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Identification and characterization of the *carAB* genes responsible for encoding carbamoylphosphate synthetase in *Halomonas eurihalina*

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Abstract *Halomonas eurihalina* is a moderately halophilic bacterium which produces exopolysaccharides potentially of great use in many fields of industry and ecology. Strain F2–7 of *H. eurihalina* synthesizes an anionic exopolysaccharide known as polymer V2–7, which not only has emulsifying activity but also becomes viscous under acidic conditions, and therefore we consider it worthwhile making a detailed study of the genetics of this strain. By insertional mutagenesis using the mini-Tn*5* Km2 transposon we isolated and characterized a mutant strain, S36 K, which requires both arginine and uracil for growth and does not excrete EPS. S36 K carries a mutation within the *carB* gene that encodes the synthesis of the large subunit of the carbamoylphosphate synthetase enzyme, which in turn catalyzes the synthesis of carbamoylphosphate, an important precursor of arginine and pyrimidines. We describe here the cloning and characterization of the *carAB* genes, which encode carbamoylphosphate synthetase in *Halomonas eurihalina*, and discuss this enzyme's possible role in the pathways for the synthesis of exopolysaccharides in strain F2–7.

Keywords Carbamoylphosphate synthetase · Exopolysaccharide · *Halomonas eurihalina* · Halophilic bacteria

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Introduction

Halomonas eurihalina is a moderately halophilic bacterium capable of growing within salt concentrations of between 1% and 20% (w/v). It is commonly found in hypersaline soils where it plays an important ecological role (Quesada et al. 1990). Strains F2–7 and H96 of this species are of particular interest to biotechnologists because they produce the anionic exopolysaccharides (EPS) V2–7 and H96, respectively, both of which are very efficient emulsifiers and are also capable of forming gels under acidic conditions. This latter feature is very unusual among exopolysaccharides (Calvo et al. 1995, 1998; Béjar et al. 1998; Quesada et al. 2003) and renders them potentially useful in many industrial fields, including food processing and cosmetics manufacture (Sutherland 1999).

Very few genetic studies have been carried out into halophilic bacteria in general (Cánovas et al. 1997, 2000; Llamas et al. 1997, 1999, 2000, 2002; Mellado et al. 1995, 1997; Vargas et al. 1995) although the biotechnological importance of these microorganisms makes them ideally suitable for such research. As far as *H. eurihalina* is concerned, its taxonomy, physiology and ecology have been studied in some depth but details of its genetic make-up are still largely unknown and so we have established a transposon mutagenesis procedure for *H. eurihalina* (Llamas et al. 2000).

Carbamoylphosphate synthetase (CPSase) catalyzes the synthesis of carbamoylphosphate from CO_2, ATP, and glutamine or ammonium chloride. Carbamoylphosphate is an important precursor of arginine and pyrimidines (Cunin et al. 1986). In all prokaryotes studied to date, CPSase activity is contained in a complex encoded by two genes, *carA* and *carB* (Ahuja et al. 2001; Kilstrup et al. 1988; Kwon et al. 1994; Piette et al. 1984). The small subunit of this enzyme complex, encoded by *carA*, is a glutamine amidotransferase. The large subunit, encoded by *carB*, can by itself catalyze the synthesis of carbamoylphosphate from

ammonia, bicarbonate, and ATP. The expression of the *carAB* operon is subject to control by arginine and pyrimidines. The enzyme is also subject to allosteric control by intermediates during the biosynthesis of pyrimidines and arginine, and by IMP and phosphoribosyl-1-pyrophosphate (Abdelal and Ingraham 1975).

A null mutation in *carA* and *carB* produces an auxotrophic phenotype for both arginine and pyrimidines. Nevertheless, certain missense mutations in both genes result in auxotrophy for arginine alone in *Salmonella typhimurium* and *Escherichia coli* (Abdelal et al. 1978; Mergeay et al. 1974).

On the basis of our results with the mutant S36 K, which produces smooth colonies and requires both arginine and uracil for growth, we describe here the characterization of the *carAB* genes responsible for encoding carbamoylphosphate synthetase in strain F2–7 of *H. eurihalina*.

Materials and methods

Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids are listed in Table 1. *H. eurihalina* strains were grown at 32°C in SW minimal medium (Nieto et al. 1989) and MD complex medium (Quesada et al. 1987), both modified with a balanced mixture of sea salts to a final concentration of 2% (w/v) (Rodríguez-Valera et al. 1981). *E. coli* derivates were grown at 37°C in Luria-Bertani broth (LB) (Sambrook and Russell 2001). Antibiotics (Sigma-Aldrich) were added to the different media at the following concentrations: ampicillin 100 μg/ml; streptomycin, 50 μg/ml; kanamycin, 50 μg/ml; tetracycline, 15 μg/ml; and rifampicin, 50 μg/ml. The pH values of the media were adjusted to 7.2 with NaOH 1 M. Solid media contained an additional 20 g/l of Bacto-agar (Difco).

