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Evidence for a Functional Quorum-Sensing Type AI-1 System in the Extremophilic Bacterium *Acidithiobacillus ferrooxidans*†

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Acidithiobacillus ferrooxidans **is one of the main acidophilic chemolithotrophic bacteria involved in the bioleaching of metal sulfide ores. The bacterium-mineral interaction requires the development of biofilms, whose formation is regulated in many microorganisms by type AI-1 quorum sensing. Here, we report the existence and characterization of a functional type AI-1 quorum-sensing system in *A. ferrooxidans*. This microorganism produced mainly acyl-homoserine lactones (AHL) with medium and large acyl chains and different C-3 substitutions, including 3-hydroxy-C_8-AHL, 3-hydroxy-C_{10}-AHL, C_{12}-AHL, 3-oxo-C_{12}-AHL, 3-hydroxy-C_{12}-AHL, C_{14}-AHL, 3-oxo-C_{14}-AHL, 3-hydroxy-C_{14}-AHL, and 3-hydroxy-C_{16}-AHL. A quorum-sensing genetic locus that includes two open reading frames, *afeI* and *afeR*, which have opposite orientations and code for proteins with high levels of similarity to members of the acyl synthase (I) and transcriptional regulator (R) protein families, respectively, was identified. Overexpression of AfeI in *Escherichia coli* and the associated synthesis of AHLs confirmed that AfeI is an AHL synthase. As determined by reverse transcription-PCR, the *afeI* and *afeR* genes were transcribed in *A. ferrooxidans*. The transcription levels of the *afeI* gene were higher in cells grown in sulfur and thiosulfate media than in iron-grown cells. Phosphate starvation induced an increase in the transcription levels of *afeI* which correlated with an increase in AHL levels. Two *afe* boxes which could correspond to the AfeR binding sites were identified upstream of the *afeI* gene. This is the first report of a functional type AI-1 quorum-sensing system in an acidophilic chemolithotrophic microorganism, and our results provide a very interesting opportunity to explore the control and regulation of biofilm formation during the bioleaching process.**

Quorum sensing (QS) is a widespread phenomenon that enables bacterial cells to establish cell-cell communication and to regulate the expression of specific genes in response to local changes in cell density (6, 48, 49). QS provides the means to coordinate the activities of cells so that they function as a multicellular unit and communicate with eukaryotic hosts (6, 20, 48, 49). In gram-negative bacteria, depending on the autoinductor (AI) molecule, two QS processes have been described: type AI-1, which is involved mainly in intraspecies communication, and type AI-2, which is related to interspecies communication (20, 49).

The type AI-1 QS regulatory system is composed of four elements: (i) a transcriptional regulator (protein family R); (ii) a *cis*-acting DNA palindromic sequence; (iii) an acyl-homoserine lactone (AHL), which is the signaling molecule or autoinducer (AI-1); and (iv) the AHL synthase protein (protein family I), which synthesizes the AI (6, 20, 48, 49). It is currently accepted that AI-1 diffuses freely between the cellular and external environments and complexes with the R protein only at a high cell density. The AHL-R complex binds through the R carboxyl domain to the specific site which corresponds to a palindromic sequence centered at about position −40 with respect to the transcriptional start sites of the target genes (15, 48, 49).

Acidithiobacillus ferrooxidans is an acidophilic gram-negative bacterium that is capable of oxidizing ferrous iron or reduced sulfur compounds to obtain energy for growth. Its energetic metabolism is directly involved in biomining processes. For this reason, there is great interest in understanding the molecular mechanisms of this peculiar physiological kind of life (34). During bioleaching *A. ferrooxidans* adheres to solid substrates by means of extracellular polymeric substances, such as exo- or lipopolysaccharides (36). *A. ferrooxidans* is also able to develop biofilm structures and exhibits morphological modifications during the cellular adhesion process (7, 13, 22, 36). AHL-mediated gene regulation has been shown to influence exopolysaccharide production and biofilm formation in many proteobacteria (12, 16, 21, 24, 49).

The purpose of the present work was to determine whether *A. ferrooxidans* possesses a functional type AI-1 quorum-sensing system. Different types of AHLs were found in the growth medium of *A. ferrooxidans* cultures. An *A. ferrooxidans* quorum-sensing locus was identified and designated *afeIR*. The *afeI* and *afeR* genes were expressed in *A. ferrooxidans*, and it was demonstrated that AfeI is an AHL synthase. Our results suggest that there is functional type AI-1 quorum sensing in *A. ferrooxidans* which could be part of a regulon controlling some physiological functions, such as exopolysaccharide synthesis and biofilm formation.

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† Dedicated to the memory of Jean-Noël Guiliani.

MATERIALS AND METHODS

Bacterial strains and growth media. *A. ferrooxidans* ATCC 23270 was grown in ferrous iron-containing modified 9K medium at pH 1.5 as described previously (4), and organisms were grown on elemental sulfur at pH 2.5 with 5% (wt/vol) sulfur prills (3, 4). *A. ferrooxidans* was grown on thiosulfate at pH 4.6 in DSMZ medium 71 containing 20 mM thiosulfate as described previously (32). *Escherichia coli* strain BL21(DE3) and derivatives of this strain were grown in Luria-Bertani (LB) medium (37).

Preparation of crude AHL extracts and LC-MS-MS analysis. The AHLs and oxo-AHLs were extracted and characterized as described previously (29). Briefly, after the bacterial cells were removed from the media of grown cultures by centrifugation, the supernatants were extracted twice with 1 volume of high-performance liquid chromatography (HPLC)-grade dichloromethane. The dichloromethane extracts were dried over anhydrous magnesium sulfate, filtered, and evaporated to dryness. Residues were dissolved in 1 ml of HPLC-grade acetonitrile and analyzed by using reverse-phase liquid chromatography coupled with positive-ion electrospray ionization and ion trap mass spectrometry (LC-MS-MS) (29). The 3-hydroxy-AHLs were analyzed using the same protocol. These compounds were identified by comparison with synthetic 3-hydroxy-AHLs based on three criteria: the MS-MS fragmentation product ions ($[M+H-H_2O]^+$ and m/z 102), their relative intensities, and the HPLC retention times.

AHL bioassays. The different bioassay steps were performed as described previously (39). A 250-μl portion of an overnight culture of the *Agrobacterium tumefaciens* NTL4(pZLR4) AHL reporter strain grown in LB medium with gentamicin was inoculated into AB minimal glucose medium (ABm) and grown for 8 h at 30°C with shaking. The culture was then mixed with an equal volume of 1.5% TOP agar containing 80 μg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal), and the preparation was used as an overlay on ABm agar plates. The dichloromethane extracts obtained from the *A. ferrooxidans* media were spotted at the center of the overlaid ABm agar plates and incubated overnight at 30°C.

