Microbiological Research 167 (2012) 317–325

Contents lists available at SciVerse ScienceDirect

Microbiological Research

journal homepage: www.elsevier.de/micres

A *Pseudomonas aeruginosa* PAO1 acetylcholinesterase is encoded by the *PA4921* gene and belongs to the SGNH hydrolase family

Diego G. Sánchez[a], Lisandro H. Otero[a], C. Magdalena Hernández[b], Ana L. Serra[a], Sergio Encarnación[b], Carlos E. Domenech[a], Ángela T. Lisa[a,*]

[a] Universidad Nacional de Río Cuarto, Río Cuarto, Córdoba, Argentina
[b] Centro de Ciencias Genómicas, Universidad Nacional Autónoma de México, A.P. 565-A, Cuernavaca, Morelos, Mexico

ARTICLE INFO

Article history:
Received 15 June 2011
Received in revised form
19 November 2011
Accepted 21 November 2011

Keywords:
Pseudomonas
Cholinesterase
PA4921 gene
SGNH hydrolase family

ABSTRACT

Through the use of molecular and biochemical experiments and bioinformatic tools, this work demonstrates that the PA4921 gene of the *Pseudomonas aeruginosa* PAO1 genome is a gene responsible for cholinesterase (ChoE) activity. Similar to the acetylcholinesterase (AchE) of *Zea mays*, this ChoE belongs to the SGNH hydrolase family. In mature ChoE, i.e., without a signal peptide, ^{18}Ser, ^{78}Gly, ^{127}N, and ^{268}H are conserved aminoacyl residues. Acetylthiocholine (ATC) and propionylthiocholine (PTC) are substrates of this enzyme, but butyrylcholine is an inhibitor. The enzyme also catalyzes the hydrolysis of the artificial esters *p*-nitrophenyl propionate (*p*NPP) and *p*-nitrophenyl butyrate (*p*NPB) but with lower catalytic efficiency with respect to ATC or PTC. The second difference is that *p*NPP and *p*NPB did not produce inhibition at high substrate concentrations, as occurred with ATC and PTC. These differences plus preliminary biochemical and kinetic studies with alkylammonium compounds led us to propose that this enzyme is an acetylcholinesterase (AchE) or propionylcholinesterase. Studies performed with the purified recombinant enzyme indicated that the substrate saturation curves and the catalytic mechanism are similar to those properties described for mammalian AchEs. Therefore, the results of this work suggest that the *P. aeruginosa* ChoE is an AchE that may also be found in *Pseudomonas fluorescens*.

1. Introduction

Cholinesterases (ChoEs) are serine hydrolases that catalyze the hydrolysis of choline esters and are classified according to their substrate specificities, e.g., acetylcholine acetylhydrolase or acetylcholinesterase (AchE) (EC 3.1.1.7), acylcholine acylhydrolase, propionylcholinesterase, and butyrylcholinesterase (EC 3.1.1.8). AchEs are widely distributed in animals and are present in nervous tissues and muscles, where they are responsible for the hydrolysis of acetylcholine. AchEs are also present in the membranes of noncholinergic tissues and in biological fluids (Cousin et al. 1996). AchEs have also been described in plants (Sagane et al. 2005). These AchEs belong to the lipase (GDSL) family and are not related to the family of animal α/β hydrolases (Sagane et al. 2005). In invertebrates, the described ChoE activities generally exhibit poor substrate specificities and highly variable kinetic behaviors (Talesa et al. 1993). In addition, ChoE activities have been detected in the fungi *Aspergillus* and *Penicillium* spp. (Lobanok et al. 1993). In prokaryotes, enzymes with ChoE activities have been reported in *Pseudomonas fluorescens* (Rochu et al. 1998), *Pseudomonas aeruginosa* (Tani et al. 1975; Garber and Nachshon 1980; Lisa et al. 1983), *Pseudomonas aurantiaca* (Velikanov et al. 1975), *Arthrobacter ilicis* (Mohapatra and Bapuji 1998), and *Aeromonas hydrophila* (Nieto et al. 1991).

In previous works, we reported that *P. aeruginosa* grown in media containing only choline, betaine, dimethylglycine or carnitine as one or the sole source of carbon and nitrogen synthesize ChoE, acid phosphatase (PchP), and hemolytic phospholipase C (PlcH) activities (Lisa et al. 1983, 2007; Lucchesi et al. 1989, 1995; Domenech et al. 1991). These three proteins, ChoE, PchP, and PlcH act as a system to acquire nutrients to meet the metabolic needs of the pathogen. The relationship between the catalytic activities of PlcH and PchP in obtaining choline and inorganic phosphate was described in our previous report (Lisa et al. 2007). Choline uptake occurs through various specific and nonspecific transporter systems (Malek et al. 2011). Inside cells, choline is metabolized to produce nitrogen, carbon and energy for bacterial growth (Kortstee 1970; Lisa et al. 2007; Wargo et al. 2008). Based on the catalytic action of *P. aeruginosa* ChoE (acetylcholine hydrolysis), prior study of the kinetic properties of this enzyme (Domenech et al. 1991), and the high acetylcholine concentration in the corneal epithelium (Pesin and Candia 1982), it has been suggested that ChoE may be

* Corresponding author at: Departamento de Biología Molecular, Universidad Nacional de Río Cuarto, 5800 Río Cuarto, Córdoba, Argentina. Tel.: +54 358 4676232; fax: +54 358 4676232.
E-mail addresses: tlisa@exa.unrc.edu.ar, teresita.lisa@gmail.com (Á.T. Lisa).

0944-5013/$ – see front matter © 2011 Elsevier GmbH. All rights reserved.
doi:10.1016/j.micres.2011.11.005

considered a pathogenic factor that contributes to corneal infection by *P. aeruginosa* (Domenech et al. 1991). Bacterial ChoE studies have described some of its biochemical properties; however, the gene that encodes the bacterial ChoE enzyme has never been located in bacterial genomes. Therefore, the aims of this work were the identification of the gene encoding ChoE in *P. aeruginosa* and the biochemical characterization of this ChoE to determine whether it is also an AchE.

2. Materials and methods

2.1. Bacterial strains and growth conditions

P. aeruginosa PAO1 wild-type (WT), *P. aeruginosa* PAO1-Δ*PA1342*, *P. aeruginosa* PAO1-Δ*PA4921* (Δ*choE*) and *P. fluorescens* Pf-5, *Pseudomonas putida* KT2440 and *Pseudomonas syringae* DC3000 strains were cultured in Luria-Bertani (LB) medium (Sambrook and Russell 2001) or high phosphate basal salt medium (HPi-BSM) supplemented with 20 mM choline, as has been previously described (Lucchesi et al. 1989, 1995; Domenech et al. 1991). *Escherichia coli* DH5α and BL21-CodonPlus were cultured in LB. The deletion mutant strains PAO1-Δ*choE* and PAO1-Δ*PA1342* were constructed by the methods of Choi and Schweizer (2005). Liquid cultures were routinely incubated at 37 °C with shaking. Antibiotics were used in growth media as follows: for *E. coli*, 100–150 μg/mL ampicillin, 30 μg/mL kanamycin, 15 μg/mL gentamycin, and for *P. aeruginosa*, 60 μg/mL gentamycin and 200 μg/mL carbenicillin.

2.2. Chemicals

Acetyl- (ATC), propionyl- (PTC), butyrylthiocholine iodide (BTC), *p*-nitrophenyl propionate (*p*NPP) and *p*-nitrophenyl butyrate (*p*NPB), choline chloride, butyrylcholine iodide, decamethonium bromide, procaine hydrochloride, atropine sulfate, trimethylamine hydrochloride (T3MA), tetraethylammonium bromide (T4EA), tetramethylammonium chloride (T4MA), and 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) were purchased from Sigma–Aldrich (USA). Cupric sulfate and potassium ferricyanide were purchased from Mallinckrodt (USA). Dimethyl sulfoxide was from Sintorgan (Argentine). All other reagents were of an analytical grade and were obtained from various sources. All mass spectrometry (MS) reagents were HPLC grade.

