Appl Microbiol Biotechnol (2013) 97:3729–3737
DOI 10.1007/s00253-012-4229-3

ENVIRONMENTAL BIOTECHNOLOGY

AHL signaling molecules with a large acyl chain enhance biofilm formation on sulfur and metal sulfides by the bioleaching bacterium *Acidithiobacillus ferrooxidans*

Alex González · Sören Bellenberg · Sigde Mamani · Lina Ruiz · Alex Echeverría · Laurent Soulère · Alain Doutheau · Cecilia Demergasso · Wolfgang Sand · Yves Queneau · Mario Vera · Nicolas Guiliani

Received: 24 February 2012 / Revised: 29 May 2012 / Accepted: 5 June 2012 / Published online: 3 July 2012

Abstract Biofilm formation plays a pivotal role in bioleaching activities of bacteria in both industrial and natural environments. Here, by visualizing attached bacterial cells on energetic substrates with different microscopy techniques, we obtained the first direct evidence that it is possible to positively modulate biofilm formation of the extremophilic bacterium *Acidithiobacillus ferrooxidans* on sulfur and pyrite surfaces by using Quorum Sensing molecules of the *N*-acylhomoserine lactone type (AHLs). Our results revealed that AHL-signaling molecules with a long acyl chain (12 or 14 carbons) increased the adhesion of *A. ferrooxidans* cells to these substrates. In addition, CARD-FISH experiments demonstrated that C14-AHL improved the adhesion of indigenous *A. ferrooxidans* cells from a mixed bioleaching community to pyrite. Finally, we demonstrated that this improvement of cell adhesion is correlated with an increased production of extracellular polymeric substances. Our results open up a promising means to develop new strategies for the improvement of bioleaching efficiency and metal recovery, which could also be used to control environmental damage caused by acid mine/rock drainage.

Keywords Biomining · Biofilm · *Acidithiobacillus ferrooxidans* · Quorum sensing · Homoserine lactone · Atomic force microscopy

Alex González and Sören Bellenberg contributed equally to this work.

Electronic supplementary material The online version of this article (doi:10.1007/s00253-012-4229-3) contains supplementary material, which is available to authorized users.

A. González · S. Mamani · L. Ruiz · N. Guiliani (✉)
Laboratory of Bacterial Communication, Department of Biology, Faculty of Sciences, University of Chile, Santiago, Chile
e-mail: nguilian@uchile.cl

S. Bellenberg · W. Sand · M. Vera
Biofilm Centre Aquatische Biotechnologie, Universität Duisburg–Essen, Essen, Germany

S. Mamani
CNRS, Institut de Microbiologie de la Méditerranée, Laboratoire de Chimie Bactérienne, Université de la Mediterranée, Faculté des Sciences de Luminy, Marseille, France

A. Echeverría · C. Demergasso
Biotechnology Center, Universidad Católica del Norte, Antofogasta, Chile

L. Soulère · A. Doutheau · Y. Queneau
ICBMS, Chimie Organique et Bioorganique, INSA Lyon, UMR 5246 CNRS, Université Lyon 1, Villeurbanne, France

Introduction

Biomining is an industrial process, in which leaching microorganisms are used for recovering valuable metals, such as copper or gold, from sulfidic ores. Nowadays, about 10 % of the extracted copper in the world comes from bioleaching (Demergasso et al. 2010). However, due to an insufficient understanding of the microbiological processes, most biohydrometallurgical plants operate far from optimum conditions. On the other hand, natural bioleaching processes generate acid mine/rock drainage (AMD/ARD), a worldwide problem of water contamination due to the release of sulfuric acid, ferric iron, and other toxic heavy metals

formed during the weathering of metal sulfides. AMD occurs in working and abandoned sulfide-bearing mines. Research on bioleaching must consider as goals the enhancement of bioleaching rates and their inhibition in order to mitigate AMD/ARD. Comprehension of the physiology and the adaptive molecular mechanisms of bioleaching microorganisms is thus an extremely important issue.

Acidithiobacillus ferrooxidans is an acidophilic, chemolithoautrophic Gram-negative bacterium that has an important role in metal leaching and AMD production (Xie et al. 2009; Mykytczuk et al. 2010). It belongs to the consortia of microorganisms related with bioleaching processes, and is often found to be abundant in mesophilic environments (Davis et al. 2000; Gonzalez-Toril et al. 2003). This bacterium is able to oxidize ferrous iron and/or reduced sulfur compounds to obtain energy for growth. Its energetic metabolism is responsible for metal solubilization (Rawlings 2002). Bacterial attachment and biofilm formation on minerals increases leaching activities due to the formation of a reaction space between the metal sulfide surface and the cell (Rohwerder and Sand 2007). These physiological steps are mediated by extracellular polymeric substances (EPS), which are composed of polysaccharides, proteins, and lipids (Gehrke et al. 1998; Harneit et al. 2006; Sand and Gehrke 2006). Biofilm development is a complex process that can be regulated at different levels through diverse mechanisms. In many Gram-negative bacteria, EPS production and biofilm formation are regulated by cell to cell communication mechanisms named Quorum Sensing (QS) (Riedel et al. 2001; Marketon et al. 2003; Labbate et al. 2007; Decho et al. 2011). Thus, QS is a target for biotechnology applications in different industrial fields (Choudhary and Schmidt-Dannert 2010).

Cell-to-cell signaling is mediated by diffusible molecules named autoinducers (AIs). These molecules allow the regulation of cellular processes in a cell-density-dependent manner. *A. ferrooxidans* possesses a functional type AI-1 QS system that involves acyl-homoserine lactone (AHLs) as autoinducer molecules (Farah et al. 2005; Rivas et al. 2005). It is commonly accepted that the QS system of *A. ferrooxidans*, like that of other Gram-negative bacteria, is composed of four molecular elements (Valenzuela et al. 2007): an AHL synthase (AfeI), a transcriptional regulator (AfeR) that binds AHLs, an *afe*-box which is the target of the binary complex [AfeR-AHL] and the different AHL-signaling molecules synthesized by AfeI. In addition, a second AHL synthase named Act has been identified (Valenzuela et al. 2007; Rivas et al. 2007). However, it cannot be considered as a part of a functional QS system since to date neither an AHL receptor nor a regulatory box have been associated with this second AHL synthase. Additionally, the transcription of *act* increased when *A. ferrooxidans* cells were grown in a soluble energetic substrate in which biofilm formation is impaired (Rivas et al. 2007).

