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Catabolic Ornithine Transcarbamylase of *Halobacterium halobium* (*salinarium*): Purification, Characterization, Sequence Determination, and Evolution

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***Halobacterium halobium* (*salinarium*) is able to grow fermentatively via the arginine deiminase pathway, which is mediated by three enzymes and one membrane-bound arginine-ornithine antiporter. One of the enzymes, catabolic ornithine transcarbamylase (cOTCase), was purified from fermentatively grown cultures by gel filtration and ammonium sulfate-mediated hydrophobic chromatography. It consists of a single type of subunit with an apparent molecular mass of 41 kDa. As is common for proteins of halophilic *Archaea*, the cOTCase is unstable below 1 M salt. In contrast to the cOTCase from *Pseudomonas aeruginosa*, the halophilic enzyme exhibits Michaelis-Menten kinetics with both carbamylphosphate and ornithine as substrates with K_m values of 0.4 and 8 mM, respectively. The N-terminal sequences of the protein and four peptides were determined, comprising about 30% of the polypeptide. The sequence information was used to clone and sequence the corresponding gene, *argB*. It codes for a polypeptide of 295 amino acids with a calculated molecular mass of 32 kDa and an amino acid composition which is typical of halophilic proteins. The native molecular mass was determined to be 200 kDa, and therefore the cOTCase is a hexamer of identical subunits. The deduced protein sequence was compared to the cOTCase of *P. aeruginosa* and 14 anabolic OTCases, and a phylogenetic tree was constructed. The halobacterial cOTCase is more distantly related to the cOTCase than to the anabolic OTCase of *P. aeruginosa*. It is found in a group with the anabolic OTCases of *Bacillus subtilis*, *P. aeruginosa*, and *Mycobacterium bovis*.**

Fermentative arginine degradation via the arginine deiminase pathway is found in different groups of *Bacteria* (for reviews see references 1 and 8). The pathway consists of an arginine-ornithine antiporter and the enzymes arginine deiminase (ADI; EC 3.5.3.6), catabolic ornithine transcarbamylase (cOTCase; EC 2.1.3.3), and carbamate kinase (CK; EC 2.7.2.2). Arginine is degraded to ornithine, ammonia, and CO_2, concomitantly generating 1 mol of ATP per mol of arginine degraded. An OTCase activity is also involved in arginine biosynthesis. Organisms capable of both arginine biosynthesis and fermentative arginine degradation possess two OTCases which are differentially regulated. The ADI pathway has been most extensively studied in *Pseudomonas aeruginosa* (2–5, 16, 17, 21, 29, 32, 58). In this organism, the genes are situated in an operon and are cotranscribed.

Halobacteria are the only *Archaea* for which anaerobic growth using arginine has been reported (22). As arginine consumption was coupled to the equimolar occurrence of ornithine in the medium, it was proposed that *Halobacterium halobium* (*salinarium*) (*H. halobium* has recently been renamed *H. salinarium*, which is used for the rest of this article.) degrades arginine via the ADI pathway (22).

To study this pathway and its regulation in an archaeon, the cOTCase from *H. salinarium* was isolated and characterized. The corresponding gene was cloned and sequenced, and the deduced protein sequence was compared to the sequences of other OTCases.

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MATERIALS AND METHODS

Archaeal strain, media, and growth conditions. *H. salinarium* L33 (59) was grown aerobically in complex medium containing peptone as described previously (46). For fermentative growth, arginine was added to a final concentration of 0.5%, the medium was inoculated, and the cultures were flushed with nitrogen for 20 min. The flasks were then closed with a glass stopper and sealed with Parafilm. For recording, we used growth flasks with a side arm which allow turbidity measurements with a Klett photometer (Klett Manufacturing Co. Inc., New York, N.Y.). For enzyme preparation, 2 liters of medium was supplemented with arginine, inoculated with 20 ml of an *H. salinarium* L33 culture grown aerobically to the stationary phase, flushed for 1 h with nitrogen, and sealed. The cultures were grown at 37°C and 100 rpm for 6 to 8 days.

Enzymatic assay. cOTCase activity was determined by measuring the ornithine + carbamylphosphate → citrulline + phosphate reaction, the reverse of the in vivo reaction. The enzymatic activity was measured in the presence of 40 mM ornithine, 20 mM carbamylphosphate, 60 mM Tris/HCl (pH 7.2), and 3 M KCl. Citrulline formation was quantitated by using a colorimetric assay essentially as described by Oginsky (38).

Protein chemical methods. Protein concentrations were determined with the BCA-kit of Pierce (Pierce, Rockford, Ill.) with bovine serum albumin as the standard. It works well with samples containing 3 M KCl but is sensitive to high concentrations of ammonia. Therefore, samples containing ammonium sulfate were either dialyzed or concentrated with a buffer change in an Amicon concentrator.

Before electrophoretic separation, the proteins were precipitated by addition of 0.1 volume of 3 M trichloroacetic acid. The pellets were dissolved in 2 M Tris/HCl (pH 8.5), mixed with an equal volume of sample buffer, and incubated for 3 min at 100°C. Electrophoretic separation was performed with standard denaturing sodium dodecyl sulfate (SDS)-polyacrylamide (10%) gels.

Purification of cOTCase. Cells were harvested by centrifugation (4,000 × *g*, 4°C, 1 h), and the wet weight of the pellet was determined. Per milligram of wet weight, 2 ml of AcA buffer (3 M KCl, 50 mM Tris/HCl [pH 7.2]) was added and the cells were carefully resuspended. Dithioerythritol, phenylmethylsulfonyl fluoride, and DNase I were added to final concentrations of 1 mM, 5 mM, and 0.3 mg/mg (wet weight), respectively. The cells were disrupted by freezing the suspension with liquid nitrogen and slowly thawing it to room temperature. Cell debris was pelleted by centrifugation (40,000 × *g*, 4°C, 1 h).