Transposon mutagenesis and the isolation of the *carB* mutant

Transposon mutagenesis was carried out via conjugation by biparental mating as described elsewhere (Llamas et al. 2000). The donor strain was *E. coli* S17–1 λ*pir*, harboring the suicide vector pUT mini-Tn*5* Km2 (de Lorenzo et al. 1990). The recipient strain was *H. eurihalina* Rif1, a spontaneous rifampicin-resistant mutant of *H. eurihalina* F2–7. The transconjugants were isolated on SW medium (Nieto et al. 1989) containing 2% (w/v) salts (Rodríguez-Valera et al. 1981), rifampicin (50 μg/ml) and kanamycin (50 μg/ml).

Southern blot analysis

Genomic DNA of *H. eurihalina* S36 K was completely digested with *Eco*RI, *Nde*I, *Not*I, or *Xba*I. DNA fragments were separated on an agarose gel and transferred onto a nylon filter by standard techniques (Sambrook and Russell 2001). Fragments containing mini-Tn*5* were located with a digoxigenin-labeled mini-Tn*5* probe using a digoxigenin DNA labeling and detection kit from Boehringer Mannheim (Germany) and following the manufacturer's instructions.

Gene cloning and sequencing

Plasmids pE36 K, pN36 K, and pX36 K were made as follows (see Table 1, Fig. 1A): chromosomal DNA from *H. eurihalina* S36 K was completely digested with *Eco*RI, *Nde*I, or *Xba*I enzymes, ligated into pUC19 and transformed into *E. coli* XL1-Blue; transformants were then selected and purified on LB medium plates supplemented with ampicillin at 100 μg/ml, tetracycline at 15 μg/ml, and kanamycin at 50 μg/ml.

The promoter DNA sequence was obtained by inverse PCR (Ochman et al. 1988) (Fig. 1B). The chromosomal DNA of *H. eurihalina* F2–7 was completely digested by restriction endonucleases with no restriction sites within the first 500 base-pair region of the *carAB* gene sequence. The appropriate restriction enzymes were selected empirically by Southern blotting and hybridization procedures using the first 200 bp as probe. The probe was labeled with digoxigenin-11dUTP by PCR (kit from Boehringer). The following oligonucleotides (Pharmacia) were used as primers: 5′-CATATGGGAGGACGTTGCATTG-3′ (forward), 5′-CGATACTGGCCCTGGAAG AT-3′ (reverse). The labeled DNA was hybridized for 16 h at 42°C to a Southern blot of digested genomic DNA on a nylon membrane.

The DNA fragments produced by the restriction enzyme in question were diluted and ligated with T4 DNA ligase (Promega) for 16 h at 12°C under conditions favoring the formation of monomeric circles (Collins and Weissman 1984). The resulting intramolecular ligation products were then used as substrates for DNA amplification by PCR using oligonucleotide primers

Table 1 Bacterial strains and plasmids

Strain or plasmid	Genotype or description	Source or reference
Strains		
E.coli		
S17–1 λ *pir*	Tp^r Sm^r*rec*A, *thi*, *pro*, *hsd*R$^-$M$^+$ RP4:2-Tc:Mu:Km Tn7,λ *pir*	Miller and Mekalanos 1988
XL1 Blue	*sup*E44 *hsd*R17 *rec*A1 endA1 ggyrA46 *thi* relA1 *lac*$^-$ F$'$ (*pro*AB$^+$ *lac*I^q *lac*ZΔM15 Tn*10*(tet^r))	Bullock et al. 1987
H.eurihalina		
F2–7	Wild type	Quesada et al. 1990
Rif 1	Spontaneous rifampicin-resistant mutant of F2–7	Llamas et al. 1999
S36 K	miniTn*5* insertion mutant of F2–7 (Km^r)	This study
Plasmids		
pUC19	High-copy-number cloning vector, Ap^r, *bla lacZ*	Vieira and Messing 1982
pGEM-T	High-copy-number cloning vector, Ap^r, *bla lacZ*	Promega
pUT mini-Tn*5* Km2	Ap^r Km^r, *ori* R6 K, *oriT* RP4	de Lorenzo et al. 1990
pE36 K	*carAB* clone containing *Eco*RI fragment in pUC19	This study
pN36 K	*carAB* clone containing *Nde*I fragment in pUC19	This study
pX36 K	*carAB* clone containing *Xba*I fragment in pUC19	This study
pXh36 K	PCR *Xho*I fragment of the *car* promoter region cloned pGEM-T	This study