AfeI is the main AHL producer. It is capable of synthesizing nine different AHLs with diverse C-3 substitutions (oxo or hydroxy) and a range of carbons in the acyl chain oscillating between 8 and 16 (Farah et al. 2005; Valenzuela et al. 2007). Inorganic phosphate (P_i) starvation increased the transcription of *afeI* and AHL levels (Farah et al. 2005). Moreover, compared to iron-grown cells, *afeI*-transcript levels also increased in cells grown in a solid energetic substrate such as sulfur. Altogether, these informations allowed us to propose that the *A. ferrooxidans* QS system (*afeI*/*afeR*) could be involved in the production of EPS and consequently in the regulation of biofilm formation, as it has been demonstrated in other Gram-negative bacteria (Marketon et al. 2003; Parsek and Greenberg 2005; Labbate et al. 2007; Decho et al. 2011). If so, the use of synthetic AHLs and AHL analogues (For reviews, see Choudhary and Schmidt-Dannert 2010; Stevens et al. 2011; Galloway et al. 2011) in this bacterium could be used to enhance or inhibit QS-regulated phenotypes, such as EPS production and biofilm formation.

In a previous study, we showed that it is possible to modulate *A. ferrooxidans* attachment to pyrite grains by using synthetic AHLs and AHL analogues (Ruiz et al. 2008). However, these results were obtained by an indirect approach, in which the number of remaining planktonic cells was used to estimate the number of cells attached to pyrite grains after incubation with synthetic AHLs. Therefore, we decided to use a different experimental approach by directly visualizing *A. ferrooxidans* cell attachment to different solid substrates in the presence of AHL signaling molecules. Our main purpose was to identify single AHLs or mixtures of them that stimulate *A. ferrooxidans* adhesion to sulfur and metal sulfide surfaces. The results reported here show that AHLs with long acyl chains used as single molecules (C12-AHL, 3-hydroxy-C12-AHL, C14-AHL, and 3-hydroxy-C14-AHL) or as a mixture (C14-AHL/3-hydroxy-C14-AHL/3-oxo-C14-AHL) increased the adhesion to sulfur and pyrite. This was demonstrated for pure cultures of *A. ferrooxidans* type strain ATCC 23270 as well as for cells of indigenous *A. ferrooxidans* cells in a mixed community inside a semi-industrial bioleaching reactor. We also show that the mixture of C14-AHLs increased the production of capsular polysaccharides of the EPS. These results are promising for finding new means to influence bacterial leaching.

Materials and methods

Strains and media *A. ferrooxidans* ATCC 23270 was grown in basal salt solution (MAC) (Mackintosh 1978) with 2 g/L ferrous iron ions or with elemental sulfur at pH 2.5 with 5 % (wt/vol) sulfur prills or coupons (Farah et al. 2005; Vera et al. 2008). The mesophilic biomining community present in

the bioreactor of the Biotechnology Center at the Universidad Católica del Norte (Antofogasta, Chile) was grown in Norris medium supplemented with pyrite (Echeverría and Demergasso 2009). Growth of the community was checked daily by cell counting using a Petroff-Hausser chamber.

AHL-signaling molecules The different AHLs used in this study were obtained by chemical synthesis from the ICBMS, Chimie Organique et Bioorganique, UMR 5246, CNRS, Université Lyon 1, INSA, Lyon, France. Fifty-micromolar stock solutions were made in DMSO 100 %.

Biofilm formation on sulfur coupons Sulfur (S°) coupons were produced by heating elemental sulfur powder until melting. Subsequently, the liquid sulfur was poured on a cover glass and cooled. Coupons were sterilized by boiling in 9 K basal salt medium. AHLs were used at 5 μM. Coupons were added to cell cultures and daily extracted for staining and microscopic observation. Coupons were washed with Norris media basal salt solution. Then, adhered cells were fixed in 4 % *p*-formaldehyde and stained with 4,6-diamidino-2-phenylindole (DAPI) at 1 mg/mL. Finally, coupons were washed in sterile water, dried at room temperature, and imaged. Two coupons and five fields per coupon were observed for each sample.

Biofilm formation on pyrite coupons Pyrite coupons were cut from pyrite cubes (Navajun, Spain) with a diamond saw, and then cleaned and sterilized as described (Schippers et al. 1996). Biofilm formation experiments were carried out in 100-mL Erlenmeyer flasks containing 50 mL MAC. Coupons were inoculated with ferrous iron grown cells of *A. ferrooxidans* ATCC 23270 at 2×10^8 cells/mL. Flasks were incubated at 28 °C with shaking at 120 rpm. AHLs mixture was used at 5 μM. Attachment and biofilm formation was visualized by combined AFM and EFM using DAPI staining.

Quantification of Acidithiobacillus thiooxidans and Leptospirrilum ferriphilum from the biomining community Total genomic DNAs were extracted from planktonic cells of the biomining community, with or without C14-AHL induction at 5 μM. 16S rDNA genes from *A. thiooxidans* and *L. ferriphilum* were amplified by qPCR using specific primers as described previously (Remonsellez et al. 2009). Experiments were performed in triplicate.

Card-fish experiments These studies were carried out as previously described (Echeverría and Demergasso 2009). *A. ferrooxidans* cells were grown in the presence of polished coupons of pyrite. C14-AHL was used at 5 μM. After the incubation period, coupons were removed and rinsed with NaCl 0.9 % to eliminate planktonic cells. Then, adhered cells were fixed with paraformaldehyde (4 %) and dried at room temperature. Samples were covered with agarose (0.2 %) and dehydrated for 1 min in ethanol (98 %). After drying, mineral samples were incubated in hybridization buffer. Subsequently, the specific probe for the *A. ferrooxidans rrs* gene (TF359), which had been previously labeled with the horseradish peroxidase (HRP) enzyme, was added and samples were incubated for 2 h at 35 °C. Finally, hybridized samples were incubated for 10 min at 37 °C in washing buffer (3 mM NaCl; 5 mM EDTA pH 8; 20 mM Tris–HCl pH 7,5; 0.01 % *w*/*v* SDS) and incubated with tyramide (HRP substrate) labeled with CY3 (Thermo scientific). Visualization of substrate-attached cells of *A. ferrooxidans* was performed using a confocal microscope (Olympus, FV-1000 Spectral).

