Carbamoyl phosphate synthetase (CPS) is involved in the production of carbamoyl phosphate (CP) from ATP, bicarbonate and ammonia (or glutamine). CPSs are large, complex, highly regulated synthetases (CPS) from ATP, bicarbonate and ammonia (or glutamine). CP is the first committed precursor of pyrimidines and arginine biosynthesis and plays a role in the production of carbamoyl phosphate (CP) for anabolic purposes. However, in some organisms, such as pyrococci, CK may have the role of replacing CPS in vivo, raising the possibility that in some organisms, such as pyrococci, CK may have the role of making CP for biosynthetic purposes. To try to clarify if CK can replace CPS in vivo, we have expressed the CK from *P. furiosus* in a strain of *Escherichia coli* with a deletion of the *carAB* gene, which encodes CPS. We show that the expressed CK restores the ability of the CPS-deficient cells to grow in the absence of arginine and pyrimidines if ammonia and bicarbonate are present. Furthermore, the CK of *Enterococcus faecalis* [10], which is used in the latter organism to make ATP fermentatively from arginine [2,3], was also found to support the growth of CPS-deficient *E. coli* in arginine and pyrimidine-free medium. Thus, CK can replace efficiently CPS in vivo, raising the possibility that in some organisms, such as pyrococci, CK may have the role of making CP for biosynthetic purposes.

2. Materials and methods

2.1. Cell strains, plasmids and growth conditions

*E. coli* strain L814 [11], which lacks both *carA* and *carB* genes (*carA* and *carB* encode the small and large *E. coli* CPS subunits) and plasmid pLLK12 [11], which carries the *carAB* genes inserted in the Bam HI site of pUC19 and which confers ampicillin resistance, were generous gifts of Dr. Carol J. Lusty (The Public Health Research Institute, NY, USA). Plasmids pCK41 [10] and pCPS184 [9], two PET-15b (Novagen) derived plasmids carrying the genes for enterococcal and pyrococcal CK, respectively, behind the T7 promoter, and conferring ampicillin resistance, were provided by Dr. A. Marina and Dr. S. Ramón-Maíques. To allow the expression in L814 of the plasmid-encoded CKs, the cells were lysogenised for phage λDE3 using a commercial kit (λDE3 lysogenisation kit, from Novagen) and are called L814T7. For expression of pyrococcal CK, the cells were transformed also with the plasmid pSJS1240 [12,13], encoding tRNAs for rare *E. coli* codons for arginine and isoleucine and conferring spectinomycin resistance (this plasmid was a gift of Dr. S.J. Sandler, Department of Microbiology, University of Massachusetts, MA, USA). Procedures for making competent cells and for transformation with plasmids, and the composition of the minimum medium are as described earlier [9,10,14]. The medium used with transformed cells also contained 0.1 mg/ml ampicillin and, in the case of the pSJS1240 co-transformants, 0.05 mg/ml spectinomycin. When indicated, it contained also 0.6 mM isopropyl-β-D-thiogalactoside (IPTG) or the specified amount of ammonium bicarbonate. The initial inoculum consisted of cells that had been grown in Luria-Bertani medium (containing antibiotics as indicated above) to an optical density of 0.6 and that were centrifuged, washed twice and suspended in minimum medium with the indicated additions to an initial absorbance of 0.2. When IPTG was used, the cells, prior to centrifugation, were exposed for 3 h to 0.6 mM IPTG, and then were centrifuged, washed and suspended as above in minimum medium containing 0.6 mM IPTG. The growth of cells at 37°C with orbital shaking (300 cycles/min) was monitored by the optical absorbance of the culture at 600 nm.

