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*

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The *alaS* gene of *Thiobacillus ferrooxidans* has been cloned and sequenced and its expression in *Escherichia coli* and *T. ferrooxidans* analysed. The same genomic organization to that in *E. coli* (*recA-recX-alaS*) has been found in *T. ferrooxidans*. The *recA* and *alaS* genes cannot be transcribed from their own promoters in *E. coli*. In addition to the well-known homology at the protein level between AlaS proteins from various organisms, a strong homology was found between all the known *alaS* genes from bacteria, archaea and eucarya. Two regions, one of which corresponds to the catalytic core, are particularly well-conserved at the nucleotide sequence level, a possible indication of strong constraints during evolution on these parts of the genes.

Keywords: *Thiobacillus ferrooxidans*, alanyl-tRNA synthetase, RecX

INTRODUCTION

Comparative analysis of primordial proteins arisen early in evolution and study of their genes could shed some light on the organization and evolution of the genome in different organisms. Cairns-Smith *et al.* (1992) have proposed that the origin of life was mineral and it is generally agreed that the *Thiobacillus ferrooxidans* way of life is one of the most primitive. To grow this bacterium, which is well-adapted to extremely low-pH environments, only requires air, which provides carbon and nitrogen, and ferrous iron or reduced sulfur compounds from pyrite as an energy source (reviewed by Leduc & Ferroni, 1994). *T. ferrooxidans* is one of the main micro-organisms involved in bioleaching (Temple & Colmer, 1951), that is the solubilization of metals from minerals. Because of its unusual growth requirements, and despite its industrial importance, our current knowledge of the physiology, biochemistry and genetics of *T. ferrooxidans* is still very poor. Therefore, when we

unexpectedly obtained part of the alanyl-tRNA synthetase gene of *T. ferrooxidans* ATCC 33020, we decided to pursue its study because (i) aminoacyl-tRNA synthetases are key enzymes which ensure fidelity of protein biosynthesis by specifically charging tRNAs with their cognate amino acids, and they are supposed to have co-evolved with tRNAs since the origin of life (Nagel & Doolittle, 1991; Schimmel *et al.*, 1993); (ii) even though aminoacyl-tRNA synthetases are diverse in subunit composition, polypeptide size and amino acid sequence, synthetases specific for one given amino acid, but from diverse organisms, are more similar to each other than they are to enzymes specific for any other amino acid in the same organism. This supports the idea that these enzymes appeared in evolution before the divergence of the different organism groups; (iii) according to Schimmel (1991), the class-defining conserved domain of the alanyl-tRNA synthetase may reflect the primordial synthetase because this enzyme makes no contact with the tRNA anticodon (Schimmel, 1990). Instead, recognition is concentrated on the amino acid acceptor helix and is centred on a single base pair, G3-U70 (Schimmel, 1990).

Study of alanyl-tRNA synthetase from *T. ferrooxidans*

Abbreviation: RT-PCR, reverse transcription-PCR.

The EMBL/GenBank accession number for the sequence reported in this paper is X95571.

is therefore of evolutionary interest. In this paper, the *T. ferrooxidans* *alaS* gene has been sequenced and compared to other alanyl-tRNA synthetase genes. Its expression in *Escherichia coli* and *T. ferrooxidans* has also been analysed.

METHODS

Strains, plasmids and growth conditions. *T. ferrooxidans* ATCC 33020 was obtained from the American Type Culture Collection. Initial screening of the recombinant *T. ferrooxidans* chromosomal library, the propagation of cosmids and complementation analysis of the *recA* mutant were performed in *E. coli* HB101 [Δ (*gpt-proA*)62 *leuB6 thi-1 lacY1 hsdB20 recA rpsL20 ara-14 galK2 xyl-5 mtl-1 supE44 mcrB_B*]. TG1 [*supE hsdΔ5 thi Δ(lac-proAB)*, F': *traD36 proAB⁺ lacI^q lacZΔM15*] was used for phagemid propagation. AB4132 (*alaS4 thi-1 metC56 lacY1 galK2 xyl-5* or *xyl-7 ara-14 tfr-15 tsx-57 supE44*) was used for the complementation analysis of *alaS*.

The phagemid pBluescript (Stratagene) was used for preparation of clones for sequencing.

E. coli growth conditions were as described in Miller (1992). For analysing the *recA* phenotype, methyl methanesulfonate (MMS) was used at 0.01% (w/v). *T. ferrooxidans* growth conditions were as published previously (Guiliani *et al.*, 1993).