Biofilm formation on floating filters Autoclaved polycarbonate filters (GTTB, Ø 2.5 cm, 0.22 μm pore size, Millipore®) were inoculated by filtration of 10^6 ferrous grown cells as described (de Bruyn et al. 1990). Inoculated filters were immediately transferred to sterile Erlenmeyer flasks containing 50 mL basal salt MAC medium and 2 g/L iron(II) ions and incubated by floating at 28 °C without agitation, with or without the addition of AHLs mixture (5 or 0.5 μM).

Nucleic acid and lectin staining Cells attached on pyrite coupons were stained by incubation of the coupons in DAPI solution (2 mg/mL) for 10 min followed by carefully submerging in particle-free, deionized water. After air-drying, coupons were mounted on glass slides for the combined AFM and EFM observation. Polycarbonate filters with grown cells were placed on a filtration device equipped with a vacuum pump. They were washed twice with 200 μL deionized sterile water, which was removed by vacuum pressure through the filter. Then, nucleic acid staining was undertaken by covering the filters with 6 μM Syto9™ (Invitrogen©, L13152 LIVE/DEAD® BacLight) for 15 min. After one washing with water, polysaccharides of the EPS were stained by covering the filters with 50 μg/mL TRITC-ConA (Invitrogen) for 30 min. After two washing steps, stained filters were air dried in darkness. In order to prolong the fluorescence of the dyes, an antifading agent (Citifluor™ AF2) was used when mounting the filters.

Atomic force microscopy A NanoWizard®II atomic force microscope (JPK Instruments, Germany) with a Biomaterial TM Workstation was used for AFM visualization, using JPK SPM and JPK Image Processing software. All measurements were performed in air, using cantilevers without coating (μMASCH® CSC37 B; typical spring constant, 0.35 N/m; resonance frequency, 28 kHz). AFM images were obtained with a setpoint of 1.0 V and a line-rate of 0.9 Hz using the contact mode.

Epifluorescence microscopy Visualization of fluorescently stained pyrite coupons was performed using an upright epifluorescence microscope (Zeiss® AxioImager™ A1m) equipped with a HBO 100 mercury vapor lamp, using Zeiss® filterset 49, air-objectives (Zeiss® EC plan NEOFLUAR 440480/9903 and EC epiplan NEOFLUAR 422393/9900) and a digital microscope camera (Zeiss® AxioCam™ MRm). The shuttle stage of the BioMaterial™ Workstation allows the combination of EFM and AFM examinations of the same (±2 μm) specimen region (Mangold et al. 2008). Images were processed using Zeiss® AxioVision™ Software.

Confocal laser scanning microscopy A laser scanning module (LSM 510 Carl Zeiss® Jena) coupled to an inverted Axiovert 100 MBP microscope (Zeiss®) was used. All micrographs were obtained with the plan-apochromatic 100×/0.79 oil DIC objective. The microscope was operated with LSM 510 Release 3.2 software (Zeiss®). Basic visualization and image manipulation was performed using ImageJ (http://rsbweb.nih.gov/ij/).

Results

The effect of the previously tested AHLs mixture [3-hydroxy-AHLs (C10, C12, C14, C16) and oxo-AHLs (C10, C12, C14, C16)] on biofilm development by *A. ferrooxidans* ATCC 23270 was directly analyzed by DAPI staining and EFM imaging (results not shown). Substantial differences between non-treated and treated cells after 36 and 60 h of incubation with AHL mixtures were visible. In agreement with the results obtained by the indirect method (Ruiz et al. 2008), the presence of this complex AHL mixture with hydroxy- and oxo-AHLs decreased the adhesion of cells of *A. ferrooxidans* ATCC 23270 to pyrite (not shown). This could be relevant to design strategies for AMD/ARD mitigation. However, regarding the biomining process, whose efficiency can be increased by biofilm formation (Schippers and Sand 1999; Rohwerder et al. 2003), AHL-signaling molecules promoting biofilm formation are essential. Since AHLs with long acyl chains of 12 or 14 carbon atoms are the main compounds produced by *A. ferrooxidans* (Farah et al. 2005; Rivas et al. 2005; Valenzuela et al. 2007), the effect of single synthetic AHL molecules (C12-AHL, 3-hydroxy-C12-AHL, C14-AHL, and 3-hydroxy-C14-AHL) and an AHL mixture composed of three AHLs (C14-AHL/3-hydroxy-C14-AHL/3-oxo-C14-AHL) was analyzed (Figs. 1 and 2; Supplementary material Fig. S1).

Single and mixed AHLs increase the adhesion of A. ferrooxidans cells to different energetic substrates In order to determine if these single AHLs with long acyl chains were directly capable of influencing cell adhesion to mineral surfaces, two different solid substrates were used. Firstly, *A. ferrooxidans* was grown on elemental sulfur (S°) coupons as energy source in the presence of C14-AHL. Cells attached to sulfur coupons were stained after different days of incubation to visualize the biofilm formation process with DAPI (Fig. 1) or with the Live/Dead® BacLight Bacterial Viability Kit (Supplementary material Fig. S1). Compared to the control without AHL molecules, results obtained by fluorescence microscopy clearly indicated that an addition of C14-AHL (Fig. 1) and 3-hydroxy-C14-AHL (Supplementary material Fig. S1) increased the levels of adhesion of *A. ferrooxidans* cells. Similar results were obtained with C12-AHL (not shown) and 3-hydroxy-C12-AHL (Supplementary material Fig. S1). Indeed, a higher density of substrate-attached *A. ferrooxidans* cells was always observed between days 3 and 5 with these single AHL molecules (Fig. 1; supplementary material Fig. S1). Since oxo-C12-AHL and 3-oxo-C14-AHL were part of the complex mixture that decreased the levels of adhesion to pyrite (Ruiz et al 2008; this work), both compounds were also tested as single molecules to assess whether these oxo-AHLs are directly involved in the decrease of adhesion. However, no changes in the levels of adhesion of cells of *A. ferrooxidans* to sulfur coupons were observed (not shown). Secondly, adhesion of *A. ferrooxidans* cells to pyrite coupons in the presence of an AHL mixture (composed of C14-AHL, 3-hydroxy-C14-AHL, and 3-oxo-C14-AHL) was tested and visualized using combined AFM and EFM (Fig. 2). The results also revealed that the adhesion of *A. ferrooxidans* cells to pyrite coupons was increased in the presence of this mixture as compared to the control without AHL molecules (Fig. 2). No significant differences in pyrite colonization compared to the control without AHL molecules were observed, when a mixture of C12-AHL, 3-hydroxy-C12-AHL, and 3-oxo-C12-AHL was tested (results not shown).

