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Acetylornithine Transcarbamylase: a Novel Enzyme in Arginine Biosynthesis

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Ornithine transcarbamylase is a highly conserved enzyme in arginine biosynthesis and the urea cycle. In *Xanthomonas campestris*, the protein annotated as ornithine transcarbamylase, and encoded by the *argF* gene, is unable to synthesize citrulline directly from ornithine. We cloned and overexpressed this *X. campestris* gene in *Escherichia coli* and show that it catalyzes the formation of *N*-acetyl-L-citrulline from *N*-acetyl-L-ornithine and carbamyl phosphate. We now designate this enzyme as an acetylornithine transcarbamylase. The K_m values for *N*-acetylornithine and carbamyl phosphate were 1.05 mM and 0.01 mM, respectively. Additional putative transcarbamylases that might also be misannotated were found in the genomes of members of other xanthomonads, *Cytophaga*, and *Bacteroidetes* as well as in DNA sequences of bacteria from environmental isolates. It appears that these different paths for arginine biosynthesis arose very early in evolution and that the canonical ornithine transcarbamylase-dependent pathway became the prevalent form. A potent inhibitor, N^{α}-acetyl-N^{δ}-phosphonoacetyl-L-ornithine, was synthesized and showed a midpoint of inhibition at approximately 22 nM; this compound may prove to be a useful starting point for designing inhibitors specific to this novel family of transcarbamylases.

The enzymatic biosynthesis of L-arginine is accomplished by a complex, highly interconnected pathway with at least eight steps to produce arginine from L-glutamate via a series of acetylated amino acid intermediates (Fig. 1). One of the essential enzymes in the pathway, ornithine transcarbamylase (OTCase) (EC 2.1.3.3), catalyzes the formation of citrulline from ornithine and carbamyl phosphate. Protein sequences within the OTCase family show strong conservation across diverse phylogenetic domains ranging from archaea to mammals (25, 26). This suggests that the role of OTCase in arginine biosynthesis was established at a very early stage of evolution, an assertion that is further supported by phylogenetic comparison of the OTCase sequences to the paralogous aspartate transcarbamylases (ATCases) involved in pyrimidine synthesis. Thus, it appears that these two enzyme families already existed at the point of the last universal common ancestor (7).

While exploring the arginine biosynthesis pathway in the anaerobe *Bacteroides fragilis*, we identified and solved the crystal structure of a transcarbamylase-like protein (the product of a gene denoted *argF′* [GI:22218874]) that is essential for arginine biosynthesis (16). However, repeated attempts to detect enzymatic carbamylation of a variety of substrates using the native or recombinant ArgF′ protein were unsuccessful. The *B. fragilis* ArgF′ protein shares only limited sequence homology with other OTCases (38% and 34% similarity to *Escherichia coli* ArgF and human OTCase, respectively) (16). By comparison, the amino acid sequence of the *B. fragilis* aspartate transcarbamylase (ATCase) catalytic subunit shares a much higher similarity (70.3%) with *E. coli* ATCase. Further analysis of the *B. fragilis* ArgF′ protein sequence revealed that a conserved Ser-Met-Gly (SMG) motif, which is present in all OTCases, was absent. This motif is part of a flexible loop that moves to cradle L-ornithine when it binds to OTCase (17, 19). This difference leads us to hypothesize that the *B. fragilis* ArgF′ protein represents a new class of transcarbamylase.

A more recent analysis of microbial genome sequences available at NCBI using BLAST allowed us to identify several other transcarbamylase-like genes that also lacked the SMG motif in *Xanthomonas axonopodis*, *Xylella fastidiosa*, *Bacteroides thetaiotaomicron*, *Cytophaga hutchinsonii*, *Tannerella forsythensis*, *Prevotella ruminicola*, and other eubacteria. Thus, the *argF′* genes are not rare.

In the current study, we cloned the *argF′* gene from *Xanthomonas campestris*, expressed it in *E. coli*, and characterized its enzymatic activity. Evidence is provided herein that the gene annotated as *argF* in the genome of *X. campestris* does not encode an OTCase but rather encodes an enzyme that belongs to a new family of transcarbamylases that catalyze the transfer of a carbamyl moiety to *N*-acetyl-L-ornithine (*N*-acetylornithine) to produce *N*-acetyl-L-citrulline (*N*-acetylcitrulline), defining a new pathway for arginine biosynthesis.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth media. *E. coli* DH5α was used for routine cloning. Strain C6.1 (14), lacking the two OTCase genes *argI* and *argF*, was used for complementation experiments. LB broth was the rich medium, and M63 with 0.5% glucose and supplements, as required, was the defined medium. The vectors pTRC99 and pET28a (Novagen) were used for the cloning of the *argF′* and *argE* genes from *X. campestris*.

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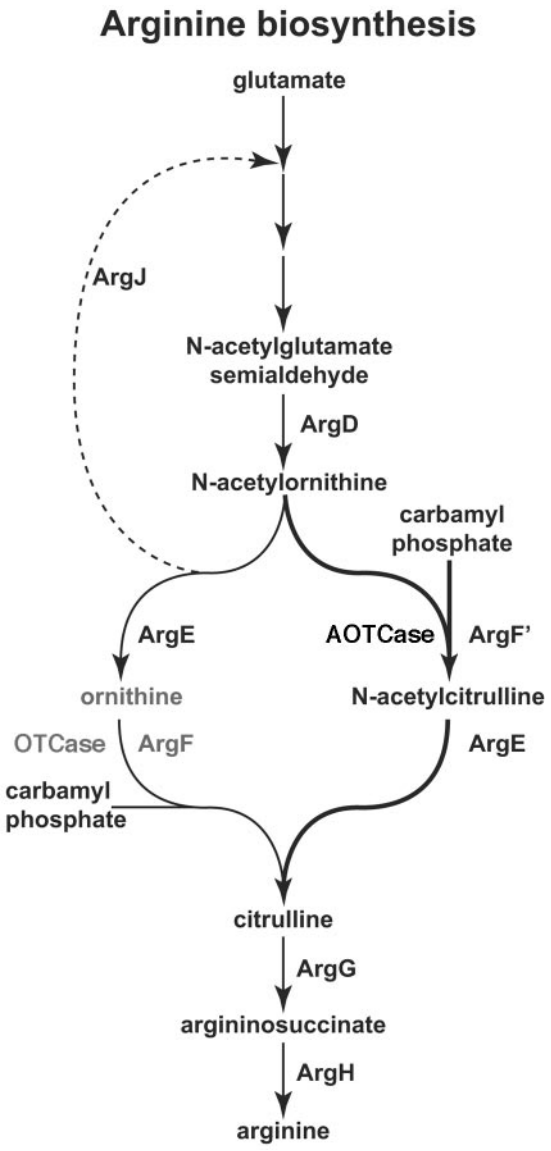

FIG. 1. Comparison of conventional and proposed arginine biosynthetic pathways. The novel arginine biosynthesis pathway inverts the order of transcarbamylation and deacetylation. *N*-Acetylornithine is converted to *N*-acetylcitrulline, which is subsequently deacetylated. The dashed line indicates the ArgJ recycling mechanism seen in many prokaryotes.