A 25-ml volume of the supernatent was applied to a gel filtration column (2.6 by 90 cm; Ultrogel AcA54; Serva, Heidelberg, Germany) previously equilibrated with AcA buffer. The elution was performed with the same buffer at a flow rate of 20 ml/h. The cOTCase eluted after about 100 ml with the void volume. The fractions containing enzymatic activity were pooled.

The volume of the eluate of the gel filtration column was determined and prepared for ammonium sulfate-mediated hydrophobic chromatography (33) by very slow addition of 3 volumes of buffer 1 (3.0 M ammonium sulfate, 1.0 M NaCl). Thereby, the ammonium sulfate concentration was adjusted to 2.2 M.

A column (5 by 6 cm) containing DE52 cellulose (Whatman Ltd., Maidstone, United Kingdom) was equilibrated with buffer 2 (2.2 M ammonium sulfate, 1.5 M NaCl, 50 mM Tris/HCl [pH 7.2]). The sample was applied, and the column was washed with buffer 2 until the optical density at 280 nm reached the baseline level again. Elution was performed with a binary gradient of decreasing ammonium sulfate concentrations (2.2 to 1.0 M) and increasing sodium chloride concentrations (1.5 to 2.7 M). The flow rate was 300 ml/h, and the concentration gradient was 2 mM/ml. It was important to keep the ionic strength high. In the presence of ammonium sulfate, the second salt needs to be sodium chloride, not potassium chloride (the predominant salt inside halobacteria), because sodium sulfate is more soluble than potassium sulfate, which would precipitate. Under these conditions, the cOTCase was eluted at an ammonium sulfate concentration of about 1.8 M, slightly earlier than the rest of the proteins. Aliquots of all fractions with enzymatic activity were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE), and only fractions in which the cOTCase was more than 95% pure were pooled.

To determine whether contaminating bands seen in SDS-PAGE were oligomers of the cOTCase or impurities (see Results and Discussion), an additional gel filtration with Ultrogel AcA34 was done. A column (2.6-cm diameter, 95-cm length) was equilibrated with AcA buffer, and the sample was applied. Elution was performed in the same buffer with a flow rate of 16 ml/h. Fractions with enzymatic activity were pooled.

Characterization of the enzyme. Analytical gel filtration was performed with a Sephacryl S-200 column (1.6 by 75 cm; flow rate, 60 ml/h; Pharmacia, Freiburg, Germany) to determine the native molecular weight. For the cOTCase, AcA buffer was used for equilibration and elution. For the calibration proteins (cytochrome *c*, chymotrypsinogen A, ovalbumin, bovine serum albumin, aldolase, catalase, and ferritin), this buffer and 200 mM sodium phosphate buffer (pH 7.2) were used.

To determine the enzymatic activities at different pHs, Tris was replaced with BisTrisPropane (Sigma, Steinheim, Germany).

Protein sequence determination. Fifty micrograms of cOTCase was cleaved with cyanogen bromide at room temperature overnight (250 μg of OTCase per ml, 100 mg of cyanogen bromide per ml, 70% formic acid). By repeated addition of double-distilled water and evaporation, the formic acid was eliminated. The sample was applied to a reversed-phase high-pressure liquid chromatography column (Lichrospher RP18; Merck, Darmstadt, Germany), and peptides were eluted with a linear gradient of 0 to 80% acetonitrile (in the presence of 0.1% trifluoroacetic acid). The N-terminal sequences of four peptides and the N terminus of the protein were determined with a sequencer (ABI 473; Applied Biosystems, Weiterstadt, Germany) in accordance with the manufacturer's instructions.

Cloning and sequencing of the *argB* gene. We used general molecular biological techniques in accordance with Sambrook et al. (42) or manufacturer instructions for cloning and sequencing of the *argB* gene. Restriction enzymes and other DNA-modifying enzymes were purchased from Boehringer (Mannheim, Germany), Pharmacia (Freiburg, Germany), and United States Biochemicals/Amersham (Braunschweig, Germany). Oligonucleotides were synthesized with an Applied Biosystems 394 DNA Synthesizer. PCRs for probe construction were performed with *Taq* polymerase (Amersham) and the following oligonucleotides: 1, ATG GAG CAC CTC/G GTC/G GAC ATC AAC/T G; 2, CTC/T AAG CTG CCC/G AAG GTC TAG CTG G. Single-stranded DNA probes were labeled in a two-step procedure. First a double-stranded DNA fragment was amplified in a standard PCR. After purification (Quiaex kit; Quiagen, Hilden, Germany), this fragment was used in a second, linear PCR including primer 2 and DIG-11-dUTP (Boehringer). In this reaction, the following concentrations were used: 20 μM DIG-11-dUTP, 30 μM dATP and dTTP, and 45 μM dGTP and dCTP.

Halobacterial genomic DNA was isolated as described by Rosenshine et al. (40). It was cleaved with different restriction enzymes and separated by agarose electrophoresis, and DNA fragments were transferred to nylon membranes (Quiabrane; Quiagen) by vacuum blotting. Hybridization was performed for 2 days at 37°C in a buffer containing 20% formamide, 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 3× Denhardt's solution, 0.5% SDS, 0.2% sodium laurylsarcosine, and 5% dextran sulfate. Signals were visualized with the DIG Luminescent Detection Kit (Boehringer). After preparative digestion with *Eco*RI and electrophoretic separation, a DNA fragment mixture of about 3.3 kbp was isolated with Prep-A-Gene (Bio-Rad, Munich, Germany) and ligated into the dephosphorylated (shrimp alkaline phosphatase; United States Biochemicals, Bad Homburg, Germany) vector Bluescript pKS+ (Stratagene, Heidelberg, Germany). *Escherichia coli* XL1 Blue MRF′ (Stratagene) was transformed by electroporation (10).