Bioinformatic tools. The BLAST program (2) was used to search for open reading frames (ORFs) encoding LuxR and LuxI homologs in the genome sequence of *A. ferrooxidans* ATCC 23270. The search for *afe* boxes was done at the Genomic and Bioinformatic Center, Catholic University of Chile (www.cgb.cl). Palindromic sequences were detected in the intergenic region of the *afeIR* locus by using the algorithm bl2seq from the BLAST software (41). Hidden Markov models (HMM) were constructed with the results obtained by using the HMMER v2.3.2 software (14). Hidden Markov models were compared with the genome sequence to identify *afe* boxes.

For homology modeling and evaluation of AfeR and AfeI protein structures, 130 models were constructed for each protein with the MODELLER program (28). The templates were chain C of the TraR structure of *A. tumefaciens* (PDB ID no. 1L3L), determined by X-ray diffraction at 1.66-Å resolution, and the LasI structure of *Pseudomonas aeruginosa* (PDB ID no. 1RO5), determined by X-ray diffraction at 2.3-Å resolution (17, 44, 50). All models were evaluated using the Verify-3D program (27). The models with the highest three-dimensional profile scores were finally selected.

Cloning the *afeI* gene. The *afeI* gene was amplified from *A. ferrooxidans* chromosomal DNA by PCR using the following primers: 5′-CATATGCAGGT TATAACCGGGCCA-3′ (5′ end) and 5′-CGGTTAGTCCAGATCTATCCAG C-3′ (3′ end). The 5′ end primer included an NdeI restriction site (underlined). The PCR was performed by using 3 min at 95°C, followed by 30 cycles of 30 s at 95°C, 30 s at 56°C, and 40 s at 72°C and finally 3 min at 72°C. The PCR fragment was purified from agarose gels with Wizard PCR Preps from Promega and cloned in pCRT7-NT-TOPO (Invitrogen) according to the manufacturer's recommendations. The different plasmid constructions were checked by automatic DNA sequencing at the Biotechnology Center of the Faculty of Sciences, University of Chile.

RNA manipulations. *A ferrooxidans* ATCC 23270 total RNA was prepared from thiosulfate-, iron- or sulfur-grown cells by a modified hot-phenol method as described previously (19, 45). Primer extension was performed with the Superscript II RNase H reverse transcriptase (Invitrogen) by using 15 μg of total RNA and the AfeIRT2 primer (5′-GGAAAGATCTCGCCCAACAG-3′) labeled with [γ-^{32}P]ATP. The sequencing reaction was performed with the *fmoI* DNA cycle sequencing system as described by Promega.

The following synthetic oligonucleotides were used in reverse transcription (RT)-PCR assays for the reverse transcription step: 5′-CGGTTAGTCCAGAT CTATCCAGC-3′, 5′-CGATCACGACAGCAACCCGAGCA-3′, and 5′-GATG TTGCTTCGTGGGAATC-3′ for the *afeI*, *afeR*, and *orf3* genes, respectively. For PCRs, the following primers were used: for *afeI*, 5′-CATATGCAGGTTATAA CCGGGCCA-3′ and 5′-CGGTTAGTCCAGATCTATCCAGC-3′; for *afeR*, 5′-CATATGGCGTCCGAAATGGCGCGT-3′ and 5′-AGGTCAACATGCCGCC CATC-3′; and for *orf3*, 5′-CGGACAAAAGATGCACCAGA-3′ and 5′-GGTA GCCTGTTCTTATCCGA-3′. The RT step was carried out with 1 μg of DNase I-treated total RNA. PCRs were done using 3 μl and 5 μl of the *afeI* and *afeR* RT reaction mixtures, respectively. The following program was used to perform the PCRs: an initial denaturation step of 95°C for 3 min; 40 cycles of 30 s at 95°C, 30 s at 60°C, and 30 s at 72°C; and a final extension step of 72°C for 3 min. For each RT-PCR experiment, a control RT reaction without reverse transcriptase was carried out to check for the absence of genomic DNA contamination in the RNA preparations used. RT-PCR products were checked by electrophoresis in a 1% agarose gel in 0.5× Tris-acetate-EDTA buffer.

Macroarray analysis. Macroarray production was performed manually by using the colony copier VP381 (V&P Scientific). The PCR products were printed onto Inmobilon-NY$^+$ membranes (Millipore). The different steps for expression analysis were performed as described previously (1). The *afeI* and *afeR* genes were amplified with the following oligonucleotide pairs: 5′-CATATGCAGGTT ATAACCGGGCCA-3′ and 5′-CGGTTAGTCCAGATCTATCCAGC-3′ for the *afeI* gene and 5′-CATATGGCGTCCGAAATGGCGCGT-3′ and 5′-CGAT CACGACAGCAACCCGAGCA-3′ for the *afeR* gene. Exposed PhosphorzImager screens were scanned with a PhosphorImager (Molecular Imager FX Systems, Bio-Rad) at a resolution of 50 μm/pixel. To normalize and quantify the results, we used a "spiked" RNA (*exp-1* of *Prunus persica*) as an internal control for the labeling reaction and hybridization steps.

Nucleotide sequence accession number. The nucleotide sequence of the *afeIR* locus has been deposited in the EMBL database under accession number no. AJ879454.

RESULTS AND DISCUSSION

Characterization of AHLs produced by *A. ferrooxidans*. Extracts obtained from the media of grown cultures were analyzed with a bioassay in solid medium using the following two reporter strains: *A. tumefaciens* NTL4(pZLR4), which is specific for AHLs having medium and long acyl chains; and *Chromobacterium violaceum* CV026, which is specific for AHLs having short acyl chains and is inhibited by long-chain AHLs. No positive results were obtained with *C. violaceum*, while the bioassays with NTL4(pZLR4) revealed the characteristic formation of a blue halo (not shown). This screening procedure showed that *A. ferrooxidans* produces AHLs with medium and/or long acyl chains. To determine precisely the chemical structure of the AHLs synthesized, the extracts were analyzed by LC-MS-MS.

The dichloromethane extracts obtained from *A. ferrooxidans* grown in different media contained AHLs with diverse C-3 substitutions and only even numbers of carbons in the acyl chain. 3-Hydroxy-C_{10}-, 3-hydroxy-C_{12}-, 3-hydroxy-C_{14}-, and 3-hydroxy-C_{16}-AHLs were present in iron-grown cells; C_{12}-, C_{14}-, 3-oxo-C_{12}-, 3-oxo-C_{14}-, 3-hydroxy-C_8, 3-hydroxy-C_{10}-, 3-hydroxy-C_{12}-, 3-hydroxy-C_{14}-, and 3-hydroxy-C_{16}-AHLs were present in sulfur-grown cells; and C_{12}-, C_{14}-, 3-oxo-C_{12}, 3-oxo-C_{14}-, 3-hydroxy-C_8-, 3-hydroxy-C_{10}-, 3-hydroxy-C_{12}-, 3-hydroxy-C_{14}-, and 3-hydroxy-C_{16}-AHLs were present in thiosulfate-grown cells (Table 1). In our experimental conditions, *A. ferrooxidans* was able to produce nine different kinds of AHLs which included all the known types of C-3 substitutions (oxo and hydroxyl), and all of them had medium or long acyl chains with an even number of carbons. For the five types of 3-hydroxy-AHL and independent of the medium, the large acyl chains with 12 or 14 carbons were the predominant forms.