Materials. Unless otherwise noted, all chemicals were purchased from Sigma Chemical Company (St. Louis, MO). *N*-Acetylcitrulline was custom synthesized and purified by Chiral Quest Company (Monmouth Junction, NJ). N^{δ}-phosphonoacetyl-L-ornithine (PALO) was synthesized previously (10). N^{α}-Acetyl-N^{δ}-phosphonoacetyl-L-ornithine (PALAO) was custom synthesized by IMI TAMI Institute for Research and Development, Ltd. (Haifa, Israel).

Sequence alignment and phylogenetic analyses. A query using the keyword 2.1.3.3 (the EC number for OTCase) with NCBI Entrez (30) returned 474 protein sequences. Preliminary sequence data from additional microbial genomes were obtained from The Institute for Genomic Research (http://www.tigr.org). Sequences that appeared truncated or incomplete were discarded. The remaining genes were then screened for the presence or absence of the SMG motif using the EMBOSS module FUZZPRO (13). Using the *B. fragilis* ArgF′ sequence in a BLAST search, we identified additional candidates from the Sargasso Sea environmental sequencing project (27). A preliminary multiple sequence alignment using six *Bacteroidetes* and xanthomonad sequences was generated using the GCG module PILEUP (5), and islands of homologous sequences were determined.

Cloning the *X. campestris argF′* gene. The *X. campestris argF′* gene (GI: 21113386) was PCR amplified from genomic DNA of strain ATCC 33913 (ATCC) using Hot Start Turbo *Pfu* DNA polymerase (Invitrogen) with primers 5′-GACATATGTCACTGAAGCACTTCTTGAACACC-3′ and 5′-GCGGATCCTCACGGGCGGCTCTGACCCAC-3′ to introduce NdeI and BamHI sites (underlined) for in-frame cloning. The amplified products were cloned into vector pCR4.1 using a Zero-blunt Topo cloning kit (Invitrogen) and transformed into *E. coli* DH5α cells. A plasmid with the correct insert was identified by restriction enzyme analysis, and the sequence of the insert was verified. Plasmid DNA was digested with NdeI and BamHI, and the fragments were ligated into a pET28a expression vector (Novagen) using T4 DNA ligase and then transformed into *E. coli* DH5α cells, The resulting pHIS-$ArgF_{xc}$ plasmid DNA was purified using a QIAGEN High Speed MidiPrep kit. For complementation studies, *X. campestris argF′* was amplified using primers 5′-GACCATGGCATGAATGTCACTGAAGCACTTCTTGAACACC-3′ and 5′-GCGGATCCTCACGGGCGGCTCTGACCCAC-3′ to introduce NcoI and BamHI sites (underlined). The PCR product was digested with both enzymes and ligated into the cloning vector pTRC99a, which had been digested with NcoI and BamHI; the resulting plasmid was named pRAG299. After sequence verification, pRAG299 was used to complement C6.1 cells as described below. All digestions and ligation reactions were carried out using NEB enzymes.

The *X. campestris argE* gene that is annotated as an ornithine deacetylase (GI:21113380) was cloned in an analogous manner from genomic DNA using primers 5′-GACATATGACCGATCTACTCG-3′ and 5′-GCGAATTCCAGTGCGAGCCGTTGATG-3′ to amplify the open reading frame and introduce NdeI and EcoRI restriction sites (underlined) suitable for cloning into pET28a. The PCR product was cloned into vector pCR4.1 using a Zero-blunt Topo cloning kit (Invitrogen) and transformed into *E. coli* DH5α. After sequence verification of the insert, plasmid DNA was digested with NdeI and EcoRI, and the fragments were ligated into a pET28a expression vector that had been digested with NdeI and EcoRI and then transformed into *E. coli* DH5α. The resulting pHIS-$ArgE_{xc}$ plasmid DNA was purified using a QIAGEN High Speed MidiPrep kit.

Complementation of OTCase-deficient *E. coli* by *X. campestris argF′*. *E. coli* C6.1, a K-12 strain lacking both OTCase genes *argF* and *argI* (14), was transformed with either pRAG299 or the parental pTRC99A plasmid and grown in M63 medium with 0.5% glucose and 50 μg/ml proline and arginine overnight with shaking at 37°C. Dilutions were made into the same medium or in medium lacking arginine but containing 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside). Incubation was continued at 37°C with shaking, and samples were taken at suitable intervals for A_{600} measurement.

Protein purification: purification of *X. campestris* ArgF′ protein. Ten milliliters of an LB culture of BL21(DE3) pHis$ArgF_{xc}$ grown overnight with 50 μg/ml kanamycin was diluted 1:100 in 1 liter of the same medium. The cultures were incubated at 25°C until they reached an A_{600} of 0.4 to 0.6. At this point, IPTG was added to a final concentration of 0.2 mM to induce *argF′* expression, and incubation continued for 12 h. Cells were harvested by centrifugation, suspended in 40 ml of sonication buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10% glycerol, and 10 mM β-mercaptoethanol, pH 7.4), and lysed by sonication using a Bransonic Sonifer at approximately 50% maximal output for 10 min on ice. The lysates were cleared by centrifugation at 14,000 × *g* for 15 min. The supernatant fluid was filtered through a 0.22-μm filter and then loaded at a flow rate of 1 ml/min onto a 5-ml HisTrap Ni-affinity column (Amersham Biosciences) equilibrated with the sonication buffer. Weakly bound or unbound proteins were removed by washing the column with the same buffer containing 50 mM imidazole until the absorbance at 280 nm returned to baseline. The imidazole concentration was then increased to 250 mM to elute the protein of interest. Protein-containing fractions were pooled; dialyzed into a buffer containing 20 mM Tris-HCl, 50 mM NaCl, 1 mM EDTA, and 5 mM β-mercaptoethanol (pH 8.0); and then loaded onto a DEAE HighTrap column equilibrated with the same buffer running at 3.0 ml/min. A linear gradient of 20 column volumes with a solution containing 20 mM Tris-HCl, 250 mM NaCl, 1 mM EDTA, and 5 mM β-mercaptoethanol (pH 8.0) as the second buffer was used to elute the protein. The eluted protein was dialyzed and stored in a solution containing 20 mM Tris-HCl, 50 mM NaCl, 1 mM EDTA, and 5 mM β-mercaptoethanol (pH 8.0). The purified protein was concentrated using a Millipore 30,000-molecular-weight-cutoff spin filter to approximately 16 mg/ml and stored at 4°C. Protein purity was verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12% polyacrylamide gel) followed by Coomassie blue staining; a single band of the expected molecular mass (37.8 kDa) was observed. For all procedures, protein concentration was determined by the Bradford method using the Bio-Rad protein assay dye reagent with bovine serum albumin as a standard (1).

Purified recombinant *X. campestris* ornithine deacetylase (product of the *argE* gene) was overexpressed and isolated as described above for the *X. campestris* ArgF′ protein. Recombinant *B. fragilis* ArgF′ was obtained as previously described (16).

