ICEAfe1, an Actively Excising Genetic Element from the Biomining Bacterium *Acidithiobacillus ferrooxidans*

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Key Words

Integrative and conjugative element · Horizontal gene transfer · *Acidithiobacillus ferrooxidans*

Abstract

Integrative conjugative elements (ICEs) are self-transferred mobile genetic elements that contribute to horizontal gene transfer. An ICE (ICEAfe1) was identified in the genome of *Acidithiobacillus ferrooxidans* ATCC 23270. Excision of the element and expression of relevant genes under normal and DNA-damaging growth conditions was analyzed. Bioinformatic tools and DNA amplification methods were used to identify and to assess the excision and expression of genes related to the mobility of the element. Both basal and mitomycin C-inducible excision as well as expression and induction of the genes for integration/excision are demonstrated, suggesting that ICEAfe1 is an actively excising SOS-regulated mobile genetic element. The presence of a complete set of genes encoding self-transfer functions that are induced in response to DNA damage caused by mitomycin C addition-

ally suggests that this element is capable of conjugative transfer to suitable recipient strains. Transfer of ICEAfe1 may provide selective advantages to other acidophiles in this ecological niche through dissemination of gene clusters expressing transfer RNAs, CRISPRs, and exopolysaccharide biosynthesis enzymes, probably by modification of translation efficiency, resistance to bacteriophage infection and biofilm formation, respectively. These data open novel avenues of research on conjugative transformation of biotechnologically relevant microorganisms recalcitrant to genetic manipulation.

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Introduction

Acidithiobacillus ferrooxidans is an environmental acidophilic, chemolithoautotrophic Gram-negative γ -proteobacterium (although some discrepancies have been reported [Williams et al., 2010]) that obtains its energy from the oxidation of ferrous ions or reduced sulfur com-

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pounds [Rawlings, 2001]. It belongs to the consortium of microorganisms that participate in the bioleaching of minerals with economical interest, such as copper. Although a couple of gene-specific knock-outs have been reported in the literature [Liu et al., 2000; Wang et al., 2012], many more unsuccessful attempts to genetically manipulate *A. ferrooxidans* have hampered the development of a genetic system for this microorganism and delayed our understanding of its physiology and genetics. Progress in this respect has resulted mainly from bioinformatic, transcriptomic and proteomic approaches [Quatrini et al., 2009; Valdés et al., 2008; Valenzuela et al., 2006] and from gene analysis in heterologous systems [Quatrini et al., 2005; Salazar et al., 1994, 2001]. Recent comparative genomic analyses between sequenced strains of *A. ferrooxidans* have shown that these genomes have acquired large genomic islands and/or integrative conjugative elements (ICEs) through horizontal gene transfer (HGT) [Holmes et al., 2009; Levicán et al., 2009; Orellana and Jerez, 2011].

HGT plays an essential role in bacterial evolution [O'Brien and Fraser, 2005]. As a consequence, many bacterial genomes contain, in addition to core genes, accessory genes acquired by HGT. ICEs are mobile genetic elements that, like temperate bacteriophages, integrate into and replicate with the host chromosome. Also, like conjugative plasmids, ICEs are transferred by conjugation to the new host contributing to HGT [Wozniak and Waldor, 2010]. Integration of ICEs into the host chromosome is promoted by an integrase encoded in the element that catalyzes a site-specific recombination through single-crossover between a circular form of the element and chromosomal target sequences (*attP* and *attB* sites, respectively) [Esposito and Scocca, 1997]. As a consequence, two ICE-chromosome junctions are formed one on each side of the integrated element (*attL* and *attR* sites). A tRNA gene is often the integration target site [Hou, 1999]. The integrase also catalyzes the excision of the ICE from its chromosomal localization in a reaction often facilitated by an excisionase encoded in the element [Lewis and Hatfull, 2001]. In this reaction, site-specific recombination occurs between *attL* and *attR* of the integrated element, reconstituting *attB* and *attP*, and generating the circular form of the ICE. This circular element is an intermediate in the conjugal transfer between bacteria.