To determine whether some of these AHLs were produced in a cell density-dependent manner, as the quorum sensing paradigm requires, the presence of these compounds was analyzed in the three media obtained from *A. ferrooxidans* cul-

TABLE 1. Identification of the different AHLs produced by *A. ferrooxidans*[a]

	AHL		oxo-AHL		hydroxy-AHL				
Substrate	C_{12}	C_{14}	C_{12}	C_{14}	C_8	C_{10}	C_{12}	C_{14}	C_{16}
Iron	ND	ND	ND	ND	ND	+	+	+	+
Sulfur	+	+	+	T	+	+	+	+	+
Thiosulfate	+	+	+	T	+	+	+	+	+

[a] C_8, R—$CH_3(CH_2)_4$; C_{10}, R—$CH_3(CH_2)_6$; C_{12}, R—$CH_3(_2)_8$; C_{14}, R—$CH_3(CH_2)_{12}$; ND, not detected; T, trace.

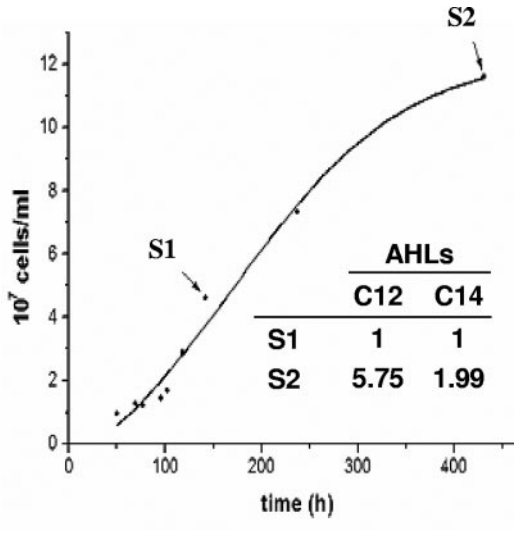

FIG. 1. Production of acyl-AHLs by *A. ferrooxidans* cells grown in sulfur. The correlation between growth phase and AHL amount was analyzed. AHLs were extracted from the culture medium with dichloromethane during the early exponential (S1) and stationary (S2) phases. The relative quantities of the large-acyl-chain AHLs produced are shown in the inset.

tures in the early exponential and early stationary growth phases. Of the nine AHLs synthesized in *A. ferrooxidans* grown in sulfur medium, only two (C_{12}-AHL and C_{14}-AHL) showed a detectable increase in the early stationary phase (Fig. 1, inset). Similar results were obtained with C_{12}-AHL and 3-hydroxy-C_8-, C_{10}-, and C_{12}-AHLs in thiosulfate medium (results not shown). On the other hand, in iron medium, the 3-hydroxy-AHL levels appeared to be low and constant (result not shown).

It is commonly accepted that AHL synthases can synthesize more than one type of AHL. Some *Rhizobium* spp. produce high numbers of AHLs by using AHL synthase-encoding genes located in the bacterial chromosome and/or plasmids (16). On the other hand, the AHL synthase SinI from *Sinorhizobium meliloti* Rm1021 is able to synthesize five different kinds of AHLs which involve different types of C-3 substitutions, a monounsaturated acyl chain, and the largest characterized acyl chain (C_{18}) (16).

Family I proteins catalyze the synthesis of AHLs from both substrates, *S*-adenosylmethionine (SAM) and acylated acyl carrier protein (acyl-ACP). In addition to the putative structural specificity of each AHL synthase, the capacity to synthesize different kinds of AHLs has been associated with the available pool of acyl-ACP substrates in each microorganism (47). Recently, for *Erwinia* sp., it has been postulated that growth conditions could also affect the acyl-ACP availability (8). This could explain why C_{12}- and C_{14}-AHLs and oxo-C_{12}- and oxo-C_{14}-AHLs were produced only in sulfur- and thiosulfate-grown cells. However, the way in which the energy source (iron versus sulfur or thiosulfate) could affect the nature of the AHLs is still unknown, since no information relating external acidic pH, energy metabolism, and cell wall metabolism is currently available for *A. ferrooxidans*. Therefore, the nature of the pool of acyl-ACPs in *A. ferrooxidans* is unknown. Our results are the first results to suggest that hydroxy-acyl-ACPs with medium and large acyl chains are present in *A. ferrooxidans* irrespective of the energy source. With regard to the second precursor, little is known about sulfur metabolism and the related pool of SAM in bacteria living under extreme conditions, such as acid pH. Recently, it was suggested that *A. ferrooxidans* could regulate sulfur assimilation in a manner comparable to the manner described for other bacteria (43). Since the first step in sulfur assimilation corresponds to sulfate uptake, the pool of SAM in *A. ferrooxidans* should depend on sulfate availability. Sulfate is present at high concentrations in all the different media used to grow *A. ferrooxidans*, and it cannot be assumed that sulfate is a limiting factor. Therefore, AHL synthesis could not be affected under the three growth conditions that we employed.

Identification and characterization of AfeI, a LuxI homolog. Some bacteria possess various loci involved in AHL synthesis (16, 20, 49). On the other hand, three AHL synthase families have been characterized (16). To determine how many loci for AHL production were present in the genomic sequence of *A. ferrooxidans* ATCC 23270, a search for ORFs encoding AHL synthases was performed. The amino acid sequences of LuxI (accession no. AAA27552) and AinS (AAP33508) from *Vibrio fischeri* and HdtS (AAG30826) from *Pseudomonas fluorescens*, which belong to the known AHL synthase families 1, 2, and 3, respectively (16), were used as queries in the tblastn search.

Orthologs were found only for AHL synthases belonging to families 1 and 3. The first gene coded for a putative protein with 53% similarity to HdtS, the first identified member of the third AHL synthase family. In *P. fluorescens*, HdtS directs the synthesis of three AHLs, C_6-AHL, C_{10}-AHL, and *N*-(3-hydroxy-7-*cis*-C_{14})-AHL (25).