Chemical syntheses. *N*-Acetylcitrulline was chemically synthesized by Chiral Quest Co. Its structure and purity were verified by infrared spectroscopy and nuclear magnetic resonance (NMR) analysis. PALAO, a potential competitive inhibitor of ArgF′ activity, was chemically synthesized by IMI TAMI Institute of Research and Development Ltd. Its structure and purity (>95%) were verified by NMR analysis.

Colorimetric transcarbamylase enzyme assays. Transcarbamylase activity was measured by using a modification of the colorimetric assay to detect a ureido group (10), except that *N*-acetylornithine was substituted for L-ornithine. Fresh color reagent (1 part 0.8% 2,3-butadione monoxime [wt/vol] dissolved in 5% acetic acid [vol/vol] mixed with 2 parts 0.5% antipyrine dissolved in 18.1 N sulfuric acid) kept on ice was used to terminate the reactions. Samples were then wrapped in foil and placed on a shaker overnight. To develop the final color, the samples were uncovered and incubated for 20 min at 42°C with illumination. Details on optimizing the color development conditions were described previously by Pastra-Landis et al. (12). Initial reaction mixtures to determine the function of the ArgF′ protein contained 4 mM of *N*-acetylornithine and 4.8 mM carbamyl phosphate in 1 ml of a 50 mM Tris-HCl, pH 8.3, buffer. All assays were performed at room temperature (25°C). The reaction was initiated with carbamyl phosphate, allowed to proceed for 5 min at room temperature, and then quenched by the addition of 1 ml of antipyrine:butadione monoxime color reagent. The chromophore in the assay produced by commercially synthesized and purified *N*-acetylcitrulline had an absorption coefficient that was 93.4% of that of citrulline.

Assay of enzymatic products by liquid chromatography mass spectrometry (LC-MS). Two micrograms of acetylornithine transcarbamylase (AOTCase) was reacted with 1 mM carbamyl phosphate and 5 mM *N*-acetylornithine at 25°C in a 1-ml volume, and the reaction was terminated with the addition of 1 ml of 30% trichloroacetic acid (TCA) after 5 min. As a control, 2 μg of AOTCase that had been boiled for 5 min was used under identical reaction conditions. Samples were centrifuged for 10 min at 14,000 × *g* to remove the precipitated protein. Ten microliters of the reaction solution was analyzed using an Agilent LC-MS 1100 and conditions described previously by Caldovic et al. for *N*-acetylglutamate synthase activity determination (2). Protonated *N*-acetylornithine has an *m*/*z* of 175.1, citrulline has an *m*/*z* of 176.1, *N*-acetylcitrulline has an *m*/*z* of 218.1, PALO has an *m*/*z* of 255.0, and PALAO has an *m*/*z* of 297.2.

Detection of reaction products using ninhydrin. Ninhydrin preferentially reacts with free amino groups to produce a detectable product with a maximal absorbance at 570 nm. Ten micrograms of purified *X. campestris* ArgF′ was incubated for 5 min at room temperature with 5 mM *N*-acetylornithine–4.8 mM carbamyl phosphate in a 1-ml volume. One hundred microliters of the reaction mixture was removed and diluted to 500 μl with water, and the reaction was terminated with 500 μl of 7% sulfosalicylic acid. Nine hundred microliters of color reagent was added to the remainder to terminate the reaction and for quantitation of the product by the colorimetric assay. The sulfosalicylic acid-treated sample was analyzed using a Beckman amino acid analyzer according to the manufacturer's instructions. Separate injections of *N*-acetylornithine and carbamyl phosphate plus enzyme at concentrations identical to those used in the enzyme assay as well as citrulline and *N*-acetylcitrulline were used as controls and standards. Citrulline and *N*-acetylornithine have easily distinguishable different elution times, while *N*-acetylcitrulline elutes near phosphoethanolamine and has a markedly reduced absorption coefficient (23).

Comparison of phosphate and *N*-acetylcitrulline production. In order to monitor the two products of the AOTCase reaction, the rates of phosphate production and *N*-acetylcitrulline production were compared to show that they occurred in a 1:1 ratio. Phosphate production was measured using a method described previously by Fiske and Subbarow (4), incorporating modifications described previously by Spector et al. (20), to reduce the breakdown of carbamyl phosphate. A total of 0.02 μg of *X. campestris* ArgF′ was incubated for 5 min at 25°C in 1 ml of a solution containing 12.5 mM Tris, pH 8.3, 5 mM *N*-acetylornithine, and 0.1 mM carbamyl phosphate. The reaction was initiated by the addition of 100 μl of 1 mM carbamyl phosphate. Standards ranging from 0 to 100 nmol of potassium phosphate in 1 ml of 12.5 mM Tris, pH 8.3, were used. The reactions were terminated by the timed addition of 100 μl of 30% ice-cold TCA, followed by the timed addition of 200 μl of 10 mM ammonium molybdate (dissolved in 2.5 N H_2SO_4) and 50 μl of Fiske and Subbarow reducer (1-amino-2-naphthol-4-sulfonic acid–sodium sulfite–sodium bisulfate mixture, 1 g in 6.3 ml water; Sigma Chemical Co.). Samples were allowed to develop on ice for 5 min, and their absorbance at 660 nm was read. Baselines were established using samples where was enzyme was substituted with buffer. The average of the baseline values calculated from the standard curve was subtracted from the values for each of the enzyme-containing samples. Identical concentrations of components and reaction conditions were used for *N*-acetylcitrulline determination, and the reactions were also quenched with the timed addition of 100 μl of 30% ice-cold TCA. This was followed by the timed addition of 1 ml antipyrine:butadione monoxime color reagent. Samples were then processed in the same manner as described above for the AOTCase colorimetric assay. Samples where enzyme was substituted by buffer were used to establish a baseline for *N*-acetylcitrulline production.

NMR confirmation of enzymatically produced *N*-acetylcitrulline. Three milligrams of purified ArgF′ protein was transferred into buffer containing 50 mM sodium phosphate and 100 mM NaCl, pH 7.4, using three rounds of centrifugation with a Millipore Microcon YM30 spin filter according to the manufacturer's directions. A total of 15.4 μg of the buffer-exchanged protein was reacted with 4.8 mM carbamyl phosphate and 5 mM *N*-acetylornithine dissolved in the same buffer at pH 8.3. After overnight incubation at 25°C, the enzyme was removed by centrifugation through a Millipore Microcon YM30 filter. Two micrograms of carbamyl phosphate, *N*-acetylornithine, and chemically synthesized *N*-acetylcitrulline were dissolved in the phosphate buffer used for the assay for use as standards. The enzymatically and chemically synthesized *N*-acetylcitrulline, carbamyl phosphate, and *N*-acetylornithine were subjected to three rounds of lyophilization and resuspension in D_2O prior to analysis to suppress the residual proton (^1H) water signals.