General DNA techniques. General DNA techniques were performed according to Ausubel *et al.* (1991). Ultrapure cosmid DNA was prepared using Qiagen plasmid kits. Digestions with restriction endonucleases were carried out as recommended by the manufacturer. DNA fragment blunt-ending and ligation were performed with the DNA blunting kit from Amersham. Preparation of competent *E. coli* and transformation were performed according to Chun *et al.* (1989).

Colony and Southern hybridization with degenerate oligonucleotides. Two oligonucleotides were designed from the amino acid sequences KDGFY and FDITKK (amino acids 115–121 and 87–93, respectively) present in the three rusticyanins characterized from three different strains of *T. ferrooxidans*. The sequence of the first oligonucleotide, Rus115, was 5'-TARCCRAAYTTTRCCRTCYTT-3' (64-fold degeneracy), the third nucleotide of each codon being changed according to the codon usage of *T. ferrooxidans* (Rawlings *et al.*, 1991). The second oligonucleotide, Rus87, was 5'-CCYTTYTTNGTDAITRCRAA-3' (192-fold degeneracy). Rus115 and Rus87 were purchased from Appligene. Rus115 was labelled with [γ -³²P]ATP (Ausubel *et al.*, 1991) and used for colony hybridization under stringent conditions (Ausubel *et al.*, 1991) of *E. coli* HB101 colonies transfected with a *T. ferrooxidans* ATCC 33020(pHC79) cosmid library kindly provided by D. E. Rawlings, University of Cape Town, South Africa.

Southern hybridization experiments were performed on cosmid DNA cleaved with different restriction enzymes. After electrophoresis, the DNA was denatured and transferred to a positively charged nylon membrane (Hybond-N+ from Amersham) by the semi-dry capillary method (Sambrook *et al.*, 1989). Prehybridization and hybridization were carried out under stringent conditions. Non-radioactive oligonucleotide labelling of Rus115 and detection were performed with the ECL 3'-oligolabelling system and ECL detection reagents purchased from Amersham.

DNA sequencing. Both strands of the 1.9 kb *Pst*I and of the 3.5 kb *Hind*III-*Kpn*I DNA fragments were sequenced after convenient subcloning of overlapping fragments had been performed, and nested deletions generated by combined *Exo*III/*Exo*VII digestions as previously described (Blasco *et al.*, 1989). When necessary, DNA fragments were amplified by PCR between two convergent oligonucleotides designed from the known nucleotide sequence, and were either directly sequenced according to Salles *et al.* (1992), or subcloned in pBluescript KS or SK(+) or (−) and sequenced. The dideoxy chain-termination method was used to sequence DNA using [³⁵S]dATPαS and the T7 Sequencing kit (Pharmacia).

PCR reactions were performed with *Taq* polymerase according to the manufacturer's recommendations in a Minicycler (MG research). All synthetic oligonucleotides for PCR and sequencing were purchased from Genset.

The DNA sequences were compiled, analysed and compared with the EMBL database sequences with the UWGCG package (Devereux *et al.*, 1984), CLUSTAL, BLAST (Altschul *et al.*, 1990) or BioScan programs through the WWW Nestcape facilities. RNA secondary structures were analysed with the RNAFOLD and SQUIGGLS programs in the UWGCG package (Devereux *et al.*, 1984).

RNA preparation. *T. ferrooxidans* total RNA was prepared from a 500 ml culture grown on FeSO₄ medium by a slightly modified hot phenol method (Aiba *et al.*, 1981). After washing the cells with 9K basal salts, the pellet was resuspended in 500 μl 0.02M sodium acetate (pH 5.5), 0.5% SDS, 1 mM EDTA. The lysed cells were extracted twice at 60 °C with phenol equilibrated with 0.02 M sodium acetate (pH 5.5), 0.5% SDS, 1 mM EDTA. RNA was precipitated with ethanol and resuspended in 200 μl 10 mM Tris (pH 8). DNA-free RNA was then obtained with the High Pure RNA isolation kit from Boehringer, omitting the lysozyme step.

Reverse transcription-PCR (RT-PCR). RT-PCR was performed with the Promega Access system. One microgram of total RNA from *T. ferrooxidans* was denatured at 94 °C for 2 min in the presence of both oligonucleotides. Immediately, reverse transcription and PCR amplification were carried out according to Promega recommendations with the following program: 48 °C for 1 h; 94 °C for 5 min; 30, 45 or 60 cycles of 45 s at 94 °C, 30 s at 62 °C, 30 s at 72 °C; 72 °C for 3 min. Oligonucleotides used were: *alaS*21, 5'-CGTTGTGTTTGCACCCGC-3'; *alaS*33, 5'-TCGTGCCCTCCAGCCCCC-3'; RA3, 5'-GCCGAAGAGGTGGCAGAGC-3'; RA5, 5'-GCCGGGCATAATCTCTGGCG-3'; RX1, 5'-GATTGACAGCGGGTGAGGGG-3'.