The second gene coded for a protein that is 64% similar to protein BveI from *Burkholderia cepacia*, a member of the LuxI family. The deduced protein of *A. ferrooxidans* was designated AfeI, and it had 183 amino acids, a molecular mass of 19.9 kDa, and a theoretical isoelectric point of 5.77. Based on the recently solved structure of the LasI protein of *P. aeruginosa* (17), we constructed an AfeI model structure (Fig. 2). Our modeling approach took advantage of the higher level of similarity between AfeI and LasI (56%) (Fig. 2A). The amino acid sequence of AfeI revealed the presence of a threonine residue (Fig. 2A) which is involved in the oxo-C_3 substitution in the AHLs synthesized by LuxI, EsaI, and LasI of *V. fischeri*, *Pantonea stewwartii* subsp. *stewartii*, and *P. aeruginosa*, respectively

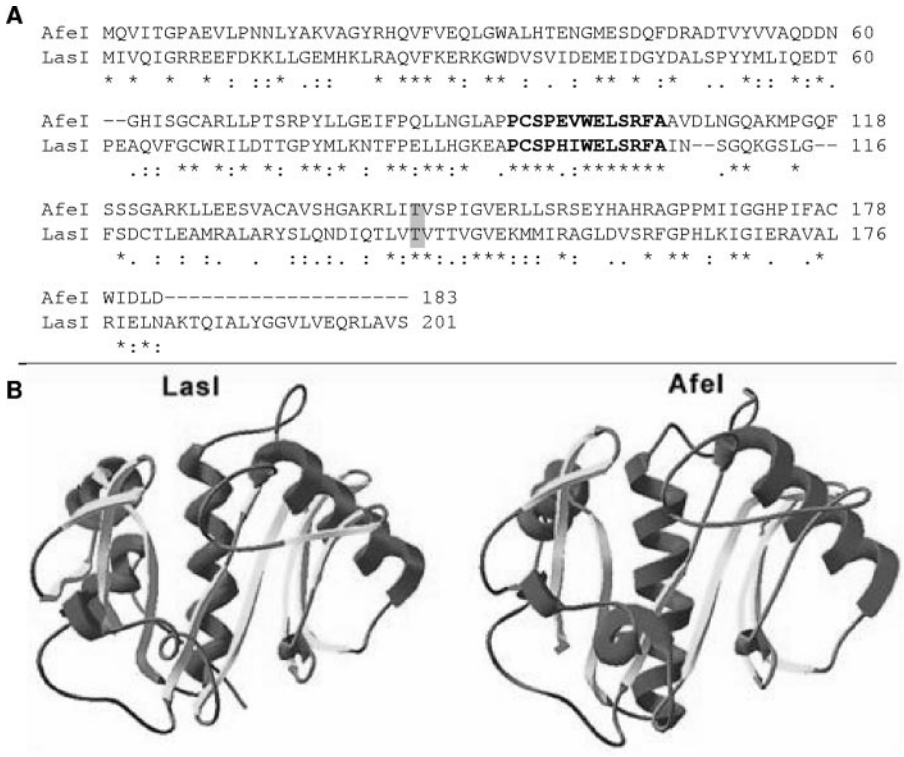

FIG. 2. Structure modeling of AfeI. (A) LasI and AfeI amino acid sequence alignment used for homology modeling. Boldface type indicates the most conserved region in the two proteins. Identical residues are indicated by asterisks, and similar residues are indicated by colons. A residue involved in oxo substitution (47) is indicated by a gray box. (B) AfeI modeled structure (right) based on LasI solved structure (left).

(47). In agreement with this, we also characterized oxo-AHLs in the growth media of *A. ferrooxidans*. The highest three-dimensional profile score yielded an AfeI structure model with high structural similarity to the LasI structure (Fig. 2B).

To confirm that the *afeI* gene product was an AHL synthase, *afeI* was overexpressed in an *E. coli* strain which does not produce AHLs. The *afeI* gene was cloned in plasmid pCRT7-NT-TOPO and transformed into the *E. coli* BL21(DE3) strain. The recombinant strain was induced with 0.4 M isopropyl-β-D-thiogalactopyranoside (IPTG) for 1 h, and the media of the induced bacteria were extracted with dichloromethane and analyzed by LS-MS-MS (Fig. 3). C_{12}-AHL and C_{14}-AHL (Fig. 3A) and five different hydroxy-AHLs (C_8, C_{10}, C_{12}, C_{14}, and C_{16}) (Fig. 3B) were characterized, while in the control *E. coli* strain carrying the same vector without the insert no AHLs were found (results not shown). The more abundant hydroxy-AHLs were C_{10}, C_{12}, and C_{14}, in agreement with the results obtained for *A. ferrooxidans* cells. This result definitively confirmed that AfeI is an AHL synthase able to synthesize AHLs and hydroxy-AHLs with large acyl chains (including C_{16}), depending on the available pool of acyl-ACPs. No other homoserine lactone synthase-encoding gene (except the *htdS*-like gene, whose function in the quorum-sensing pathway is still unknown) was identified in the available genome sequence of *A. ferrooxidans*. In *S. meliloti*, the AHL synthase SinI is responsible for the synthesis of a series of long-chain AHLs ranging in size from C_{12}-AHL to C_{18}-AHL, including some oxo-AHLs and a monounsaturated AHL (16). Therefore, we concluded that AfeI could be responsible for the synthesis of various long-chain AHLs in *A. ferrooxidans*.

Organization and transcription of the *afeI-afeR* locus. At a position 746 bp downstream of the *afeI* gene and in the opposite orientation, we found an ORF which exhibited 59% similarity to BviR, an R protein family member from *B. cepacia*. The deduced protein was designated AfeR and had 214 amino acids, a molecular mass of 23.7 kDa, and an isoelectric point of 9.89. Despite the low level of amino acid identity with TraR (less than 25%), we constructed a protein structure model for AfeR, which was very similar to the TraR structure (results not shown). The high level of structural similarity between the two proteins strongly suggests that AfeR is a transcriptional regulator of the R protein family, and, most likely, it acts in a dimeric form by recognizing *lux*-type boxes. A third ORF designated *orf3* was identified in the *afeIR* intergenic region. *orf3* had the same orientation as *afeI*, and it coded for a protein with 51% similarity to a hypothetical protein from *B. cepacia* R18194. The corresponding genes (*afeI*, *orf3*, and *afeR*) formed the *A. ferrooxidans* quorum-sensing locus (*afeIR*) shown in Fig. 4A. Despite the fact that *A. ferrooxidans* is a γ-proteobacterium, higher similarity scores for AfeI and AfeR (69% and 66%, respectively) were obtained with the genomic data for other members of the *Burkholderiaceae* belonging to the β-proteobacterial subdivision. This is in agreement with the idea of coevolution and acquisition of the *IR* regulatory cassettes by horizontal transmission (18).