One-dimensional ^1H NMR was performed using a Bruker NMR spectrometer (DRX-500; Bruker, Billerica, MA) equipped with a tri-nuclei inverse probe with a Z gradient at 500.13 MHz at 25°C ± 0.5°C. Chemical shifts were calibrated with an external reference of 3-trimethylsilyl propanesulfonic acid, sodium salt, set as 0 ppm at the same temperature. NMR line assignments were based on the similarities (within 2 Hz) in the one-dimensional ^1H chemical shifts between the model compounds (chemically synthesized *N*-acetylcitrulline and *N*-acetylornithine) and the enzymatically synthesized product at the same pH, ionic strength, and temperature. Two-dimensional NMR-correlated spectroscopy (COSY) with solvent presaturation was carried out using a Bruker NMR spectrometer (AV-400) fitted with a broadband inverse probe with a Z gradient at 400.13 MHz at 25°C. The relaxation delay was 2.5 s. The two-dimensional matrix for COSY was 2,000 by 512, yielding digital-free induction decay resolutions of 2.1 Hz and 8.6 Hz, respectively. Linear prediction was used for processing of the data, giving a matrix size of 2,000 by 1,000.

AOTCase catalyzed production of *N*-acetylornithine via arsenolysis. The reverse reaction, formation of *N*-acetylornithine or ornithine from *N*-acetylcitrulline or citrulline, respectively, was performed in a manner similar to that described previously by Sainz et al. (15), except that the formation of *N*-acetylornithine or ornithine was detected by LC-MS analysis. Two micrograms of *X. campestris* ArgF′ protein or human OTCase was incubated for 3 h at 37°C in 1 ml containing 20 mM *N*-acetylcitrulline, or 20 mM citrulline, and 20 mM sodium arsenate in 150 mM imidazole buffer, pH 6.8. Control reactions were performed with buffer replacing arsenate or by omitting the enzyme. The reactions were terminated by the addition of a 1:1 volume of 30% TCA, and samples were centrifuged for 10 min at 14,000 × *g* to remove precipitated protein and analyzed using the LC-MS as described above.

Inhibition assays of *X. campestris* ArgF′ protein. PALAO or PALO varied between 9 nM and 90 μM in a series of 1-ml assay reaction mixtures containing 0.2 μg of purified *X. campestris* AOTCase, 1 mM carbamyl phosphate, and 5 mM *N*-acetylornithine in 50 mM Tris-HCl buffer, pH 8.3. The reactions were allowed to proceed for 5 min at room temperature and were then terminated and processed as described above for the colorimetric assay.

Deacetylation of acetylornithine and acetylcitrulline by *X. campestris* ArgE protein. The activity of the ArgE protein from *X. campestris* was determined using a Biochrom 30 amino acid analyzer to measure the formation of ornithine and citrulline from *N*-acetylornithine and *N*-acetylcitrulline, respectively. The method described previously by Vogel and McLellan (28) was adapted for a 500-μl assay mixture volume. Twenty micrograms of purified enzyme was incubated for 10 min at 37°C in a 500-μl mixture containing 80 mM potassium phosphate, 20 μM $CoCl_2$, and 80 μM glutathione, containing a range of 0.1 to 6 mM *N*-acetylornithine or *N*-acetylcitrulline, pH 7.0. Samples containing 6 mM of substrate and enzyme that had been boiled for 10 min, or with the enzyme omitted, were used as controls. The reactions were initiated by the addition of enzyme and allowed to proceed for 20 min. Four hundred microliters of the reaction mixture was terminated with the addition of an equal volume of 7% sulfosalicylic acid, and deacetylated amino acids were detected using the amino acid analyzer.

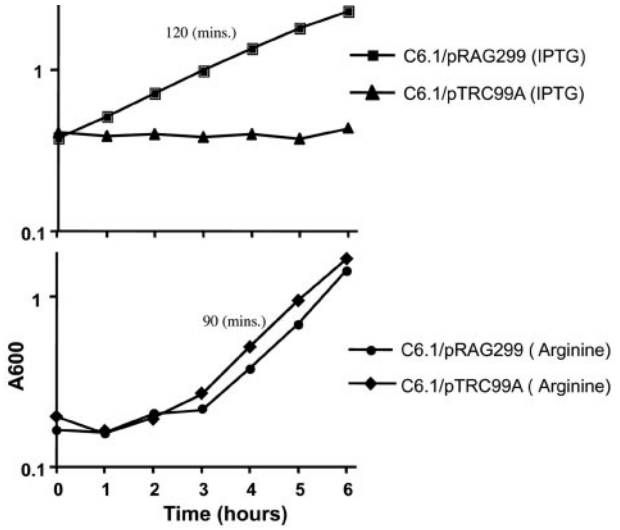

FIG. 2. Expression of the *X. campestris argF′* gene rescues *E. coli* C6.1 from arginine auxotrophy. *E. coli* ΔOTCase strain C6.1 transformed with pRAG299 containing the gene for *X. campestris argF′*, or the parental plasmid pTRC99a, was grown as described in Materials and Methods and diluted into either minimal medium lacking arginine but containing IPTG to induce expression (upper panel) or minimal medium supplemented with arginine (lower panel). The values listed along the graphs are the calculated doubling times.

RESULTS

Complementation of *E. coli argF* mutants by *argF′*$_{xc}$. Plasmid pRAG299, containing the cloned *argF′* genes from *X. campestris*, was introduced into the *E. coli* arginine auxotroph C6.1, which lacks both genes for OTCase (*argI* and *argF*). Upon induction of *argF′* gene expression by IPTG, plasmid-containing cells were now capable of growth in the absence of arginine (Fig. 2). Thus, the ArgF′ protein from *X. campestris* functions in *E. coli* and provides a reaction product that can be used for arginine biosynthesis. However, no OTCase catalytic activity could be detected in these cells (see below).

Catalytic activity of ArgF′$_{xc}$. When transcarbamylase activity was assayed with carbamyl phosphate and ornithine as substrates in crude *E. coli* lysates from cells containing the expressed *X. campestris* ArgF′$_{xc}$ protein, no significant citrulline production was detected (Table 1). Since the complementation experiments indicated that cells expressing the *X. campestris* protein could synthesize arginine, we tested other substrates that could be used by the ArgF′ protein in the crude lysates. Only *N*-acetyl ornithine and carbamyl phosphate were found to serve as precursors to produce a reactive ureido group. Thus, the *X. campestris* ArgF′ enzyme carbamylates-acetyl-ornithine rather than ornithine and is an *N*-acetyl-L-ornithine transcarbamylase (AOTCase).

Properties of purified *X. campestris* AOTCase. *X. campestris* AOTCase, purified as described above, showed Michaelis-Menten kinetics for both substrates with a K_m of 1.05 ± 0.23 mM and a V_{max} of 65.28 ± 4.77 μmol/min/mg for *N*-acetyl-ornithine and a K_m and V_{max} of 0.01 ± 0.001 mM and 50.68 ± 1.03 μmol/min/mg, respectively, for carbamyl phosphate. Compared to the affinity of the human OTCase for ornithine (K_m, 0.11 mM ± 0.02 mM), the affinity of AOTCase for *N*-acetyl-ornithine is almost 10-fold less, while the affinity for carbamyl phosphate is approximately fivefold greater (11).