Colony screening was performed as described by Buluwela et al. (7) in a buffer that was the same as that described above, except that the formamide concentration was 50%. Small-scale plasmid isolations were done by the LiCl boiling method (60), and large-scale isolations were done with Quiagen columns. DNA sequences were determined by using the PRISM Ready Reaction Dye Deoxy Terminator Cycle Sequencing Kit and a 370A sequencer (both from Applied Biosystems). The cloned fragment was sequenced on both strands by gene walking. The programs Analysis and Seqed (Applied Biosystems) were used for analysis of the data.

Computer programs, sequence alignments, and construction of phylogenetic trees. The software packages of the Genetics Computer Group (18) and the Protein Identification Resource (37) were used on a Microvax computer (Digital Equipment). Fifteen OTCase sequences were copied from the available databases. For the initial protein sequence alignment, the Pileup program (18) was used and the alignment was manually edited with the help of the Lineup multiple sequence editor (18). The DNA sequences were aligned in accordance with the protein sequence alignment. The software package of Felsenstein (15) and the PAUP program (48) were used for construction of evolutionary trees.

Nucleotide sequence accession number. The sequence described here has been deposited in the EMBL database under the name HHARGB and accession no. X81712.

RESULTS AND DISCUSSION

Fermentative growth. First, growth conditions were tested to ensure that the cultures used for protein isolation grew fermentatively. No growth was detected in complex medium without inclusion of arginine. Addition of increasing amounts of arginine led to anaerobic growth with increasing growth yields. To test whether arginine fermentation can be used by *H. salinarium* as the sole energy source, cultures were grown anaerobically by serial dilution for 100 generations in the presence of arginine. No growth rate reduction was detected throughout the experiment. The generation time was determined to be 8 h, which is about a factor of 2 slower than aerobic growth in the early exponential growth phase.

One representative of each halobacterial phenon, as defined by Torreblanca et al. (51), was tested for arginine fermentation to clarify whether this pathway is unique to *H. salinarium*. The representatives of phenons A to D and M were able to grow via arginine fermentation, in addition *Halobacterium* sp. strains GRA and GRB (13).

Purification of cOTCase. Most halophilic soluble proteins are not stable in low salt concentrations; therefore, high ionic strength was used throughout the purification. If possible, potassium chloride, the predominant salt in the cytoplasm of halobacteria, was used. Gel filtration was applied as a first purification step, leading to sixfold enrichment. The cOTCase was found in the void volume with a yield of more than 80%. As a second step, ammonium sulfate-mediated hydrophobic chromatography (33) was used. When only the first fractions with OTCase activity were pooled, this second step already resulted in an almost pure protein. The yield was 30%, and the purification was about fivefold. A further gel filtration step with a matrix that retards the cOTCase revealed that minor bands coeluted exactly with the cOTCase, so that they probably were aggregation products. A gel with aliquots of each purification step is shown in Fig. 1. The OTCase can be seen as a single band with an apparent molecular mass of 41 kDa.

During fermentative growth, the cOTCase is a major protein. This can be seen by gel electrophoretic analysis (Fig. 1) and by the fact that 30-fold enrichment was sufficient to obtain a pure protein. This high abundance can be expected from an enzyme involved in an energy-generating process with a low yield (1 mol of ATP per mol of arginine) and is in accordance with the results obtained with *P. aeruginosa* (3).

Determination and alignment of peptide sequences. The cOTCase was cleaved with cyanogen bromide, and four peptides were isolated by reversed-phase high-pressure liquid chromatography. The N-terminal sequences of these peptides and the N-terminal sequence of the undegraded protein were determined. The sequence of one of the peptides turned out to be included in the N-terminal sequence of the protein. The peptide sequences, which comprise about 30% of the protein sequence, are indicated in the deduced protein sequence in Fig. 2. To determine the positions of these peptides in the

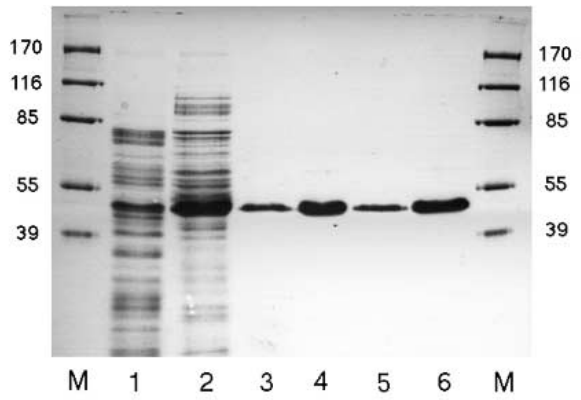

FIG. 1. Gel electrophoretic analysis. Aliquots of the protein mixtures at different stages of protein isolation were analyzed by SDS-PAGE. Lanes: M, marker proteins (the sizes are indicated); 1, soluble proteins of the crude extract; 2, pooled fractions after gel filtration on AcA54; 3 and 4, 1 and 5 μg, respectively, of the pooled fractions after ammonium sulfate-mediated hydrophobic chromatography; 5 and 6, 1 and 5 μg, respectively, of the pooled fractions after gel filtration chromatography on AcA34.

protein sequence, a multiple sequence alignment of the cOTCase from *P. aeruginosa* and anabolic OTCases from different organisms was constructed. All peptide sequences of *H. salinarium* showed enough similarities to the eubacterial and eucaryotic enzymes to allow their integration into the alignment (data not shown).

Cloning and sequence determination of the *argB* gene. Two degenerated oligonucleotide primers were derived from the N-terminal sequence and one of the internal peptide sequences (Fig. 2). Only major codons according to the halobacterial codon usage table (44) were included. With genomic DNA of *H. salinarium* L33 as the template, a PCR fragment of the calculated length was obtained. It was used as a homologous probe in Southern analyses, and with different restriction enzymes, in each case one major hybridization signal and one minor hybridization signal were found (data not shown). *H. salinarium* is able to grow in synthetic medium in the presence of ornithine (data not shown) and thus possesses an anabolic enzyme. Therefore, the minor signals could originate from the gene for the anabolic OTCase.