Intergenic sequence analysis of the *afeIR* locus revealed two

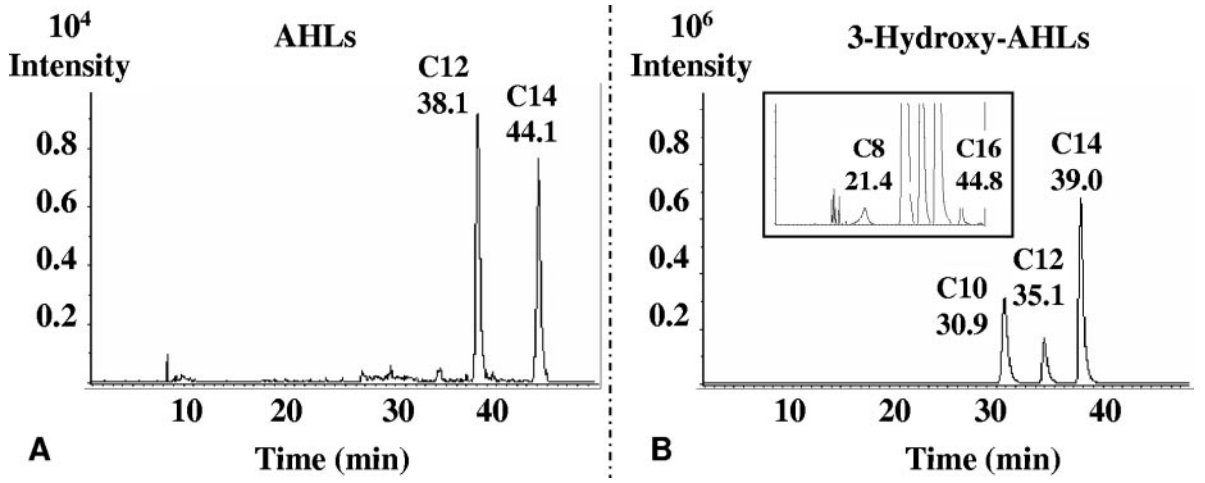

FIG. 3. Characterization of the AHLs produced by AfeI in *E. coli* carrying the *afeI* gene. *E. coli* transformed with plasmid pCRT7-NT-TOPO with the *afeI* gene was grown in LB medium. After the cells were removed, the growth medium was extracted with dichloromethane, and the presence of AHLs was analyzed by LS-MS-MS. The different MS-MS spectra of the selected ion *m/z* 102, which is specific for the lactone ring of the homoserine lactone, are shown. AHLs (A) and hydroxy-AHLs (B) were characterized. The large amounts of hydroxy-C_{10}, hyrodxy-C_{12}-, and hydroxy-C_{14}-AHLs (B) made it necessary to present this spectrum with two different intensity scales (10^6 for the common size and 10^4 for the inset). The designation of each AHL and the different elution times are indicated in each spectrum.

palindromic sequences, PS1 and PS2 (Fig. 4A). PS2 was located 36 bp upstream of *orf3* and 168 bp from *afeR*, and its function is unknown. The analysis of the PS1 sequence revealed a hierarchical organization (Fig. 4B). Each arm of the 30-bp palindromic sequence could be subdivided into two identical palindromic sequences whose sizes (15 bp) were similar to that of the known *lux* box. The nucleotide sequences of the two *afe* boxes were identical except for the three central bases (Fig. 4B), suggesting that these bases are not essential for AfeR binding but could play a role as a physical spacer for the binding of the dimeric form of AfeR. In the 3′ end of each *afe* box, a purine base was conserved. In addition, each *afe* box could be sufficient to bind a dimeric form of AfeR, as suggested by the modeling of the $(AfeR)_2$-*afe* box complex based on the $(TraR\text{-}AHL)_2$-DNA structure data (not shown). We designated these boxes *afe* box 1 and *afe* box 2 (Fig. 4B) since they could correspond to the typical type AI-1 QS regulator binding sites located close to a putative −35 transcriptional site and could play roles similar to those of *lux* and *tra* boxes (15, 48).

To study if the *afeIR* locus was functional in *A. ferrooxidans*, gene expression was analyzed by RT-PCR. Total RNA was prepared from planktonic cells of *A. ferrooxidans* grown in thiosulfate medium. The results clearly showed that *afeI*, *orf3*, and *afeR* were expressed in *A. ferrooxidans* (Fig. 4C). Nevertheless, in our experimental conditions *orf3* and *afeI* were not cotranscribed.

As the AHLs produced in the presence of iron, sulfur, or thiosulfate were different (Table 1), we analyzed the transcription levels of the *afeI* gene in the different media in DNA macroarray experiments. The *afeI* transcription levels in thiosulfate and sulfur media were similar. Compared to iron-grown cells, transcription of the *afeI* gene was increased 19- and 18-fold in sulfur- and thiosulfate-grown cells, respectively (Table 2). In iron medium, the transcription level was very low. This could explain the lack of detection of C_{12}- and C_{14}-AHLs and oxo-C_{12}- and oxo-C_{14}-AHLs (Table 1). Interestingly, the increase in the transcription levels of the *afeI* gene was not related to the transcription levels of *afeR* since the expression of this gene did not change when iron- and sulfur-grown cells were compared (results not shown).

To highlight the *afe* box function, the 5′ end of the *afeI* gene transcript was determined by primer extension analysis with RNA samples prepared from *A. ferrooxidans* cells grown in thiosulfate as the energy source. Two transcriptional initiation sites were determined with oligonucleotide AfeIRT2 (Fig. 4D). The first site had a double transcription initiation site (G and A), while the second site had a single transcription initiation site (A) (Fig. 4B). The two transcription initiation sites were located 17 or 18 bp and 28 bp, respectively, upstream of the translational start codon of *afeI* (Fig. 4B). An *E. coli* σ^{70}-type promoter (5′-TTGTCA-16 bp-TAGATT-3′) was identified and correctly positioned upstream of the second transcription initiation sites (Fig. 2B). As observed for the *lux* and *tra* boxes, *afe* box 1 overlapped the −35 transcriptional region determined (Fig. 4B).

Based on the *lux* box-*tra* box models, we decided to investigate the presence of the *afe* boxes (Fig. 4B) in the entire genome sequence of the ATCC 23270 strain. By using hidden Markov models constructed with the HMMER v2.3.2 software (14), we characterized various putative *afe* boxes (not shown). However, our results are preliminary results which only suggest the existence of a quorum-sensing regulon in *A. ferrooxidans*. Proteomic and transcriptomic studies like those performed with other bacteria (*P. aeruginosa*, *B. cepacia* H111, and *S. meliloti*) should help demonstrate the existence of a QS regulon in *A. ferrooxidans* (5, 10, 30, 35, 38, 46).

Phosphate starvation activates transcription of *afeI*. Different results have revealed that type AI-1 QS influences exopolysaccharide production and biofilm formation in many proteobacteria (16, 21, 24, 49), and phosphate and polyphosphate metabolism has been linked to biofilm formation and the quo-