The reaction product of AOTCase is *N*-acetylcitrulline. To prove that the reaction product of AOTCase is indeed *N*-acetylcitrulline, we used information from the colorimetric assay, ninhydrin reactivity, LC-MS, and NMR analysis. The colorimetric assay detects the ureido group common to the side chains of both citrulline and *N*-acetylcitrulline. Ninhydrin preferentially reacts with free amines and would therefore react strongly with citrulline but weakly with *N*-acetylcitrulline, which has a blocked alpha amino group. LC-MS was used to calculate the mass of the product. When 10 μg of purified ArgF$_{xc}$ protein was added to 4.8 mM carbamyl phosphate and 5 mM *N*-acetylornithine, 136 nmol of a product with a reactive ureido compound in the colorimetric assay was produced. This product was not citrulline, based on amino acid analysis (Fig. 3). Analysis of the same enzyme reaction by LC-MS (Agilent 1100) revealed a peak with a mass equal to that of protonated *N*-acetylcitrulline (218.1 *m/z*). LC-MS analysis of chemically synthesized *N*-acetylcitrulline by the same methods yielded the same mass ion (Fig. 4). All the proton NMR peaks of the chemically synthesized *N*-acetylcitrulline had chemical shifts identical to those seen for the enzymatically generated product (Fig. 5). The peaks seen between 3.5 and 3.8 ppm in the enzymatically produced *N*-acetylcitrulline were determined to be due to a separate impurity in the sample by the absence of correlating peaks in two-dimensional COSY-NMR (Fig. 5). Comparison of carbon-proton ^{13}C-^{1}H heteronuclear single quantum correlation NMR measurements of the enzymatic and chemically synthesized *N*-acetylcitrulline also showed identical correlations between the ^{1}H and ^{13}C signals and also indicated that these additional peaks did not correlate with any of the carbons of *N*-acetylcitrulline (data not shown).

TABLE 1. Comparison of transcarbamylase activity in crude cell lysates and purified enzymes[a]

Enzyme	Activity (nmol/min/mg ± SEM)			
	Crude lysate		Purified enzyme	
	Citrulline	*N*-Acetylcitrulline	Citrulline	*N*-Acetylcitrulline
X. campestris ArgF′	3.7 ± 0.04	63.9 ± 0.6	ND	13911.4 ± 394.9
Homo sapiens OTCase	588.3 ± 4.9	22.2 ± 0.9	15353.6 ± 113.4	192.8 ± 8.9
B. fragilis ArgF′	2.5 ± 0.03	0.9 ± 0.1	ND	314.9 ± 6.8

[a] Ornithine and acetylornithine transcarbamylase activity in purified protein and crude lysates of *E. coli* overexpressing *X. campestris* ArgF′, human OTCase, or *B. fragilis* ArgF′. Lysates boiled for 5 min were used as negative controls and showed no activity. A total of 4.8 mM carbamyl phosphate and 5 mM *N*-acetylornithine or ornithine were used in a 1-ml volume under the conditions described in Materials and Methods. Means ± standard errors of the means (n = 3) are shown. ND, product formation was below the limits of detection.

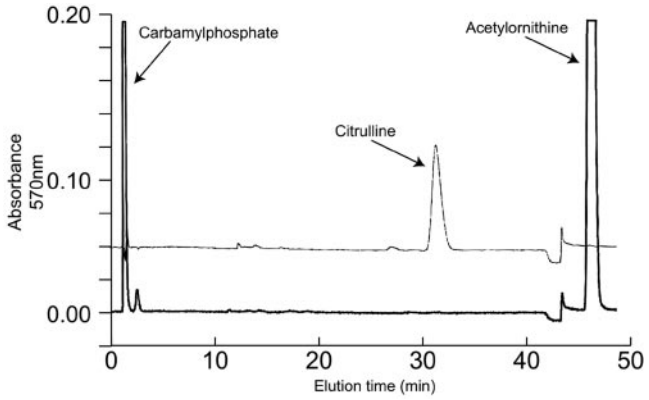

FIG. 3. The amino group of the enzymatically synthesized product is blocked. The enzyme reaction described in Materials and Methods was analyzed using an amino acid analyzer for ninhydrin reactivity to determine if the reaction *N*-acetylornithine + carbamyl phosphate produced *N*-acetylcitrulline or citrulline. Only peaks corresponding to carbamyl phosphate and *N*-acetylornithine were seen (thick trace). Based on the colorimetric transcarbamylase assay, 136 nmol of a ureido-containing compound was formed in the enzyme reaction. For comparison, the absorbance and retention profile of 110 nmol of citrulline from the amino acid analyzer is shown as a thin trace that is vertically offset by approximately 0.05 absorbance units.

Both phosphate and *N*-acetylcitrulline are produced in equal amounts. The rates of inorganic phosphate and *N*-acetylcitrulline production were measured and compared. Phosphate and *N*-acetylcitrulline were produced at the same rate under the conditions used, 37.74 ± 2.33 nmol/min/mg and 37.21 ± 1.15 nmol/min/mg ($n = 10$), respectively, as would be expected for a group transfer reaction, supporting the following proposed enzyme reaction: *N*-acetylornithine + carbamyl phosphate→phosphate + *N*-acetylcitrulline.

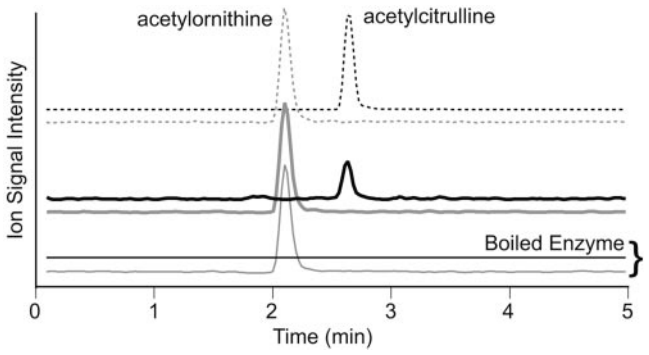

FIG. 4. LC-MS analysis of enzymatically synthesized *N*-acetylcitrulline. The enzymatic product of the *X. campestris* AOTCase reaction (thick solid black line) has the same mass ion and retention time as chemically synthesized *N*-acetylcitrulline (dashed black line). Traces of 5 mM *N*-acetylcitrulline (topmost trace, dashed black line) and 5 mM *N*-acetylornithine (second from top, dashed gray line) are shown for reference. Samples with boiled (thin solid traces) and unboiled (thick solid traces) enzymes were analyzed by individually extracting the ions corresponding to *N*-acetylornithine (gray) and *N*-acetylcitrulline (black) from their total ion traces. Traces are shown offset vertically. The vertical scale of the extracted ion trace for enzymatically synthesized *N*-acetylcitrulline was multiplied by a factor of 10 for clarity.