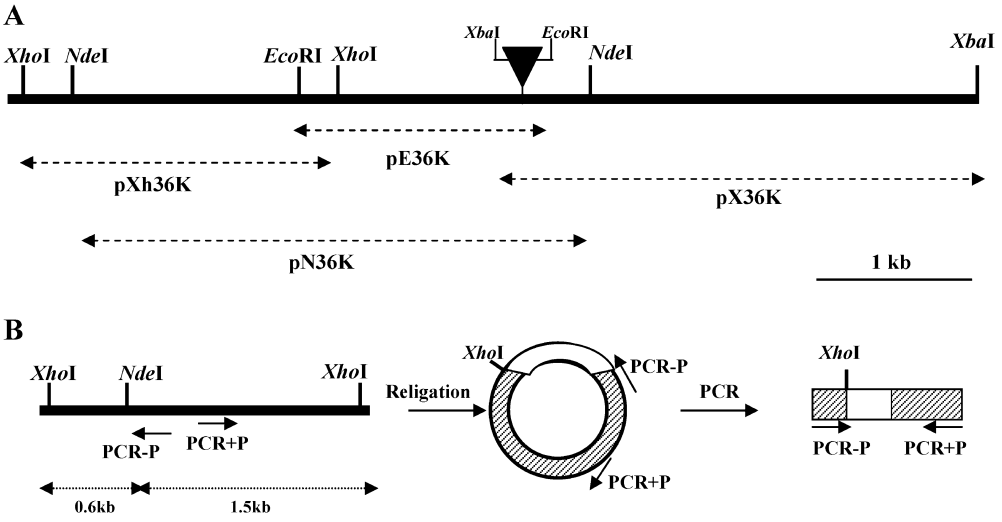

Fig. 1 **A** Scheme of cloning strategy: pN36 K, pE36 K, and pX36 K are obtained by fusion of the pUC19 plasmid and *Eco*RI, *Nde*I, and *Xba*I restriction fragments. Transposon (mini-Tn*5* Km2) insertion site is indicated. **B** Scheme of inverse PCR to obtain the promoter DNA from the *H. eurihalina* S36 K mutant. *Shaded boxes* denote known nucleotide sequences. pXh36 K is obtained by the fusion of the pGEM-T plasmid and 1.5 kb PCR fragment

homologous to the ends of the known DNA sequence, but facing in opposite directions. The chromosomal DNA of *H. eurihalina* strain F2–7 was amplified by PCR with 0.5–1 μg of DNA to a final volume of 50 μl containing 20 pM of each primer, 100 μM dNTP, and 2.5 U of AmpliTaq DNA polymerase (Perkin-Elmer). Amplification was performed for 30 cycles at 94°C for 30 s, 60°C for 30 s; and 72°C for 45 s.

The amplified fragment was purified with microcon PCR Kit (Millipore), ligated into pGEM-T plasmid and transformed into *E. coli* XL1-Blue.

DNA sequences were determined by the dideoxynucleotide chain termination method of Sanger et al. (1977) with both universal and synthetic oligonucleotide primers and double-stranded plasmid templates (Mierendorf and Pfeffer 1987).

Preparation of cell extracts

H. eurihalina F2–7 and its mutant S36 K were grown in MD minimal medium (Quesada et al. 1987). The following compounds were added as required: arginine at 1,000 μg/ml and uracil at 20 μg/ml. Exponentially growing cultures were harvested by centrifuge at an optical density of 0.8 at 600 nm. The cells were washed and resuspended in 0.1 M K_2PO_4H/KPO_4H_2 buffer (pH 7.6) containing 0.5 mM EDTA. The cells were ruptured by passage through a French press cell at 9,000 psi and centrifuged at 27,000 *g* for 30 min. Samples were then dialyzed against 0.1 M potassium phosphate buffer (pH 7.6). The protein concentration was determined by Bradford's method (Bradford 1976) with bovine serum albumin as standard.

Enzyme assays

Carbamoylphosphate synthesis was determined in the presence of ornithine by measuring the formation of citrulline in an assay coupled with ornithine transcarbamylase (Rubino et al. 1986). The reaction mixture contained 0.05 M Hepes/NaOH, pH 7.6, 100 mM KCl, 10 mM ATP, 20 mM magnesium acetate, 20 mM $NaHCO_3$, and 10 mM glutamine (or 300 mM NH_4Cl) to a final volume of 0.25 ml. The reaction was initiated by the addition of appropriate quantities (2–10 μg) of enzyme (cell extracts). After incubation for 10 min at 37°C the reaction was ended by adding 75 μl of 0.5 M EDTA and the carbamoylphosphate formed was converted into citrulline by adding 1 μmol of ornithine and 2 units of ornithine transcarbamylase (Sigma). After 10 min at 37°C, citrulline was quantified by absorbance at 464 nm, using an absorption coefficient of 37,800 M^{-1} cm^{-1}.

Nucleotide sequence accession number

The nucleotide sequence reported in this paper has been submitted to the EMBL Data Library and assigned accession number AJ431666.

Results and discussion

Isolation and characterization of strain S36 K

Strain S36 K of *Halomonas eurihalina* is an EPS-deficient mutant, which we obtained by insertional mutagenesis via the mini-Tn*5* Km2 transposon (Llamas et al. 2000). Southern hybridization analysis revealed that only one mini-transposon was inserted into the transconjugant S36 K (data not shown). The mutant thus obtained produced smooth, translucent colonies in SW (2% w/v) medium during the first 3 days of incubation but after the third day some of them tended to become mucous. In SW medium it grew more slowly (0.38 h^{-1}) than the wild strain (0.77 h^{-1}) and did not grow at all in synthetic MD medium with glucose as the sole carbon source and $(NH_4)_2HPO_4$ as the sole nitrogen source. Its optimum growth rate was observed when arginine (1,000 μg/ml) plus uracil (20 μg/ml) were added to the MD medium.