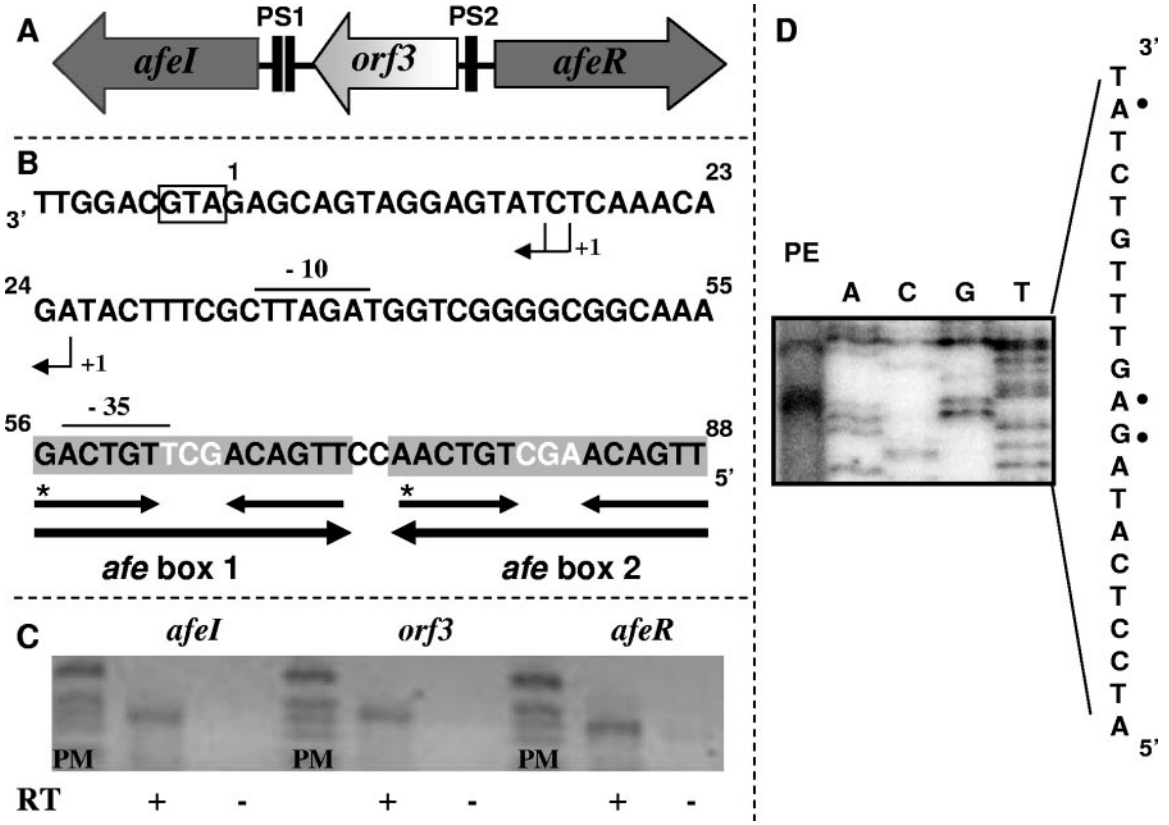

FIG. 4. Quorum-sensing genetic locus of *A. ferrooxidans*. (A) Schematic map of the quorum-sensing locus of *A. ferrooxidans* composed of three genes: *afeR* encoding the transcriptional regulator, *afeI* encoding the AHLs synthase, and *orf3* having an unknown function. Two palindromic sequences (PS1 and PS2) were located (solid boxes). (B) Nucleotide sequence of the putative *afe* boxes. The large palindromic sequence (PS1) was conformed by a 32-bp palindromic sequence (large arrows) which was built over internal, hierarchical and smaller palindromic sequences (small arrows) called *afe* box 1 and *afe* box 2. The box at the 3′ end indicates the translational start codon for *afeI*. +1 indicates the transcriptional initiation sites identified; overlining indicates the *E. coli* σ^{70}-type promoter; and asterisks indicate the purine base transition at the 3′ ends of the *afe* boxes. (C) Transcription analysis of the quorum-sensing genetic locus by RT-PCR analysis. RT reactions were carried out with 1 μg of total RNA from thiosulfate-grown *A. ferrooxidans* cells and were performed with (+) and without (−) the Moloney murine leukemia virus reverse transcriptase in order to exclude amplification due to genomic DNA contamination. RT-PCR products were analyzed by 1% agarose gel electrophoresis. (D) Determination of transcriptional initiation sites for the *afeI* gene by a primer extension experiment. The relevant DNA sequence (complementary to the sequence shown in panel B between nucleotides 8 and 27) is shown on the right, and the positions of the possible start sites are indicated by solid dots.

rum-sensing regulatory pathway (26, 33, 40). On the other hand, in *Serratia* sp. strain ATCC 390006, mutation of the *pstS* gene, which belongs to the Pho regulon and whose product is part of a high-affinity phosphate transporter, mimicked phosphate limitation and caused a three- to fourfold increase in transcription of the AHL synthase-encoding gene (*smaI*) of *Serratia* type AI-I QS (40).

During bioleaching *A. ferrooxidans* adheres to solid substrates by means of extracellular polymeric substances, such as exo- or lipopolysaccharides, and develops biofilm structures during the cellular adhesion process (7, 13, 22, 36). Previous immunological results have demonstrated that the amounts of lipopolysaccharides which are part of the polysaccharide matrix involved in ore colonization are increased in phosphate-starved *A. ferrooxidans* cells (3), and a *pho* regulon has been described in *A. ferrooxidans* (45). Therefore, the effect of phosphate starvation on the transcription of the *afeI* gene was investigated by using a DNA macroarray analysis.

TABLE 2. Effect of the energy source on the transcription levels of the *afeI* gene

	Medium		
	Iron	Sulfur	Thiosulfate
afeI spot intensity[a]	0.68	13.22	12.49

[a] Relative intensity.

Transcription of the *afeI* gene was increased 20-fold when *A. ferrooxidans* cells were cultured in a low-phosphate medium ($-P_i$ medium; P_i concentration, 0.22 mM) compared to a high-phosphate medium ($+P_i$ medium; P_i concentration, 22 mM) (Table 3). The overexpression of the *afeI* gene in phosphate-starved cells was directly correlated with increases in the C_{12}-AHL, C_{14}-AHL, and hydroxy-AHL levels (Table 3). These results are in agreement with those obtained with the *smaI* gene of *Serratia* sp. (40). Therefore, the AfeIR quorum-sensing

TABLE 3. Effect of the phosphate starvation on *afeI* expression and AHL synthesis in thiosulfate-grown cells

	+ Pi	– Pi
afeI spot intensity[a]	7.57	152.52
C_{12}-AHL	1	5.01
C_{14}-AHL	1	3.39
Hydroxy-AHL[b]	1	2.31

[a] Relative intensity.
[b] Average values including hydroxy-C_{10}, hydroxy-C_{14}, and hydroxy-C_{16}-AHLs.

system appears to be modulated by P_i availability. Slater et al. (40) suggested that the expression of *smaI* could be enhanced through the two-component regulatory system PhoR-PhoB. In *A. tumefaciens* phosphate-starved cells, biofilm formation is positively affected through the PhoR-PhoB regulatory pathway (11). Nevertheless, how these different regulatory levels are related and affect biofilm formation in *A. ferrooxidans* is still an open question.

Recently, preliminary evidence for the occurrence of homoserine lactone signal production in archaea has been reported (31). Some biomining microorganisms, such as *Ferroplasma* type II and *Lepotspirillum ferrooxidans* type II, are also able to form a natural and mixed acidophilic biofilm (42). Therefore, the existence of an AHL communication system in *A. ferrooxidans* and its impact on the biofilm structure and abundance of bacteria and archaea in biomining microbial communities are very relevant.