For each RT-PCR experiment, three controls were performed: one without template as a negative control to detect any contamination, one with genomic DNA as a control for PCR amplification, one with RNA but without reverse transcriptase to check that there were no DNA traces in the RNA preparation. In each case, PCR was performed for 30 and 45 cycles, and when no amplification product was detected, for 60 cycles.

RESULTS

Molecular cloning of the *T. ferrooxidans* *alaS* region

Two degenerate oligonucleotides, Rus115 and Rus87, designed on the basis of two highly conserved segments in rusticyanins were used to screen a *T. ferro-*

oxidans(pHC79) cosmid library. Out of 1100 colonies tested, 19 gave a positive signal with Rus115 under stringent conditions. They also responded positively with the second oligonucleotide, Rus87. The cosmid DNA was prepared from each clone, digested with *EcoRI* and probed with Rus115. All contained only one fragment which responded positively, the size of the fragment ranging from 8 to 18 kb (data not shown). When digested with other restriction enzymes, 15 cosmids were found to contain a single 1.9 kb *PstI* fragment hybridizing with Rus115, the other four containing a 2 kb *PstI* fragment (data not shown).

Therefore, the 1.9 kb *PstI* DNA fragment of one of these cosmids, pNG4, was cloned in pBluescript SK(+) and sequenced. No ORF corresponding to the rusticyanin protein could be found. On the contrary, comparison of the nucleotide sequence with the EMBL database sequences, revealed the 3' end of the *recA* gene, previously sequenced by Ramesar *et al.* (1988, 1989), and the 5' end of a previously uncharacterized gene showing a high degree of similarity with the nucleotide sequence of the *alaS* genes of *E. coli* (67.5% identity) (Putney *et al.*, 1981), *Sinorhizobium* (*Rhizobium*) *meliloti* (68%) and *R. leguminosarum* biovar *viciae* (68%) (Selbitschka *et al.*, 1991). The AlaS protein is an alanyl-tRNA synthetase which catalyses the specific attachment of alanine to its cognate tRNA. Since nearly all the 19 different cosmids contained the same 1.9 kb *PstI* fragment, we can exclude joining of two different non-contiguous fragments during library construction. This was confirmed by PCR amplification of *T. ferrooxidans* DNA with oligonucleotides corresponding to *recA* and *alaS* (see Fig. 4).

Expression of the *recA* and *alaS* genes in *E. coli*

To confirm that we had indeed cloned a *T. ferrooxidans* *alaS* gene, complementation studies were carried out using two different mutants of *E. coli*: a *recA* strain and an *alaS*^{ts} strain. The former is sensitive to UV irradiation and to MMS treatment while the latter, which carries an allele encoding a thermosensitive AlaS protein, can grow at 30 °C but fails to develop at 42 °C. Surprisingly, while nearly all of the cosmids contained the same *PstI* fragment, only cosmid pNG12 could restore (i) resistance of the *recA* mutant to UV or MMS and (ii) growth of the *E. coli* *alaS*^{ts} mutant at 42 °C. This result showed that a *T. ferrooxidans* *alaS* gene is present on pNG12 and indicated that it can be transcribed and translated into a functional AlaS protein in *E. coli*.

The restriction analysis of the three cosmids, pNG3, 4 and 12, indicated that they all carried both *recA* and *alaS* genes (Fig. 1) even though only pNG12 complemented the *E. coli* *recA* and *alaS* strains. By comparison of the restriction maps of pNG4 and of the *recA* region (Ramesar *et al.*, 1989), and accounting for the size of the *E. coli* AlaS protein, it was deduced that the *recA* and *alaS* genes should be located on a 5 kb *KpnI* fragment. When this fragment was cloned from pNG4 into pUC19

Fig. 1. Restriction maps of the *recA* region (Ramesar *et al.*, 1988, 1989), and pNG3, pNG4 and pNG12. The phenotype conferred by these cosmids upon *recA* and *alaS* mutants of *E. coli* is indicated on the top right. The limits of the *recA* gene have been deduced from the *recA* sequence (Ramesar *et al.*, 1989). Boxes represent pHC79. The direction of transcription from the *tet* promoter (*Ptet*) is shown below pHC79. B, E, H, K, P and V represent *Bgl*II, *Eco*RI, *Hind*III, *Kpn*I, *Pst*I and *Eco*RV restriction sites. Arrows under pNG3, 4 and 12 indicate the restriction fragments hybridizing with the Rus115 oligonucleotide.