2.2. Enzyme activity assays and electrophoretic methods

Cells isolated by centrifugation were sonicated in 20 mM Tris–HCl, 0.5 M sucrose and 1 mM EGTA (pH 7.5), with protease inhibitors (0.1 mM PMSF, 1 mM benzamidine, 1× protease inhibitor cocktail), and the extracts were centrifuged at 15,000 g for 30 min at 4°C. The supernatants were collected and the protein concentrations were determined following the method of Lowry et al. [15]. E. coli [10] and *P. abyssi* [11] CPS activities were determined following the formation and release of 14CO2 from [1-14C]bicarbonate in the presence of 0.1 mM ATP and 10 mM NH4Cl. Measurement of carB activity was performed using the 14C-labeled commercial kit (Novagen) for the detection of carB activity. Electrophoretic analyses were performed with a 15% polyacrylamide gel and immobilised proteins were stained with a Coomassie blue solution.
pH 8, containing 1 mM ethylene diamine tetraacetic acid and 0.3 mM phenylmethylsulphonyl fluoride, and the extract was clarified by centrifugation and was desalted by centrifugal gel filtration [15] in the same buffer. CK activity was assayed at either 37 or 60°C, in the direction of CP synthesis, by conversion of the CP to citrulline with ornithine transcarbamylase [9]. One enzyme unit corresponds to the production of 1 μmol CP min⁻¹ at the specified assay temperature. Protein in the extracts was assayed according to Bradford [16] using bovine serum albumin as a standard. Extracts were subjected to SDS-PAGE in 10% polyacrylamide gels followed by Western blotting and immunostaining with alkaline phosphatase using as first antibody a monoclonal (mAbCK2 [10]) or a polyclonal mouse antibody recognising enterococcal and pyrococcal CK, respectively. Densitometric quantification of stained bands was carried out by comparison with the purified target enzymes, run in parallel tracks, using a scanner and the Signamul program (Sigma).

3. Results and discussion

Since E. coli has a single CPS that produces the CP needed for making both arginine and pyrimidines [17], E. coli strain L814T7, which lacks this CPS [11], cannot grow in minimum medium (Fig. 1A,B, open circles) except when arginine and uracil are added (Fig. 1B, closed circles). Supplementation with only one of these compounds does not result in growth of these cells. Transformation with plasmid pLLK12, which encodes E. coli CPS, restores the ability of the cells to grow in the absence of arginine and uracil (Fig. 1A, inverted triangles). Transformation with pCK41 (Fig. 1, squares) or with pCPS184 (Fig. 1, upright triangles), which encode the CKs from E. faecalis and P. furiosus, respectively, results in growth in the absence of uracil, if arginine is added. However, growth does not occur when uracil is added and arginine is absent. Thus, it appears that the expressed enterococcal or pyrococcal CKs (revealed in Fig. 2 by SDS-PAGE and Coomassie-staining) supply enough CP to cope with the demands of pyrimidine synthesis but not enough to cope with the larger requirements of arginine synthesis.

In these experiments IPTG was used and growth was delayed in the cells transformed with pCK41 and, particularly, with pCPS184 (Fig. 1B), even in the presence of added arginine and uracil, suggesting that the IPTG-promoted massive expression of these CKs has some toxic effect that delays growth. Consequently, IPTG was not used in further experiments, particularly since pilot assays demonstrated some expression of the plasmid-encoded proteins in the absence of IPTG (see below).

In the above experiments CP appeared to be synthesised in vivo by CK in the absence of a substantial source of carbamate, the substrate used by CK to make CP. At the concentrations in the medium of HCO₃⁻ (0.2 mM, derived from air [18]) and NH₃ (0.15 mM; derived from the ammonium sulphate present), carbamate should be present in trace amounts (0.06 μM, calculated from the equilibrium: K = [NH₃][HCO₃⁻]/[carbamate] = 0.53 M⁻¹ [3]) with respect to Kₐ values for carbamate of the CKs from E. faecalis (77 μM [3]) and P. furiosus (5–7 μM; [9] and unpublished results). Thus, to increase the concentration of carbamate, 10–80 mM ammonium bicarbonate was added to the minimum medium (Fig. 3, top panel). This addition did not induce any growth (monitored after 18 h) of the non-transformed cells and it did not impair or promote the growth of the cells transformed with plasmid pLLK12, which encodes E. coli CPS, except at the highest salt concentration, at which growth was strongly inhibited. In contrast, the addition of the salt enabled the cells transformed with the CK-encoding plasmids to grow in minimum medium, although the growth was also inhibited at the highest salt concentration tested. The highest cell densities after 18 h of culture were attained at concentrations of ammonium bicarbonate of 20–40 mM with pCK41 and of 40–50 mM with pCPS184, and were approximately two-fold lower with pCPS184 than with pCK41, with which they were similar to those attained by transformation with pLLK12. A plot of the cell density of the pCK184-transformed cells versus the calculated carbamate concentration (Fig. 3, lower panel) suggests mid-saturation by approxi-
approximately 7 μM carbamate and saturation by 50 μM carbamate, in agreement with the expectations for the carbamate dependency of pyrococcal CK. Thus, being saturated by carbamate, it appears that the rate of CP production by the expressed pyrococcal enzyme limits the growth of the pCPS184-transformed cells and causes the lower culture densities found with these cells than with the pCK41 and pLLK12-transformed cells.