Obviously, further studies with *A. ferrooxidans* are necessary (i) to determine if AfeR is a positive transcriptional regulator or negative transcriptional regulator or both, as in the case of AhyR of *Aeromonas hydrophila* (23), (ii) to understand the biological significance of both *afe* boxes upstream of the *afeI* gene, (iii) to understand the role of the product of the *orf3* gene, and (iv) to identify all the genes which form the quorum-sensing regulon. Since no genetic transfer techniques are currently available for *A. ferrooxidans*, strategies using *afeI* mutants or any kind of gene cloning are not possible. However, the use of proteomic and transcriptomic analyses of mimicked null mutants obtained with quorum-sensing inhibitors (9) should be a successful global approach for identification of all the components of the quorum-sensing regulon in *A. ferrooxidans*.

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REFERENCES

1. **Acosta, M., S. Beard, J. Ponce, M. Vera, J. C. Mobarec, and C. A. Jerez.** 2005. Identification of putative sulfurtransferase genes in the extremophilic *Acidithiobacillus ferrooxidans* ATCC 23270 genome: structural and functional characterization of the proteins. OMICS **9:**13–29.
2. **Altschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25:**3389–3402.
3. **Amaro, A. M., M. Seeger, R. Arredondo, M. Moreno, and C. A. Jerez.** 1993. The growth conditions affect *Thiobacillus ferrooxidans* attachment to solids, p. 577–585. *In* A. E. Torma, M. L. Apel, and C. L. Brierley (ed.), Biohydrometallurgical technologies. The Minerals, Metals and Material Society, Warrendale, Pa.
4. **Amaro, A. M., D. Chamorro, M. Seeger, R. Arredondo, I. Peirano, and C. A. Jerez.** 1991. Effect of external pH perturbations on in vivo protein synthesis by the acidophilic bacterium *Thiobacillus ferrooxidans*. J. Bacteriol. **173:**910–915.
5. **Arevalo-Ferro, C., M. Hentzer, G. Reil, A. Gorg, S. Kjelleberg, M. Givskov, K. Riedel, and L. Eberl.** 2003. Identification of quorum-sensing regulated proteins in the opportunistic pathogen *Pseudomonas aeruginosa* by proteomics. Environ. Microbiol. **5:**1350–1369.
6. **Bassler, B.** 2002. Small talk: cell-to-cell communication in bacteria. Cell **109:**421–424.
7. **Blais, J., R. Tyagi, N. Meunier, and J. Auclair.** 1994. The production of extracellular appendages during bacterial colonization of elemental sulphur. Process Biochem. **29:**475–482.
8. **Brader, G., S. Sjoblom, H. Hyytiainen, K. Sims-Huopaniemi, and E. T. Palva.** 2005. Altering substrate chain length specificity of an acylhomoserinelactone synthase in bacterial communication. J. Biol. Chem. **280:**10403–10409.
9. **Castang, S., B. Chantegrel, C. Deshayes, R. Dolmazon, P. Gouet, R. Haser, S. Reverchon, W. Nasser, N. Hugouvieux-Cotte-Pattat, and A. Doutheau.** 2004. *N*-Sulfonyl homoserine lactones as antagonists of bacterial quorum sensing. Bioorg. Med. Chem. Lett. **14:**5145–5149.
10. **Chen, H., M. Teplitski, J. B. Robinson, B. G. Rolfe, and W. D. Bauer.** 2003. Proteomic analysis of wild-type *Sinorhizobium meliloti* responses to *N*-acyl homoserine lactone quorum-sensing signals and the transition to stationary phase. J. Bacteriol. **185:**5029–5036.
11. **Danhorn, T., M. Hentzer, M. Givskov, M. R. Parsek, and C. Fuqua.** 2004. Phosphorus limitation enhances biofilm formation of the plant pathogen *Agrobacterium tumefaciens* through the PhoR-PhoB regulatory system. J. Bacteriol. **186:**4492–4501.
12. **Davies, D. G., M. R. Parsek, J. P. Pearson, B. H. Iglewski, J. W Costerton, and E. P. Greenberg.** 1998. The involvement of cell-to-cell signals in the development of a bacterial biofilm. Science **280:**295–298.
13. **DiSpirito, A., M. Silver, L. Voss and, O. Tuovinen.** 1982. Flagella and pili of iron-oxidizing thiobacilli isolated from uranium mine in northern Ontario, Canada. Appl. Environ. Microbiol. **43:**1196–1200.
14. **Eddy, S. R.** 1998. Profile hidden Markov models. Bioinformatics **14:**755–763.
15. **Fuqua, W. C., and S. Winans.** 1996. Conserved *cis*-acting promoter elements are required for density-dependent transcription of *Agrobacterium tumefaciens* conjugal transfer genes J. Bacteriol. **178:**435–440.
16. **Gonzalez, J. E., and M. M. Marketon.** 2003. Quorum sensing in nitrogen-fixing rhizobia. Microbiol. Mol. Biol. Rev. **67:**574–592.
17. **Gould, T. A., H. P. Schweizer, and M. E. Churchill.** 2004. Structure of the *Pseudomonas aeruginosa* acyl-homoserinelactone synthase LasI. Mol. Microbiol. **53:**1135–1146.
18. **Gray, K., and J. Garey.** 2001. The evolution of bacterial LuxI and LuxR quorum sensing regulators. Microbiology **147:**2379–2387.
19. **Guiliani, N., A. Bengrine, F. Borne, M. Chippaux, and V. Bonnefoy.** 1997. Alanyl-tRNA synthetase gene of the extreme acidophilic chemolithoautotrophic *Thiobacillus ferrooxidans* is highly homologous to *alaS* genes from all living kingdoms but cannot be transcribed from its promoter in *Escherichia coli*. Microbiology **143:**2179–2187.
20. **Henke, J. M., and B. L. Bassler.** 2004. Bacterial social engagements. Trends Cell Biol. **14:**648–656.
21. **Huber, B., K. Riedel, M. Hentzer, A. Heydorn, A. Gotschlich, M. Givskov, S. Molin, and L. Eberl.** 2001. The *cep* quorum-sensing system of *Burkholderia cepacia* H111 controls biofilm formation and swarming motility. Microbiology **147:**2517–2528.
22. **Karamanev, D.** 1991. Model of the biofilm structure of *Thiobacillus ferrooxidans*. J. Biotechnol. **20:**51–64.
23. **Kirke, D. F., S. Swift, M. J. Lynch, and P. Williams.** 2004. The *Aeromonas hydrophila* LuxR homologue AhyR regulates the N-acyl homoserine lactone synthase, AhyI, positively and negatively in a growth phase-dependent manner. FEMS Microbiol. Lett. **241:**109–117.
24. **Labbate, M., S. Y. Queck, K. S. Koh, S. A. Rice, M. Givskov, and S. Kjelleberg.** 2004. Quorum sensing-controlled biofilm development in *Serratia liquefaciens* MG1. J. Bacteriol. **186:**692–698.
25. **Laue, B. E., Y. Jiang, S. R. Chhabra, S. Jacob, G. S. Stewart, A. Hardman, J. A. Downie, F. O'Gara, and P. Williams.** 2000. The biocontrol strain