A 3.3-kbp *Eco*RI fragment corresponding to one of the major signals was cloned, and its sequence was determined by gene walking. The entire *argB* gene was found, together with about 400 bp downstream and 2 kbp upstream. For the gene sequence and the deduced protein sequence, see Fig. 2. The GC content of 62% and the codon usage of the *argB* gene are typical of protein-coding genes of halophilic *Archaea* (44). It codes for a protein of 295 amino acids with a calculated molecular mass of 32 kDa. The cOTCase has an amino acid composition which is characteristic of soluble halophilic proteins; i.e., it contains 20% acidic residues (D+E), and the preference of acidic residues, D+E minus K+R, is 14%, compared with 2% for proteins of enteric bacteria (45). The great fraction of acidic residues leads to the difference between the calculated molecular mass (32 kDa) and the apparent molecular mass, determined by SDS-PAGE (41 kDa), which is typical of halophilic proteins. This can be explained by reduced binding of the anionic detergent in comparison with nonhalophilic marker proteins.

About 20 nucleotides downstream of the *argB* gene, there is a stretch of T residues (Fig. 2), a sequence motif which has been proposed to be involved in transcriptional termination (6). No open reading frame could be detected about 400 bp downstream of the gene.

Upstream of the *argB* gene, one entire open reading frame and one partial open reading frame exhibiting the halobacterial codon usage were found. The deduced protein sequences have similarities to the carbamate kinase and the ADI of *P. aeruginosa* (41). On the one hand, the close proximity of the genes for the carbamate kinase and the ADI makes the existence of an operon with a polycistronic mRNA possible. In *P. aeruginosa*, the genes of the ADI pathway are cotranscribed and the primary transcript is subsequently processed to smaller mRNAs. On the other hand, upstream of the halobacterial *argB* gene there is a sequence with strong similarity to the consensus sequence for a halobacterial promotor (9) (Fig. 2). This putative distal promotor element has the required spacing to the transcriptional start site if transcription initiates at or near the beginning of the open reading frame, as is often found for halobacterial genes. Potential ribosomal binding sites are present upstream of and inside the open reading frame (Fig. 2).

Sequence alignment and construction of a phylogenetic tree. A protein sequence alignment of the halobacterial cOTCase with the second cOTCase, whose sequence is known, and 14 anabolic OTCases was constructed. In Fig. 3, an alignment is shown which contains, for clarity, only 8 of the 16 sequences. The cOTCase of *H. salinarium* clearly belongs to this protein family. Thirty-six residues are conserved in all 15 eucaryotic and bacterial OTCases (indicated in Fig. 3). With the exception of G-124 (underlining indicates position in the alignment of Fig. 3), all residues are also conserved in the halobacterial enzyme (amino acid position numbers are underlined if they refer to the numbering of the multiple sequence alignment [Fig. 3], in contrast to the individual sequences). In addition, the motif FX(E/K)XS(G/T)RT, which in the Genetics Computer Group PROSITE database program package was chosen as a signature for carbamoyltransferases, is found in the sequence, i.e., 91-FAKPSTRT-98 (Fig. 3). The lysine residue at position 3 is characteristic of OTCases, in contrast to aspartate transcarbamylases.

The similarities of the halobacterial enzyme to the other enzymes (fraction of identical amino acids) vary from about 30 to 45% (*B. subtilis*). Unexpectedly, the similarity to the anabolic enzyme of *P. aeruginosa*, 43% identical amino acids, was much higher than that to the cOTCase (34%). Therefore, it seems that the OTCase specialization for the catabolic versus the anabolic function is a derived rather than an ancestral feature. In accordance with this view, it had been shown that single amino acid changes can convert a catabolic enzyme to an "anabolic" enzyme (4).

To get insight into the evolution of OTCases, phylogenetic trees were constructed. For tree construction, the protein sequence alignment was used as a template for alignment of the corresponding gene sequences. The GC contents of the OTCase genes vary greatly, from 36% (*Saccharomyces cerevisiae*) to 69% (*M. bovis*). It has been shown that the GC content of codon position 3 is most sensitive to the GC content of the genome (36). Therefore, to minimize the effect of GC content on tree topology, phylogenetic trees were calculated by using only the first two codon positions. The distance matrix, maximum-likelihood, and parsimony methods, the latter including bootstrap analysis, were used for calculation. The different methods yielded similar trees, which were also in accordance with the results of a calculation based on protein sequence data. A typical tree is shown in Fig. 4. The results of the different methods deviate in the topology of the group composed of the OTCases of *H. salinarium*, *B. subtilis*, *P. aerugi-*