to give pNG22, the *T. ferrooxidans* *recA* gene restored UV and MMS resistance to an *E. coli* *recA* mutant, but only partial complementation of the *E. coli* *alaS*^{ts} mutant was observed. Ramesar *et al.* (1989) reported that even when preceded by the upstream 2.2 kb, the expression of the *T. ferrooxidans* *recA* gene in *E. coli* could only be achieved from a vector-borne promoter. On cosmid pNG12, 2 kb of the *recA* upstream region separated the gene from the *Ptet* promoter carried by the cosmid vector, while 0.5 kb of the same region is present between the gene and the *Plac* promoter on plasmid pNG22. All these results suggest that the *recA* and *alaS* promoters either are located more than 2.2 kb upstream from *recA*, or are not recognized by the *E. coli* RNA polymerase.

Among the 19 cosmids analysed, one would expect that some cosmids would have, upstream from *recA* gene, a region larger than 2.2 kb likely to carry this promoter. Indeed, the two cosmids, pNG3 and 4, have been shown by restriction mapping to carry more than 25 kb upstream from *recA* (Fig. 1). In pNG4, the genes are in the same orientation as the vector-borne promoter *Ptet*, while in pNG3, they are in the opposite orientation (Fig. 1). Internal deletions of pNG4 were generated by partial *Eco*RV digestion and recircularization to place these genes at 1.3 kb from *Ptet* (Fig. 1). As expected, complementation of *E. coli* *recA* and *alaS* mutants was observed, indicating that the genes were functional in

Fig. 2. Schematic representation of *alaS* genes from *T. ferrooxidans* (Tf), *R. leguminosarum* (Rl), *Sin. meliloti* (Rm), *E. coli* (Ec), *Haem. influenzae* (Hi), *M. capricolum* (Mc), *M. genitalium* (Mg), *C. elegans* (Ce), *A. thaliana* (At), *B. mori* (Bm), *S. cerevisiae* (Sc), *H. sapiens* (Hs) and *Sulfolobus acidocaldarius* (Sa). Vertical numbers above the line represent positions in the *T. ferrooxidans* AlaS amino acid sequence. Vertical numbers below the line correspond to the position in the AlaS protein of the organism indicated. Where *alaS* has been partly sequenced, the size of the determined sequence is shown in parentheses. Horizontal numbers represent the percentage identity between regions of the *alaS* genes from *T. ferrooxidans* and from the organism indicated. The highly conserved regions are shown in bold lines and are noted above the *T. ferrooxidans* *alaS* gene with the class-defining motifs indicated in parentheses.

cosmid pNG4 but were not transcribed. All these results strongly suggest that the *recA* and *alaS* promoters are not recognized by the *E. coli* RNA polymerase.

Analysis of the *T. ferrooxidans* *alaS* gene

The 3' end of *alaS* is likely located on the 3.2 kb *HindIII*-*KpnI* DNA fragment which overlaps the 1.9 kb *PstI* fragment already sequenced. This fragment was blunt-ended with T4 polymerase and cloned into the *SmaI* site of pBluescript SK(-), and the nucleotide sequence determined.

A large ORF of 2631 bp, positions 672–3302, encoding a putative 877 amino acid polypeptide is present on the same strand as *recA*. This 95.5 kDa protein shares a high degree of sequence identity with the (partial) alanyl-tRNA synthetase not only from *E. coli* (57.4%) (Putney

et al., 1981), *Rhizobium leguminosarum* (65.5%) (Selbitschka *et al.*, 1991), *Sin. meliloti* (66.2%) (Selbitschka *et al.*, 1991), *Haemophilus influenzae* (53.8%) (Fleischmann *et al.*, 1995) but also from organisms as distantly related as *Mycoplasma capricolum* (39.9%) (P. Bork and others, unpublished results; P. Gillevet and others, unpublished results), *Mycoplasma genitalium* (33.1%) (Fraser *et al.*, 1995), *Sulfolobus acidocaldarius* (39.2%) (Ramirez *et al.*, 1994), *Arabidopsis thaliana* (37.9%) (H. Mireau and others, unpublished results), *Saccharomyces cerevisiae* (39.5%) (Ripmaster *et al.*, 1995), *Bombyx mori* (36.6%) (Chang & Dignam, 1990), *Caenorhabditis elegans* (33.5%) (Wilson *et al.*, 1994) and *Homo sapiens* (38.7%) (Shiba *et al.*, 1995). The identity is not restricted to those regions including the residues shown to be important for enzyme activity in *E. coli* (see below), but occurs along the full length of the proteins. Some similarity is also

found at the nucleotide sequence level (Fig. 2 and Discussion).