C14-AHL increases adhesion to pyrite by indigenous A. ferrooxidans cells from a biomining community To analyze if this behavior also occurs with indigenous *A. ferrooxidans* cells, a single C14-AHL was added to a culture of a bioleaching community. This community had previously been extracted from a semi-industrial reactor and grown on pyrite coupons. Substrate-attached *A. ferrooxidans* cells were imaged by staining with the TF359 specific 16S DNA probe and then visualized by fluorescence microscopy (Fig. 3). In agreement with the previous results obtained with pure cultures of *A. ferrooxidans*, the presence of a C14-AHL clearly increased the adhesion of indigenous *A. ferrooxidans* cells to pyrite compared to the biotic control without AHL molecules. In addition, to analyze the effect of C14-AHL on other bacterial species from this bioleaching community,

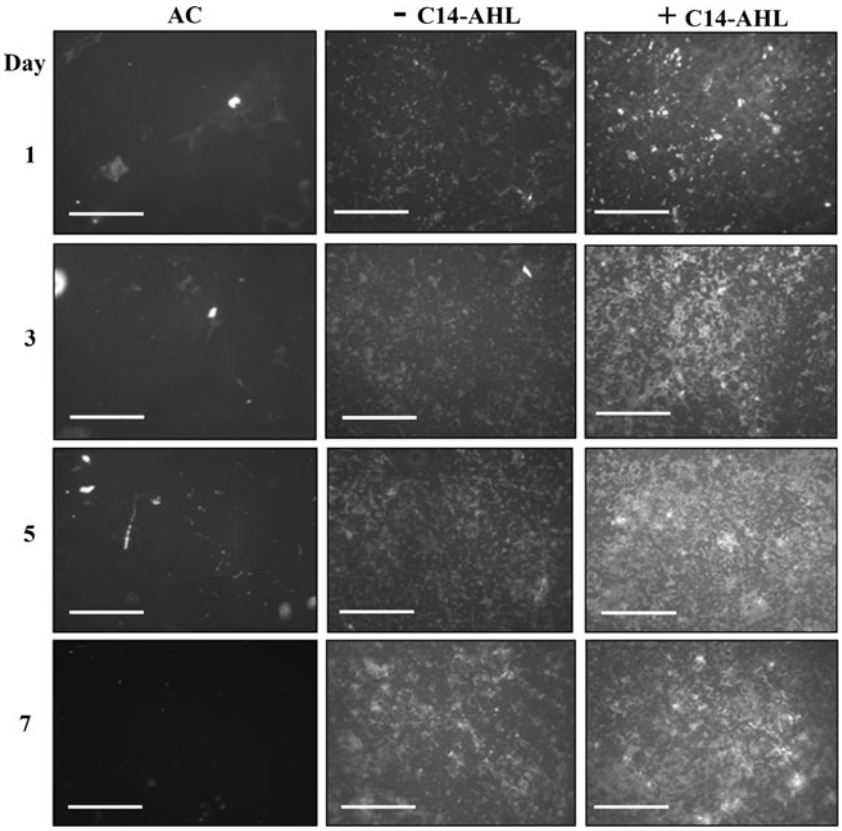

Fig. 1 C14-AHL promotes the adhesion of *A. ferrooxidans* cells to sulfur coupons. *A. ferrooxidans* cells were grown on S° in the absence (−) or presence (+) of 5 μM C14-AHL. S° coupons were extracted during growth at different days, immediately stained with DAPI, and visualized by fluorescence microscopy. *AC* abiotic control for unspecific staining. *Size bars* represent 50 μm

planktonic cells of indigenous *A. thiooxidans* and *L. ferriphillum* were assessed by qPCR (Fig. 4). Comparing with the control without C14-AHL, no significant change was observed for *A. thiooxidans*, whereas the number of *L. ferriphillum* planktonic cells was considerably reduced (tenfold) suggesting that the adherence of this microorganism was enhanced.

A. ferrooxidans EPS formation is enhanced by a C14-AHLs mixture In order to elucidate whether the enhanced attachment to surfaces due to the addition of QS molecules is related to an enhancement of EPS production, we tested the formation of biofilms by *A. ferrooxidans* on floating polycarbonate surfaces as described (Bellenberg et al. 2011a). Whereas on pyrite and sulfur surfaces interactions with the attached bacteria were strong enough to be visualized after the first days of biofilm formation, the staining and washing procedures released many cells from the polycarbonate surfaces, impairing good visualizations before 6 days of incubation. Cells were stained with Syto9 and the polysaccharide moieties of the EPS were made

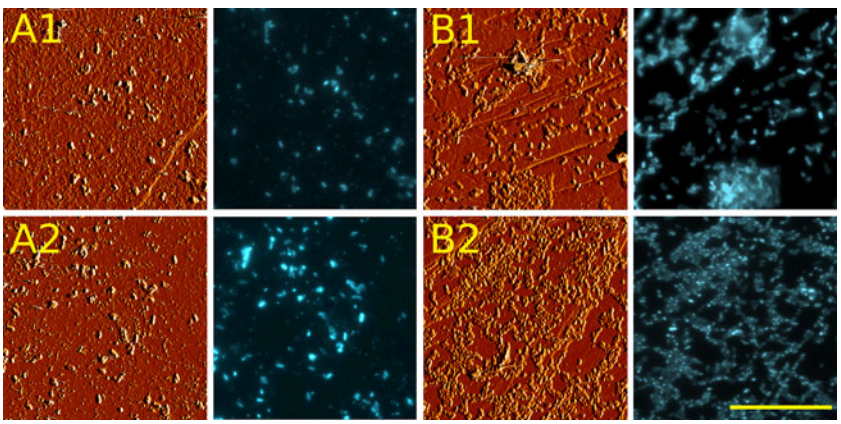

Fig. 2 AHLs mixture of C14-, 3-oxo-C14, and 3-hydroxy-C14-AHL enhances *A. ferrooxidans* biofilm formation on pyrite coupons. *A. ferrooxidans* iron(II)-grown cells were incubated on pyrite coupons without (**A**) and with 5 μM C14, 3-oxo-C14, and 3-hydroxy-C14-AHLs mixture (**B**). Coupons were imaged after 3 days (*1*) and 6 days (*2*). Paired corresponding AFM (*left*) and EFM images (*right*) are shown in image margins of 50 μm each. Size bar represents μm.

