinI<sup>-</sup>. On the other hand, *E. coli* containing the vector plasmid but lacking *act* does not express  $\beta$ -galactosidase (streak a). The positive-control *S. meliloti* Rm41 wild type, which produces AHLs of various chain lengths from C<sub>12</sub> to C<sub>16</sub> (streak c), induces the expression of  $\beta$ -galactosidase in *S. meliloti* SinI<sup>-</sup> (streak c). (B) Same as panel A except that the reporter strain used is *A. tumefaciens* NT1, which responds mainly to shorter substituted AHLs and AHL chain lengths of C<sub>4</sub> to C<sub>14</sub>.

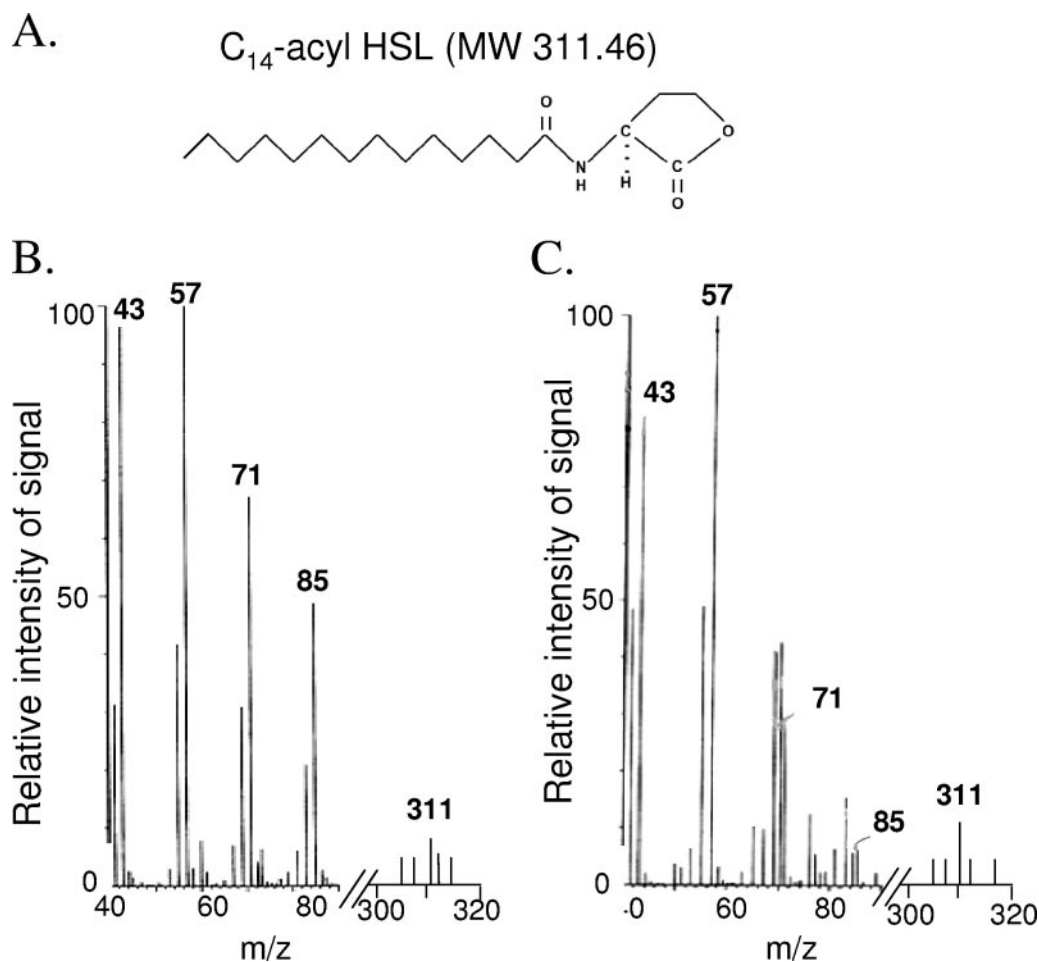


FIG. 3. Identification by MS of an AHL in the extracellular supernatant derived from a culture of *E. coli* JM109 containing pAf-act. (A) Structure of a standard unsubstituted AHL of chain length  $C_{14}$ . MW, molecular weight; HSL, homoserine lactone. (B and C) Mass spectra of a supernatant extract from *E. coli* JM109 pAf-act (B) and a synthetic standard of unsubstituted  $C_{14}$ -AHL (C) (the scale to the right of the line break on the  $x$  axis has been amplified five times to show the  $m/z$  at 311). The characteristic mass-to-ion charge ratios ( $m/z$ ) of the AHL are indicated.