As with other alanyl-tRNA synthetases, the *T. ferrooxidans* enzyme, TfAlaS, contains the conserved class II motifs, which are localized in the amino terminal half of the primary structure (reviewed by Schimmel, 1990; Eriani *et al.*, 1990; Ribas de Pouplana *et al.*, 1993; Lu & Hill, 1994; Davis *et al.*, 1994). In each motif, the invariant residue is present as well as several semi-invariant residues of the strongly conserved core. In addition to the class-II-defining motifs, TfAlaS contains the zinc-binding 'cysteine-histidine box' which was shown to be important for tRNA recognition in EcAlaS, the *E. coli* enzyme (Miller *et al.*, 1991; Miller & Schimmel, 1992; Wu *et al.*, 1994). Furthermore, the residues important for the catalytic activity of EcAlaS, those required for tRNA recognition (G174, A410), adenylate transfer to the 3' end of tRNA^{ala} (D235) and the cognate tRNA aminoacylation (K74, C665, G674, G677) are also present in TfAlaS (Schimmel, 1990; Miller *et al.*, 1991; Filley & Hill, 1993; Wu *et al.*, 1994; Shi *et al.*, 1994). Note that residues C665, G674 and G677 are not located in the catalytic core of the enzyme but in its 'dispensable' part (Schimmel, 1990; see Discussion).

The overall G + C content of the *T. ferrooxidans* *alaS* (62.3 mol %) gene does not differ significantly from other *T. ferrooxidans* ATCC 33020 genes (59 mol %) and the codon usage is typical for ATCC 33020. The G + C content in the codon third position is 71.7 mol % in agreement with other *T. ferrooxidans* genes (74.3 mol %). A possible ribosome-binding site reading AcGGAaG, at positions 661–667, is present 4 bp upstream from the *alaS* putative translational initiation codon but no obvious σ^{70} promoter consensus sequence is apparent in the 400 bp region upstream from the *alaS* ORF. In *E. coli*, a palindromic sequence involved in the autogenous repression of the gene is located upstream from the initiation codon of the *alaS* gene (Putney & Schimmel, 1981). No such sequence can be found upstream from the *T. ferrooxidans* *alaS*. However, this region contains several indirect repeats which are potentially capable of forming stem-loop structures. Finally, no characteristic rho-independent transcription termination site is present at the 3' end of *alaS*, but three pairs of complementary inverted repeats centred at position 3330.5 ($\Delta G = -23.5 \text{ kcal mol}^{-1} / -98.3 \text{ kJ mol}^{-1}$), 3361 ($\Delta G = -17.8 \text{ kcal mol}^{-1} / -74.5 \text{ kJ mol}^{-1}$) and 3365.5 ($\Delta G = -7 \text{ kcal mol}^{-1} / -29.2 \text{ kJ mol}^{-1}$) lie downstream of the TAG termination codon and could be a rho-dependent transcription terminator.

Analysis of the *T. ferrooxidans* *recX* gene

Downstream of the *recA* gene, De Mot *et al.* (1994) reported the existence of a putative regulatory gene, *recX*, conserved in some Gram-negative and -positive bacteria. Using the *T. ferrooxidans* *recA* sequence determined by Ramesar *et al.* (1989), De Mot *et al.*

(1994) found the 5' end of an ORF which overlaps the *recA* gene and which encodes a putative polypeptide presenting some homology with RecX.

When determining the sequence downstream of *recA*, we corrected the sequence determined by Ramesar *et al.* (1989); therefore, we have re-examined the potential ORFs in this region. Two potential coding regions, ORF1 and ORF2 are located between *recA* and *alaS* at position 211–609 and 330–599, respectively. None corresponds to that proposed by De Mot *et al.* (1994) deduced from the Ramesar *et al.* (1989) nucleotide sequence. Analysis of the codon usage and G + C content in the codon third position of these two ORFs indicated that both could correspond to typical *T. ferrooxidans* genes. A search for homologous polypeptides in the protein databases, gave no positive match for the putative polypeptide encoded by ORF1, but revealed a significant similarity between the polypeptide encoded by ORF2 and the *Pseudomonas aeruginosa* (44.3% identity) (Sano, 1993) and *Pseudomonas fluorescens* (37.9%) (De Mot *et al.*, 1994) RecX proteins and the *E. coli* OraA protein (35%) (Zaitsev *et al.*, 1994). Realignment of (partial) RecX sequences from different Gram-negative and -positive bacteria (*Azotobacter vinelandii*, *Legionella pneumophila*, *Methylobacterium clara*, *Mycobacterium leprae*, *Pseudomonas putida* and *Yersinia pestis*) with the putative protein corresponding to *T. ferrooxidans* ORF2 also shows some significant similarities. Furthermore, the putative ORF2-encoded polypeptide is quite basic with a high proportion of arginine residues and has a calculated pI of 11.36, features previously noticed for RecX proteins. All of these data suggest that ORF2 is the *recX* homologue of *T. ferrooxidans*.