Knowledge of the impact of ICEs on their host biology derives mainly from the analysis of pathogenicity islands [Schmidt and Hensel, 2004]. Less is known about the organization of ICEs from environmental microorganisms and their role in the adaptation to variable environmental

conditions or colonization of new ecological niches [Bordeleau et al., 2010; Dobrindt et al., 2004].

In this report, we analyze the genetic organization and excision capabilities of ICEAfe1, a 291-kbp genetic element present in the *A. ferrooxidans* type strain ATCC 23270. Excision levels and potential conjugal transfer of ICEAfe1 are consistent with the measured levels of mRNAs from genes involved in these processes. Identification of mobile genetic elements in industrially relevant acidophilic microorganisms, and characterization of the genes that they carry, will contribute to our current understanding of their genetics and ecophysiology in the short run.

Results and Discussion

ICEAfe1, a Predicted ICE Encoding All Genes Required to Be Functional

A putative ICE is inserted in the chromosome of *A. ferrooxidans* type strain ATCC 23270 in a tRNA^{Ala} gene with recognizable flanking 48-bp direct repeats, 291 kbp apart from each other (from bp 909,225 to bp 1,200,495; fig. 1a). Bioinformatic whole genome comparison of the two available sequences (ATCC 23270: GenBank CP001219 and ATCC 53993: GenBank CP001132) revealed that this element is not present in the ATCC 53993 strain [Holmes et al., 2009; Levicán et al., 2009]. A total of 352 open reading frames (ORFs) were originally annotated in this genomic segment [Valdés et al., 2008]. As in most mobile genetic elements [Hsiao et al., 2005], approximately 64% of the ORFs encode proteins with unknown or hypothetical functions and several others encode transposases (Tn5648 [Peters and Craig, 2001], two ISAfe3 and two ISAfe8 [Valdés et al., 2008]). ORFs with assigned functions include integration/excision, conjugative DNA processing, transfer, maintenance and regulation proteins (table 1), indicating that this element is an ICE. Being the first ICE identified in *A. ferrooxidans* we have named it ICEAfe1. To our knowledge, this element together with the mycobacteriophage Ms6 of *Mycobacterium* spp. [Freitas-Vieira et al., 1998] and a putative genomic island in the bioleaching acidophile *Leptospirillum* sp. group II [Boyd et al., 2009] are the only mobile genetic elements that use a tRNA^{Ala} gene as integration target site.

Integration-Excision Functions

The predicted protein products of the first two genes adjacent to the tRNA^{Ala} gene are an integrase Int (COG4974; AFE_0995) that belongs to the tyrosine re-

Fig. 1. Schematic representation of the integration and excision of ICEAfe1 (**a**) and PCR assays to detect the corresponding DNA species (**b**). **a** The diagram represents the ICEAfe1 in its integrated (top) and excised (middle) forms. The empty chromosomal site that remains after the excision is also shown (bottom). ICEAfe1 and chromosomal DNA are shown as solid line and empty bar,

respectively. The tRNA^{Ala} and the 48-bp direct repeat are indicated by a gray arrow and a gray bar, respectively. Primers used for PCR assays are shown as small gray arrowheads above the target sequence. **b** PCR products obtained with each pair of primers indicated in **a**.

Table 1. Relevant ORFs encoded in ICEAfe1

Functional category	ORFs, n (% of total)	Relevant ORFs ^a
DNA replication, recombination and repair	28 (8)	AFE1145, AFE1199, AFE1230, AFE1260
Cell envelope	19 (5.4)	AFE1339, AFE1341–AFE1344, AFE1346, AFE1349, AFE1351, AFE1354–AFE1355, AFE1357, AFE1366
Type IV conjugative transfer system	21 (5.9)	See online suppl. table S1
Mobile and extrachromosomal element functions	31 (8.8)	AFE0995, AFE0996, AFE1361–AFE1362, AFE1098–AFE1099
Unknown function or hypothetical	224 (64)	
Total	352	