2.3. Steps to obtain a pure P. aeruginosa ChoE protein spot to perform MALDI-TOF

Various steps were necessary to perform the matrix-assisted laser desorption ionization/time-of-flight mass spectrometry (MALDI-TOF) analyses. Initially, *P. aeruginosa* wild-type was employed. However, after discovering that the spot from the 2D-PAGE that contained the ChoE activity was contaminated with another protein (PA1342), all steps were performed with the deletion mutant, *P. aeruginosa* PAO1-Δ*PA1342*, and there were: (i) *Ammonium sulfate precipitation*: periplasmic proteins from *P. aeruginosa* grown in HPi-BSM supplemented with 20 mM choline were isolated as previously described (Ames et al. 1984). The extracts, which contained 0.5–0.6 U/mg protein of ChoE activity, were precipitated with 90% (wt/v) ammonium sulfate and then suspended and dialyzed against 10 mM HCl-Tris buffer (pH 8.0). (ii) *Protein separation using an alkylammonium column*: fractions containing 1 mg of dialyzed proteins were applied to a Q-Sepharose column (15 mm × 200 mm; Sigma) equilibrated with 10 mM Tris–HCl buffer (pH 8.0). (iii) *Denaturation of protein extracts and two-dimensional polyacrylamide gel electrophoresis* (2D-PAGE). The ChoE activity found in the flow-through fraction, which was 20% of the initial protein with a specific activity of approximately 3 U/mg, was precipitated with 15% (v/v) trichloroacetic acid for 45 min (4 °C). Precipitated proteins were desalted by five washes with 1 mL of cold acetone. Before 2D-PAGE, the pellet was dried and mixed with 7 M urea, 2 M thiourea, 4% (w/v) 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate, 2 mM tributylphosphine, 2% (v/v) ampholytes, and 60 mM dithiothreitol, as previously described (Salazar et al. 2010). (iv) *Renaturation and specific staining of ChoE*: the denatured ChoE obtained in step (iii) was renatured by washing the gel three times with 10 mM Tris–HCl buffer (pH 8.0) and 5% (v/v) Triton X-100, as previously described (Lacks and Springhorn 1980). Detection of ChoE activity on the polyacrylamide gel was performed according to Karnovsky and Roots (K&R) staining (Karnovsky and Roots 1964), utilizing 33 mg/mL potassium ferricyanide and 0.5 mM ATC. (v) *Preparation of the sample for MALDI-TOF analysis*: the K&R positive spot was selected and excised manually. Both portions of the gel (the spot and remainder gel) were fixed with water/methanol/acetic acid (50:40:10) and completely discolored by repeated washings using 40 mM EDTA (pH 8.0). The gels were stained with colloidal Coomassie G-250, and the selected spot was prepared for mass spectrometry analysis as described below.

2.4. Proteomic experiments

The selected spots from preparative 2-D PAGE was excised and processed automatically using the Proteineer SP spot picker and DP digestion robots (Bruker Daltonics, Billerica, MA) following the manufacturer's instructions, as previously described (Encarnación et al. 2005). The excised spots were deposited into a 96-well digest adapter, digested using a DP Chemical kit (Bruker Daltonics, Billerica, MA), and automatically spotted onto the MALDI target. Mass spectra were obtained using a Bruker Daltonics Autoflex (Bruker Daltonics Bellerica, MA, USA) operated in delayed extraction and reflectron mode. The spectra were externally calibrated using a peptide calibration standard (Bruker Daltonics 206095). The MASCOT server 2.0 search engine was used to compare protein fingerprints against the NCBI database (release 2011-01) using the following parameters: TAXON-Bacteria (Eubacteria), mass tolerance up to 100 ppm, no missed cleavage allowed, carbamidomethyl cysteine as the fixed modification and oxidation of methionine as the variable modification.

2.5. DNA manipulations and primers

DNA manipulations and PCR amplifications were performed as previously described by Choi and Schweizer (2005) and Sambrook and Russell (2001). Chromosomal and plasmid DNA purifications were performed using the QIAamp DNA and QIAprep Mini-Spin kits (Qiagen), respectively. The specific primers utilized for the deletion mutants were as follows: UpF4921-Gw: 5′-TACAAAAAAGCAGGCTtgggttcttcacc aagtc-3′, UpR4921-Gm: 5′-TCAGAGCGCTTTTGAAGCTAATTCGttgaagacctggttgc gaca-3′, DnF4921-Gm: 5′-AGGAACTTCAAGATCCCCAATTCGttttcatgcagcggat cgct-3′, and DnR4921-Gw: 5′-TACAAGAAAGCTGGGTaccagatgcag-ttcaacacc-3′ for the PAO1-Δ*4921* strain; UpF1342-Gw: 5′-TACAAAAAAGCAGGCTttcggcaagcacga tactct-3′, UpR1342-Gm: 5′-TCAGAGCGCTTTTGAAGCTAATTCGgtgccggtttccttgat ctt-3′, DnF1342-Gm: 5′-AGGAACTTCAAGATCCCCAATTCGaagggcctgaacct-caactt-3′ and DnR4921-Gw: 5′-TACAAGAAAGCTGGGTtcaatcggttgc-agacactg-3′ for the PAO1-Δ*1342* strain. The common primers utilized were as followed: Gm-F: 5′-CGAA TTAGCTTCAAAAGCGCTCTGA-3′; Gm-R: 5′-CGAATTGGGGATC-TTGAAGTT CCT-3′; GW-*attB*1: 5′-GGGGACAAGTTTGTACAAAAAA-GCAGGCT-3′; GW-*attB*2: 5′-GGGGACCACTTTGTACAAGAAAGCTG-GGT-3′. The following primers were used for cloning the *choE*

gene: Up4921: 5′-ggaattccatatgcacacatcccc gctgct-3′ and Dn4921: 5′-accctgaccctgaagtgatg-3′. Sequences in capital letters are common for all genes amplified and overlap with the Gm or *attB* primer sequences. Lower-case letters indicate gene-specific sequences used for amplification of genes indicated by their PA annotation number. The underline sequences shown site of restriction enzyme.

2.6. Expression and purification of wild-type recombinant ChoE

To overexpress the ChoE protein, the gene was amplified without the sequence that encodes the signal peptide (the N-terminal 20 amino acids, as predicted by SignalP v.3.0) (Bendtsen et al. 2004) using the primers Up4921 and Dn4921 and DyNAzyme EXT DNA polymerase (Thermo SCIENTIFIC). The amplicon was cloned into the pCR2.1-TOPO vector (Invitrogen), sequenced, and subcloned into a pET15b vector (Novagen) using *Nde*I and *BamH*I (Genbiotech). The recombinant protein, an N-terminal His-tagged fusion, was expressed in *E. coli* BL21-CodonPlus (Novagen) as follows. The recombinant strain was grown in 1 L of LB medium containing 150 μg/mL ampicillin until it reached an $OD_{550} \cong 0.6$–0.7. Isopropyl β-D-1-thiogalactopyranoside (IPTG) (0.4 mM) was then added to induce protein expression. After 12–16 h at 20 °C, the culture was harvested at 8000 × *g* for 10 min, and the cells were broken with sonication. The soluble fraction was collected and purified using Ni-NTA beads (Promega) according to the manufacturer's instructions. Samples were analyzed by 12% (w/v) SDS-PAGE. The purified enzyme was dialyzed against 10 mM Tris–HCl buffer (pH 8.0) containing 30% (v/v) glycerol and stored at −20 °C. The specific activity of the recombinant enzyme preparation was 2428 U/mg proteins.