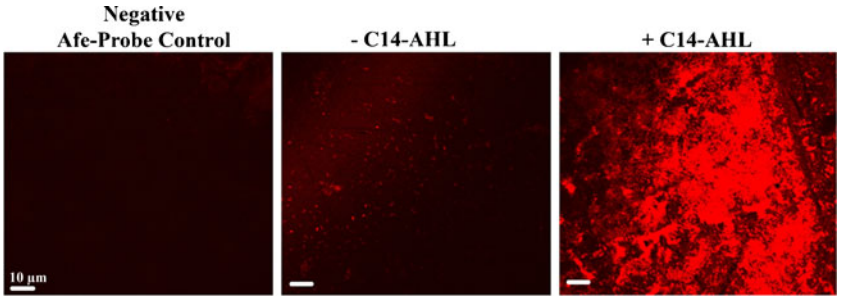

Fig. 3 C14-AHL improves the adhesion to pyrite by *A. ferrooxidans* cells from a biomining community present in a semi-industrial reactor. A biomining community was grown in pyrite (coupons) in a semi-industrial reactor in the absence or presence of 5 μM C14-AHL. *A. ferrooxidans* cells were detected by using a specific 16S DNA probe. A negative control for unspecific binding to pyrite of the DNA probe was also performed. *Size bars* represent 10 μm

visible by using the fluorescently labeled lectin ConcanavalinA (ConA). This lectin recognizes α-D-mannosyl and α-D-glucosyl groups present in the EPS of *A. ferrooxidans* (Gehrke et al. 1998; Bellenberg et al. 2011a). Double-stained samples were imaged by CLSM. Results revealed that the addition of the C14-AHLs mixture (0.5 μM) strongly enhanced the ConA signal after 6 days of incubation, as compared to the control without an addition of AHLs (Fig. 5) or cultures treated with 5 μM (not shown).

Discussion

A better understanding of the mechanisms involved in control and regulation of the biofilm development during bioleaching processes is of great significance for the development of control strategies for this process in the biomining industry and also for management of the environmental problems generated by AMD/ARD. A functional QS system has been characterized in *A. ferrooxidans* (Farah et al. 2005). Since biofilm formation processes are controlled by QS mechanisms in many Gram-negative bacteria (Riedel et al. 2001; Labbate et al. 2007; Choudhary and Schmidt-Dannert 2010; Decho et al. 2011) and previous evidence suggested that QS could influence biofilm formation in this bacterium too (Farah et al. 2005; Ruiz et al. 2008), microscopical biofilm formation studies were performed to identify single AHLs and AHL mixtures that positively regulate biofilm formation on mineral surfaces by *A. ferrooxidans* cells.

A. ferrooxidans is able to synthesize at least nine different AHLs with diverse C-3 substitutions including unsubstituted, hydroxy and oxo AHLs. However, the biological role of each AHL is still an open question. Since AHLs with long acyl chains of 12 (C12) or 14 (C14) carbon atoms are the predominant forms in *A. ferrooxidans* and were found to be overproduced by cells grown on reduced inorganic sulfur compounds (Farah et al. 2005; Valenzuela et al. 2007), the role of C12- and C14-AHLs with diverse C-3 substitutions on adhesion to solid substrates such as elemental sulfur or pyrite was tested. In our experimental conditions, the addition of synthetic unsubstituted AHLs (C12 or C14) or 3-hydroxy-AHLs (C12 or C14) or a C14-AHL mixture (composed of

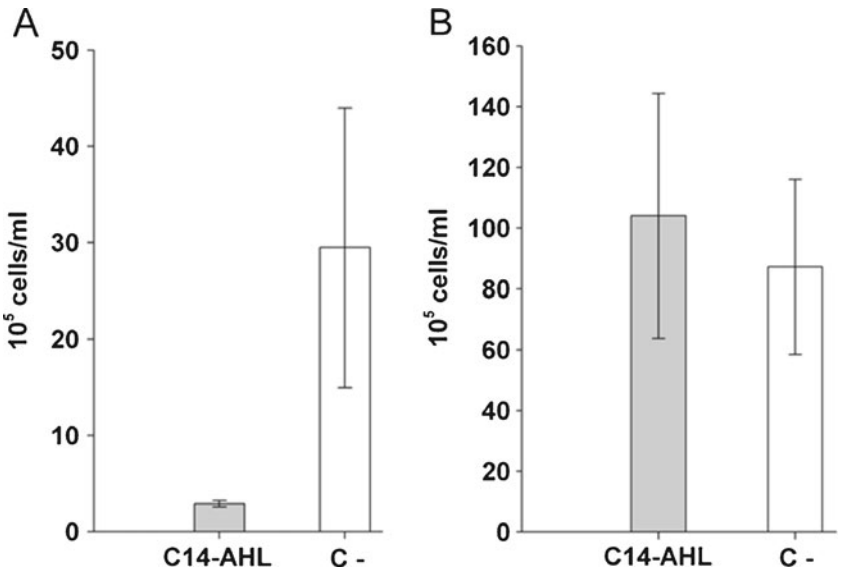

Fig. 4 C14-AHL promotes cell attachment to minerals by *L. ferriphilum* cells from a biomining community. A biomining community was grown in industrial mineral mainly composed of pyrite in absence (C-) or presence of 5 μM C14-AHL. *L. ferriphilum* (**a**) and *A. thiooxidans* (**b**) planktonic cells were quantified by qPCR using specific primers for 16S rDNA genes of each species