^a According to NCBI nomenclature.

combinase family [Esposito and Scocca, 1997] and an excisionase Xis (COG1257; AFE_0996). Next to *int* and *xis*, there are genes encoding for orthologs of the *cI* (AFE_0998) and *Cro* (AFE_0999) phage regulators (COG2932 and COG1609, respectively), the relative levels of which are known to control the lysogenic/lytic switch of phages such as lambda [Court et al., 2007] and to modulate the excision and transfer of some ICEs [Bea-ber et al., 2004; Bellanger et al., 2007; McGrath et al., 2005]. All these proteins are likely candidates to control ICEAfe1 integration into, and SOS-regulated excision out of, the *A. ferrooxidans* chromosome.

Conjugative Transfer Functions

Twenty-one ORFs present in ICEAfe1 encode bacterial type IV secretion system functions possibly involved in the conjugative transfer of this genetic element. Predicted ORFs are organized as four isolated genes and four gene clusters (TRAI-TRAIV; online suppl. table S1; see www.karger.com/doi/10.1159/000346669 for all online suppl. material) whose general architecture closely resembles those of mobile genetic elements such as R391, SXT, pMERPH and R997 [Böltner and Osborn, 2004]. Cluster I encodes transfer genes and clusters II–IV encode transferosome components for pilus assembly. Isolated genes include the relaxase, the coupling protein, an additional VirB4-like ATPase and a product of unknown function similar to the central domain of TraN (online suppl. table S1). Most of the conjugation proteins encoded by ICEAfe1 resemble the Tra-type proteins of the IncF plasmids [de la Cruz et al., 2010; Frost et al., 1994; Lawley et al., 2003]. Occurrence and predicted gene clustering and motif conservation of all these gene products suggest that ICEAfe1 encodes functions for the required channel across the bacterial envelope and the extended F-type pilus for secretion of the ICEAfe1 nucleoprotein complex or relaxosome.

Stabilization Functions

At least three modules consisting of pairs of genes encoding a stable toxin (T) and a labile antitoxin (A) that belong to the type II systems [Gerdes et al., 2005] are predicted in ICEAfe1 according to TADB database (AFE_1098/AFE_1099, AFE_1361/AFE_1362 and AFE_1367/AFE_1368) [Shao et al., 2011]. All these putative TA systems are transcribed in *A. ferrooxidans* ATCC 23270 under basal growth conditions [Quatrini et al., 2009; P. Bustamante (data not shown)]. TA modules are likely candidates for stable maintenance of ICEAfe1 as it is the case with the *mosTA* gene pair in the SXT ICE

[Wozniak and Waldor, 2009] and other TA systems in previously characterized genomic islands [Pandey and Gerdes, 2005].

Accessory or Adaptation Functions

An entire set of 37 putative tRNA genes clustered in a 10-kbp segment was found within ICEAfe1 [Levicán et al., 2009]. All twenty tRNA species are represented in this cluster. Some of the encoded tRNAs have been shown to be functional in aminoacylation [Levicán et al., 2005, and data not shown]. Existence of tRNA genes has been suggested to contribute to better adapt the translation machinery of host bacteria to the expression of genes encoded in integrated mobile genetic elements, particularly in cyanophages [e.g. Bailly-Bechet et al., 2007; Enav et al., 2012]. Whether the presence of tRNA genes in ICEAfe1 alters the proteome of *A. ferrooxidans* is still to be explored. Also a Clustered Regularly Interspaced Short Palindromic Repeat locus (CRISPR) is encoded in ICEAfe1 [Holmes et al., 2009]. This locus is likely to have a role in bacterial immunity against bacteriophages and/or other types of mobile genetic elements [Bhaya et al., 2011]. The genes present in the distal region of ICEAfe1 encode functions that are predicted to be involved in the biosynthesis and export of exopolysaccharides rich in glucose, galactose, mannose and glucuronic acid (EPS/CPS) of the Wzy-dependent class [Cuthbertson et al., 2009]. It corresponds to a gene locus encoding for the EPS/CPS export apparatus, several glycosyltransferases and sugar inter-conversion functions. These genes represent a biosynthesis pathway described previously in *A. ferrooxidans* that could be linked to biofilm formation in this model bacterium [Barreto et al., 2005]. The presence of these genes in ICEAfe1 might contribute to the colonization of new ecological niches upon transfer of this mobile genetic element to other microorganisms [Rohwerder et al., 2003]. How all these genes might contribute to the fitness of *A. ferrooxidans* and other possible recipient microorganisms in both natural and experimental settings are interesting questions requiring further detailed studies.