2.7. Enzymatic assay and kinetic and inhibition studies

A standard assay for measuring ChoE activity was performed as previously described using ATC as the substrate (Ellman et al. 1961; Domenech et al. 1981). Briefly, in a final volume of 0.5 mL, the assay mixture contained 100 mM sodium phosphate buffer (pH 7.0), 0.4 mM ATC and 0.33 mM DTNB. PTC and BTC were also tested as substrates under identical conditions. The reaction mixture was pre-incubated at 37 °C for 3 min, and then 10 μL (4.8 ng) of the diluted recombinant purified enzyme (1:2500) was added. When necessary, inhibitors were added to the reaction mixture before the enzyme. In the assays performed to obtain kinetic data, special care was taken to work with initial velocity values following a linear OD_{412} increment for the first 1–3 min, and the results were expressed per second of reaction. One unit of ChoE activity was defined as the amount of enzyme that released 1 μmol of thiocholine per minute at 37 °C.

*p*NPP and *p*NPB were used as substrates to determine esterase activity using a modification of a previously described procedure (Yang et al. 2002). *p*NPP and *p*NPB were first solubilized with dimethyl sulfoxide and diluted to 1.5 mM in a mixture assay containing, in a final volume of 0.5 mL, 100 mM sodium phosphate buffer (pH 7.0) and 4.8 ng or 120 ng of recombinant purified enzyme when the substrate was *p*NPP or *p*NPB, respectively. The activity was determined by measuring the rate of release of *p*-nitrophenol at 410 nm for the first 1–3 min of reaction. The extinction coefficient (ε) for *p*-nitrophenol under these conditions was of 6700 M^{-1} cm^{-1}. One unit of ChoE activity (as esterase) was defined as the amount of enzyme that released 1 μmol of *p*-nitrophenol per minute at 37 °C.

As in our previous studies (Otero et al. 2011; Beassoni et al. 2011), the kinetic data were analyzed by nonlinear least-squares regression using the DYNAFIT program (Kuzmic 1996) to assess the best-fitting mechanism (model discrimination analysis) and to calculate the corresponding constants. The reaction velocity for ATC and PTC was given by

$$v = \frac{V_{max\,1}([S]/K_m) + V_{max\,2}([S]^2/K_m \cdot K_{si})}{1 + ([S]/K_m) + ([S]^2/K_m \cdot K_{si})}$$

where $V_{max1} = k_{cat1}[E_T]$ and $V_{max2} = k_{cat2}[E_T]$.

The reaction velocity for *p*NPP, *p*NPB as well as ATC or PTC in the presence of Cho and T4MA was given by:

$$v = \frac{V_{max\,1}[S]}{K_m + [S]}$$

where $V_{max1} = k_{cat1}[E_T]$.

The effects of various alkylammonium compounds on enzymatic activity were determined with 0.4 mM ATC, PTC or 1 mM of *p*NPP and in the presence of the respective effectors at 1 mM, which were added before the enzyme.

2.8. Other methods

Protein concentrations were determined as previously described (Bradford 1976) or by spectrophotometric measurements at 280 nm using the theoretical molar extinction coefficient, $\varepsilon = 49{,}390\ M^{-1}\ cm^{-1}$, which was calculated using the "ProtParam" tool assuming that all cysteine residues are reduced (Gasteiger et al. 2005).

2.9. Bioinformatics analysis

ClustalX and ClustalW version 2.0 (Larkin et al. 2007) were used for multiple ChoE alignments. Phylogenetic analyses were performed using software described elsewhere (Tamura et al. 2007). To determine the conserved domains, another program, a CDD server (conserved domain database) was used, as described previously (Marchler-Bauer et al. 2009). The ChoE sequences of different organisms were obtained from the NCBI database (http://www.ncbi.nlm.nih.gov) and from the *Pseudomonas* genome database V_2 (http://www.pseudomonas.com). Comparisons of protein sequences were obtained using BLASTP searches (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Altschul et al. 1997, 2005).

3. Results

3.1. Identifying a protein with ChoE activity in P. aeruginosa and the gene encoding it

Using the *P. aeruginosa* PAO1-Δ*PA1342* strain, a protein with ChoE activity was identified, which is encoded by the *PA4921* gene (gi: 15600114). This result was obtained by following the steps described in the Section 2, as illustrated in Fig. 1(A)–(C) and the analysis of the excised K&R (from Karnovsky and Roots 1964) positive spot by MALDI-TOF was shown in Fig. 1D. The full-length *PA4921* gene is 924 nucleotides long and encodes a protein of 307 amino acids. The SignalP server indicated that this protein contained a 20-residue signal peptide. Therefore, without the signal peptide, the enzyme contains 287 amino acid residues, which corresponds to a molecular weight of 31,505.5 and a theoretical pI of 7.32. To verify that *PA4921* encodes a protein with ChoE activity, the *PA4921* gene was amplified from *P. aeruginosa* PAO1-WT chromosomal DNA using specific oligonucleotides, Up4921 and Dn4921 (see Section 2). The PCR-amplified fragments containing the whole *PA4921* gene were cloned into a pTOPO plasmid and subcloned in pET15b for expression in *E. coli* BL21-CodonPlus, which has no basal ChoE activity. The IPTG-induced cultures of the resulting strain expressed high