with DCM. The extract was subjected to preparative fractionation with Sep-Pack  $C_{18}$ . Five fractions were recovered (F1 to 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 AHL- $C_{14}$ , AHL- $C_6$ , and AHL- $C_8$ . Fraction F5 contained, as the main product, a compound identified as an unsubstituted AHL of  $C_{14}$  in chain length (Fig. 3); however, the production of minor amounts of other classes of AHLs cannot be discounted. Extracts derived from *E. coli* JM109 containing the cloning vector pKK223-3 but without *act* did not yield a product with a mass spectrum related to AHL (data not shown), indicating that the presence of *act* is required for the production of AHL. It is concluded that Act catalyzes the formation mainly of an unsubstituted AHL of chain length  $C_{14}$ .

#### DISCUSSION

The detection by a reporter strain of bacteria of AHLs produced by the enzyme expressed from a cloned copy of *act* and the demonstration by GC/MS that these AHLs are enriched in molecules of  $C_{14}$  in chain length provide experimental evidence that Act has AHL synthase activity. Homology-

based sequence similarity analyses, including the identification of functional domains and motifs, suggest that Act is related to the LPA acyltransferase family, which includes NlaB from *Neisseria meningitidis* (35) and PlsC from *E. coli* (28). In *E. coli*, the LPA acyltransferase PlsC catalyzes the transfer of an acyl chain from either acyl coenzyme A (acyl-CoA) or acyl-acyl carrier protein onto LPA to produce phosphatidic acid, which, like LPA, is a critical phospholipid intermediate in cell membrane biosynthesis. Act possesses two motifs, KHQSAWET and PEGTR, which are highly conserved in prokaryotic and eukaryotic LPA acyltransferases and which may constitute an acyl-CoA/acyl-acyl carrier protein binding site. This raises the possibility that Act is involved in phospholipid biosynthesis, and it was shown recently that HdtS exhibits LPA acyltransferase activity (EC 2.3.1.51) involved in cell membrane biosynthesis (5). However, amino acid residues outside these motifs may alter the activity or specificity of these acyltransferases (35, 42) and could allow the transfer of an acyl group from acetyl-CoA to a substrate such as *S*-adenosylmethionine to form AHL as found in the classical LuxI system.

The experimental evidence that HdtS is involved in both the

acylation of LPA in cell membrane biosynthesis (5) and the acylation of *S*-adenosylmethionine in AHL biosynthesis (reference 15 and this paper) suggests that it has a dual function. Coordinated expression of cell membrane biosynthesis and the production of AHLs could be a means for cells to communicate that cell division is taking place and that cell density increases could be occurring. A relationship has also been established between cell membrane fluidity changes due to phospholipid decreases resulting from stress and the activation of AHLs and QS (2).

Bioinformatic prediction and experimental validation demonstrate that *act* is found in a transcriptional unit together with the predicted genes *glyQ*, *glyS*, and *gph* predicted to encode glycyl-tRNA synthetase  $\alpha$  chain (EC 6.1.1.14), glycyl-tRNA synthetase  $\beta$  chain (EC 6.1.1.14), and a phosphatase (general function), respectively. This locus appears to be highly conserved, including the gene order, in a wide range of proteobacteria (data not shown).

The role of the predicted phosphatase in this locus is not known. However, it has been shown that in eukaryotic systems a phosphatidate phosphatase (EC 2.3.1.15) carries out the conversion of 1,2-diacyl-*sn*-glycerol 3-phosphate (one of the potential products of Act) to 1,2-diacyl-*sn*-glycerol, which is subsequently used for phospholipid biosynthesis (43). It is possible that the predicted phosphatase encoded by *gph* assumes this role in bacteria, even though it exhibits no sequence similarity to its eukaryotic counterpart.