Cloning and identification of the gene affected by transposon insertion

To identify the gene affected by transposon insertion into *H. eurihalina* S36 K we cloned the chromosomal regions flanking mini-Tn*5*. Suitable size-restriction fragments were selected by Southern hybridization analysis using a digoxigenin-labeled mini-Tn*5* probe [fragment of 1.8 kb (pUT-mini-Tn*5* digested by *Eco*RI and *Xba*I)]. None of the enzymes used had restriction sites within the transposon sequence.

The chromosomal DNA of S36 K was completely digested with *Eco*RI, *Nde*I, or *Xba*I and then ligated and transformed into *E. coli* XL1-Blue. Plasmid DNA was isolated from these recombinant transformants, pE36 K, pN36 K, and pX36 K (Fig. 1A) and subjected to DNA sequencing with a universal primer and a synthetic oligonucleotide primer (5′-GCCGCACTTGTG-TATAAGAGTC-3′) based on the sequence determined for the O-end of the mini-Tn*5* transposon (de Lorenzo et al. 1990). This allowed us to determine the nucleotide sequences of the chromosomal regions adjacent to the transposon in these plasmids. The total chromosomal nucleotide sequence was obtained using synthetic oligonucleotides.

The nucleotide sequence of these recombinant plasmids shows two complete ORFs (EMBL data library). The first extends from a TTG triplet, at what we take to be position 1, and terminates with a TGA triplet at position 1,145. It has a coding capacity of 382 amino acids, yielding a polypeptide with an estimated molecular mass of 41 kDa. The second ORF extends from an ATG triplet at position 1,205 and terminates at position 4,504. It has a coding capacity of 1,091 amino acids, yielding a polypeptide with an estimated molecular mass of 120 kDa. Derived amino-acid sequencing for these two ORFs revealed a high degree of homology with the CarA and CarB proteins of various other microorganisms, including *Pseudomonas* spp., *Salmonella typhimurium*, and *Escherichia coli* (Table 2). These proteins represent the small and large subunits of the carbamoylphosphate synthetase enzyme (Kwon et al. 1994; Kilstrup et al. 1988; Nyunoya and Lusty 1983; Piette et al. 1984). Thus we tentatively identified these ORFs as being *carA* and *carB*, respectively. An analysis of the 57-nucleotide intergenic region between *carA* and *carB* showed no significant homology with any of these DNA sequences.

Location of the transposon insertion site

The transposon was inserted into the large subunit of the carbamoylphosphate synthetase, which is encoded by the *carB* gene. This enzyme catalyzes the synthesis of carbamoylphosphate, a precursor for the biosynthesis of pyrimidines and arginine. Using PCR we amplified and subsequently sequenced a 500-bp fragment from the chromosomal DNA of the wild F2–7 strain, which contained the complete nucleotide sequence (see Materials and Methods section). The following oligonucleotides were used as primers to locate the mini-Tn*5* insertion site: 5′-TATCGACCGTTGGTTCCTGG-3′ (forward), 5′-GGCGTCTGGCA CCGAACTG-3′ (reverse) (Pharmacia). A comparison of the sequence of this fragment with the S36 K chromosomal sequence showed that the mini-Tn5 insertion was located at position 2,877 (EMBL Data Library, number AJ431666) (Fig. 2).

Some microorganisms, such as *Lactococcus lactis* (Martinussen and Hammer 1998) and *Pseudomonas aeruginosa* (Park et al. 1997), can degrade arginine via the arginine deaminase pathway, thus forming carbamoylphosphate. Since *Halomonas eurihalina* is phylogenetically related to *Pseudomonas*, we strongly suspect that this biochemical pathway also exists in *H. eurihalina*. To test whether arginine was a precursor for carbamoylphosphate, which could subsequently be used in the biosynthesis of pyrimidine, we inoculated S36 K into the MD medium both in the presence and absence of arginine and/or uracil. Both arginine and uracil caused a moderate increase in the growth of strain S36 K.

Mutations resulting in double auxotrophy for arginine and uracil have also been obtained in *P. aeruginosa* (Loutit 1952). Evidence based on the isolation of double auxotrophs for arginine and pyrimidine suggests the existence of a single CPSase in *Halomonas eurihalina*. This is the type of organization most commonly found among prokaryotes and occurs widely in Gram-negative bacteria, especially *Enterobacteriaceae*.