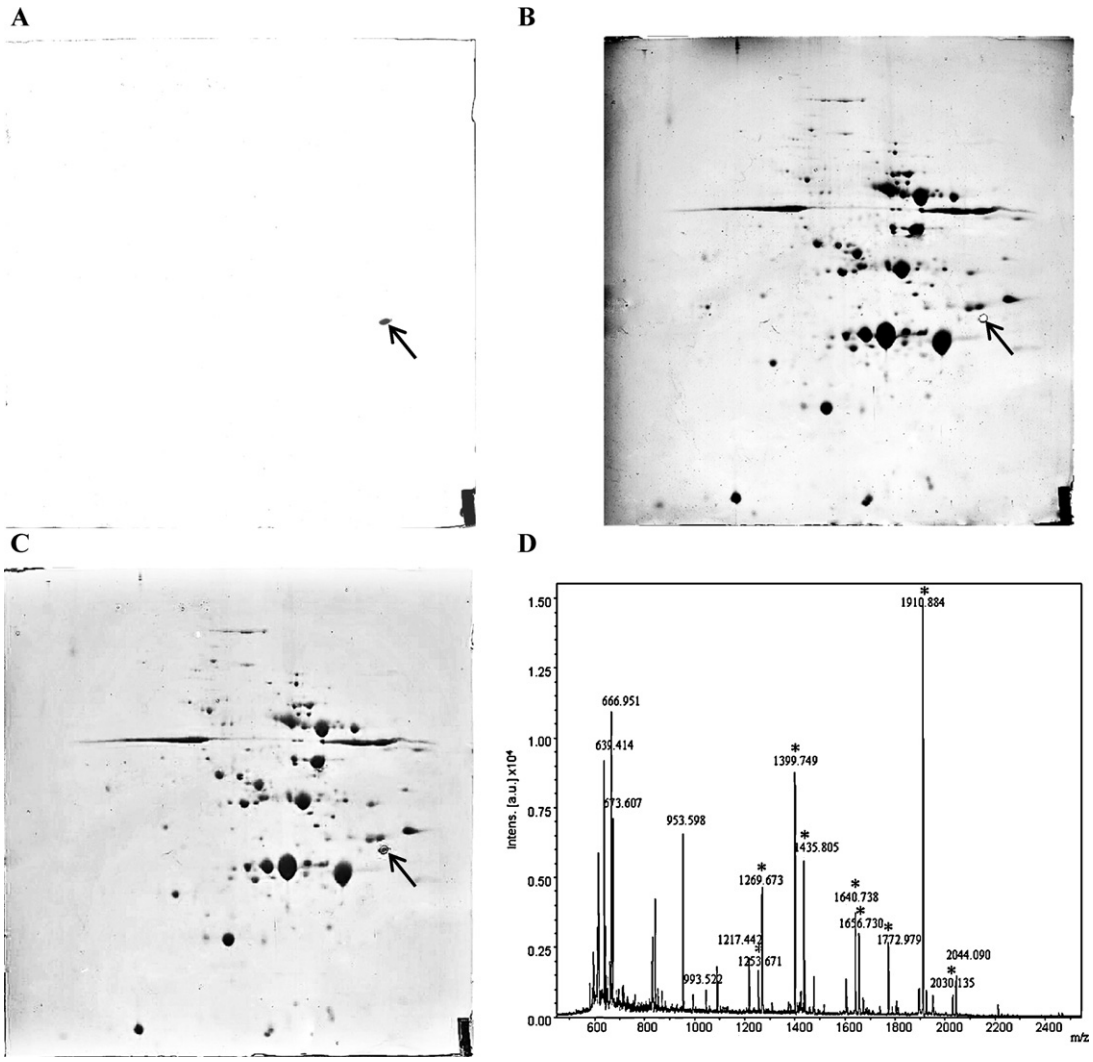


Fig. 1. Separation and identification of a *P. aeruginosa* protein with ChoE activity. Protein (1 mg) obtained from the periplasmic space of *P. aeruginosa* PAO1-WT grown in choline and treated as described in Section 2 was subjected to 2D-PAGE. (A) Identification of the ChoE activity by K&R staining after renaturation from SDS-PAGE. The arrow indicates the location of the K&R positive stain. (B) Coomassie Brilliant Blue staining after the K&R spot was excised. The gel was fixed, and the K&R reagent was removed. The arrow indicates the hole where the spot was excised. (C) Figure obtained by scanning overlapping figures (A and B). (D). MALDI-TOF mass spectrum of an *in situ* digested spot isolated from a 2-D gel detected by K&R staining (after renaturation from the SDS-PAGE) for ChoE activity. Monoisotopic peptide masses (matching masses are marked with asterisks) were obtained, and a search was performed against the eubacterial database, as described in Section 2. The peptide was identified as a hypothetical protein, PA2G_05031 [*Pseudomonas aeruginosa* 2192] (gi|254244186), with a 128 score value, 35% sequence coverage, and 9 mass values matched against 18 mass values searched.

levels of recombinant protein with ChoE activity compared with the non-induced controls. Purified recombinant ChoE (rChoE), at a concentration of approximately 1.2 mg/mL of protein, displayed specific activities of 2428 ± 124 U/mg protein, 3540 ± 132 U/mg protein, 3544 ± 90 U/mg protein and 60.44 ± 3 U/mg protein with ATC, PTC, *p*-NPP and *p*NPB as substrates, respectively.

As shown in Fig. 2A, Lane 1, the enzyme obtained from the periplasmic extract of *P. aeruginosa* PAO1-WT, grown in choline as a carbon and nitrogen source, exhibited a unique K&R positive band of ≅31 kDa. In a periplasmic extract of the PAO1-Δ*PA4921* strain grown under the same induced culture conditions of the *P. aeruginosa* PAO1-WT strain, no K&R positive band was detected (Fig. 2A, Lane 2). In addition, the molecular mass of rChoE differed from that of the enzyme obtained from the periplasmic extract of *P. aeruginosa* PAO1-WT. The higher molecular weight of rChoE is because it has 21 additional residues that were added by the pET15b plasmid (Fig. 2A, Lane 3). As shown in Fig. 2B, Lane 4, the rChoE protein was apparently homogeneous, as judged by a gel stained with Coomassie R-250, with a molecular weight coinciding with the protein detected by K&R staining. Thus, through these experiments, the ChoE activity of the overexpressed protein and the absence of activity in the PAO1-Δ*PA4921* mutant demonstrated that the *P. aeruginosa* PAO1 *PA4921* gene encodes a protein with ChoE activity.

3.2. Phylogeny of the P. aeruginosa ChoE

To evaluate the phylogenetic relationships of the *P. aeruginosa* ChoE, its amino acid sequence was analyzed using a multiple protein alignment with different organisms that contain ChoE activity such as *P. fluorescens*, in which this activity was determined using a crude extract, but the gene encoding it has not been identified; however, there is a protein with a 53% identity to *P. aeruginosa* PA4921 that was included in the Clustal analysis. From the ClustalW alignments, evolutionary relationships and a phylogenetic tree were obtained (Fig. 3). Phylogenetic analysis showed that the *P. aeruginosa* ChoE clustered in the same monophyletic group as the *P. fluorescens* protein (Fig. 3).

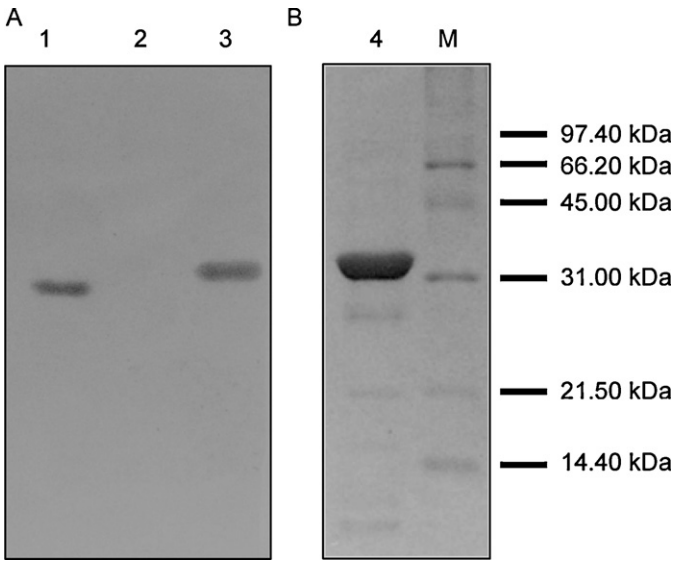


Fig. 2. SDS-PAGE (12%) of the His-tagged rChE expressed in *E. coli* and proteins from periplasmic extracts of *P. aeruginosa* PAO1-WT and PAO1-Δ*pa*4921. (A) Detection by K&R staining (after renaturation from the SDS-PAGE) for ChoE activity: Lane 1, periplasmic space protein (30 μg) from wild-type *P. aeruginosa* PAO; Lane 2, protein (30 μg) from the periplasmic space of *P. aeruginosa* PAO1-Δ*pa*4921; Lane 3, purified His-tagged rChoE protein (20 ng) obtained from *E. coli* BL21-CodonPlus. (B) Lane 4, Coomassie Brilliant Blue staining of purified His-tagged rChoE protein (10 μg) expressed in *E. coli*. Lane M, molecular mass standards (BioRad Low Range).

3.3. The P. aeruginosa ChoE belongs to the SGNH hydrolase family

A phylogenetic analysis using the CDD server (Marchler-Bauer et al. 2009) indicated that the *P. aeruginosa* ChoE belongs to the SGNH hydrolase family (Fig. 4), which is depicted by four invariant residues: Ser, Gly, Asn, and His in Blocks I, II, III, and V, respectively (Mohapatra and Bapuji 1998). The catalytic triad is formed by Ser/Asp/His in blocks I and V, jointly comprising the active site of the hydrolase with the conserved residues Gly/Asn in blocks II and III. As shown in Fig. 4, all of the proteins included in the analysis contained the characteristic four domains of the SGNH hydrolase family. However, with the exception of proteins from various strains of *P. aeruginosa* (96–100% identity) and PFL 2697 of *P. fluorescens* Pf5 (≈53% identity), all the other proteins that were analyzed share a low homology (<30% identity) with the PA4921 protein (Fig. 4). Experimentally, we detected ChoE activity in the periplasmic extracts of *P. fluorescens* Pf-5 grown on HPi-BSM with choline, which showed a K&R positive spot after the renaturation of the enzyme (see Section 2). In *P. putida* and *P. syringae*, the periplasmic extracts did not contain a protein capable of catalyzing the hydrolysis of ATC (data not shown).