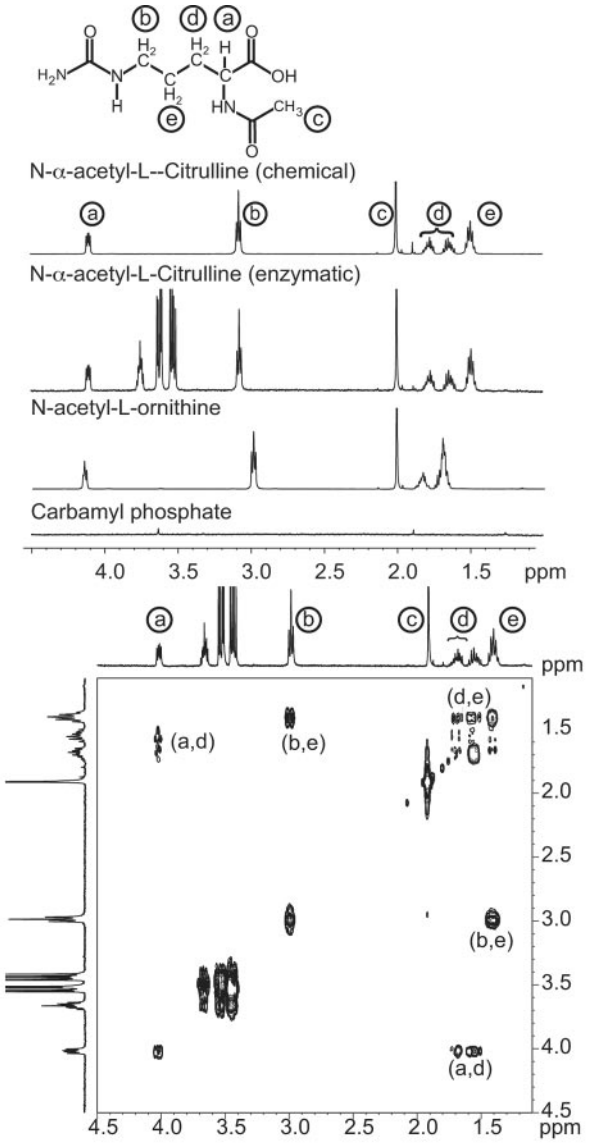

FIG. 5. COSY-NMR spectra show the enzymatically generated compound to be *N*-acetylcitrulline. (Top) The chemical structure of acetylcitrulline with the protons seen in the labeled NMR spectra. ^{1}H-NMR comparison of chemically and enzymatically synthesized *N*-acetylcitrulline, *N*-acetylornithine, and carbamyl phosphate. (Bottom) Two-dimensional COSY-NMR of the enzymatically synthesized *N*-acetylcitrulline sample. The self-correlation peaks can be seen on the diagonal from lower left to upper right. Coupled peaks are seen off the diagonal. Protons that are coupled are identified by tracing horizontally and vertically off a point on the diagonal to a coupled peak and then returning vertically or horizontally to a new peak on the diagonal. The peaks between 3.5 and 3.8 ppm are not coupled to any other groups in *N*-acetylcitrulline and are likely due to a contaminant from the enzyme preparation.

Specificity of the reverse reaction. *X. campestris* ArgF′ is able to use arsenolysis to convert *N*-acetylcitrulline to *N*-acetylornithine but does not convert citrulline to ornithine, indicating that it has preferential specificity for the acetylated form of the product (Fig. 6).

***X. campestris* ArgE can deacetylate *N*-acetylcitrulline and *N*-acetylornithine.** In the range of substrates used (0.1 to 6

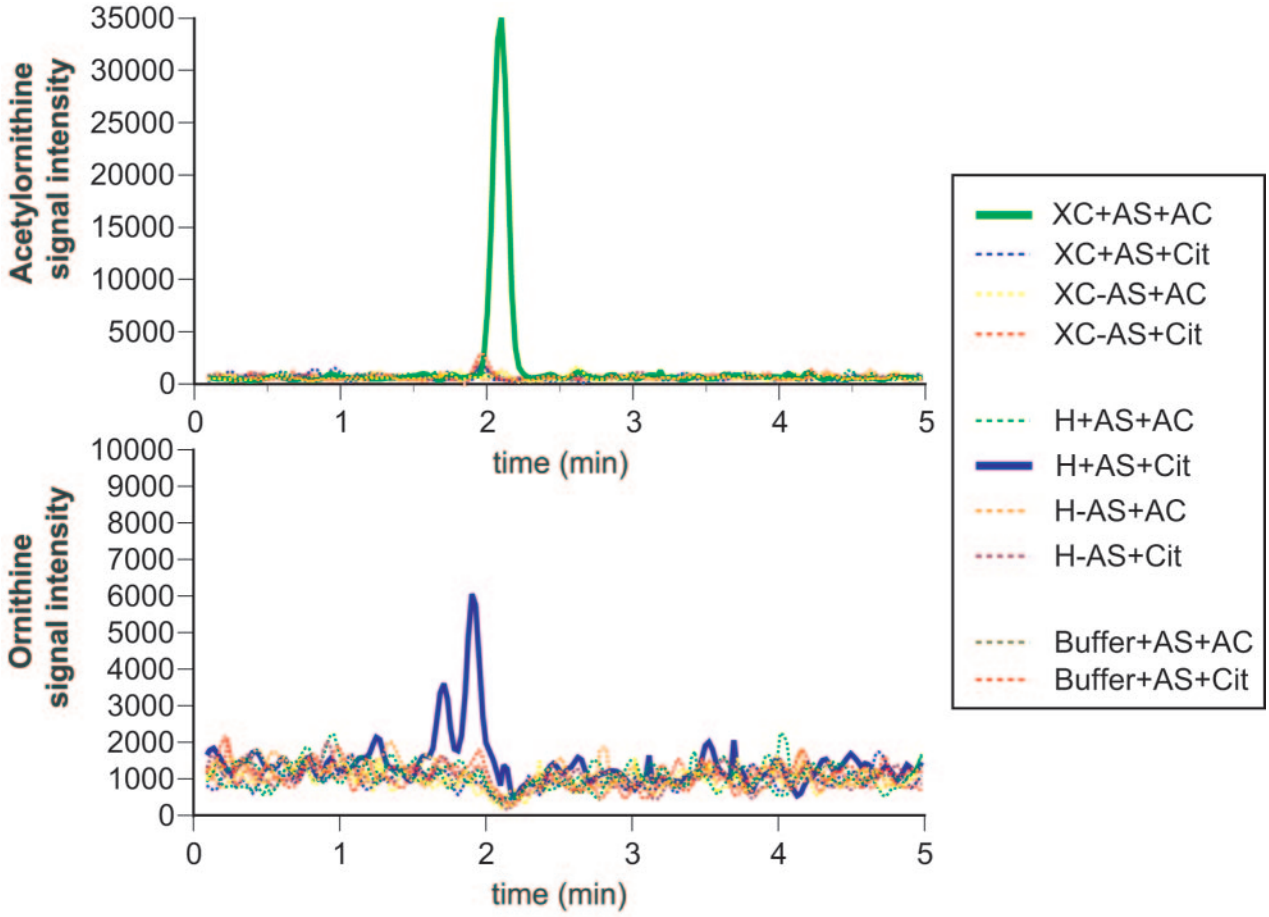

FIG. 6. Arsenolysis of *N*-acetylcitrulline and citrulline. *X. campestris* AOTCase (XC) and human OTCase (H) were incubated with *N*-acetylcitrulline (+AC) or citrulline (+Cit) in the presence and absence of arsenate (+AS and −AS, respectively) as described in Materials and Methods, and samples were analyzed using LC-MS. The total ion data were collected, and the signals corresponding to either *N*-acetylornithine or ornithine were extracted from the same data set. The upper panel shows the intensity of the 175 mass ion signal, corresponding to *N*-acetylornithine. The lower panel shows the intensity of the 133 mass ion signal, corresponding to ornithine. The ionization efficiency of ornithine is markedly lower than that of *N*-acetylornithine. Signals corresponding to *N*-acetylcitrulline and citrulline were also extracted from the data set, and the decreases in *N*-acetylcitrulline or citrulline peak areas relative to the buffer blanks were used to estimate the amounts of product formation. These peaks represent 1.8 μmol of *N*-acetylornithine and 1.6 μmol of ornithine.