The conservation of *glyQS* in the *act* locus is more enigmatic. In addition to its role in phospholipid metabolism, 1,2-diacyl-*sn*-glycerol feeds into a pathway for the biosynthesis of glycine which may connect it with the requirement for coordinated expression of glycine tRNA synthetase. An alternative hypothesis is that glycine tRNA synthetase can act as a glycine donor in the cross-linking of peptidoglycan via a characteristic pentaglycine interpeptide bridge, as has been demonstrated in the gram-positive bacteria *Staphylococcus*, *Weissella*, *Streptococcus*, *Renibacterium*, and *Bifidobacterium* spp. and the gram-negative bacteria *Treponema* and *Borrelia* spp. (31). The coordinated formation of peptidoglycan and cell membrane constituents during cell division and cell membrane and cell wall repair has been established (9, 37), potentially requiring the coordinated expression of glycine tRNA synthetase and phospholipid biosynthesis.

*act* is more highly expressed when cells are grown in iron-containing medium than when they are grown in sulfur-containing medium, which is the converse of what has been observed for the LuxIR QS genes of *A. ferrooxidans* (8, 27). This demonstrates that *A. ferrooxidans* has two distinct AHL-based mechanisms for QS. The function of these two systems may depend upon environmental signals that are related to the ability of *A. ferrooxidans* to colonize and use different solid sulfur- and iron-containing minerals. Both QS systems mediate their effects through the biosynthesis of AHLs. Intercellular communication signals in the environment inhabited by *A. ferrooxidans* would need to be acid stable, and AHLs produced by *A. ferrooxidans* have been shown to be active in acid medium (8, 27). The stability of other QS signals in extremely acidic conditions is unknown.

Although a candidate promoter for the *act* operon was detected, a search for known transcription factor binding sites

was not successful; therefore, details as to the mechanism(s) that differentially regulates the expression of *act* and *luxI* await further study. Other organisms are known to produce and respond to more than one QS signal. For example, *P. aeruginosa* produces two AHLs that bind and activate respectively LasR and RhlR transcription factors, both of which are members of the LuxR family of helix-turn-helix transcription factors (23). *Vibrio harveyi* produces and responds to three autoinducers, and this sensory information converges to control the expression of bioluminescence, biofilm formation, type III secretion, and protease production (41).

The physiological responses controlled by the putative Act QS system of *A. ferrooxidans* remain to be investigated. In other bacteria QS has been shown to be involved in production of antibiotics and virulence factors, conjugation, transformation, swarming behavior, and biofilm formation (20); coaggregation (11); and the production of biosurfactants (6). It has been suggested that biofilm formation in *A. ferrooxidans* might be under the control of the LuxIR system (8, 27). Given that *A. ferrooxidans* forms biofilms on different iron- and sulfur-containing mineral surfaces such as pyrite (FeS<sub>2</sub>) and chalcocite (Cu<sub>2</sub>S) (24, 30), perhaps via extracellular polysaccharide formation (1), the possible roles of the LuxIR and Act systems in biofilm formation on different minerals become an attractive option, especially as they could help to explain the opposite responses of the LuxIR and ActS systems to iron and sulfur, respectively. This suggests the presence of signaling cascades, and future work will be directed towards the discovery of gene targets of the LuxIR and Act systems and how they might be integrated to produce appropriate physiological responses dictated by environmental signals.

#### ACKNOWLEDGMENTS

The work was supported by Fondecyt grant no. 1050063 and a Microsoft Sponsored Research Award. M.S. thanks the Millennium Scientific Initiative through Millennium Nucleus EMBA grant P04/007-F and USM grant 130522 for financial support.

We thank Stephen Winans for providing cultures of *Agrobacterium tumefaciens* NT1 and Juan Gonzalez for providing *Sinorhizobium meliloti* Rm41 and Rm41 SinI<sup>-</sup>. We thank Myriam González from Universidad Técnica Federico Santa María, Valparaíso, for assistance with the GC/MS experiments.

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