3.4. Biochemical characterization of the P. aeruginosa ChoE activity

A preliminary biochemical characterization of this enzyme included the utilization of ATC, PTC, BTC, *p*NPP and *p*NPB as substrates. Under identical experimental conditions, BTC and BC were not substrates for this enzyme. Saturation curves for the *P. aeruginosa* ChoE using ATC and PTC as substrates are shown in Fig. 5A. After reaching saturation (Michaelis–Menten curves; Fig. 5, inset), both substrates had an inhibitory effect on the enzyme's activity at high substrate concentrations. Although the true K_m values obtained with ATC and PTC were practically identical, the true catalytic constants (Table 1) indicated that the enzyme was catalytically more efficient when using PTC as a substrate. The saturation curves with *p*NPP or *p*NPB (Fig. 5B) illustrate that, contrary to the observed behavior with ATC or PTC, neither compound caused inhibition at high substrate concentration. Considering the K_M and the catalytic efficiency (k_{cat}/K_M) values, it is clear that ATC and PTC were better substrates than *p*NPP and *p*NPB. In addition, *p*NPP was a better substrate than *p*NPB (Table 1A).

The differing behavior of the enzyme against substrates containing or lacking the N-trimethylammonium moiety led us to perform experiments to confirm the presence of an N-methylammonium

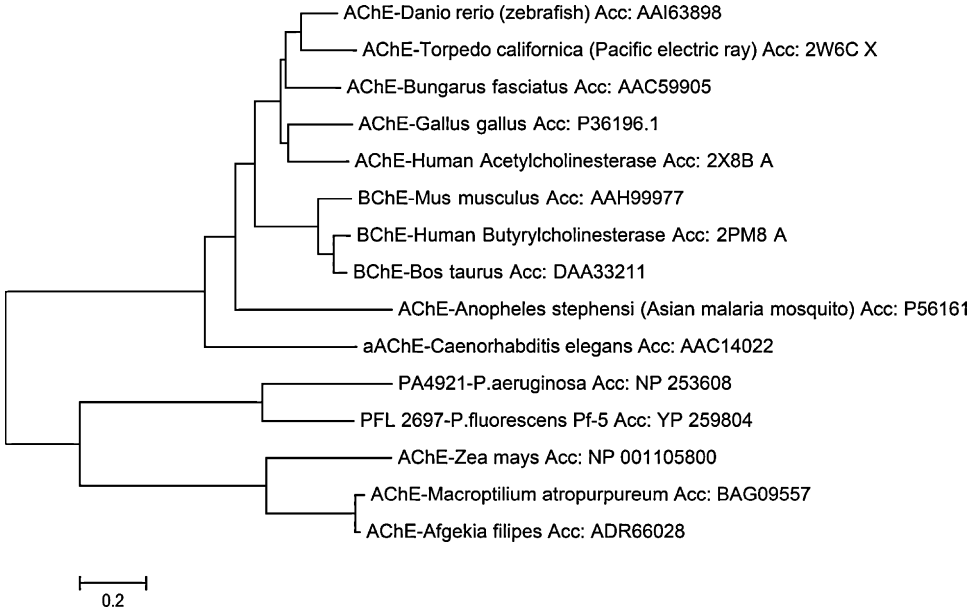


Fig. 3. Phylogenetic representation of sequences based on the ClustalW analysis of AChE and BuChE enzymes from the various organisms listed in the figure, including their accession numbers. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei 1987). The optimal tree is shown, with a sum of branch lengths of 6.69772272. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method (Zuckerkandl and Pauling 1965) and reported in the units of the number of amino acid substitutions per site. The analysis involved 16 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 254 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura et al. 2007).

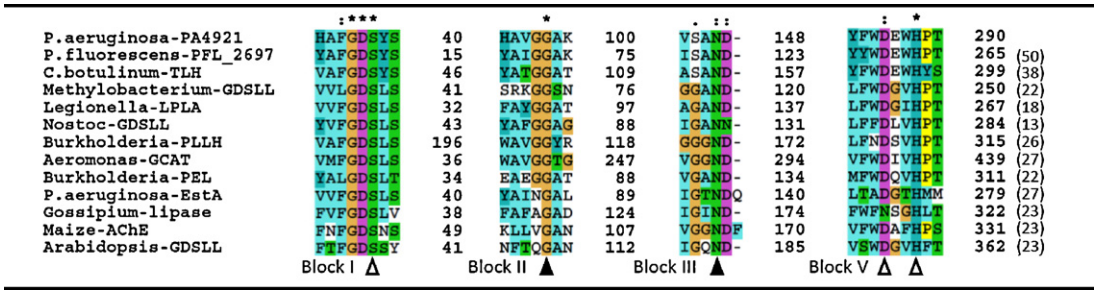


Fig. 4. ClustalX analysis of the amino acid sequences of the *P. aeruginosa* ChoE (PA4921) using selected members of the SGNH hydrolase family. The amino acid sequence identities between the *P. aeruginosa* ChoE and each gene product (i.e., the score against the *P. aeruginosa* ChoE) are indicated as percentages, in brackets, in the right column. Dashes indicate gaps introduced to improve the alignment. Numbers indicate the amino acid position in the sequence. The putative catalytic triad of the active site Ser/Asp/His residues (blocks I and V) is indicated by white triangles, and the residues indicated as black triangles (blocks II and III) are also contained in the active site of the SGNH hydrolase family. In the mature *P. aeruginosa* AChE, the catalytic triad is ^{18}Ser/^{265}Asp/^{268}His. For the dyad, the positions are ^{78}Gly/^{127}Asn. The abbreviations and accession numbers for the proteins used in this analysis are as follows: *P. aeruginosa*-PA4921, *P. aeruginosa* PAO1 ChoE (this study); *P. fluorescens PFL_*2697; *Clostridium*-TLH, *Clostridium botulinum D str. 1873* thermolabile hemolysin (ACT33708); Methylobacterium-GDSLL, *Methylobacterium extorquens* GDSL family lipase (YP_001637603); Legionella-LPLA, *Legionella pneumophila* 130b lysophospholipase A (AAN63820); *Nostoc-GDSLL*, *Nostoc punctiforme*, GDSL family lipase (YP_001869641); *Burkholderia-PLLH*, *Burkholderia dolosa* phospholipase/lecithinase/hemolysin (ZP_04947073); *Burkholderia*-PEL, *Burkholderia pseudomallei* putative exported lipase (YP_108832); *Pseudomonas*-EstA, *P. aeruginosa* PAO1 autotransporter esterase EstA (ZP_04937329); *Gossypium*-lipase, *Gossypium hirsutum* lipase (ABX75139); Maize-ChE, *Zea mays* acetylcholinesterase (NP_001105800); Arabidopsis-GDSLL, *Arabidopsis thaliana* GDSL-motif lipase (NP_188038).

binding site in the ChoE protein. Although the figure was not shown, it was clear that the presence of choline increased the K_M and k_{cat} values when the enzyme activity was measured with ATC or *p*NPB (Table 1B). However, the increase of the k_{cat} value did not compensate for the decreased affinity of the enzyme for both substrates to maintain the same catalytic efficiency (Table 1B). The equivalent experiment performed with T4MA indicated that the effect produced by choline was not only due to its alkylammonium moiety because T4MA decreased the K_M value for ATC and slightly increased it for *p*NPP. The other kinetic constants (Tables 1B and 2) show that T4MA is a true inhibitor of ChoE activity.

In addition, the results shown in Table 2 indicate that with ATC, PTC or *p*NPP as a substrate, the alkylammonium compounds tested were inhibitors of this enzymatic activity, whereas choline was an activator of enzymatic activity when ATC or PTC were used as substrate (Table 2). Inhibition curves were obtained with different concentrations of substrate and inhibitor and analyzed with the DYNAFIT program (data not shown); as expected, they indicated that butyrylcholine is a competitive inhibitor with a K_i value of 0.008 mM. Striking effects were observed with ATC, PTC or *p*NPP as substrates because the T4EA ion was much more effective than the T4MA ion at inhibiting ChoE activity (Table 2).

