A Glance on *Pseudomonas aeruginosa* Phosphorylcholine Phosphatase, an Enzyme whose Synthesis Depends on the Presence of Choline in its Environment

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This paper reports the discovery, plus kinetic, biochemical, biophysical and molecular characteristics of a *P. aeruginosa* phosphorylcholine phosphatase (PchP) whose synthesis depends on choline and derivatives in the culture medium. PchP is the product of the PA5292 gene in the *P. aeruginosa* PAO1 genome and it is principally transcribed as a monocistronic 1 Kb mRNA. PChP belongs to the haloacid dehalogenases hydrolase superfamily which contains totally three conserved motifs. In mature PChP, motifs I, II and III are the aminoacyl residues ^{31}DMDNT^{35}, ^{166}S and K^{242}/^{261}GDTPDSD^{267}, respectively. After site directed mutagenesis on these motifs, the more important residues for catalysis were identified. PchP is involved in the pathogenesis of *P. aeruginosa* through the coordinated and sequential action of hemolytic phospholipase C and PchP on phosphatidylcholine or sphyngomyelin and phosphorylcholine, respectively.

Keywords *Pseudomonas aeruginosa*; phosphorylcholine phosphatase; choline

Pseudomonas aeruginosa is an opportunistic Gram-negative bacterium capable of surviving in a wide variety of environments and causing a wide spectrum of diseases in insects, plants and animals. In our laboratory, we started to work with *P. aeruginosa* because we were interested in obtaining a purified cholinesterase (ChE), described by [1], to continue our studies related to the action of organophosphate and alkylammonium compounds on acetylcholinesterases [2,3]. To obtain *P. aeruginosa* ChE, bacteria were grown in a basal salt minimal medium with acetylcholine, choline or betaine as a carbon source. In one of our first experiments, we found that an acid phosphatase (AcPase) was synthesized in parallel to a ChE activity. The distinctive characteristic of this AcPase activity, with respect to other AcPases described in different organisms was that its activity, measured with p-nitrophenylphosphate (*p*-NPP) in the presence of Mg^{2+} at pH 5, was inhibited by choline, betaine or acetylcholine. The AcPase activity, like ChE, was practically nonexistent when *P. aeruginosa* was grown in the same saline medium with acetate, lactate, glycerol, succinate or glucose as a carbon source [4]. The discovery of an AcPase with unusual and unique properties led us to continue the study of this enzyme. Our first aim was to determine if a similar AcPase activity could be detected in the presence of choline or betaine in the culture medium in other microorganisms. We found that *P. aeruginosa* AcPase induced by choline metabolites was different from other AcPases produced by other Gram-negative bacteria, such as *Escherichia coli*, *Shigella flexneri*, *Salmonella typhimurium*, *Proteus mirabilis* and *Enterobacter liquefacciens* [5]. These bacteria have in common that they are unable to utilize choline as carbon or nitrogen sources. *Rhizobium meliloti*, however, is a bacterium capable of metabolizing choline [6], but also the AcPase activity found in this bacterium was different to that found in *P. aeruginosa* [7, 8]. In the earlier taxonomic study carried out by Stanier et al., [9], it was shown that bacteria of the *Pseudomonas* genus were able to metabolize choline or other alkylammonium compounds. In our case, we tested all of the choline, betaine, dimethylglycine, sarcosine, glycine, ethanolamine, methylethanolamine, and dimethylethanolamine. We observed that choline, betaine, dimethylglycine and sarcosine were utilized as nutrients by *P. aeruginosa* at similar rates; dimethylaminoethanol and methylethanolamine did not