mM), *X. campestris* ArgE deacetylated both *N*-acetylcitrulline and *N*-acetylornithine with almost equal velocity and showed apparent first-order rate constants of 0.0290 min^{-1} and 0.0292 min^{-1}, respectively.

PALAO is a potent inhibitor of AOTCase. Acetic anhydride treatment of PALO led to the formation of a compound with the expected mass of protonated PALAO (*N*-acetylated PALO, *m/z* 297) (Fig. 7). PALAO was tested for inhibition of AOTCase activity in the presence of *N*-acetylornithine and carbamyl phosphate. As shown in Fig. 8, PALAO, but not PALO, showed pronounced inhibition. Under the conditions used (1 mM carbamyl phosphate, 5 mM *N*-acetylornithine, pH 8.3), half-maximal inhibition occurred at approximately 22.4 nM PALAO.

DISCUSSION

In all previously studied prokaryotic and eukaryotic species capable of the de novo synthesis of arginine via acetylated intermediates, deacetylation of *N*-acetylornithine to produce ornithine precedes the transcarbamylation reaction that yields citrulline. The AOTCase described in this paper catalyzes the formation of a new intermediate in a novel arginine biosynthetic pathway found in bacteria that do not possess the canonical *argF* gene.

Purified ArgF′ enzyme from *Xanthomonas* is unable to catalyze the OTCase reaction (formation of citrulline from ornithine) but instead catalyzes the formation of *N*-acetylcitrulline from *N*-acetylornithine (Fig. 1). The *X. campestris* AOTCase shares 49.8% similarity to the *B. fragilis* ArgF′ protein. Despite the fact that the *argF′* gene from *B. fragilis* ($argF'_{bf}$) was the first of these transcarbamylases to be identified, we are still unable to measure the activity of this enzyme in extracts of *B. fragilis* or from *E. coli* cells expressing the $argF'_{bf}$ gene using ornithine or *N*-acetylornithine as the second substrate. Expression of the related *C. hutchinsonii argF′* gene showed detectable AOTCase activity in crude lysates, but the specific activity for *N*-acetylornithine of both the lysates and purified protein was very low.

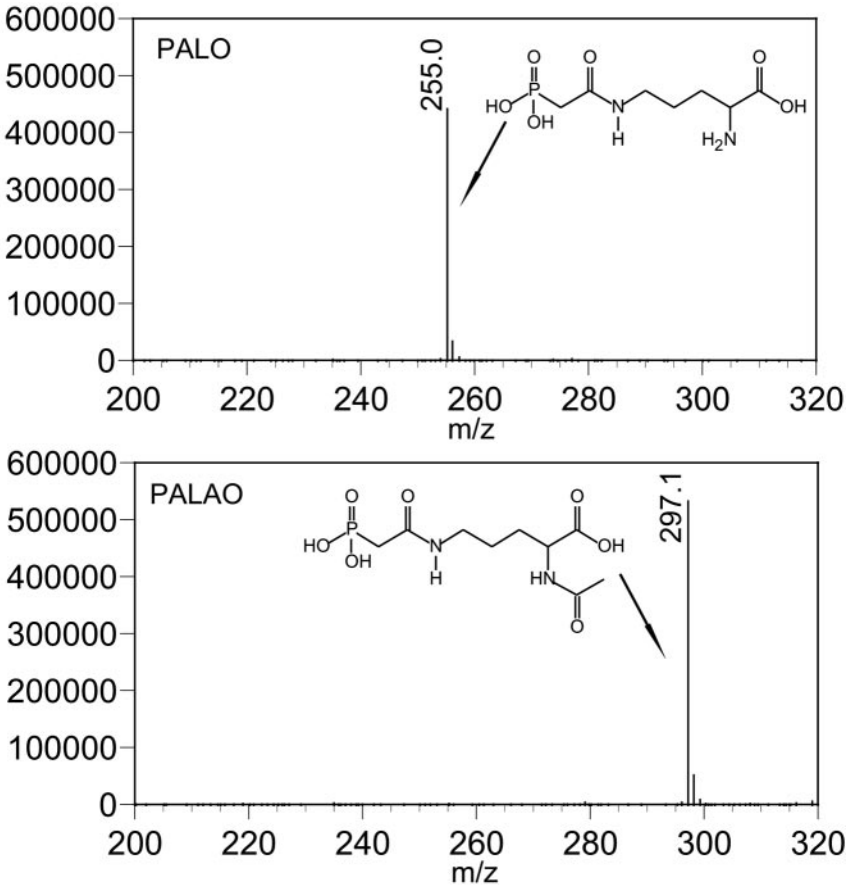

FIG. 7. LC-MS analysis of PALAO. PALAO synthesized by the acetylation of PALO (*m*/*z* 255.0) has a predicted *m*/*z* of 297.1 that is detectable by LC-MS.

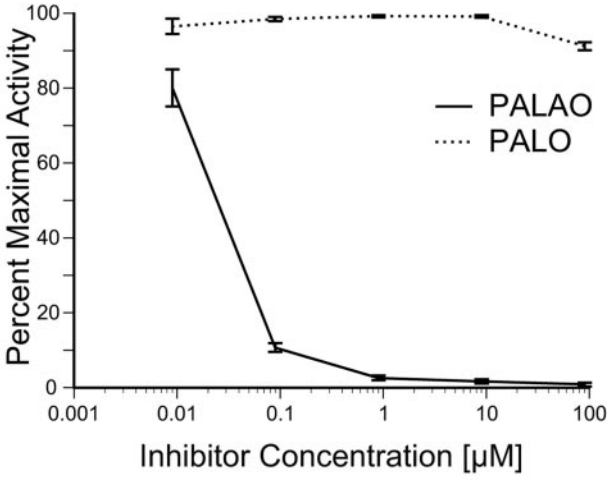

FIG. 8. Inhibition of *X. campestris* AOTCase by PALAO. The addition of PALAO (solid line) to the enzymatic reaction showed marked inhibition of AOTCase, while little inhibition was seen if PALO (dashed line) was used at the same concentrations. The averages of two independent assays are plotted, with error bars denoting the range. Maximal activity was the amount of *N*-acetylcitrulline formed in the assay under the same conditions without an inhibitor.