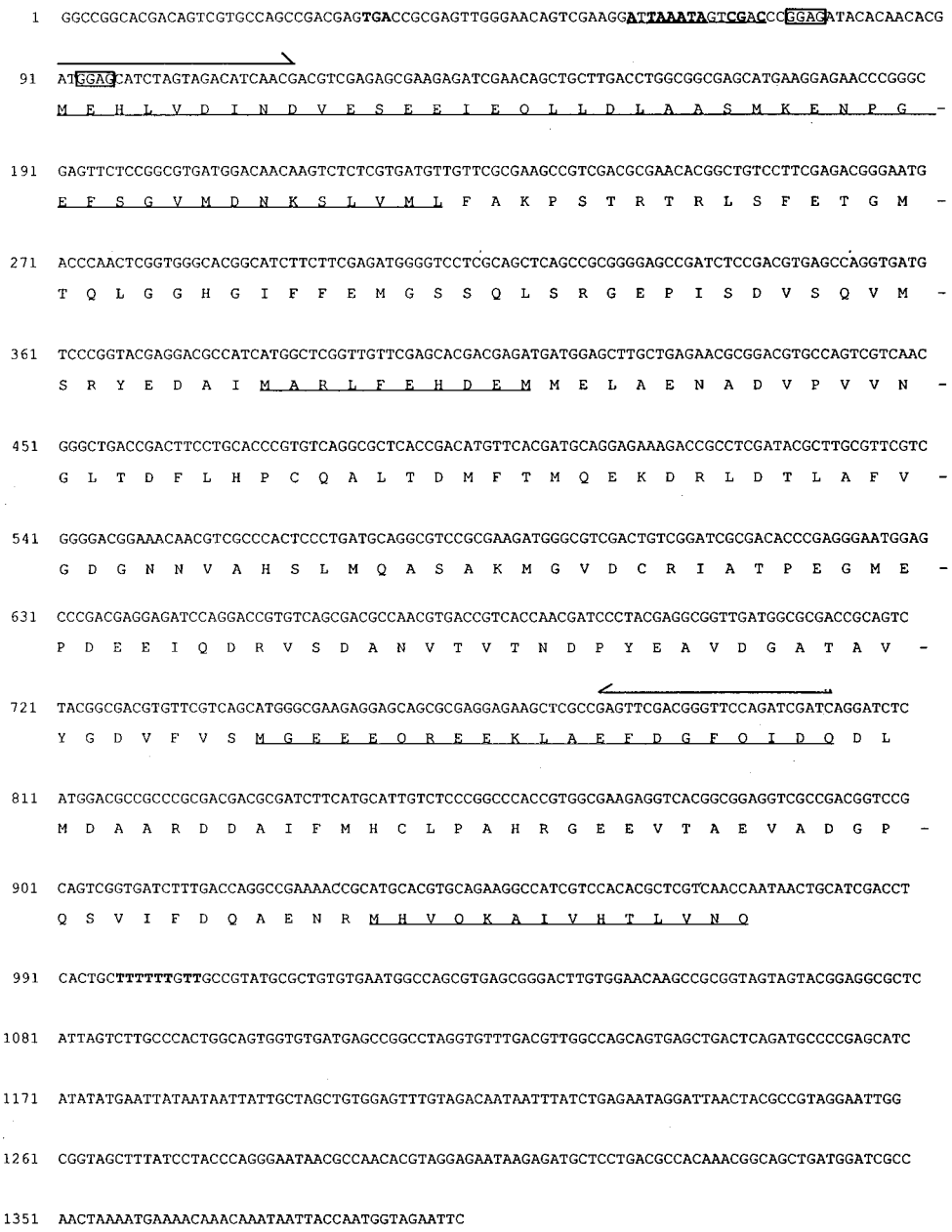

FIG. 2. The *argB* gene and the deduced sequence of the cOTCase protein. The regions of the deduced polypeptide sequence verified by protein sequencing are underlined. A putative promoter element is underlined, and the bases matching the halobacterial consensus promoter (9) are in boldface. Potential ribosomal binding sites are boxed. The T residues possibly involved in transcriptional termination are in boldface. The two primers used for probe construction are indicated by arrows above the sequence. The stop codon of an open reading frame upstream of the *argB* gene is in boldface.

nosa (anabolic), and *M. bovis*. The parsimony method of Felsenstein placed the halobacterial enzyme next to the anabolic OTCase of *P. aeruginosa*, whereas the other methods found the anabolic OTCase of *B. subtilis* to be the closest relative. On the other hand, all methods were in agreement on the following points (Fig. 4). (i) There are three groups based on eucaryotic sequences, the sequences of gram-negative proteobacteria, and a mixed group of sequences of two gram-positive enzymes, one gram-negative enzyme, and one archaeal enzyme. (ii) The halobacterial cOTCase was never found as the deepest branch within the mixed group. (iii) The eucaryotic sequences are subdivided into two groups as shown in Fig. 4. (iv) The sequence of *P. syringae* forms the deepest branch of gram-negative *Proteobacteria.*

In this tree, the *Bacteria* are not monophyletic. The phylogeny of the living world has been studied intensively by using different molecules crucial for central metabolism, and it is clear that *Archaea*, *Bacteria*, and *Eucarya* form monophyletic groups termed domains (for reviews, see references 39 and 61). Therefore, within the OTCase protein family the anabolic OTCase of *P. aeruginosa* and the cOTCase of *H. salinarium* deviate from the evolution of organisms. It has been shown before that the similarities between OTCases is not straightforward but, e.g., on the basis of immunological methods, the