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support bacterial growth and poor or scarce growth was detected with ethanolamine and glycine [5]. The publication of the *P. aeruginosa* PAO1 genome (http://v2.pseudomonas.com) [10], plus a recent proteomic work [11], confirmed definitively that *P. aeruginosa* metabolizes choline through two oxidation steps catalyzed by choline dehydrogenase and betaine aldehyde dehydrogenase, plus a demethylation step catalyzed by the action of glycine betaine transmethylase [12]. At present, the gene responsible for the synthesis of a dimethylglycine dehydrogenase has not been identified in *P. aeruginosa*. However, the genes involved in the synthesis of sarcosine oxidase and those acting in the glycine cleavage system were described in this bacterium [10]. Therefore, it may be concluded that choline metabolism in *P. aeruginosa* occurs through the steps shown in Figure 1. The second point of our interest was to determine which of the choline metabolites were involved in the synthesis of *P. aeruginosa* AcPase. Therefore, with the exception of betaine aldehyde, all of the substrates showed in Figure 1 were employed. AcPase was synthesized when bacteria were grown in culture media with choline, betaine or dimethylglycine as carbon, nitrogen or carbon and nitrogen sources. Poor or scarce inductions of AcPase and ChE were observed with sarcosine. Carnitine, other ammonium quaternary compound, resembles choline in inducing the synthesis of AcPase because the breakdown of carnitine in *P. aeruginosa* occurs via the formation of betaine [13] (Fig. 1). These findings were in good agreement with those described by Kleber [14], who showed that, in some members of the *Pseudomonas* genus, carnitine is metabolized through the action of L-carnitine dehydrogenase to form betaine. Another distinctive characteristic of *P. aeruginosa* AcPase with respect to other acid phosphatases from different organisms was that its activity, measured with the sodium salt of *p*-nitrophenylphosphate (*p*-NPP), was inhibited in vitro by choline, betaine, phosphorylcholine (Pch) and other alkylammonium compounds [5]. The clear importance of the alkylammonium moiety contained in different compounds that act as effectors of the AcPase activity, led us to perform kinetic experiments with various alkylammonium ions. Tetramethylammonium was more effective than tetraethylammonium for inhibiting the enzyme activity. After testing the two series of compounds, one of them with an alcohol group and the other with a carboxylic group, it was shown that in both series the inhibitory power as well as the affinity for the enzyme decreased with the loss of methyl groups. After these results, it was indubitable that this AcPase contained an anionic site with affinity for a trimethyl substrate [15]. This conclusion was confirmed by performing saturation curves with Pch and phosphorylethanolamine in the presence of 2 mM Mg2+, at pH 5.0. Kinetic data taken from these saturation curves led us to conclude that phosphorylcholine was a better substrate than phosphorylethanolamine for this AcPase, K_{Mapp} values were near 0.2 mM and 0.7 mM, respectively. With all together our results, we proposed that this AcPase might be considered a phosphorylcholine phosphatase (PchP), an enzyme capable to catalyze the hydrolysis of phosphorylcholine to produce inorganic phosphate plus choline [16]. The finding of a PlcH in a high Pi medium was in total disagreement with previous findings of other authors, who indicated that PlcH plus an alkaline phosphatase (AlkPase) activity may only be synthesized under low Pi concentrations [18,19]. However, three years later, our finding was confirmed [20]. We demonstrated that the PlcH induced by choline or derivatives in a high Pi medium also catalyzed the hydrolysis of phosphatidylcholine, lysophosphatidylcholine or sphingomyelin [17, 21] as it was shown for the PlcH synthesized in a low Pi medium [19]. Like PchP, the AlkPase could also utilize Pch as a substrate. Therefore, PlcH plus PchP or AlkPase acting sequentially may produce choline plus Pi (Fig. 2). Since PlcH and PchP are synthesized in a "high Pi medium," the proposed role for PlcH and AlkPase as a phosphate scavenging mechanism [18] was discarded. We proposed instead that, through the coordinated and sequential action of PlcH and PchP, the bacteria may breakdown the choline-containing phospholipids of the host cells to cover its metabolic needs as carbon or nitrogen sources rather than a Pi source. According to the results of that moment, one limitation to explain an in vivo action with the intervention of PlcH and PchP was the optimal pH for each enzyme; the PlcH acts at the neutral-alkaline zone and PchP at acidic pH. After testing the optimal pH for PchP with Pch as a substrate it was found again, that this enzyme could work with practically identical catalytic efficiency in a broad range of pH from 5.0 to 8.0 [22,23]. Therefore, we proposed that choline might be considered a factor capable of increasing the pathogenicity of *P. aeruginosa* through the action of PlcH and PchP on membrane phospholipids. Since the human lung is a target organ of *P. aeruginosa* to produce pulmonary illnesses, the proposed mechanism was that PlcH