ICEAfe1 Excises from the Chromosome by a Phage-Type Mechanism

The capacity of ICEAfe1 to excise from its chromosomal location was investigated. Polymerase chain reaction (PCR) primers pairs, A1A2 and A3A4 (fig. 1a; table 2), were designed to amplify *attL* and *attR* sites, respectively, when ICEAfe1 is inserted in the chromosome at the tRNA^{Ala} gene. Primer pair A2 and A3 should render a PCR product only if ICEAfe1 is excised from the chro-

mosome as a circular intermediate. All three PCR products were obtained when the DNA from *A. ferrooxidans* ATCC 23270 was used as template (fig. 1b, top and middle), confirming the existence of the integrated and excised form of the element. The existence of an empty *attB* chromosomal site was evidenced using primers A1 and A4 (fig. 1b, bottom). As expected, in the case of *A. ferrooxidans* ATCC 53993 only the integration target site *attB* was detected using this approach. Sequencing of the obtained PCR products confirmed the identity of all amplicons and evidenced that site-specific recombination, occurring within the 48-bp direct repeats flanking ICEAfe1, gave rise to precisely excised products without indels (data not shown). Primers A1-A2 and A3-A4, as well as 10 other pairs designed to amplify internal sequences were used to detect the presence of ICEAfe1 in 12 other strains from *A. ferrooxidans*. None of them gave a positive signal for the entire ICEAfe1.

Determination of ICEAfe1 excision levels out of the chromosome was carried out by real-time PCR (fig. 2), quantifying the empty *attB* chromosomal site that resulted after the ICEAfe1 excision. The results revealed that when *A. ferrooxidans* was grown to logarithmic phase in medium supplemented with ferrous ion as energy source, there were approximately 0.034 ± 0.001 *attB* sites per 100 chromosomes (fig. 2a). Excised ICEAfe1 was higher (0.12 ± 0.01 *attB* sites per 100 chromosomes) when the bacterium was grown in medium supplemented with sulfur. There were no major differences between phases of growth in both culture media [compare logarithmic (L) vs. stationary (S) in fig. 2a]. However, >300-fold increase in *attB* sites was observed when these cells were treated with mitomycin C (MMC, a DNA damage inducer; 19.34 ± 0.11 vs. 0.06 ± 0.03 *attB* sites in MMC and control cultures, respectively; fig. 2b). These results further suggest that a phage-type mechanism underlies ICEAfe1 site-specific excision and this process might be regulated by the SOS response. Since we detected a higher copy number of *attP* than *attB* sites in all measured conditions (data not shown), we cannot rule out the eventual replication of excised ICEAfe1, as in other ICEs [Lee et al., 2010; Ramsey et al., 2006].

ICEAfe1 Excision and Conjugative Transfer Genes Are Transcriptionally Active

Transcriptional profiling of *A. ferrooxidans* ATCC 23270 using microarray analysis (online suppl. fig. S1) [Quatrini et al., 2009] and qRT-PCR (data not shown) revealed that the genes encoding the integrase, excisionase, *cI*, *Cro* as well as some of the conjugal transfer pro-