CPSase activity

The specific activities of CPSase preparations from *H. eurihalina* F2–7 and its mutant S36 K were determined. In the S36 K mutant, CPSase activity was only about

Table 2 Homologies of ORF1 and ORF2 from *H. eurihalina* S36 K

Protein	Microorganism	(aa)[a]	% Identity (positives)	Accession number	Reference
CPSase (carA)	*Pseudomonas aeruginosa*	378	72 (80)	P38098	Kwon et al. 1994
CPSase (carA)	*Salmonella typhimurium*	382	68 (77)	P14845	Kilstrup et al. 1988
CPSase (carA)	*Escherichia coli*	382	68 (77)	P00907	Piette et al. 1984
CPSase (carA)	*Pseudomonas stutzeri*	384	68 (77)	P38099	Kwon et al. 1994
CPSase (carB)	*Pseudomonas aeruginosa*	1,073	84	U81259	Kwon et al. 1994
CPSase (carB)	*Escherichia coli*	1,073	77 (85)	P00968	Nyunoya and Lusty 1983
CPSase (carB)	*Salmonella typhimurium*	1,075	76 (85)	P14846	Kilstrup et al. 1988
CPSase (carB)	*Bacillus caldolyticus*	1,065	55 (73)	P96495	Ghim et al. 1994
CPSase (carB)	*Bacillus subtilis*	1,027	45 (64)	P18185	O'Reilly et al. 1994
CPSase (carB)	*Lactobacillus plantarum*	1,058	48 (65)	P77886	Elagoz et al. 1996

[a] Number of amino acids

30% of that in the wild strain (1.125 nmol/mg/min for wild type, 0.35 nmol/mg/min for S36 K). These results confirm that the expression of the *carAB* operon is affected by the insertion of the mini-Tn*5* transposon.

The CPSase enzyme from *H. eurihalina* is inhibited by arginine and uracil (data not shown) as are the monomeric CPSases from *E. coli* (Meister and Powers 1978), *S. typhimurium* (Abdelal and Ingraham 1975), and *Serratia marcescens* (Crane and Abdelal 1980).

Structure of the *carAB* operon of *H. eurihalina*

The *carA* and *carB* genes of *H. eurihalina* are adjacent, as they are in *P. stutzeri* (Kwon et al. 1994). To determine whether both genes are transcribed by a single promoter located before the *carA* gene we sequenced the region upstream of the gene. The promoter sequence was obtained by inverse PCR as described above (see Materials and Methods; Fig. 1B). By Southern blotting and hybridization analysis we selected the *Xho*I restriction endonuclease using a 200-bp, digoxigenin-labeled fragment as probe. The *Xho*I fragment (2.1 kb) was re-ligated and amplified by inverse PCR, followed by ligation and transformation. DNA plasmids of this recombinant, pXh36 K, were isolated and sequenced with universal primers. A preliminary gene-bank search of the nucleotide DNA sequence showed one truncated ORF and a 246-bp fragment where the promoter region is located. The partial ORF had a coding capacity of 82 amino

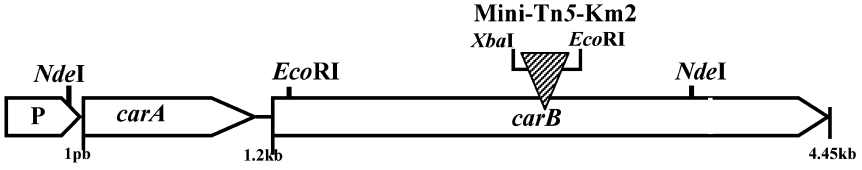

Fig. 2 Physical map of the *carAB* operon, encoding the carbamoylphosphate synthetase in *H. eurihalina*