4. Discussion

The above molecular experiments, the utilization of bioinformatic tools and the preliminary enzymatic characterizations provide conclusive evidence that the *PA4921* gene of the *P. aeruginosa* PAO1 genome encodes the ChoE enzyme previously studied by our group (Lisa et al. 1983; Domenech et al. 1991; Lucchesi et al. 1989, 1995). The high levels of ChoE activity in *E. coli* expressing this protein plus the absence of this enzymatic activity in *P. aeruginosa* strain PAO1-Δ*PA4921* confirms this conclusion. In our previous studies (Lisa et al. 1983; Domenech et al. 1991; Lucchesi et al. 1995), we determined some of the biochemical and physiological properties related to the synthesis of ChoE; however, the gene encoding the ChoE in *P. aeruginosa* was not determined. A BLASTP search was performed among various AchE sequences including the AchE sequences of the maize (*Zea mays*) (Sagane et al. 2005) and *P. aeruginosa* genomes, but no homologous proteins were found. Because this result was not in agreement with our biochemical and microbiological results, we decided to utilize other methods to locate the *choE* gene in *P. aeruginosa*.

The first step was to find an appropriate and sensitive method to detect, after 1- or 2-D PAGE (including renaturation of the enzyme), a protein spot with ChoE activity after staining using the K&R method (Karnovsky and Roots 1964). Such a spot was isolated by the treatment of periplasmic extracts of wild-type *P. aeruginosa* and analyzed by MALDI-TOF; the predominant peptides corresponded principally to a protein encoded by the *PA1342* gene of the *P. aeruginosa* PAO1 genome. This gene was cloned, and the protein was expressed in *E. coli*. However, this protein was not capable of catalyzing the hydrolysis of ATC or PTC and was not detected by the K&R staining after PAGE. We concluded that the PA1342 protein and the ChoE have similar or identical mobility in 2D-PAGE. The PA1342 protein was therefore the predominant protein, and the ChoE was a minor component with a high specific activity in the excised K&R-positive spot. For this reason, the next experimental step was

Table 1
Kinetic constants of ChoE obtained with ATC, PTC, *p*NPB and *p*NPP (A). Effect of 1 mM choline or tetramethylammonium (T4MA) when the enzyme activity was measured with ATC or *p*NPP (B). The constants values ± SD represent an average from at least three independent experiments experiments.

	K_M, mM ± SD	k_{cat1}, s^{-1} ± SD	k_{cat2}, s^{-1} ± SD	K_{SI}, mM ± SD	k_{cat1}/K_M, s^{-1} mM^{-1}
(A) Substrate					
ATC	0.12 ± 0.006	1176 ± 39	175 ± 15	1.63 ± 0.14	9800
PTC	0.13 ± 0.011	1683 ± 81	369 ± 18	0.75 ± 0.11	12,946
*p*NPP	0.34 ± 0.027	1495 ± 46	[a]	[a]	4397
*p*NPB	0.65 ± 0.052	28.2 ± 1.5	[a]	[a]	
(B) Substrate + effector					
ATC + choline	0.29 ± 0.03	1624 ± 56	[b]	[b]	5600
*p*NPP + choline	0.91 ± 0.08	2136 ± 112	[b]	[b]	2347
ATC + T4MA	0.04 ± 0.007	473 ± 17	[b]	[b]	11,825
*p*NPP + T4MA	0.47 ± 0.06	788 ± 48	[b]	[b]	1677

[a] There are not inhibition by high substrate concentration and a second site was not detected for these substrates.
[b] In the presence of 1 mM choline or T4MA there are not inhibition was observed by high concentration of substrate (ATC or *p*NPP).

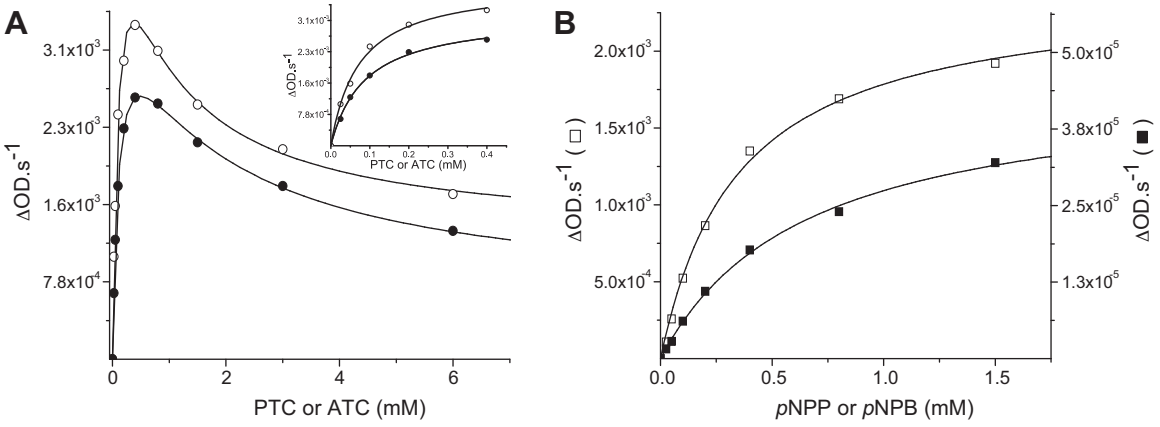


Fig. 5. Kinetic characterization of the *P. aeruginosa* ChoE: (A) Saturation curves for the ChoE activity using different concentrations of ATC (filled circles) or PTC (open circles). Insets in the figure A show the same data in conditions there was not enzyme inhibition by high substrate concentrations. The statistics of least-squares fit were: mean square = 4.17422×10^{-10} (ATC) and 1.60355×10^{-9} (PTC); root-mean-square deviation = 2.04309×10^{-5} (ATC) and 4.00444×10^{-5} (PTC); number of data points = 11; optimized parameters = 4; degrees of freedom = 7; percentage confidence interval level = 99%. (B). Saturation curves for esterase activity of ChoE were determined by using different concentrations of *p*NPP (open squares) or *p*NPB (filled squares). The statistics of least-squares fit were: mean square = 1.29882×10^{-9} (*p*NPP), and 1.49207×10^{-10} (*p*NPB); root-mean-square deviation = 3.60392×10^{-5} (*p*NPP), and 1.2215×10^{-5} (*p*NPB); number of data points = 8; optimized parameters = 2; degrees of freedom = 6; percentage confidence interval level = 99%. Figures A and B were obtained using the DYNAFIT program by nonlinear least-squares regression.

Table 2
Effects of alkylammonium compounds and cholinesterases classic inhibitors on ChoE activity. Percentages of residual activity determined in the presence of different effectors in 100 mM sodium phosphate buffer, pH 7.0.

	% Residual activity		
	ATC (0.4 mM)	PTC (0.4 mM)	*p*NPP (1 mM)
Effectors (5 mM)			
Choline	145	162	98
Trimethylamine	92	79	84
Tetramethylammonium	39	52	36
Tetraethylammonium	4	3	5
Decamethonium	38	44	25
Atropine	25	32	38
Procaine	45	37	31

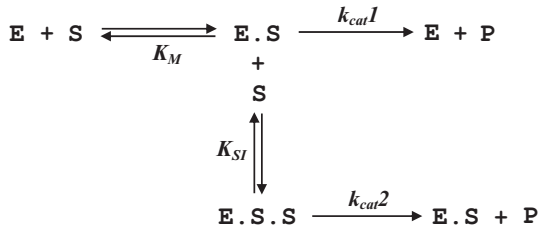
the deletion of the *PA1342* gene. Using this deletion mutant, *P. aeruginosa* strain PAO1-Δ*PA1342*, the procedures described above were performed to obtain a single K&R-positive spot without the contaminating protein. To achieve this goal, a sample enriched in ChoE protein was obtained from a Q-Sepharose column, and the enzyme was precipitated with trichloroacetic acid followed by washings with ice-cold acetone. This treatment plus the renaturation of the enzyme after 2D-PAGE and the utilization of K&R staining to detect the enzymatic activity in the gel provided the necessary amount of enzyme to perform the MALDI-TOF assay, which ultimately led to the identification of the *PA4921* gene.