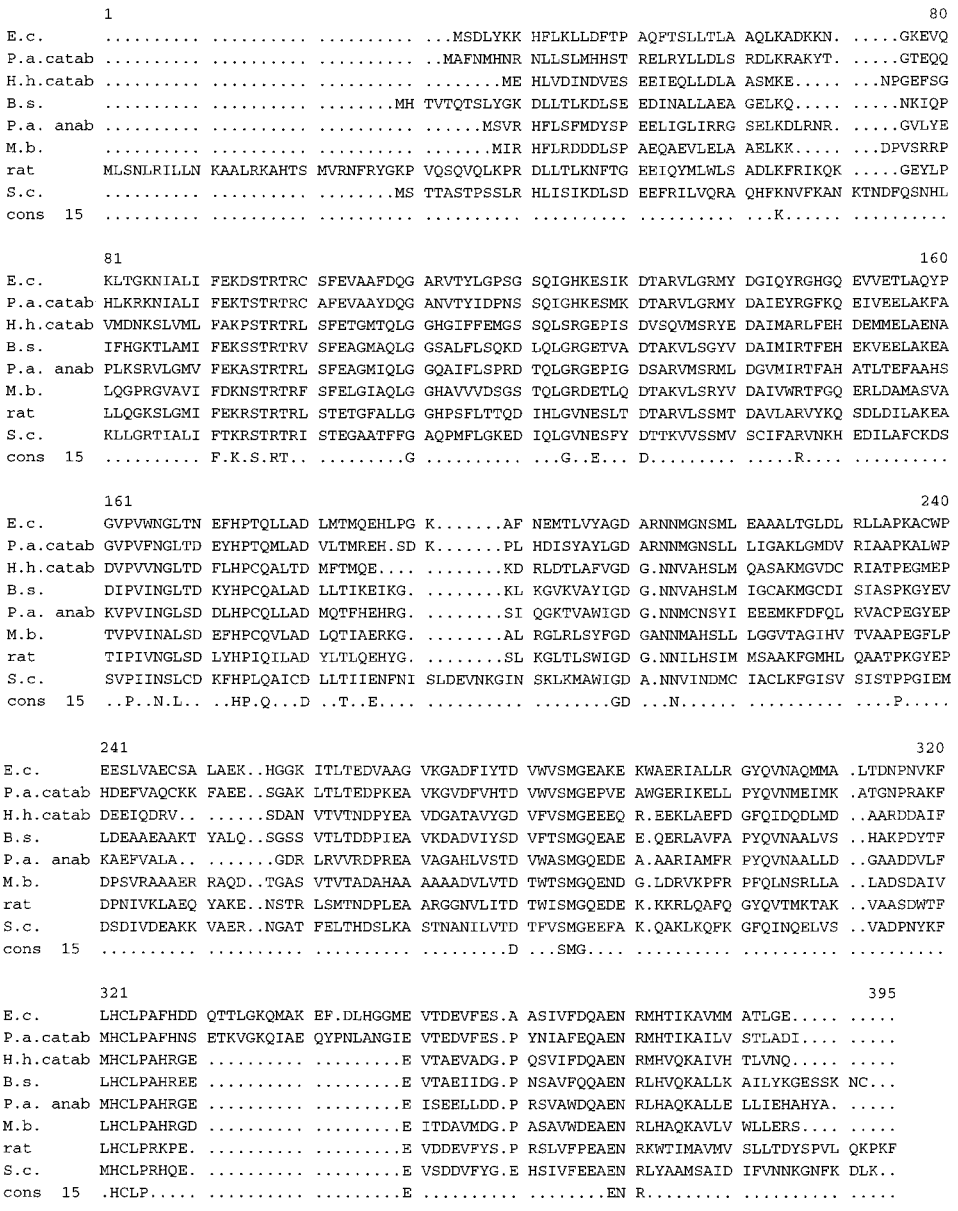

FIG. 3. OTCase protein sequence alignment. Eight of 16 OTCases in a multiple sequence alignment are shown. The bottom line (cons 15) represents a consensus sequence of the 15 nonarchaeal sequences. Abbreviations: E.c., *E. coli*; P.a.catab, cOTCase of *P. aeruginosa*; H.h.catab, cOTCase of *H. salinarium*; B.s., *B. subtilis*; P.a. anab, anabolic OTCase of *P. aeruginosa*; M.b., *M. bovis*; S.c., *S. cerevisiae.*

catabolic enzymes from *P. aeruginosa* and *Aeromonas formicans* are more related to the anabolic enzyme of *E. coli* than to the anabolic enzymes from the respective organisms (14, 52).

Three explanations for the OTCase tree seem possible. (i) The halobacterial cOTCase is not typical of archaeal OTCases, and because of the short branch length between the eucaryal and bacterial domains this sequence has been misplaced. (ii) *H. salinarium* acquired the cOTCase gene by lateral gene transfer from an ancestor of gram-positive *Bacteria*. The same would be true of the gene for the anabolic OTCase of *P. aeruginosa*. (iii) The ancestor of the domains *Archaea*, *Bacteria*, and *Eucarya* possessed two gene copies, which later randomly specialized in the anabolic or catabolic function (or one copy was lost). Therefore, the OTCase tree shown in Fig. 4 includes paralogous as well as orthologous genes.

More sequences are needed to clarify the evolution of the OTCase protein family, especially archaeal sequences and both anabolic and catabolic sequences from several organisms.

Quaternary structure. The native molecular mass of the cOTCase was determined by analytical gel filtration by using nonhalophilic marker proteins. The marker proteins were applied to the gel filtration under conditions of high and low salt concentrations, and they eluted identically under both conditions. Because of its instability at low ionic strength, the cOTCase could only be investigated in a high salt concentration. The molecular mass was determined to be 200 kDa.

By denaturating gel electrophoresis it had been shown that the cOTCase consists of a single type of subunit (Fig. 1). The molecular mass calculated on the basis of the gene sequence is 32 kDa. Therefore, the native enzyme functions as a homohexamer. This is in good agreement with the known OTCases of other organisms. In general, anabolic OTCases were found