With the information that *X. campestris* ArgF′ is an AOTCase, we were recently able to solve the structure of this protein liganded to the reaction product *N*-acetylcitrulline (18). Although this enzyme can catalyze the reverse reaction, the formation of *N*-acetylornithine from *N*-acetylcitrulline, we do not yet know whether AOTCase serves in a catabolic capacity for the organism(s), as is the case with certain OTCases (22).

Distribution of AOTCase in bacteria. Our search for homologous *argF′* genes in various genomes indicates that the AOTCase protein is not rare and is found in several eubacterial species. Of 474 sequences annotated as OTCase (EC 2.1.3.3) in the databases, 56 do not contain the SMG motif that is nearly universally seen in OTCase when queried using the EMBOSS module FUZZPRO. This group was aligned using PILEUP (5), and the cluster associated with *B. fragilis* was used for further analysis. Ten complete *argF′* genes from the following organisms were found and annotated as potential carbamoyltransferases: *Bacteroides thetaiotaomicron*, *Cytophaga hutchinsonii*, several pathovars of *Xylella fastidiosa*, *Xanthomonas campestris*, *Xanthomonas axonopodi*s, *Prevotella ruminicola*, and *Tannerella forsythensis. Xanthomonas* and *Xylella*, members of the *Xanthomonadales*, are a subclass of the γ-*Proteobacteria* group, primarily known as plant pathogens that damage a wide variety of economically important crops. Other species included *Cytophaga*, an aerobic gram-negative soil bac-

terium that has been taxonomically classified with *Bacteroides* in the *Cytophaga-Flavobacterium-Bacteroides* group. Although the ArgF′ protein from *Cytophaga hutchinsonii* was able to produce acetylcitrulline, the specific activity of the enzyme was quite low, and the closely related ArgF′ from *Bacteroides fragilis* had no detectable AOTCase activity in crude lysates or in the purified form. Distinct differences in the positions of insertions and gaps were seen in the alignments that also correlated with the phyla division between the xanthomonads and *Bacteroidetes*; the effects of these changes on substrate specificity and function are being investigated. Of the many OTCase candidates retrieved using a BLAST query of *X. campestris* AOTCase against the Sargasso Sea environmental isolate collection, seven appear to be more closely related to AOTCases than the canonical OTCases. Alignment of these seven sequences show that three of them (GI:43643189, GI:44587056, and GI:44518502) cluster with the xanthomonads, while the other four (GI:43262406, GI:44636812, GI:44286498, and GI:43259584) are more closely related to *Bacteroides*. The identification of these AOTCase-like genes in these environmental samples suggests that this mode of arginine biosynthesis may be common. Although *Chlorobium* and fibrobacteria are considered to be close relatives of *Bacteroides* and are included in the *Cytophaga-Flavobacterium-Bacteroides* group, the ArgF sequences identified from genomic data for *Chlorobium tepidum* and *Fibrobacter succinogenes* have the SMG motif and appear to be canonical OTCases, sharing 49% and 53% amino acid similarity with *E. coli* OTCase, respectively (3).

Evolutionary considerations. The degree of OTCase sequence conservation across all branches of the tree of life is quite high, with clearly recognizable sequence motifs for both carbamyl phosphate and ornithine binding. In addition to OTCase and ATCase, which are the best-characterized members of this superfamily, several transcarbamylases that catalyze the transfer of the carbamyl moiety to canaline, oxamate, or putrescine have been described (8, 24, 29). Extensive work on the evolutionary history of the transcarbamylase superfamily has been reported previously by Labedan et al. (7). Those authors suggested that the last universal common ancestor to all extant life today had both a pyrimidine biosynthetic pathway utilizing ATCase and an OTCase-dependent arginine biosynthetic pathway. Although the newly identified AOTCase protein in *X. campestris* reported herein represents a newly discovered class of transcarbamylases, it may in fact represent an ancient arginine biosynthetic pathway. Our findings indicate that the topology of the transcarbamylase phylogenetic tree is more complex, with a cluster of sequences that we now have shown to be AOTCase-like, located at the earliest branch to the root of the ATCase/OTCase tree. The genomes of *Xanthomonadales* and *Bacteroidetes* contain only *argF′* genes and no other *argF* homologs; thus, these genes play an essential role in arginine biosynthesis in these organisms. Since the AOTCase family arose at one of the earliest branch points from the root of the ATCase/OTCase tree described previously by Naumoff et al. (11), the existence of this alternate arginine biosynthetic pathway pushes back the date of the last universal common ancestor. It therefore appears that these two paths of arginine biosynthesis arose very early in evolution and that organisms utilizing the canonical OTCase-dependent mode went on to dominate the biosphere. Despite the similarities between the *B. fragilis* and *X. campestris* genes, *B. fragilis* ArgF′ does not catalyze the AOTCase reaction. Although the ArgF′ derived from another member of the *Bacteroidetes*, *Cytophaga hutchinsonii* (GI:48855246), was found to be able to catalyze the AOTCase reaction, the specific activity of the purified protein was extremely low (data not shown), suggesting either protein instability or that another ornithine derivative is the preferred substrate in the *Bacteroidetes* branch.

In organisms that use AOTCase for arginine biosynthesis, an additional modification of the canonical pathway is necessary to deacetylate one of the downstream metabolites. The *X. campestris* ArgE protein was able to produce citrulline from *N*-acetylcitrulline, which could fulfill this role. Generally, ArgE deacetylases have a fairly broad range of substrates, including N^{α}-acetyl-L-methionine and N^{α}-formyl-L-methionine (6), and it would appear that *E. coli* ArgE protein may also be able to deacetylate acetylcitrulline, forming a likely basis for the complementation of *argI*/*argF*-deficient *E. coli* by *X. campestris argF′*. In the absence of a gene product able to acetylate L-citrulline, the AOTCase-dependent arginine metabolic pathway would be unidirectional, and its sole purpose would be to synthesize arginine. However, greater metabolic flexibility is achieved with a bidirectional canonical OTCase-dependent pathway. Indeed, many bacteria can utilize arginine as a carbon and energy source via OTCase when excess arginine is present in their environment (9).

AOTCase, a new specific target for inhibition. Specific inhibitors of AOTCase-dependent arginine biosynthesis may be useful for managing the spread of *Xylella* and *Xanthomonas* infestations of crops. Preparations of PALAO (acetylated PALO) showed pronounced inhibition of AOTCase. Acetylation of another structurally similar, potent inhibitor of OTCase (22), the active derivative of phaseolotoxin, N^{δ}-(*N′*-sulfodiaminophosphinyl)-L-ornithine, would also be expected to inhibit AOTCase. These and other inhibitors of AOTCase activity may provide tools for combating a variety of plant and animal pathogens.

The discovery of this novel class of transcarbamylases raises new questions about the events in the evolution of the arginine biosynthetic pathway, the answers to which will be a guide to a better understanding of the nature of the last universal common ancestor.

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