Table 2. Bacterial strains and primers used in this study

Bacterial strain or primer	Description or sequence
<i>A. ferrooxidans</i> strains	
ATCC 23270	Type strain
ATCC 53993	Kindly provided by A. Borole (Oak Ridge National Laboratory, USA)
Primers for PCR, qPCR and qRT-PCR	
A1	5' TCGGTGACGTAGAGAAAGC 3'
A2	5' ATTCTGTTTGCTGAGGGTCG 3'
A3	5' CTTCCGGTGGCATGATCTTC 3'
A4	5' CTGGGAGAGCGCTTGAATG 3'
attL1	5' AGGTTTGACATCATCCGCATG 3'
attL2	5' AAGCCAAACGCACTGTAACCA 3'
attR1	5' GTAGATTATGGCTTTGGCAAGC 3'
attR2	5' AGATGCATGCTTTGCGTTTCG 3'
fur1	5' TCAAACCTGCGTAAGCACTCG 3'
fur2	5' TTCGCATTTTTGAGGACAGC 3'
qPCRint.f	5' GAACTCCACGATAGCCTCAAG 3'
qPCRint.r	5' CTCGAAAGTGATCCTGTAGCC 3'
qPCRxis.f	5' CGCAAAATTCCTCAACCTCAG 3'
qPCRxis.r	5' CATTTTGCCTCCGTGTTTCC 3'
qPCRcl.f	5' GGTTTAGGTGATGGTGAGGTAG 3'
qPCRcl.r	5' TTGATGTACGGCTCCATTGAG 3'
qPCRcro1	5' AAATCCTTGGGCGGTCTAC 3'
qPCRcro2	5' AGCTCTTGCCGGGTAATTGA 3'
qPCRtraA.f	5' AACAAACCCCTATCGCCTGAT 3'
qPCRtraA.r	5' AGTTTGACAGAGTGTGCTCGA 3'
qPCRtraG.f	5' TTCTTACTCCGCTTCGAGTG 3'
qPCRtraG.r	5' CTGGCTATTGATCAACGACG 3'
qPCRtraB.f	5' TCTACCAATGGTATTGCGCC 3'
qPCRtraB.r	5' TATTGCTGACCTTCACCGTG 3'
rpoC1	5' AATGCGGTGTTGAGGTAACC 3'
rpoC2	5' AGGTACTGGTCTTCGGTAAG 3'

teins, are transcribed in both logarithmic and stationary cultures grown in the presence of ferrous ion or sulfur as energy source. A marked increase in the level of the mRNA encoding the excisionase upon treatment of the cells with MMC (fig. 3a) is consistent with the elevated excision of ICEAfe1 in this culture condition (fig. 2b). Also, the repression of *cI* and induction of *cro* orthologs in MMC cultures (fig. 3a) are in agreement with a phage-type mechanism for integration/excision of ICEAfe1 [Court et al., 2007]. Similar changes in the expression profiles were obtained for the conjugal transfer genes. Upon MMC treatment of the cell culture, the mRNA level of these genes was enhanced (fig. 3b). These results show that conditions that enhanced the excision of ICEAfe1 also promote the expression of the type IV secretion system encoded within the ICEAfe1 and further suggest that ICEAfe1 may be capable of self-transfer to ade-

Fig. 2. Quantification of ICEAfe1 excision. **a** Excision of ICEAfe1 in logarithmic (L) and stationary (S) growth phases, either with Fe²⁺ or S⁰ as energy source. **b** Effect of MMC treatment on the ICEAfe1 excision.

Fig. 3. Quantification of the relative levels of mRNA for *int*, *xis*, *cI*, *cro* (**a**) and some *tra* genes (**b**). The log₂ ratio of the level of mRNAs from cultures in the presence of MMC versus control cultures is shown. Data were normalized to the expression level of *rpoC* gene. *Int* = Integrase; *xis* = excisionase; *cI* = λ-like repressor; *tra* = conjugative genes: *traA_p*, *traB_F* and *traG_F*.

quate recipient cells. However, these experiments remain a challenge until adequate selectable markers and conjugation protocols are developed for this microorganism.