Sequence comparisons and alignments revealed that the *P. aeruginosa* ChoE was related to members of the SGNH hydrolase family, of which the first member described was the rhamnogalacturonan acetylesterase of *Aspergillus aculeatus* (Mølgaard et al. 2000). SGNH hydrolases are members of a diverse family of lipases and esterases, and the tertiary folds of these proteins are substantially different from those of the α/β hydrolase family (see Mølgaard et al. (2000) for a comparison of the topologies in both families of proteins). The SGNH hydrolase family contains the Ser/His/Asp(Glu) catalytic triad of the α/β hydrolase family, which includes animal AchEs and other serine hydrolases (Mølgaard et al. 2000; Shafferman et al. 1992). However, the SGNH hydrolase family is characterized by having four completely conserved blocks containing Ser, Gly, Asn and His residues (S, G, N, and H, respectively) located in blocks I, II, III and V, respectively, that are perfectly preserved in the PA4921 protein. Therefore, in the mature *P. aeruginosa* ChoE (without the signal peptide), the conserved aminoacyl residues of this family are ^{18}Ser, ^{78}Gly, ^{127}N, ^{268}H, and the catalytic triad is formed by ^{18}Ser, ^{268}H, ^{265}D.

The phylogenetic tree, which shows the evolutionary relationships among various AchEs, indicated that ChoEs from *P. aeruginosa*, *P. fluorescens* and *Z. mays* belong to the SGNH family. When considering *P. fluorescens,* our results are contradictory to previously reported results (Rochu et al. 1998) that described an enzyme with a cholinesterase-like activity in *P. fluorescens* strain ATCC no. 11150. A BLASTP comparison of the N-terminal peptide sequenced by Rochu et al. (1998) against the *P. fluorescens* genome showed that it has 100% identity with a soluble extracellular substrate-binding protein encoded by the PFL 1048 gene of *P. fluorescens* Pf5 and the PFLU 1213 gene of *P. fluorescens* SBW25, corresponding to a protein identified as a putative ABC transporter/substrate-binding protein. Therefore, this purified protein, and its sequenced peptide (Rochu et al. 1998), could not be a protein with AchE activity. This discrepancy may exist because the protein purified by Rochu et al. is a major component that co-purifies with AchE, which is a minor component with a high specific activity. However, bioinformatic analysis seems to indicate that in *P. fluorescens*, ChoE activity is encoded by the PFL 2697 gene.

The results of the kinetic and inhibition studies performed with *P. aeruginosa* ChoE confirmed our previous results and those of other studies (Domenech et al. 1981; Tani et al. 1975) and indicate that this activity is capable of catalyzing the hydrolysis of ATC and PTC. The catalytic hydrolysis of *p*-NPP or *p*-NBP also occurred, but the catalytic efficiency was much lower than that observed with ATC or PTC. Therefore, it is possible that the esterase activity dependent on *PA4921* gene expression corresponds to an AchE or PchE activity. On the basis of k_{catl}, k_{cat2} or k_{catl}/K_M, PTC is a better substrate than ATC, and based on the kinetic data, the *P. aeruginosa* ChoE may be considered a PchE. However, assuming that ChoE is a virulence factor, the scarcity of propionylcholine in higher organisms, which has little to no significance in animals as an endogenous or a false neurotransmitter, led us to propose that the *P. aeruginosa* ChoE has a more suitable location within the AchE family.

The inhibition by high ATC or PTC concentrations and the presence of two different catalytic constants, $k_{catl} > k_{cat2}$, led us to propose the catalytic mechanism illustrated in Scheme 1. Here, Ache can bind a second substrate molecule, as occurs with animal AchEs, which is responsible for the inhibition at high substrate concentrations, or, as is otherwise seen, for the entry of a second substrate molecule to produce a ternary complex SES



Scheme 1. Catalytic mechanism of the AChE using ATC or PTC as a substrate. The proposed model was obtained using the DYNAFIT program by nonlinear least-squares regression. The following abbreviations are used: enzyme (E), substrate (S), and product (P).

(Substrate/Enzyme/Substrate), which is less effective than the ES complex for product formation. This catalytic mechanism is consistent with the behavior of animal AchEs described by other authors, as recently reviewed (Dvir et al. 2010). Therefore, according to these reports, it is possible that the *P. aeruginosa* AchE (as occurs with the *P. aeruginosa* PchP) (Beassoni et al. 2011; Otero et al. 2011; Domenech et al. 2011) may also contain a catalytic site formed by two sub-sites: the esteratic site and the anionic site. In addition, it is also possible that the entry of a second molecule of acetylcholine could occur in a peripheral site, as has been shown in animal AchEs (Dvir et al. 2010). At this time, it is not possible to study this mechanism in more detail because the structure of this enzyme is not known.

Finally, as expected, to a greater or lesser extent, all of the alkylammonium compounds were inhibitors of the *P. aeruginosa* enzyme. The most striking result was the high enzymatic inhibition produced by T4EA compared with the inhibition produced by T4MA. This effect may be due to differences in the long carbon chains or their hydrophobicity, resulting in different interactions in a hydrophobic region of the enzyme located in the vicinity of the active site. In conclusion, although more work is required to better understand the *P. aeruginosa* AchE, the results of our preliminary biochemical characterizations, molecular experiments, and the utilization of bioinformatic tools emphasize that in addition to identifying the *PA4921* gene, which is responsible for the synthesis of AchE, we also provided strong evidence that this activity may also be found in other prokaryotes, as represented here by *P. aeruginosa* and, by extension, in *P. fluorescens.*

Acknowledgements

ATL is a Career Member of the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET). CED is an honorary "Profesor Consulto" at the UNRC and is a retired Career Member of the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET). DGS and LHO would like to acknowledge fellowship support from CONICET and the Ministerio de Ciencia y Técnología-Córdoba (MinCyT-Cba). DGS and ATL are grateful to Drs. E. Morett and L. Olvera by introduce them to Dr. S. Encarnación with the MALDI-TOF experiments. This work was supported by grants from SECYT-UNRC, MinCyT-Cba and CONICET of Argentina. ATL acknowledges support from Red "Bio-Bio-Mol II", which is financed by the Secretaría de Políticas Universitarias (SPU) of Argentina.

References

Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, et al. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 1997;25(17):3389–402.

Altschul SF, Wootton JC, Gertz EM, Agarwala R, Morgulis A, Schäffer AA, et al. Protein database searches using compositionally adjusted substitution matrices. FEBS J 2005;272(20):5101–9.

Ames GF, Prody C, Kustu S. Simple, rapid, and quantitative release of periplasmic proteins by chloroform. J Bacteriol 1984;160(3):1181–3.

Beassoni PR, Otero LH, Boetsch C, Domenech CE, Gonzalez-Nilo FD, Lisa AT. Site-directed mutations and kinetic studies show key residues involved in alkylammonium interactions and reveal two sites for phosphorylcholine in *Pseudomonas aeruginosa* phosphorylcholine phosphatase. Biochim Biophys Acta: Proteins Proteomics 2011;1814(7):858–63.

Bendtsen JD, Nielsen H, von Heijne G, Brunak S. Improved prediction of signal peptides: SignalP 3.0. J Mol Biol 2004;340(4):783–95.

Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal Biochem 1976;72(2):248–54.

Choi KH, Schweizer HP. An improved method for rapid generation of unmarked *Pseudomonas aeruginosa* deletion mutants. BMC Microbiol 2005;5:30.

Cousin X, Créminon C, Grassi J, Méflah K, Cornu G, Saliou B, et al. Acetylcholinesterase from Bungarus venom: a monomeric species. FEBS Lett 1996;387(2–3):196–200.

Domenech CE, Garrido MN, Lisa AT. *Pseudomonas aeruginosa* cholinesterase and phosphoryl choline phosphatase: two enzymes contributing to corneal infection. FEMS Microbiol Lett 1991;82:131–5.