Expression of *recA*, *recX* and *alaS* in *T. ferrooxidans*

To determine if the *alaS* gene is transcribed as a single transcription unit or is part of a larger transcription unit including the *recA* and *recX* genes, we have investigated its expression in *T. ferrooxidans*. Total RNA was isolated from *T. ferrooxidans* cells grown on ferrous iron or sulfur medium, and the *alaS* specific mRNAs were analysed by Northern blotting with different non-radioactive DNA or RNA probes labelled with DIG-dUTP or DIG-UTP, respectively. In all cases, only two bands corresponding to ribosomal RNA were detected (data not shown). A similar result was obtained with the only other *T. ferrooxidans* aminoacyl-tRNA synthetase gene characterized, *tyrZ*, encoding tyrosyl-tRNA synthetase (O. Salazar, J. Salazar, E. Jedlicki & O. Orellana, unpublished results). This could suggest that the *alaS* gene is expressed so weakly that its transcript cannot be detected.

To determine whether the *alaS* gene is transcribed in *T. ferrooxidans*, RT-PCR was used with oligonucleotides *alaS*21 and *alaS*33 that hybridize at the beginning of the *alaS* coding region. As can be seen in Fig. 3(a), an amplification product of the expected size was obtained

Fig. 3. Analysis of *alaS* and *recX* transcription in *T. ferrooxidans* ATCC 33020 by RT-PCR. *alaS* and *recX* mRNA were detected by reverse transcription from 1 µg *T. ferrooxidans* total RNA and PCR amplification with the following oligonucleotides: (a) *alaS*33 and *alaS*21 (45 amplification cycles; 3% agarose gel); (b) RA3 and *alaS*21 (45 cycles; 1.5% agarose); (c) RX1 and *alaS*21 (60 cycles; 3% agarose); (d) RA3 and RA5 (45 cycles; 2% agarose). Locations of the different oligonucleotides relative to the *recA*, *recX* and *alaS* ORFs are shown. Lanes: 1, no template; 2, chromosomal DNA from ATCC 33020 strain as template; 3, total RNA from ATCC 33020 strain as template; 4, total RNA from ATCC 33020 strain as template but without reverse transcriptase; 5, DNA molecular mass markers (Boehringer).

showing unambiguously that the *alaS* gene is expressed in *T. ferrooxidans*.

In *E. coli*, expression of the *alaS* and *recA* genes, as judged by complementation of *recA* and *alaS*^{ts} mutants, can only be detected when the genes are expressed from a vector-borne promoter. To determine whether *recA*, *recX* and *alaS* are in a single transcription unit, we have looked for transcripts extending from *recA*, or *recX*, to *alaS* in *T. ferrooxidans* by RT-PCR. No amplification product could be detected even after 60 cycles of amplification between the *alaS*21 and RA3 or RX1 primers located at the beginning of *alaS* coding region and at the end of the *recA* and *recX* ORFs, respectively (Fig. 3b, c). The absence of transcripts extending from the end of the *recX* coding region to the *alaS* gene indicates that the *T. ferrooxidans alaS* gene is transcribed independently from *recX*, and therefore from *recA*, and that the *alaS* transcriptional start site is located in the *recX-alaS* intergenic region.

The existence of the *recX* gene has been deduced from comparisons of *recA* downstream regions from various micro-organisms (De Mot *et al.*, 1994). Furthermore, it has been shown that the *recA* gene from some of these bacteria can be cloned in a *recA* mutant of *E. coli* only if *recX* is also present on the insert (De Mot *et al.*, 1994). However, until now, there has been no proof that *recX* is transcribed. To address this question, RT-PCR was performed with the oligonucleotides RA3 and RA5 hybridizing to the end of the *recA* coding region and within the *recX* ORF, respectively. As shown in Fig. 3(d), an amplification product of the expected size was

obtained, indicating that *recX* is transcribed with *recA* in *T. ferrooxidans*.