To monitor the expression of the plasmid-encoded CKs, cell extracts were subjected to immunoblotting at the end of the 18 h incubation. Fig. 4 illustrates the lack of CK in non-transformed cells and the presence of a substantial amount of the expected CK in the pCK41 and pCPS184-transformed cells. In the former cells the enterococcal enzyme, quantified densitometrically in the blots (Fig. 5), represented approximately 6% of the protein at 10 mM ammonium bicarbonate and decreased to about 4% at 40 mM ammonium bicarbonate. In the pCPS184-transformed cells the pyrococcal enzyme accounted for 20% of the protein at 10 mM ammonium bicarbonate and decreased at higher salt concentrations to about 5% of the protein. These results suggest that at increasing cell density plasmid-encoded CK expression is decreased, but in any case they show that there is CK expression in the cells in which growth was observed. However, any connection between CK expression and growth would be invalidated if there were substantial endogenous background CK activity in E. coli. In fact, there are three putative endogenous CK genes in E. coli [10], and the expression of these cannot be excluded by the experiments shown thus far, since the corresponding protein products may not cross-react with our highly specific antibodies. CK activity assays were therefore used to rule out the existence of substantial endogenous CK. No substantial CK activity, assayed at either 37 or 60°C, was detected in the cell extracts used for immunoblotting of the non-transformed cells (detection limit, 0.03 U/mg protein in the extract), whereas substantial CK activity was found, paralleling the levels of immunodetected CK protein, in the pCK41- and pCPS184-transformed cells (Fig. 5, top and bottom panels, respectively) exhibiting, as expected, high thermostability in the pCPS184-transformed cell extracts (tested by heating the extract for 5 min at 90°C, data not shown). Furthermore, the ratio between the CK activity and CK protein levels yield estimates for the specific activities of the enterococcal and pyrococcal enzymes, respectively, of 668 (37°C) and 15.3 (60°C) U/mg, in excellent agreement with previous estimations with the purified enzymes of 630 [3] and 16.7 [9] U/mg protein. Thus, the activity observed is due to the expression of the plasmid-encoded enzyme, and is associated with the ability of the CPS-deficient cells to grow in the absence of arginine and uracil, thus allowing the conclusion that CK can replace CPS in vivo.

Our results are relevant in the context of the in vivo synthesis of CP in P. furiosus and P. abyssi. At the high temperatures of the normal living environment of these microorganisms the CK activity in these archaea was estimated to represent, in the direction of CP synthesis, approximately 10 μmol h⁻¹ mg protein⁻¹ [9], a value not exceeding the activity of the pyrococcal enzyme that supported the growth of E. coli at 37°C in our experiments (10–3.5 μmol h⁻¹ mg protein⁻¹) at 37°C, depending on the ammonium bicarbonate concentration, Fig. 5; applying a 13-fold reduction in the activity for the decrease in the temperature from 60 to 37°C [9], supporting the possibility that a CK is responsible in these pyrococci for the synthesis of CP. In fact, as previously discussed [9], the concentration of carbamate may be relatively high in the habitat of these pyrococci, given the finding...
of high concentrations of CO₂ (the true reactant, rather than bicarbonate, in the chemical synthesis of carbamate) and of 0.6–1 mM ammonia in their environment. Furthermore, a CK gene but no classical CPS gene has been found in the entire genome of *Pyrococcus horikoshii* [19] (although a putative classical CPS gene has been identified in the genome of *P. furiosus*; http://www.genome.utah.edu/sequence.htm). Pending a definitive answer on the role of pyrococcal CK that may possibly be provided only by knocking out the CK gene in these pyrococci, the present results stress the possibility that a physiological function of CK in some organisms may be to make CP for biosynthetic purposes rather than to use it for making ATP. To our knowledge the pyrococci would be the first organisms in which a CPS would not be utilised for the production of CP for biosynthetic purposes.

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**References**