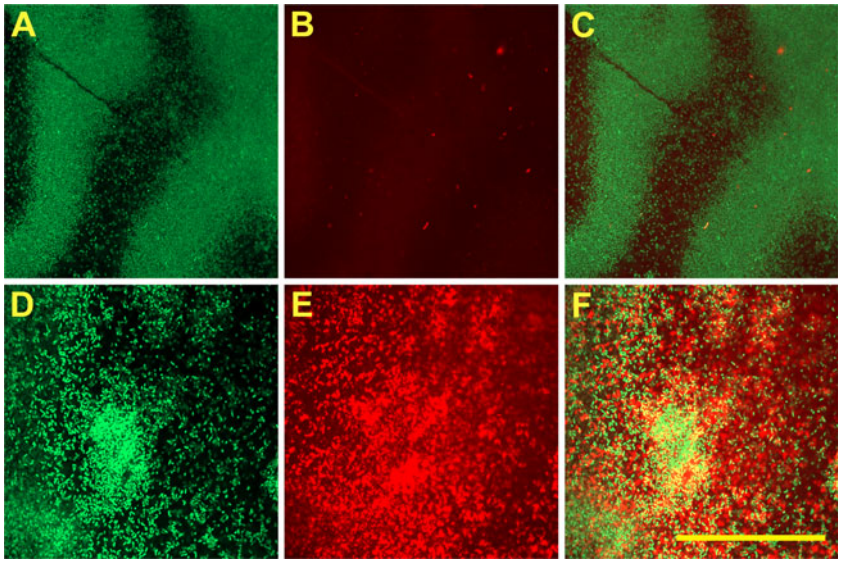

Fig. 5 *A. ferrooxidans* polysaccharides from EPS are induced by the C14- and 3-hydroxy-C14-AHL mixture. *A. ferrooxidans* biofilms were grown on floating filters inoculated with 10^6 cells each and incubated without agitation at 28 °C. After 6 days of incubation, filters were double stained. The *upper panel* shows a control filter with no addition of the AHL mixture. The *lower panel* shows a filter incubated with 0.5 μM hydroxy-C14-AHL and C14-AHL mixture. CLSM images for Syto9 (**a**, **d**), ConA (**b**, **e**), and merge (**c**, **f**) are shown. *Size bars* represent 20 μm

equal amounts of the unsubstituted, the 3-hydroxy- and the 3-oxo substituted C14-AHLs compounds) resulted in a stimulation of *A. ferrooxidans* biofilm formation on sulfur (Fig. 1; supplementary material Fig. S1) or on pyrite (Fig. 2). These findings suggested that AfeR, the quorum-sensing regulatory protein in *A. ferrooxidans* (Farah et al. 2005; Barreto et al. 2005), is able to recognize AHLs with different acyl chain lengths. This is in agreement with the results reported by Welch et al. (2000) which showed that CarR from *Erwinia carotovora* is also capable of binding AHLs with different acyl chain lengths.

Biofilm formation is one of several phenotypic changes linked to an increase in the production of EPS (Carlier et al. 2009; Flemming and Wingender 2010; Janczarek 2011). Previously, we reported that planktonic *A. ferrooxidans* cells grown with ferrous iron do not have significant amounts of exopolymers in extracellular polysaccharides detectable by specific lectin binding assays. After 24 h of biofilm formation, the same cells had an increased ConA lectin signal, indicating that the induction of EPS and capsular polysaccharide (CPS) biosynthesis occurs simultaneously with the formation of micro colonies on the pyrite surface (Bellenberg et al. 2011a). However, despite the fact that gene clusters for the formation of EPS precursors (Barreto et al. 2005) and capsular polysaccharide biosynthesis and export systems (Bellenberg et al. 2011b) have been identified, the molecular mechanisms involved in the induction of the biosynthesis of EPS/CPS in *A. ferrooxidans* have not been characterized. Here, we report the first evidence connecting a specific regulatory pathway such as QS with EPS/CPS production in a bioleaching bacterial specie. Cells grown on floating filters oxidizing ferrous iron showed an increased ConA signal in the presence of the C14-AHLs mixture (Fig. 5) as compared to the control cells without exogenous AHLs. A greater enhancement of EPS/CPS production was obtained with 0.5 μM of the C14-AHLs mixture compared to 5 μM. However, it is unknown why a lower concentration of this mixture resulted in a clear visualization of EPS/CPS production on floating filters whereas cell adherence on minerals such as pyrite and sulfur was observed at 5 μM. Nevertheless, it cannot be ruled out that the different physico-chemical properties of polycarbonate-floating filters and mineral surfaces may be involved in this phenomenon. Independently, this result obtained with the polycarbonate-floating filters clearly indicates that the production of EPS/CPS was strongly induced by the external addition of AHLs. Presumably, the addition of AHLs reduced the time needed to reach a threshold concentration that would be required for autoinduction and EPS production.

Adhesion to copper sulfide minerals during bioleaching is a selective process in which microorganisms attach preferentially to growth-favoring minerals such as pyrite (Echeverría and Demergasso 2009). Thus, the action of the autoinducer could be faster especially in cells, which are electrostatically or hydrophobically interacting with pyrite or sulfur surfaces, respectively. In consequence, increased cell densities prevail at these interfaces. This means of cell trapping might result in the preferential activation of regulons controlled by QS in cells attached to solid substrates, as compared to the planktonic cell subpopulation. This activation would contribute to establish the EPS/CPS matrix and the biofilm phenotype observed in pyrite grown cultures. A similar regulation mechanism for the initiation of biofilm formation has been described for *Vibrio cholerae* (Waters et al. 2008). In agreement, a bioinformatic study identified a QS regulon in *A. ferrooxidans*, in which several genes involved in EPS biosynthesis are present (Banderas and Guiliani, submitted).

The current knowledge of AHL or other autoinducer/inhibitor compounds produced by bacterial species sharing habitats with *A. ferrooxidans* such as other Acidithiobacilli and *Leptospirillum* species is limited. However, the effect of

the single C14-AHL on the amount of *L. ferriphillum* planktonic cells (Fig. 4) suggests that some of the nine different AHL molecules produced by *A. ferrooxidans* are being sensed by other bacterial species belonging to the biomining community and that cross-communication may be taking place between members of bioleaching communities as has been described in mixed biofilms of other Gram-negative bacteria (Riedel et al. 2001). On the other hand, it is in agreement with recently published data, which revealed that mineral-adapted cultures of *A. ferrooxidans* and *L. ferriphilum* have similar attachment characteristics on sulfide minerals (Africa et al. 2012).

Nevertheless, further studies are necessary to clearly understand and characterize all the molecular steps induced by the addition of AHL-signaling molecules on biofilm formation by *A. ferrooxidans* and other *Acidithiobacillus* sp. To this end, we recently characterized a functional c-di-GMP pathway that could also be involved in the regulation of biofilm formation (Ruiz et al. 2012). Both pathways are linked in several bacterial species (Ryan et al. 2006; Waters et al. 2008; Ueda and Wood 2009; Zhang 2010; Kozlova et al. 2011) and c-di-GMP levels and extracellular QS signals are connected to polysaccharide production and biofilm formation (Ueda and Wood 2009).