DISCUSSION

While trying to clone the *rus* gene from *T. ferrooxidans*, we also inadvertently cloned its *alaS* gene. The hybridization experiments were repeated with the Rus115 probe after digestion of three cosmid DNAs (pNG3, 4 and 12) with various restriction enzymes (data not shown), and the Rus115 probe was deduced to hybridize with a 0.18 kb *Pst*I–*Hind*III fragment internal to the *Pst*I–*Pst*I fragment described above (Fig. 1). In fact, when the Rus115 oligonucleotide sequences were compared to that of this fragment, some of them were found to be partially complementary. As all experiments had been performed under stringent conditions, this result is clearly due to the degeneracy of the oligonucleotides used when screening the *T. ferrooxidans* genomic library.

The identification of the *T. ferrooxidans* cloned gene as encoding an alanyl-tRNA synthetase was unambiguously confirmed by the genetic complementation study which indicated that the *T. ferrooxidans* AlaS (TfAlaS) can substitute for the *E. coli* AlaS (EcAlaS). In addition, the primary structure of TfAlaS deduced from the nucleotide sequence is similar to that of all known alanyl-tRNA synthetases. This is not surprising since tRNA^{ala} are very well conserved, in particular the *T. ferrooxidans* tRNA^{ala} has a high degree of similarity to those from *E. coli* (82.9%) and *Bacillus subtilis* (84.2%) (Venegas *et al.*, 1988). Particular attention has been paid to the region encompassing residues 699–808 predicted

<i>T. ferrooxidans</i>	700	TGAALEATQRDEER-----LQAAAGLLKVP-----	AEI
<i>E. coli</i>	699	TGGAATVHDSDR-----LSVAHLLKGS-----	NNI
<i>H. influenzae</i>	698	TGGAIDLCNQRI-----LTQADILLSDV-----	NTL
<i>M. genitalium</i>	705	SSNFTNNYLKAKNQLTQLKSELKVLSTLDSI-----	FKV
<i>B. mori</i>	757	TGPAATKAIKLVLEN-----EVNNVAFIKQNESISHKEVSKKIVDLTNEISQAQISYWKDEL	
<i>H. sapiens</i>	756	TGAEAQKALRKAESELRK-----CLSVMEAKVKAQT--APKKDVQREIADLGEALATAVIPQWQDEL	
<i>S. cerevisiae</i>	752	TGTEAFEAQRLAEQFAA-----DLDAADKLPSPK-----EK-KLKEGLVKQLSISVITKNEL	
<i>A. thaliana</i>	750	TTECAFALNAASLLER-----EVEDASRAEGSAL-----EKVSALKSRVDAITPAKKADI	
<i>C. elegans</i>	781	TGKAAMCRN-----Y-----	

<i>T. ferrooxidans</i>	DQRLAQTLERLPQLEKELEKVRDEAVRAGAGLAAEAEVDGVPVLRIRLQMGDKALRDA	790
<i>E. coli</i>	ADKVRSVLERTRQLSEKELQQLKEQAAQESANLSSKATDVNGVKLVSELSGVEPKMLRTM	789
<i>H. influenzae</i>	AEKIQQLQDKAKKVEKELQQLKEAAMQAGSYFVKSARKINGSVIAQQLDGIETKSLRVM	788
<i>M. genitalium</i>	ELKELQQLRDKFLLPEKITQLRDASDTLLAKNDINQLKTKNKKVSOQALALSIKKQLSL	803
<i>B. mori</i>	RNMLGNKKQLDDKRAKALIIITQVTERAKELCLERKE--SKYIVSELKAFGNTKALDGA	877
<i>H. sapiens</i>	RETLASLKQYMDLDRASADQVRKRLKPTQSDNPN--QPLVILESGASAKALNEA	874
<i>S. cerevisiae</i>	KQKFNKIKKAVDEKVRANKENKQTLDEKVTPLQTNEN--APYLVKFIDISPNAKAITEA	865
<i>A. thaliana</i>	RTKIASIQNEVRKQKQKIAEQNLKSKVLAETEAESAASDQKTFCTIIQLDVLGDAVAQEA	864
<i>C. elegans</i>	TKSIYDLRSTD--PKERSK-----TKNNIMKRVPIADQARISIL	830

Fig. 4. CLUSTAL alignment of the putative oligomerization domain of the different alanyl-tRNA synthetases known. Gaps are indicated by dashes. The position of the amino acids in the AlaS enzymes are indicated. AlaS known to be monomeric and multimeric are denoted respectively by '*' and '*''.

to be the oligomerization domain of EcAlaS (Jasin *et al.*, 1983). As can be seen in Fig. 4, two main subgroups can be distinguished by the presence or the absence of a segment of about 30 amino acids. Interestingly, EcAlaS, which is tetrameric, does not belong to the group including *S. cerevisiae*, *B. mori* and *H. sapiens* AlaS which are monomeric. This could suggest that the first group, including *E. coli*, *Haem. influenzae*, *T. ferrooxidans* and *C. elegans* AlaS, corresponds to oligomeric synthetases, while the second corresponds to monomeric synthetases.