Domenech CE, Garrido MN, Machado de Domenech EE, Lisa AT. Acetylcholinesterase from rat red cells and cholinesterase of *Pseudomonas aeruginosa*: different types of inhibition by atropine. Mol Cell Biochem 1981;34:95–9.

Domenech CE, Otero LH, Beassoni PR, Lisa AT. Phosphorylcholine phosphatase: a peculiar enzyme of *Pseudomonas aeruginosa*. SAGE-hindawi access to research enzyme research. 2011. Article ID 561841. doi:10.4061/2011/561841 [12 pages].

Dvir H, Silman I, Harel M, Rosenberry TL, Sussman JL. Acetylcholinesterase: from 3D structure to function. Chem-Biol Interact 2010;187:10–22.

Ellman GL, Courtney KD, Andres Jr V, Feather-Stone RM. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol 1961;7:88–95.

Encarnación S, Hernández M, Contreras S, Martínez-Batallar G, Vargas MC, Mora J. Comparative proteomics using 2-D gel electrophoresis and mass spectrometry as tools to dissect stimulons and regulons in bacteria with sequenced or partially sequenced genomes. Biol Proced Online 2005;7:117–35.

Garber N, Nachshon I. Localization of cholinesterase in *Pseudomonas aeruginosa* strain K. J Gen Microbiol 1980;117:279–83.

Gasteiger E, Hoogland C, Gattiker A, Duvaud S, Wilkins MR, Appel RD, et al. Protein identification and analysis tools on the ExPASy server. In: Walker JM, editor. The proteomics protocols handbook. Totowa, NJ: Humana Press; 2005. p. 571–607.

Karnovsky MJ, Roots D. A "directcoloring" thiocholine method for cholinesterases. J Histochem Cytochen 1964;12:219–21.

Kortstee GJ. The aerobic decomposition of choline by microorganisms. I. The ability of aerobic organisms, particularly coryneform bacteria, to utilize choline as the sole carbon and nitrogen source. Arch Mikrobiol 1970;71(3):235–44.

Kuzmic P. Program DYNAFIT for the analysis of enzyme kinetic data: application to HIV proteinase. Anal Biochem 1996;237(2):260–73.

Lacks SA, Springhorn SS. Renaturation of enzymes after polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. J Biol Chem 1980;255(15):7467–73.

Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, et al. Clustal W and Clustal X version 2.0. Bioinformatics 2007;23(21):2947–8.

Lisa AT, Beassoni PR, Masssimelli MJ, Otero LH, Domenech CE. A glance on *Pseudomonas aeruginosa* phosphorylcholine phosphatase, an enzyme whose synthesis depends on the presence of choline in its environment. In: Méndez-Vilas A, editor. Communicating current research and educational topics and trends in applied microbiology. España: Badajoz; 2007. p. 255–62.

Lisa AT, Garrido MN, Domenech CE. Induction of acid phosphatase and cholinesterase in *P. aeruginosa* and their in vitro control by choline, acetylcholine and betaine. Mol Cell Biochem 1983;50:149–55.

Lobanok AG, Mikhailova RV, Zakharenko I. Production of esterases by the fungi of the genera *Aspergillus* and *Penicillium*. Prikladnaya Biokhimiya i Mikrobiologiya 1993;29:102–7.

Lucchesi GI, Lisa AT, Casale CH, Domenech CE. Carnitine resembles choline in the induction of cholinesterase, acid phosphatase, and phospholipase C and in its action as an osmoprotectant in *Pseudomonas aeruginosa*. Curr Microbiol 1995;30(1):55–60.

Lucchesi GI, Lisa AT, Domenech CE. *Pseudomonas aeruginosa* phospholipase C activity in a high phosphate medium. FEMS Microbiol Lett 1989;48(3):335–8.

Malek AA, Chen C, Wargo MJ, Beattie GA, Hogan DA. Roles of three transporters CbcXWV. BetT1, and BetT3, in Pseudomonas aeruginosa choline uptake for catabolism. J Bacteriol 2011;193(12):3033–41.

Marchler-Bauer A, Anderson JB, Chitsaz F, Derbyshire MK, DeWeese-Scott C, Fong JH, et al. CDD: specific functional annotation with the Conserved Domain Database. Nucleic Acids Res 2009;37:D205–10.

Mohapatra BR, Bapuji M. Characterization of acetylcholinesterase from *Arthrobacter ilicis* associated with the marine sponge (*Spirastrella* sp.). J Appl Microbiol 1998;84:393–8.

Mølgaard A, Kauppinen S, Larsen S. Rhamnogalacturonan acetylesterase elucidates the structure and function of a new family of hydrolases. Structure 2000;15(4):373–83.

Nieto TP, Santos Y, Rodríguez LA, Ellis AE. An extracellular acetylcholinesterase produced by *Aeromonas hydrophila* is a major lethal toxin for fish. Microb Pathog 1991;11(2):101–10.

Otero LH, Beassoni PR, Boetsch C, Lisa AT, Domenech CE. Different effects of Mg^{2+} and Zn^{2+} on the two sites for alkylammonium compounds in *Pseudomonas aeruginosa* phosphorylcholine phosphatase. Enzyme Res Online 2011., doi:10.4061/2011/826784.

Pesin S, Candia O. Acethylcholine concentration and its role in ionic transport by the corneal epithelium. Invest Ophtalmol Vis Sci 1982;22(5):651–9.

Rochu D, Rothlisberger C, Taupin C, Renault F, Gagnon J, Masson P. Purification, molecular characterization and catalytic properties of a *Pseudomonas fluorescens* enzyme having cholinesterase-like activity. Biochim Biophys Acta 1998;1385(1):126–38.

Sagane Y, Nakagawa T, Yamamoto K, Michikawa S, Oguri S, Momonoki YS. Molecular characterization of maize acetylcholinesterase. A novel enzyme family in the plant kingdom. Plant Physiol 2005;138:1359–71.

Saitou N, Nei M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 1987;4(4):406–25.

Salazar E, Díaz-Mejía JJ, Moreno-Hagelsieb G, Martínez-Batallar G, Mora Y, Mora J, et al. Characterization of the NifA-RpoN regulon in *Rhizobium etli* in free life and in symbiosis with *Phaseolus vulgaris*. Appl Environ Microbiol 2010;76(13):4510–20.

Sambrook J, Russell DW. Molecular cloning: a laboratory manual. 3rd ed. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press; 2001.

Shafferman A, Kronman C, Flashner Y, Leitner M, Grosfeld H, Ordentlich A, et al. Mutagenesis of human acetylcholinesterase. Identification of residues involved in catalytic activity and in polypeptide folding. J Biol Chem 1992;267(25):17640–8.

Talesa V, Principato GB, Giovamini E, DiGiomanni MV, Rosi G. Dimeric forms of cholinesterase in *Sipunculus nudus*. Eur J Biochem 1993;215(2):267–75.

Tamura K, Dudley J, Nei M, Kumar S. MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol Biol Evol 2007;24(8):1596–9.

Tani Y, Nagasawa T, Sugisaki H, Ogata K. Purification and characterization of the cholinesterase of *Pseudomonas aeruginosa* A-16. Agric Biol Chem 1975;39(6):1284–94.

Velikanov NL, Kolesnikova IG, Lyakh SP. Acetylcholinesterase activity of *Pseudomonas* species. Mikrobiologiia 1975;44(4):761–2.

Wargo MJ, Szwergold BS, Hogan DA. Identification of two gene clusters and a transcriptional regulator required for *Pseudomonas aeruginosa* glycine betaine catabolism. J Bacteriol 2008;190(8):2690–9.

Yang J, Koga Y, Nakano H, Yamane T. Modifying the chain-length selectivity of the lipase from *Burkholderia cepacia* KW1-56 through in vitro combinatorial mutagenesis in the substrate-binding site. Protein Eng 2002;15(2):147–52.

Zuckerkandl E, Pauling L. Evolutionary divergence and convergence in proteins. In: Bryson V, Vogel HJ, editors. Evolving genes and proteins. New York, NY: Academic Press; 1965. p. 97–166.