Since no genetic transfer techniques are currently available for *A. ferrooxidans* (Farah et al. 2005), transcriptomic analysis should help to characterize at the molecular level how AHL-signaling molecules and AfeR induce EPS/CPS production and biofilm formation in *A. ferrooxidans*. Such studies would be particularly valuable to understand whether the transcription of the putative EPS precursor biosynthetic genes (Barreto et al. 2005), capsular polysaccharide assembly and export genes whose transcription levels are up-regulated in the biofilm cell sub-population from pyrite-grown *A. ferrooxidans* cultures (Bellenberg et al. 2011b) and genes that possess *afe*-like boxes such as AFE_0233, AFE_1339, and AFE_1354 coding for putative proteins required for the biosynthesis of surface polysaccharides (Banderas and Guiliani, submitted) are regulated by the *afeR*/*afeI* QS system, by the c-di-GMP pathway, or by both.

However, our results are in agreement with the working hypothesis that the manipulation of cell surface properties allows cells to be primed for attachment to minerals (Africa et al. 2012). Additionally, this is the first direct evidence that it is possible to positively modulate EPS/CPS production and biofilm formation by an acidophilic bacterium of industrial and environmental importance by using QS-signaling molecules. Since adherence to the mineral surface and the subsequent formation of biofilms can improve bioleaching (Rohwerder et al. 2003; Rohwerder and Sand 2007), these results open up a new chemical/biological means which can be explored for improvement of the biomining process.

Acknowledgments This work was supported by grants FONDECYT 1080441, FONDECYT 1120295, and CNRS-CONICYT (PICS no. 5270). Alex González was supported by scholarships from CONICYT. Sören Bellenberg and Mario Vera acknowledge the financial support of the program of promotion of Young Scientists from University of Duisburg-Essen. We thank Dr. Michael Handford (Universidad de Chile) for proof-reading the manuscript.

References

Africa CJ, van Hille RP, Harrison STL (2012) Attachment of *Acidithiobacillus ferrooxidans* and *Leptospirillum ferriphilum* cultured under varying conditions to pyrite, chalcopyrite, low-grade ore and quartz in a packed column reactor. Appl Microbiol Biotechnol. doi:10.1007/s00253-012-3939-x

Barreto M, Jedlicki E, Holmes DS (2005) Identification of a gene cluster for the formation of extracellular polysaccharide precursors in the chemolithoautotroph *Acidithiobacillus ferrooxidans*. Appl Environ Microbiol 71:2902–2909

Bellenberg S, Leon-Morales F, Holuscha D, Krok B,Vera M, Sand W (2011a). A lectin-based approach for visualization of capsular EPS after attachment and biofilm formation by acidophilic leaching bacteria. In Biohydrometallurgy: Biotech key to unlock mineral resources value. Proceedings of the 19th international Biohydromettallurgy symposium. Central South University Press, Changsha, China

Bellenberg S, Vera M, Sand W (2011b). Transcriptomic studies of capsular polysaccharide export systems involved in biofilm formation by *Acidithiobacillus ferrooxidans. In Biohydrometallurgy:* Biotech key to unlock mineral resources value. Proceedings of the 19th international Biohydromettallurgy symposium. Central South University Press, Changsha, China.

Carlier A, Burbank L, von Bodman SB (2009) Identification and characterization of three novel EsaI/EsaR quorum-sensing controlled stewartan exopolysaccharide biosynthetic genes in *Pantoea stewartii* ssp. *stewartii*. Mol Microbiol 74:903–913

Choudhary S, Schmidt-Dannert C (2010) Applications of quorum sensing in biotechnology. Appl Microbiol Biotechnol 86:1267–1279

Davis RA, Welty AT, Borrego J, Morales JA, Pendon JG, Ryan JG (2000) Rio Tinto estuary (Spain): 5000 years of pollution. Environ Geol 39:1107–1116

De Bruyn J, Boogerd F, Bos P, Kuenen J (1990) Floating filters, a novel technique for isolation and enumeration of fastidious, acidophilic, iron-oxidizing, autotrophic bacteria. Appl Environ Microbiol 56:2891–2894

Decho AW, Frey RL, Ferry JL (2011) Chemical challenges to bacterial AHL signalling in the environment. Chem Rev 111:86–99

Demergasso C, Galleguillos F, Soto P, Serón M, Iturriaga V (2010) Microbial succession during a heap bioleaching cycle of low grade copper sulfides: does this knowledge mean a real input for industrial process design and control? Hydrometallurgy 104:382–390

Echeverría A, Demergasso C (2009) Assessment of microbial adhesion in mixed cultures to sulfide minerals using CARD-FISH techniques. Adv Mat Res 71–73:83–86

Farah C, Vera M, Morin D, Haras D, Jerez CA, Guiliani N (2005) Evidence for a functional quorum-sensing type AI-1 system in the extremophilic bacterium *Acidithiobacillus ferrooxidans*. Appl Environ Microbiol 71:7033–7040

Flemming HC, Wingender J (2010) The biofilm matrix. Nat Rev Microbiol 8:623–633

Galloway WR, Hodgkinson JT, Bowden SD, Welch M, Spring DR (2011) Quorum sensing in gram-negative bacteria: small-molecule

modulation of AHL and AI-2 quorum sensing pathways. Chem Rev 111:28–67

Gehrke T, Telegdi J, Thierry D, Sand W (1998) Importance of extracellular polymeric substances from *Thiobacillus ferrooxidans* for bioleaching. Appl Environ Microbiol 64:2743–2747

Gonzalez-Toril E, Llobet-Brossa E, Casamayor EO, Amann R, Amils R (2003) Microbial ecology of an extreme acidic environment, the Tinto River. Appl Environ Microbiol 69:4853–4865

Harneit K, Goksel A, Kock D, Klock JH, Gehrke T, Sand W (2006) Adhesion to metal sulfide surfaces by cells of *Acidithiobacillus ferrooxidans*, *Acidithiobacillus thiooxidans* and *Leptospirillum ferrooxidans*. Hydrometallurgy 83:245–254

Janczarek M (2011) Environmental signals and regulatory pathways that influence exopolysaccharide production in rhizobia. Int J Mol Sci 12:7898–7933