Concluding Remarks

The data presented in this report evidenced the existence of a remarkably large genetic element in the acidophilic bacterium *A. ferrooxidans* whose genetic organization resembles that of other well-characterized ICEs. This element is active in excision out from the host chromo-

somes both constitutively and upon induction by DNA-damaging agents. The resulting extrachromosomal excision intermediate is most likely relevant for its own dissemination and for the genetic transfer of the functions encoded in it to other potential recipient microorganisms through conjugative transfer. The presence in this element of several toxin-antitoxin modules is predicted to contribute to its maintenance in the cell in spite of active excision and dispersal.

Our findings, and those of others [Andersson and Banfield, 2008; Orellana and Jerez, 2011; Rawlings, 2005], strongly suggest that in the acidic environments where

these organisms exist, there is an active exchange of genetic material between members of the microbial community. The presence in ICEAfe1 of a large number of genes with as yet unassigned function represents a source of novel activities that, once elucidated, might contribute to a better understanding of the requirements of these organisms to adapt to their natural extreme environment. For instance, existence of a cluster of tRNA genes might contribute to the efficient translation of the genes encoded in the ICEAfe1, as has been described in bacteriophages [Bailly-Bechet et al., 2007], and the existence of a CRISPR system might be advantageous to defend the organism against viral infections [Holmes et al., 2009]. The presence in the ICEAfe1 of a number of genes predicted to encode proteins involved in the synthesis of exopolysaccharides that contribute to the biofilm formation, could enhance the persistence of these microorganisms in the environment and/or help to improve mineral dissolution [Rohwerder et al., 2003].

These and other potential advantages for cells carrying ICEAfe1 and the elucidation of environmental conditions that enhance the excision and transfer of this element are relevant topics to be addressed to improve our understanding of the physiology and ecology of these industrially and environmentally relevant microorganisms.

Experimental Procedures

Nucleotide Sequence Data Analysis

Completely sequenced genomes and predicted proteomes of *A. ferrooxidans* ATCC 23270 (GenBank CP001219) and ATCC 53993 (GenBank CP001132) were obtained from the NCBI website (<http://www.ncbi.nlm.nih.gov>). To identify exclusive regions in the genome of either strain, nucleotide sequences were cross-compared using ACT [Carver et al., 2005]. The following bioinformatic resources were used to further characterize candidate genes (and the predicted protein products) found within ICEAfe1: BLASTp and PSI-BLAST [Altschul et al., 1997], the suite of protein comparison and classification programs available in InterProScan [Mulder and Apweiler, 2007], the COG database [Tatusov et al., 2000] and the POG database [Kristensen et al., 2011]. Categorizations were made using Perl scripts and manual revision. Putative ICE sequence was searched for tRNAs using tRNAscan-SE [Schattner et al., 2005] and ARAGORN [Laslett and Canback, 2004] and CRISPRs using CRISPI [Rousseau et al., 2009], CRISPR Finder [Grissa et al., 2007] and CRT1 CRISPR Recognition Tool version 1.1 [Bland et al., 2007] which are available online. Transposases were predicted using TnpPred (<http://www.mobilomics.cl>).

Potential transmembrane regions and putative sub-cellular localization were predicted using PSORTB (<http://www.psort.org/psort/>) [Yu et al., 2010] and TMHMM (<http://www.cbs.dtu.dk/services/TMHMM/>) [Krogh et al., 2001].

Bacterial Strains and Growth Conditions

Type strain *A. ferrooxidans* ATCC 23270 and *A. ferrooxidans* ATCC 53993 were grown at 30°C in modified 9K medium [Silverman and Lundgren, 1959], pH 1.6, supplemented with 120 mM FeSO₄ × 7H₂O or in modified 9K medium, pH 3.5, supplemented with 1% S⁰.

Mitomycin C Treatment

Late exponential-phase cells grown in modified 9K medium supplemented with sulfur were collected by centrifugation, the pellet was divided in two parts and each one resuspended in a half of the original volume with modified 9K medium, pH 3.5, containing 1% S⁰. One culture served as the control and the other was treated with 2 µg/ml mitomycin C (Sigma). Both cultures were incubated for 27 h at 30°C with agitation. After that time, genomic DNA and RNA were extracted as describe below.