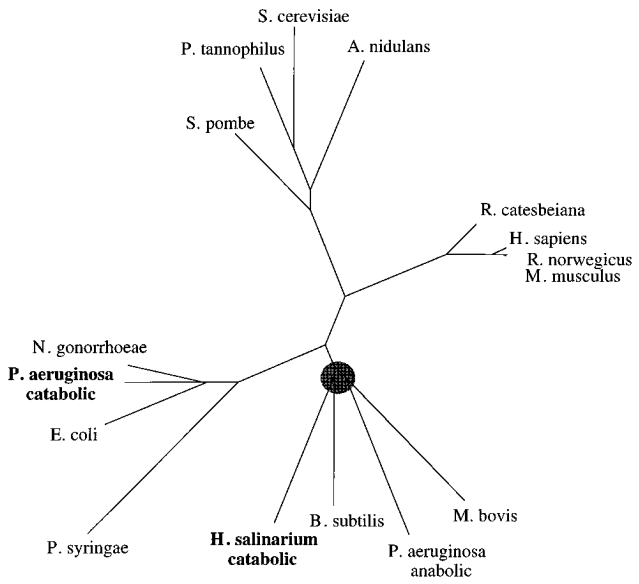

FIG. 4. Evolutionary tree of the OTCase protein family. Construction of the tree is described in the text. The shaded circle indicates that different methods yield different topologies in this region. The abbreviations and the references for the sequences are the following: S. pombe, *Schizosaccharomyces pombe* (55); P. tannophilus, *Pachysolen tannophilus* (43); S. cerevisiae, *S. cerevisiae arg3* gene (25); A. nidulans, *Aspergillus nidulans* (54); R. catesbeiana, *Rana catesbeiana* (bullfrog; 23); H. sapiens, *Homo sapiens* (24); R. norwegicus, *Rattus norwegicus* (49); M. musculus, *Mus musculus* (mouse; 57); N. gonorrhoeae, *Neisseria gonorrhoeae* (30); P. aeruginosa catabolic, cOTCase of *P. aeruginosa* (3); E. coli, *E. coli argF* gene (56); P. syringae, *Pseudomonas syringae* (34); H. salinarium catabolic, cOTCase of *H. salinarium*; B. subtilis, *Bacillus subtilis* (35); P. aeruginosa anabolic, anabolic OTCase of *P. aeruginosa* (26); M. bovis, *Mycobacterium bovis* (50).

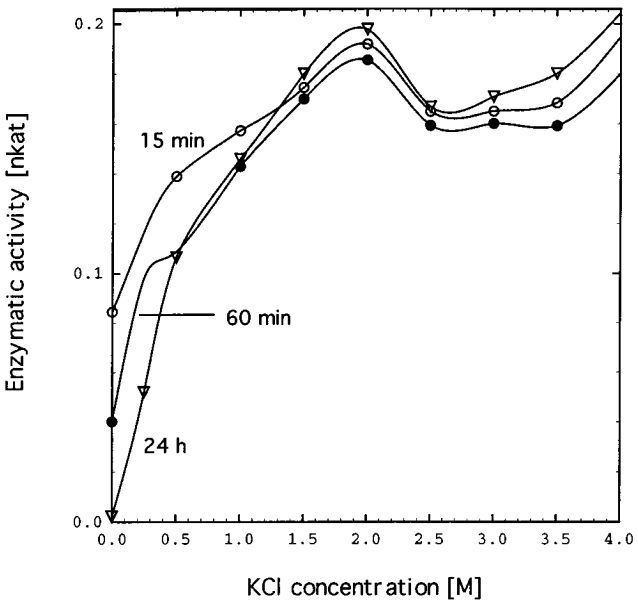

FIG. 5. Dependence of cOTCase activity and stability on the salt concentration. OTCase was brought to the indicated potassium chloride concentrations by dilution. The pH was adjusted to 7.2 by including 50 mM Tris/HCl. After 15 min, 1 h, and 24 h, the activities were determined at the respective salt concentrations.

to be trimeric enzymes with molecular weights ranging from 100,000 to 140,000 (28). For the anabolic OTCase of *E. coli*, this was verified by crystallization (27). With the exception of the enzyme of *B. licheniformis*, the catabolic enzymes are larger, with molecular weights of around 220,000 (*Streptococcus faecalis* and *Streptococcus* sp. strain D10), 360,000 (*Mycoplasma hominis*), and 420,000 (*A. formicans*, *Lactobacillus fermenti*, *P. aeruginosa*, and *P. putida*) (28). The cOTCase of *P. aeruginosa* has been crystallized and shown to be a dodecamer built of four trimers (29).

Enzyme kinetic characterization. First, the salt dependence of the cOTCase activity and stability was measured. The result is shown in Fig. 5. Like many other halophilic proteins, the cOTCase was not stable below 1 M KCl. The highest activity was found at the physiological salt concentration of 4 M KCl, but it also peaked at around 2 M. The cOTCase was stable in high-salt solutions, and the isolated enzyme could be kept for 1 year at room temperature without loss of activity.

Next, the pH dependence of the cOTCase activity was determined. Optimum activity was found at around pH 8.8 (data not shown). At the physiological pH of 7, activity was only about 30%.

For further characterization, the enzyme's kinetic behavior with both carbamylphosphate and ornithine as substrates was measured. Fig. 6 is a linear representation of the data for carbamylphosphate according to Hanes. The cOTCase clearly exhibited Michaelis-Menten saturation kinetics with both carbamylphosphate and ornithine (data not shown). The K_m values for carbamylphosphate and ornithine were found to be 0.4 and 8 mM, respectively. The effects of a number of possible allosteric regulators on the activity of the cOTCase were tested. Arginine led to slight activation; the maximal activation of the enzymatic activity was less than twofold with 50 mM arginine (data not shown). The effects of different nucleotide phosphates are summarized in Fig. 7. All nucleotide phosphates inhibited the enzyme; at a concentration of 5 mM, less

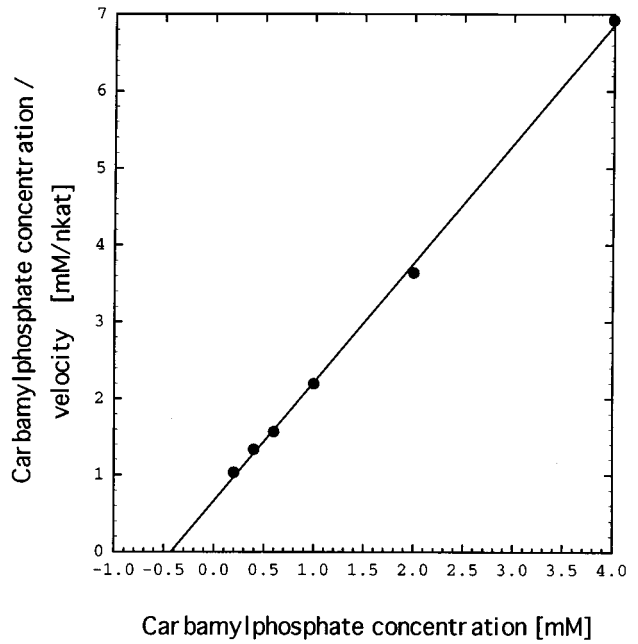

FIG. 6. Determination of the K_m value for carbamylphosphate. Enzymatic activity was measured as described in the text at the indicated carbamylphosphate concentrations. The data were transformed as described by Hanes, and the quotient of the substrate concentration and the initial velocity was plotted versus the substrate concentration. The datum points and a graph derived by linear regression are shown.