While the nucleotide sequence is well-conserved along the full length of the *alaS* coding regions of *T. ferrooxidans* and *E. coli*, the situation differs between the *alaS* gene of *T. ferrooxidans* and the *alaS* genes of eukaryotes such as *S. cerevisiae*, *A. thaliana*, *C. elegans*, *B. mori* or *H. sapiens*. Seven discrete regions, Aa, Ab, Ac, Ad, Ae, Ba and Bb, appear to be highly conserved (Fig. 2) and deserve special comment. Firstly, those located in the 5' part of the gene, up to position 947 (residue 315), encode the adenylate synthesis domain of the catalytic core (Schimmel, 1990). Interestingly, the class-defining motifs 1, 2 and 3 of the alanyl-tRNA synthetases are encoded by regions Aa, Ab and Ad respectively. Secondly, regions Ba and Bb, positions 1691–1816 (residues 565–605) and positions 2015–2073 (residues 673–691), correspond neither to the catalytic core nor to the oligomerization domain defined by Schimmel (1990). The exact function of these protein regions remains controversial since (i) they are 'dispensable' in EcAlaS (Schimmel, 1990); (ii) they are missing in *M. genitalium* while *A. thaliana* lacks Bb; (iii) mutations G674D and G677D (Schimmel, 1990) located in region Bb, affect the catalytic activity of EcAlaS. Thirdly, Schimmel *et al.* (1993) had proposed that tRNA synthetases are, like tRNA, organized into two major domains: the class-defining conserved domain containing the active site is needed for acceptor-stem recognition while the non-conserved domain provides contact outside of this acceptor stem with the distal parts of the tRNA molecule and, more precisely, the anticodon stem. Furthermore, it has recently been proposed that

the region encompassing Ba and Bb may be needed specifically for synthetase-tRNA interactions involving parts of the tRNA structure outside of the acceptor helix domain (Shiba *et al.*, 1995). Such a strong conservation at the nucleotide sequence level in *alaS* genes from organisms from all kingdoms suggests that there were strong constraints during evolution on these parts of the genes, and strengthens the idea that tRNA synthetase genes derive from a common ancestor.

The same overall genetic organization of the *recA* region exists in *T. ferrooxidans* and *E. coli*. In both cases, the *recA*–*alaS* intercistronic region contains an ORF, *oraA* in *E. coli* and *recX* in *T. ferrooxidans*, which could encode a polypeptide that aligns well with the putative RecX protein described by De Mot *et al.* (1994). Such a genetic linkage between the *recA*, *recX* and *alaS* genes seems particular to *E. coli* and *T. ferrooxidans*. In fact, *alaS* but not *recX* is present in *R. leguminosarum* and *Sin. meliloti* (Selbitschka *et al.*, 1991) while *recA* and *recX* but not *alaS* are linked in *Haem. influenzae* (Fleischmann *et al.*, 1995), and neither *recX* nor *alaS* has been found downstream of *recA* in *Streptococcus pneumoniae* (Martin *et al.*, 1995), *Synechococcus* strain PCC 7002 (Murphy *et al.*, 1990), *M. genitalium* (Fraser *et al.*, 1995), *Campylobacter jejuni* (Guerry *et al.*, 1994) and *Helicobacter pylori* (Schmitt *et al.*, 1995). The genetic arrangement of the *recA*, *recX* and *alaS* genes is then the first reported case of identical genomic organization in *T. ferrooxidans* and *E. coli* of independently transcribed genes. Indeed, all genes which have so far been reported to display the same genetic organization belong to operonic structures i.e. ribosomal RNA operons (Venegas *et al.*, 1988; Salazar *et al.*, 1989), the *cysDNC* operon involved in sulfate assimilation (Fry & Garcia, 1989), and the *atpEFHAGDC* operon encoding ATP synthase (Brown & Rawlings, 1993; Brown *et al.*, 1994). This indicates that the highly acidophilic, obligately chemolithotrophic *T. ferrooxidans* and the heterotrophic neutrophile *E. coli*, even though they belong to two different phylogenetic groups of the 'purple' bacteria and live in quite different biotopes are sufficiently related to share partly a common genomic organization. Also, in *Vibrio cholerae*, which belongs to the same subgroup of the purple bacteria as *E. coli*, the *recX* and *alaS* genes have been found 132 and 432 bp, respectively, downstream of *recA*. The AlaS sequence determined has a high degree of similarity to the other known alanyl-tRNA synthetases (N. Gupta, N. Bhasin & A. Ghosh, personal communication).

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