Kozlova EV, Khajanchi BK, Sha J, Chopra AK (2011) Quorum sensing and c-di-GMPdependent alterations in gene transcripts and virulence-associated phenotypes in a clinical isolate of *Aeromonas hydrophila*. Microb Pathog 50:213–223

Labbate M, Zhu H, Thung L, Bandara R, Larsen MR, Wilcox MD, Givskov M, Rice SA, Kjelleberg S (2007) Quorum-sensing regulation of adhesion in *Serratia marcescens* MG1 is surface dependent. J Bacteriol 189:2702–2711

Mackintosh M (1978) Nitrogen fixation by *Thiobacillus ferrooxidans*. J Gen Microbiol 105:215–218

Mangold S, Harneit K, Rohwerder T, Claus G, Sand W (2008) Novel combination of atomic force microscopy and epifluorescence microscopy for visualization of leaching bacteria on pyrite. Appl Environ Microbiol 74:410–415

Marketon MM, Glenn SA, Eberhard A, Gonzalez JE (2003) Quorum sensing controls exopolysaccharide production in *Sinorhizobium meliloti*. J Bacteriol 185:325–331

Mykytczuk NC, Trevors JT, Twine SM, Ferroni GD, Leduc LG (2010) Membrane fluidity and fatty acid comparisons in psychrotrophic and mesophilic strains of *Acidithiobacillus ferrooxidans* under cold growth temperatures. Arc Microbiol 192:10051018

Parsek MR, Greenberg EP (2005) Sociomicrobiology: the connections between quorum sensing and biofilms. Trends Microbiol 13:27–33

Rawlings DE (2002) Heavy metal mining using microbes. Annu Rev Microbiol 56:65–91

Remonsellez F, Galleguillos F, Moreno-Paz M, Parro V, Acosta M, Demergasso C (2009) Dynamic of active microorganisms inhabiting a bioleaching industrial heap of low-grade copper sulfide ore monitored by real-time PCR and oligonucleotide prokaryotic acidophile microarraymbt_112. Microbial Biotechnol 6:613–624

Riedel K, Hentzer M, Geisenberger O, Huber B, Steidle A, Wu H, Hoiby N, Givskov M, Molin S, Eberl L (2001) *N*-acylhomoserine-lactone-mediated communication between *Pseudomonas aeruginosa* and *Burkholderia cepacia* in mixed biofilms. Microbiology 147:3249–3262

Rivas M, Seeger M, Holmes DS, Jedlicki E (2005) A lux-like quorum sensing system in the extreme acidophile *Acidithiobacillus ferrooxidans*. Biol Res 38:283–297

Rivas M, Seeger M, Jedlicki E, Holmes DS (2007) Second acyl homoserine lactone production system in the extreme acidophile *Acidithiobacillus ferrooxidans*. Appl Environ Microbiol 73:3225–3231

Rohwerder T, Sand W (2007) Oxidation of inorganic sulfur compounds in acidophilic prokaryotes. Eng Life Sci 7:301–309

Rohwerder T, Gehrke T, Kinzler K, Sand W (2003) Bioleaching review part A: progress in bioleaching: fundamentals and mechanisms of bacterial metal sulfide oxidation. Appl Microbiol Biotechnol 63:239–248

Ruiz LM, Castro M, Barriga A, Jerez CA, Guiliani N (2012) The extremophile *Acidithiobacillus ferrooxidans* possesses a c-di-GMP signalling pathway that could play a significant role during bioleaching of minerals. Lett Appl Microbiol 54:133–139

Ruiz LM, Valenzuela S, Castro M, Gonzalez A, Frezza M, Soulere L, Rohwerder T, Sand W, Queneau Y, Jerez CA, Doutheau A, Guiliani N (2008) AHL communication is a widespread phenomenon in biomining bacteria and seems to be involved in mineral-adhesion efficiency. Hydrometallurgy 94:133–137

Ryan RP, Fouhy Y, Lucey JF, Crossman LC, Spiro S, He YW, Zhang LH, Heeb S, Cámara M, Williams P, Dow JM (2006) Cell-cell signaling in *Xanthomonas campestris* involves an HD-GYP domain protein that functions in cyclic di-GMP turnover. Proc Natl Acad Sci U S A 103:6712–6717

Sand W, Gehrke T (2006) Extracellular polymeric substances mediate bioleaching/biocorrosion via interfacial processes involving iron(III) ions and acidophilic bacteria. Res Microbiol 157:49–56

Schippers A, Sand W (1999) Bacterial leaching of metal sulfides proceeds by two indirect mechanisms via thiosulfate or via polysulfides and sulfur. Appl Environ Microbiol 65:319–321

Schippers A, Jozsa P, Sand W (1996) Sulfur chemistry in bacterial leaching of pyrite. Appl Environ Microbiol 62:3424–3431

Stevens AM, Queneau Y, Soulère L, Von Bodman S, Doutheau A (2011) Mechanisms and synthetic modulators of AHL-dependent gene regulation. Chem Rev 111:4–27

Ueda A, Wood TK (2009) Connecting quorum sensing, c-di-GMP, pel polysaccharide, and biofilm formation in *Pseudomonas aeruginosa* through tyrosine phosphatase TpbA (PA3885). PLoS Pathog 5: e1000483

Valenzuela S, Banderas A, Jerez CA, Guiliani N (2007) Cell–cell communication in bacteria: a promising new approach to improve bioleaching efficiency? In: Donati ER, Sand W (eds) Microbial processing of metal sulfides. Springer, The Netherlands, pp 253–264

Vera M, Pagliai F, Guiliani N, Jerez CA (2008) The chemolithoautotroph *Acidithiobacillus ferrooxidans* can survive under phosphate-limiting conditions by expressing a C-P lyase operon that allows it to grow on phosphonates. Appl Environ Microbiol 74:1829–1835

Waters CM, Lu W, Rabinowitz JD, Bassler BL (2008) Quorum sensing controls biofilm formation in *Vibrio cholerae* through modulation of cyclic di-gmp levels and repression of vpsT. J Bacteriol 190:2527–2536

Welch M, Todd DE, Whitehead NA, McGowan SJ, Bycroft BW, Salmond GPC (2000) EMBO J 19:631–641

Xie XH, Xiao SM, Liu JS (2009) Microbial communities in acid mine drainage and their interaction with pyrite surface. Curr Microbiol 59:71–77

Zhang LH (2010) A novel C-di-GMP effector linking intracellular virulence regulon to quorum sensing and hypoxia sensing. Virulence 1:391–394.