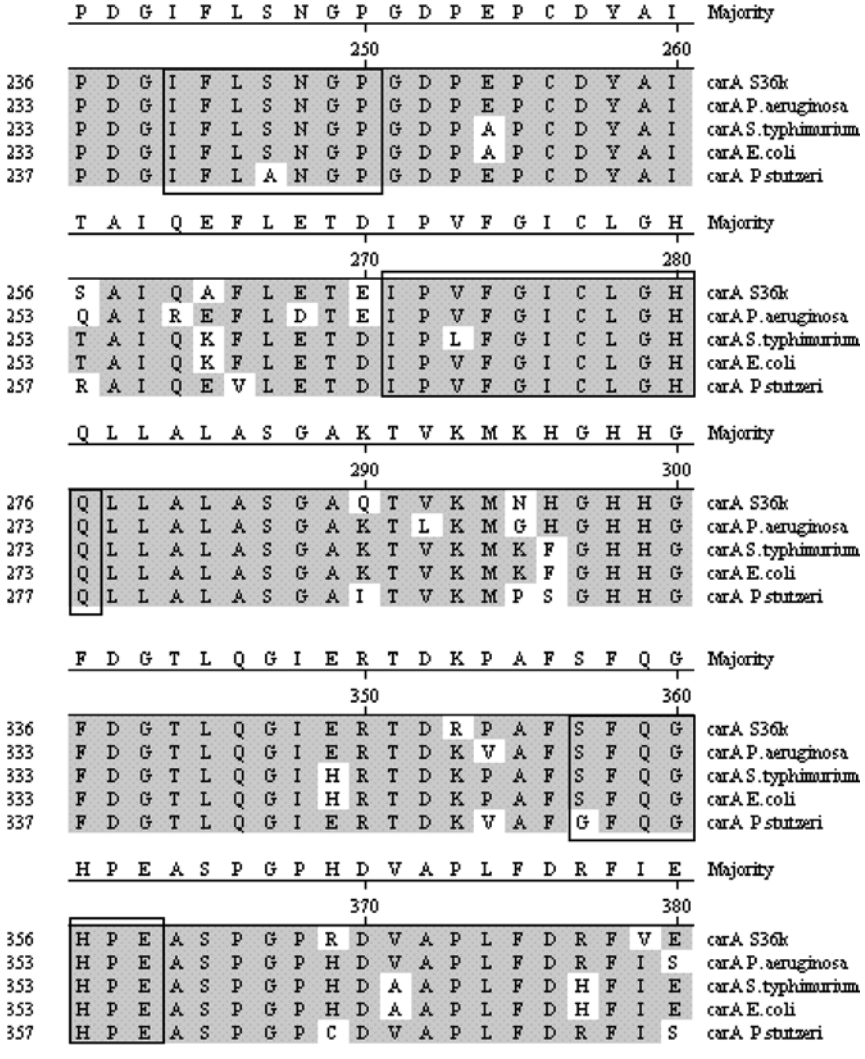

Fig. 3 Alignment of the partial amino-acid sequences of *carA*, which encode the glutamine amidotransferase domain, from *H. eurihalina* S36 K, *P. aeruginosa*, *S. typhimurium*, *E. coli*, and *P. stutzeri*. *Shaded regions* denote identical residues in two or more neighboring sequences. Boxes represent three regions of similarity between *carA* products and defined glutamine amidotransferases (Werner et al. 1985)

acids. The derived amino-acid sequence for this partial ORF showed a high degree of homology (80%) with the dipicolinate reductase of *Pseudomonas* spp. (Kwon et al. 1994).

These results suggest that the *carAB* operon is formed by two units which are transcribed as a monocistronic mRNA, as shown schematically in Fig. 2.

A comparison between the derived amino-acid sequences for *carA* in *H. eurihalina*, *P. aeruginosa*, *S. typhimurium*, *P. stutzeri* and *E. coli* (Table 2) showed significant homology throughout the structural genes. The derived sequence for *carA* in *H. eurihalina* was closest (80%) to that in *P. aeruginosa*. The glutamine amidotransferase domain (residues 240 to 380; Fig. 3) (Werner et al. 1985) is preserved in all of these microorganisms. It has also been reported that the presence of a cysteine residue at position 269 is a distinguishing feature of this domain in prokaryotic enzymes (Rubino et al. 1986). This amino acid is also preserved in *Halomonas eurihalina*.

The structure of the *carAB* operon from *Halomonas eurihalina* is significantly close to that of *Pseudomonas aeruginosa*, showing the presence of a potential leader peptide, consisting of 39 and 18 amino acids respectively, just upstream of the initiation site for the translation of *carA* (Kwon et al. 1994). Primer-extension experiments suggested that the T, located 155 nucleotides upstream of the coding sequence, was probably the 5′-terminal residue of the *carAB* transcript (data not shown).

Despite the high degree of homology of the amino-acid sequences in CPSase in *H. eurihalina* and *Pseudomonas aeruginosa*, a clear difference can be seen in the genetic arrangement of the *carA* and *carB* structural genes: in *H. eurihalina* the *carA* and *carB* genes are adjacent, as they are in *E. coli*, *P. stutzeri* and *S. typhimurium* (Piette et al. 1984; Kwon et al. 1994; Kilstrup et al. 1988), whilst in *P. aeruginosa* the *carA* and *carB* genes are separated by a 682-bp sequence encoding an ORF of 216 amino acids.

To explain the role of the carbamoylphosphate enzyme in the exopolysaccharide biosynthetic pathway, we propose the following hypothesis. The enzyme catalyzes the crucial step for the synthesis of carbamoylphosphate, an essential precursor of arginine and pyrimidines. These compounds are essential to the formation of nucleotide diphosphosugar precursors such as UDP-glucose, which participate in the first step of EPS biosynthesis. Because the expression of the *carAB* operon is affected by the insertion of a transposon in the transconjugant S36 K, the smaller quantity of carbamoylphosphate generated by the arginine deaminase pathway, is directed solely towards the synthesis of essential molecules such as DNA and RNA during the exponential growth phase and so the colonies produced are EPS-deficient. Upon reaching the stationary phase, however, when cell growth has finished, the carbamoylphosphate can be used to synthesize the nucleotide diphosphosugar precursors used in EPS biosynthesis and thus some colonies become mucous.