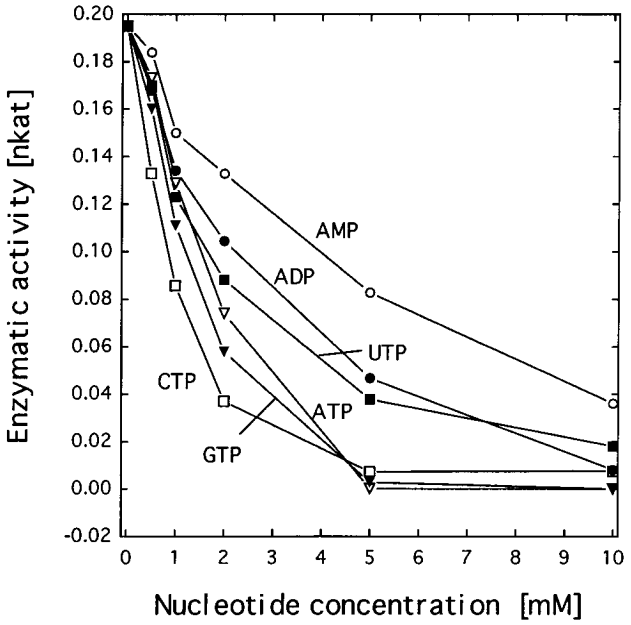

FIG. 7. Nucleotide phosphates as allosteric effectors of cOTCase. cOTCase activity was measured as described in the text. Different nucleotide phosphates were added as indicated.

than 10% activity was found. The effect of ATP was not stronger than that of GTP or CTP. Surprisingly, AMP also inhibited cOTCase activity, albeit not as strongly as ATP. Addition of phosphate led to a slight reduction of cOTCase activity to about 80%; a phosphate concentration of 10 mM maximized this effect.

Comparison to other OTCases. A halophilic OTCase has been isolated and characterized before (11, 12). In contrast to the present study, the cultures were not grown fermentatively and arginine was not included in the medium. Therefore, it is likely that the anabolic OTCase rather than the cOTCase was purified. The enzyme described by Dundas differs in several aspects from the one described here, e.g., K_m values, pH optimum, cooperativity of ornithine binding, and stability. Because of difficulties in halobacterial strain designations it is not clear which *H. salinarium* strain was used by Dundas more than 20 years ago. Therefore, the differences might also be caused by the use of different strains and direct comparison of the data is not possible.

In *P. aeruginosa*, the anabolic and catabolic enzymes have specialized properties. The anabolic enzyme can form a binary dead-end complex with citrulline; because of this kinetic mechanism, phosphorylic cleavage of citrulline is inhibited at nonsaturating citrulline concentrations and the enzyme functions only in the anabolic direction (47). The catabolic enzyme binds carbamylphosphate cooperatively, leading to a sigmoidal saturation curve with a half-saturation concentration more than 10 times higher than the K_m of the anabolic enzyme. Therefore, at physiological carbamylphosphate concentrations the catabolic enzyme is inactive in the anabolic direction. It cannot substitute for the anabolic enzyme in vivo: mutants carrying only the catabolic enzyme are auxotrophic for arginine. Mutations at two positions have been shown to abolish the allosteric properties. (i) Replacement of glutamic acid 106 (position 144) with glycine or alanine converts the cOTCase to the anabolic enzyme, the K_m value is lowered about 40 or 70 times, respectively, the saturation curve becomes hyperbolic, and the enzyme is active in the anabolic direction in vivo (4). (ii) Deletion of C-terminal isoleucine 335 (position 386) strongly reduces cooperativity (53). In contrast, the cOTCase of *H. salinarium* exhibits Michaelis-Menten kinetics with both carbamylphosphate and ornithine as substrates. The two critical residues discussed for the *Pseudomonas* enzyme are absent in the halobacterial cOTCase: at position 144, there is a methionine, as in the anabolic enzymes of *B. subtilis* and *P. aeruginosa*, and the enzyme is shorter by one amino acid at the C terminus, so the equivalent of I-386 is missing. It is not clear whether in *H. salinarium* the functional adaptation of the cOTCase to its catabolic role is missing or whether a different mechanism is utilized. The K_m value for carbamylphosphate (0.4 mM) is in the same range as the values for anabolic enzymes (*P. fluorescens*, 0.3 mM; *E. coli*, 0.36 mM). However, the K_m value for ornithine (8 mM) is 10-fold higher than that of the cOTCase of *P. aeruginosa* and this low affinity for ornithine could prevent the enzyme from functioning in the anabolic direction in vivo.

The allosteric regulation of the cOTCase is also different in *Pseudomonas* species and in *H. salinarium*. The *Pseudomonas* enzyme is activated by phosphate and AMP, which both reduce the activity of the halobacterial enzyme slightly or substantially.

It should be noted that two amino acids are conserved which have been shown by mutational studies to be important in other systems, i.e., cysteine 323, which is involved in ornithine binding (20, 31), and arginine 97, which is part of the carbamylphosphate-binding site and is crucial for the substrate binding order and induced-fit isomerization of the enzyme (19, 20, 31).

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