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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**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*
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 [Δgpr-proA] ΔleuB6 thiB-1 lacY1 hsdR20 recA rpsL20 ara-14 galK2 xyl-5 mtl-1 supE44 merB9], TG1 [supE hsdS8 thi Δlac-proAB, F- trdD36 proAB lacIq lacZAM15] was used for phagemid propagation. AB4132 (alaS thi-1 metC56 lacY1 galK2 xyl-5 or xyl-7 ara-14 tfr-15 tss-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 blunting 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 KDGKFGY 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'-TARCCRAAYTTCRCCYTCTT'G (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, Rus877, was 5'-CCYTTYTTGTDARTCRAA-3' (192-fold degeneracy). Rus115 and Rus877 were purchased from Applied. Rus115 was labelled with [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(pHCl79) 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 PsI and of the 3.5 kb HindIII–Kpn I DNA fragments were sequenced after convenient subcloning of overlapping fragments had been performed, and nested deletions generated by combined ExoIII/ExoVII 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 K5 or SK (+) or (−) and sequenced. The dideoxy chain-termination method was used to sequence DNA using [35S]dATPaS 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 UWGGCG package (Devereux et al., 1984), CLUSTAL, BLAST (Altschul et al., 1990) or BioScan programs through the WWW Nescaple facilities. RNA secondary structures were analysed with the RNAfold and squiggles programs in the UWGGCG package (Devereux et al., 1984).

RNA preparation. T. ferrooxidans total RNA was prepared from a 50 ml culture grown on FeSO4 medium by a slightly modified hot phenol method (Aliba 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 to obtain the following program: 48° 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: alaS21, 5'-CGTGTGTTGTGG-GCGCCG CGC -3'; alaS33, 5'-TCGTGCCCTCCAGCCCCGCCC-3'; RA3, 5'-GCCGAGAGGCTGCAGGAGC-3'; RA5, 5'-GCCGGGCATATCTCTGGC GCG-3'; RX1, 5'-GATTTGAC-AGCGGGTGAGGG-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 Rus877, 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 viciea (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 alasS 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 alasS 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 3 kb Kpnl fragment. When this fragment was cloned from pNG4 into pUC19 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 alasS 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 Pet 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 Pet, while in pNG3, they are in the opposite orientation (Fig. 1). Internal deletions of pNG4 were generated by partial EcoRV digestion and recircularization to place these genes at 1-3 kb from Pet (Fig. 1). As expected, complementation of E. coli recA and alas mutants was observed, indicating that the genes were functional in
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 Smal 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\(^{\text{Ala}}\) (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 C663, 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 717 mol% in agreement with other *T. ferrooxidans* genes (74.3 mol%). A possible ribosome-binding site reading AcGGAG, at positions 661–667, is present 4 bp upstream from the *alaS* putative translational initiation codon but no obvious \(^5\) 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 (AG = -23.5 kcal mol\(^{-1}\) / -98.3 kJ mol\(^{-1}\)), 3361 (AG = -17.8 kcal mol\(^{-1}\) / -74.5 kJ mol\(^{-1}\)) and 3365-5 (AG = -7 kcal mol\(^{-1}\) / -29.2 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, Methylomonas 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 pl 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-UTP 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, *tyz*, 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 alaS21 and alaS33 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.
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 Rus15 probe after digestion of three cosmids DNAs (pNG3,4 and 12) with various restriction enzymes (data not shown), and the Rus15 probe was deduced to hybridize with a 0.18 kb PstI-HindIII fragment internal to the PstI-PstI 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 identity 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 Yo) and Bacillus subtilis (84.2 Yo) (Venegas et al., 1988). Particular attention has been paid to the region encompassing residues 699-808 predicted...
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 (Selbischka 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), Syneccobacus 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 atpEHAGDC 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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