Isolation and Purification of Nucleic Acids

DNA from *A. ferrooxidans* was isolated using Wizard Genomic DNA Purification Kit (Promega) with the following modification for cell lysis: bacterial pellet was resuspended in Nucleic Lysis Solution, frozen at -80°C for 10 min and immediately heated at 80°C for 10 min. This procedure was repeated three times and then allowed to cool down to room temperature. The rest of the lysis protocol was performed as recommended by the manufacturer. RNA was isolated using RNeasy Mini Kit (Qiagen) from bacterial pellet previously stored at 4°C in RNeasy Lysis Buffer (Qiagen). The removal of genomic DNA from RNA preparations was carried out by digestion with DNase I (Fermentas). DNA and RNA quality was evaluated by 1.0% agarose gel electrophoresis and its concentration was measured by the absorbance at 260 nm in an Epoch Microplate Spectrophotometer (BioTek).

Polymerase Chain Reaction

Amplification of sequences to detect excision of ICEAfe1 from the chromosome was performed using PfuUltra II Fusion HS DNA Polymerase (Stratagene) according to the protocol provided by the manufacturer. The cycling conditions were as follows: initial denaturation (95°C, 2 min); 30 cycles consisting of denaturation (95°C, 20 s), primer annealing [TA (at the estimated primer annealing temperature), 20 s], and extension (72°C, 15 s/kbp); followed by a final extension step (72°C, 5 min). PCR products were visualized on 2% agarose gels stained with ethidium bromide. PCR products containing *attL*, *attR*, *attP* and *attB* sites were purified with SpinPrep™ Gel DNA Kit (Novagen) and sequenced by Macrogen, USA.

Amplification of DNA to generate the standard curve for qPCR was performed using Paq5000 DNA Polymerase (Stratagene) according to the protocol provided by the manufacturer. The cycling conditions were the same as before, except that the extension step in each cycle was 30 s/kbp. PCR products were purified using QIAquick PCR Purification Kit (Qiagen) and quantified by the absorbance at 260 nm in an Epoch Microplate Spectrophotometer (BioTek).

Oligonucleotides used in this study for PCR and real-time PCR are listed in table 2.

cDNA Synthesis

The first strand of cDNA to be used in the two-step qRT-PCR was prepared from 1 µg of total DNA-free RNA using random

hexamer primers and RevertAid™ Reverse Transcriptase (Fermentas) according to instructions provided by the manufacturer.

Real-Time Quantitative PCR and RT-PCR Assays

The reactions were performed in the Rotor-Gene Q PCR System (Qiagen) using the Rotor-Gene SYBR Green PCR Kit (Qiagen) and the Rotor-Gene 6000 series Software 1.7 for data analysis. Each reaction was carried out in 12.5-μl volumes containing 1× Rotor-Gene SYBR Green Master Mix, 500 nM of each oligonucleotide and 10 ng of DNA or 1 μl of 1:10 diluted cDNA sample as templates. The cycling protocol was as follows: initial activation step (95°C, 5 min) and 35 cycles of denaturation (95°C, 5 s) and combined annealing/extension (60°C, 10 s). The fluorescence data collection was performed in this last step. The PCR products were subjected to a melting curve analysis between 50 and 99°C; specific amplification was confirmed by a single peak in the melting curve. Reaction efficiency ($E = (10^{-1/\text{slope}}) - 1$) for every gene was derived from the slope of the corresponding 6-point standard curve with 10-fold dilutions of corresponding PCR amplicons for qPCR or total DNA from *A. ferrooxidans* ATCC 23270 for qRT-

PCR. The amounts of *attB* were normalized to the amount of chromosomal DNA in each sample by amplifying the *fur* gene, a chromosomal gene unaffected by ICEAfe1 excision. The amount of *int*, *xis*, *cI*, *cro* and *tra* transcripts were normalized to the amount of *rpoC* as reference genes [Nieto et al., 2009] in each sample. In each experimental condition, the reactions were carried out in duplicate in the same run from samples obtained from three independent cultures.

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