Pseudomonas fluorescens F113 produces the *Rhizobium* small bacteriocin, N-(3-hydroxy-7-*cis*-tetradecenoyl)homoserine lactone, via HdtS, a putative novel N-acylhomoserine lactone synthase. Microbiology **146:**2469–2480.
26. **Ledgham, F., C. Soscia, A. Chakrabarty, A. Lazdunski, and M. Foglino.** 2003. Global regulation in *Pseudomonas aeruginosa*: the regulatory protein AlgR2 (AlgQ) acts as a modulator of quorum sensing. Res. Microbiol. **154:**207–213.
27. **Lüthy, R., J. U. Bowie, and D. Eisenberg.** 1992. Assessment of protein models with three-dimensional profiles Nature **356:**83–85.
28. **Marti-Renom, M. A., A. C. Stuart, A. Fiser, R. Sánchez, F. Melo, and A. Sali.** 2000. Comparative protein structure modeling of genes and genomes Annu. Rev. Biophys. Biomol. Struct. **29:**291–325.
29. **Morin, D., B. Grasland, K. Vallee-Rehel, C. Dufau, and D. Haras.** 2003. On-line high-performance liquid chromatography-mass spectrometric detection and quantification of N-acylhomoserine lactones, quorum sensing signal molecules, in the presence of biological matrices. J. Chromatogr. A **1002:** 79–92.
30. **Nouwens, A. S., S. A. Beatson, C. B. Whitchurch, B. J. Walsh, H. P. Schweizer, J. S. Mattick, and S. J. Cordwell.** 2003. Proteome analysis of extracellular proteins regulated by the *las* and *rhl* quorum sensing systems in *Pseudomonas aeruginosa* PAO1. Microbiology **149:**1311–1322.
31. **Paggi, R. A., C. B. Martone, C. Fuqua, and R. E. De Castro.** 2003. Detection of quorum sensing signals in the haloalkaliphilic archaeon *Natronococcus occultus*. FEMS Microbiol. Lett. **221:**49–52.
32. **Ramirez, P., N. Guiliani, L. Valenzuela, S. Beard, and C. A. Jerez.** 2004. Differential protein expression during growth of *Acidithiobacillus ferrooxidans* on ferrous iron, sulfur compounds, or metal sulfides. Appl. Environ. Microbiol. **70:**4491–4498.
33. **Rashid, M. H., K. Rumbaugh, L. Passador, D. G. Davies, A. N. Hamood, B. H. Iglewski, and A. Kornberg.** 2000. Polyphosphate kinase is essential for biofilm development, quorum sensing, and virulence of *Pseudomonas aeruginosa*. Proc. Natl. Acad. Sci. USA **97:**9636–9641.
34. **Rawlings, D. E.** 2002. Heavy metal mining using microbes. Annu. Rev. Microbiol. **56:**65–91.
35. **Riedel, K., C. Arevalo-Ferro, G. Reil, A. Gorg, F. Lottspeich, and L. Eberl.** 2003. Analysis of the quorum-sensing regulon of the opportunistic pathogen *Burkholderia cepacia* H111 by proteomics. Electrophoresis **24:**740–750.
36. **Rohwerder, T., T. Gehrke, K. Kinzler, and W. Sand.** 2003. Bioleaching review part A: progress in bioleaching: fundamentals and mechanisms of bacterial metal sulfide oxidation. Appl. Microbiol. Biotechnol. **63:**239–248.
37. **Sambrook, J., and D. W. Russell.** 1989. Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
38. **Schuster, M., C. P. Lostroh, T. Ogi, and E. P. Greenberg.** 2003. Identification, timing, and signal specificity of *Pseudomonas aeruginosa* quorum-controlled genes: a transcriptome analysis. J. Bacteriol. **185:**2066–2079.
39. **Shaw, P. D., G. Ping, S. L. Daly, C. Cha, J. E. Cronan, Jr., K. L. Rinehart, and S. K. Farrand.** 1997. Detecting and characterizing N-acyl-homoserine lactone signal molecules by thin-layer chromatography. Proc. Natl. Acad. Sci. USA **94:**6036–6041.
40. **Slater, H., M. Crow, L. Everson, and G. P. Salmond.** 2003. Phosphate availability regulates biosynthesis of two antibiotics, prodigiosin and carbapenem, in *Serratia* via both quorum-sensing-dependent and -independent pathways. Mol. Microbiol. **47:**303–320.
41. **Tatusova, T. A., and T. L. Madden.** 1999. BLAST 2 Sequences, a new tool for comparing protein and nucleotide sequences. FEMS Microbiol. Lett. **174:** 247–250.
42. **Tyson, G. W., J. Chapman, P. Hugenholtz, E. E. Allen, R. J. Ram, P. M. Richardson, V. V. Solovyev, E. M. Rubin, D. S. Rokhsar, and J. F. Banfield.** 2004. Community structure and metabolism through reconstruction of microbial genomes from the environment. Nature **428:**37–43.
43. **Valdes, J., F. Veloso, E. Jedlicki, and D. Holmes.** 2003. Metabolic reconstruction of sulfur assimilation in the extremophile *Acidithiobacillus ferrooxidans* based on genome analysis. BMC Genomics **4:**51.
44. **Vannini, A., C. Volpari, C. Gargioli, E. Muraglia, R. Cortese, R. De Francesco, P. Neddermann, and S. Marco.** 2002. The crystal structure of the quorum sensing protein TraR bound to its autoinducer and target DNA. EMBO J. **21:**4393–4401.
45. **Vera, M., N. Guiliani, and C. A. Jerez.** 2003. Proteomic and genomic analysis of the phosphate starvation response of *Acidithiobacillus ferrooxidans*. Hydrometallurgy **71:**125–132.
46. **Wagner, V. E., D. Bushnell, L. Passador, A. I. Brooks, and B. H. Iglewski.** 2003. Microarray analysis of *Pseudomonas aeruginosa* quorum-sensing regulons: effects of growth phase and environment. J. Bacteriol. **185:**2080–2095.
47. **Watson, W. T., T. D. Minogue, D. L. Val, S. B. von Bodman, and M. E. Churchill.** 2002. Structural basis and specificity of acyl-homoserine lactone signal production in bacterial quorum sensing. Mol. Cell **9:**685–694.
48. **Whitehead, N., A. Barnard, H. Slater, N. Simpson, and G. Salmond.** 2001. Quorum sensing in Gram-negative bacteria. FEMS Microbiol. Rev. **25:**365–404.
49. **Winans, S., and B. Bassler.** 2002. Mob psychology. J. Bacteriol. **184:**873–883.
50. **Zhang, R., T. Pappas, J. Brace, P. Miller, T. Oulmassov, J. Molyneaux, J. Anderson, J. Bashkin, S. Winans, and A. Joachimiak.** 2002. Structure of a bacterial quorum-sensing transcription factor complexed with pheromone and DNA. Nature **417:**971–974.