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References

Abdelal AT, Ingraham JL (1975) Carbamoylphosphate synthetase from *Salmonella typhimurium*: regulations, subunit composition, and function of the subunits. J Biol Chem 250:4410–4417

Abdelal AT, Greigo E, Ingraham JL (1978) Arginine auxotrophic phenotype of mutations in *pyrA* of *Salmonella typhimurium*: role of N-acetylornithine in the maturation of mutant carbamoylphosphate synthetase. J Bacteriol 134:528–536

Ahuja A, Purcarea C, Guy HI, Evans DR (2001) A novel carbamoyl-phosphate synthetase from *Aquifex aeolicus*. J Biol Chem 276:45694–45703

Béjar V, Llamas I, Calvo C, Quesada E (1998) Characterization of exopolysaccharides produced by 19 halophilic strains included in the species *Halomonas eurihalina*. J Biotechnol 61:135–141

Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Bullock WO, Fernández JM, Short JM (1987) XL1-Blue: a high efficiency plasmid transforming *recA Escherichia coli* strain with betagalactosidase selection. Biotechniques 5:376

Calvo C, Ferrer MR, Martínez-Checa F, Béjar V, Quesada E (1995) Some rheological properties of the extracellular polysaccharide produced by *Volcaniella eurihalina*. Appl Biochem Biotech 55:45–54

Calvo C, Martínez-Checa F, Motam A, Béjar V, Quesada E (1998) Effect of cations, pH and sulphate content on the viscosity and emulsifying activity of the *Halomonas eurihalina* exopolysaccharide. J Ind Microbiol Biotechnol 20:205–209

Cánovas D, Vargas C, Iglesias-Guerra F, Csonka LN, Rhodes D, Ventosa A, Nieto JJ (1997) Isolation and characterization of salt-sensitive mutants of the moderate halophile *Halomonas elongata* and cloning of the ectoine synthesis genes. J Biol Chem 272:25794–25801

Cánovas D, Vargas C, Kneip S, Morón MJ, Ventosa A, Bremer E, Nieto JJ (2000) Genes for the synthesis of the osmoprotectant glycine betaine from choline in the moderately halophilic bacterium *Halomonas elongata* DSM 3043. Microbiology 146:455–463

Collins FS, Weissman SM (1984) Directional cloning of DNA fragments at a large distance from an initial probe: a circularisation method. Proc Natl Acad Sci USA 81:6812–6816

Crane CJ, Abdelal AT (1980) Regulation of carbamoylphosphate synthesis in *Serratia marcescens*. J Bacteriol 143:588–593

Cunin R, Glansdorff N, Piérard A, Stalon V (1986) Biosynthesis and metabolism of arginine in bacteria. Microb Rev 50:314–352

Elagoz A, Adbi A, Hurbert JC, Kammerer B (1996) Structure and organization of the pyrimidine byosynthesis pathway genes in *Lactobacillus plantarum*: a PCR strategy for sequencing without cloning. Gene 182:37–43

Ghim SY, Nielsen P, Neuhard J (1994) Molecular characterization of pyrimidine biosynthesis genes from the thermophilic *Bacillus caldolyticus*. Microbiology 140:479–491

Kilstrup M, Lu CD, Abdelal A, Neuhard J (1988) Nucleotide sequence of the *carA* gene and regulation of the *carAB* operon in *Salmonella typhimurium*. Eur J Biochem 176:421–429

Kwon DH, Lu CD, Walthall DA, Brown TM, Houghton JE, Abdelal AT (1994) Structure and regulation of the *carAB* operon in *Pseudomonas aeruginosa* and *Pseudomonas stutzeri*: no untranslated region exists. J Bacteriol 176:2532–2542

Llamas I, Moral A del, Béjar V, Girón MD, Salto R, Quesada E (1997) Plasmids from *Halomonas eurihalina*, a microorganism which produces an exopolysaccharide of biotechnological interest. FEMS Microbiol Lett 156:251–257

Llamas I, Béjar V, Argandoña M, Quesada E, Moral A del (1999) Chemical mutagenesis of *Halomonas eurhalina* and selection of exopolysaccharide-deficient variants. Biotechnol Lett 21:367–370

Llamas I, Argandoña M, Quesada E, Moral A del (2000) Transposon mutagenesis in *Halomonas eurihalina*. Res Microbiol 151:1–7

Llamas I, Sánchez MJ, Argandoña M, Béjar V, Quesada E, Moral A del (2002) Analysis of the genome of the moderate halophile *Halomonas eurihalina*. Curr Microbiol 45:233–239

Lorenzo V de, Herrero M, Jakubzik U, Timmis KN (1990) Mini-Tn*5* transposon derivatives for insertion mutagenesis, promoter probing, and chromosomal insertion of cloned DNA in Gram-negative eubacteria. J Bacteriol 172:6568–6572

Loutit JS (1952) Studies on mutationally deficient strains of *Pseudomonas aeruginosa*. I The production by X-rays and the isolation of mutationally deficient strains. Aust J Exp Biol Med 30:287–294

Martinussen J, Hammer K (1998) The *carB* gene encoding the large subunit of carbamoylphosphate synthetase from *Lactococcus lactis* is transcribed monocistronically. J Bacteriol 180:4380–4386

Meister A, Powers SG (1978) Glutamine-dependent carbamoylphosphate synthetase: catalysis and regulation. Adv Enzyme Regul 16:289–315

Mellado E, Asturias JA, Nieto JJ, Timmis KN, Ventosa A (1995) Characterization of the basic replicon of pCM1, a narrow-host-range plasmid from the moderate halophile *Chromohalobacter marismortui*. J Bacteriol 177:3443–3450

Mellado E, García MT, Nieto JJ, Kaplan S, Ventosa A (1997) Analysis of the genome of *Vibrio costicola*: pulsed-field gel electrophoretic analysis of genome size and plasmid contents. Syst Appl Microbiol 20:20–26

Mergeay M, Gigot D, Beckmann J, Glansdorff N, Pierard A (1974) Physiology and genetics of carbamoylphosphate synthesis in *Escherichia coli* K12. Mol Gen Genet 133:299–316

Mierendorf RC, Pfeffer D (1987) Direct sequencing of denatured plasmid DNA. Methods Enzymol 152:556–562

Miller VL, Mekalanos JJ (1988) A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in *Vibrio cholerae* requires ToxR. J Bacteriol 170:2575–2583

Nieto JJ, Fernández-Castillo R, Márquez MC, Ventosa A, Quesada E, Ruíz-Berraquero F (1989) A survey of metal tolerance in moderately halophilic eubacteria. Appl Environ Microbiol 55:2385–2390

Nyunoya H, Lusty J (1983) The *carB* gene of *Escherichia coli*: a duplicated gene coding for the large subunit of carbamoylphosphate synthetase. Proc Natl Acad Sci USA 80:4629–4633

Ochman H, Gerber AS, Hartl DL (1988) Genetic applications of an inverse polymerase chain reaction. Genetics 120:621–623

O'Reilly M, Woodson K, Dowds BCA, Devine KM (1994) The citrulline biosynthetic operon argC-F and a ribose transport operon rbs from *Bacillus subtilis* are negatively regulated by Spo0A. Mol Microbiol 11:87–98

Park SM, Lu CD, Abdelal AT (1997) Cloning and characterization of *argR*, a gene that participates in regulation of arginine biosynthesis and catabolism in *Pseudomonas aeruginosa* PAO1. J Bacteriol 179:5300–5308

Piette J, Nyunoya H, Lusty CJ, Cunin R, Weyens G, Crabeel M, Charlier D, Glansdorff N, Pierard A (1984) DNA sequence of the *carA* gene and the control region of *carAB*: tandem promoters, respectively controlled by arginine and the pyrimidines, regulate the synthesis of carbamoyl-phosphate synthetase in *Escherichia coli* K-12. Proc Natl Acad Sci USA 81:4134–4138

Quesada E, Valderrama MJ, Béjar V, Ventosa A, Ruíz-Berraquero F, Ramos-Cormenzana A (1987) Numerical taxonomy of moderately halophilic Gram-negative nonmotile eubacteria. Syst Appl Microbiol 9:132–137

Quesada E, Valderrama MJ, Béjar V, Ventosa A, Gutiérrez MC, Ruíz-Berraquero F, Ramos-Cormenzana A (1990) *Volcaniella eurihalina* gen nov, sp nov, a moderately halophilic nonmotile Gram-negative rod. Int J Syst Bacteriol 40:261–267

Quesada E, Béjar V, Ferrer MR, Calvo C, Llamas I, Martínez-Checa F, Arias S, Ruiz-García C, Páez R, Martínez-Cánovas J, Moral A del (2003) Moderately halophilic, exopolysaccharide-producing bacteria. In: Ventosa A (ed) Halophilic microorganisms. Springer, Berlin Heidelberg New York

Rodríguez-Valera F, Ruíz-Berraquero F, Ramos-Cormenzana A (1981) Characteristics of the heterotropic bacterial populations in hypersaline environments of different salt concentrations. Microb Ecol 7:235–243

Rubino SD, Nyunoya H, Lusty CJ (1986) Catalytic domains of carbamoyl phosphate synthetase. J Biol Chem 261:11320–11327

Sambrook J, Russell DW (2001) Molecular cloning: a laboratory manual, 3rd edn. Cold Spring Harbor Laboratory Press, New York

Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 74:5473–5477

Sutherland IW (1999) Microbial polysaccharide products. Biotechnol Genet Eng Rev 16:217–229

Vargas C, Fernández-Castillo R, Cánovas D, Ventosa A (1995) Isolation of cryptic plasmids from moderately halophilic eubacteria of the genus *Halomonas*. Characterization of a small plasmid from *H. elongata*: its use for shuttle vector construction. Mol Gen Genet 246:411–418

Vieira J, Messing J (1982) The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene 19:259–268

Werner M, Feller A, Pierard A (1985) Nucleotide sequence of yeast gene CPSI encoding the small subunit of arginine-pathway carbamoylphosphate synthetase: homology of the deduced amino acid sequence to other glutamine amidotransferases. Eur J Biochem 146:371–381