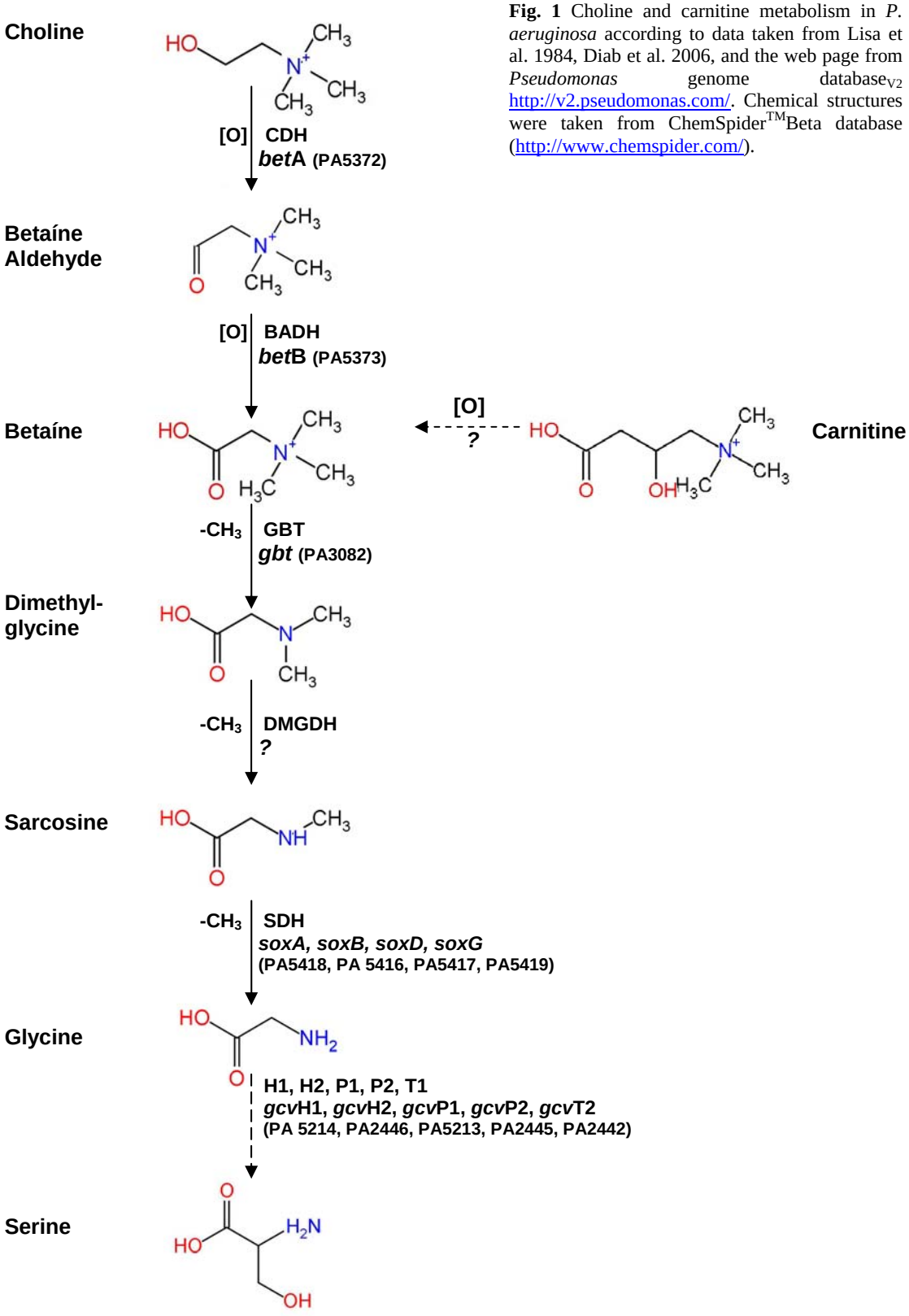

Fig. 1 Choline and carnitine metabolism in *P. aeruginosa* according to data taken from Lisa et al. 1984, Diab et al. 2006, and the web page from *Pseudomonas* genome database$_{V2}$ http://v2.pseudomonas.com/. Chemical structures were taken from ChemSpider^{TM}Beta database (http://www.chemspider.com/).

acts against dipalmitoylphosphatidylcholine (the major lipid component of lung surfactant) and produces Pch, which is hydrolyzed to choline plus Pi through the action of PchP [24]. Considering that PlcH activity is excreted into the culture medium and that PchP is a periplasmic enzyme [16], we proposed that Pch would diffuse into the periplasmic space through the hydrophilic channels formed by porin proteins. Bacterial and mammalian cells contain high and low affinity components for choline uptake. However, the formation of choline in the periplasmic space offers a "territorial" advantage for the bacterium with respect to the mammalian cells. With this strategy, choline may be immediately captured and metabolized, favoring the induction of PlcH, PchP and ChE. Additionally, if the bacteria found a hyperosmotic medium, choline may also act as an osmoprotectant through the oxidation to betaine [25, 26]. To cover another aspect related to the action of PlcH and PchP, we also decided to explore the factors that affect the activity of these enzymes. Therefore, the effect of metal ions on the AcPase was studied, since AcPase activity was dependent on Mg^{2+} and PlcH inhibited by Zn^{2+} [19,21]. We found that Mg^{2+}, Zn^{2+} and Cu^{2+} were activators of AcPase activity. The K_A value for Zn^{2+} indicated that the AcPase could catalyze the hydrolysis of *p*-NPP at a concentration twenty fold lower than that necessary to inhibit

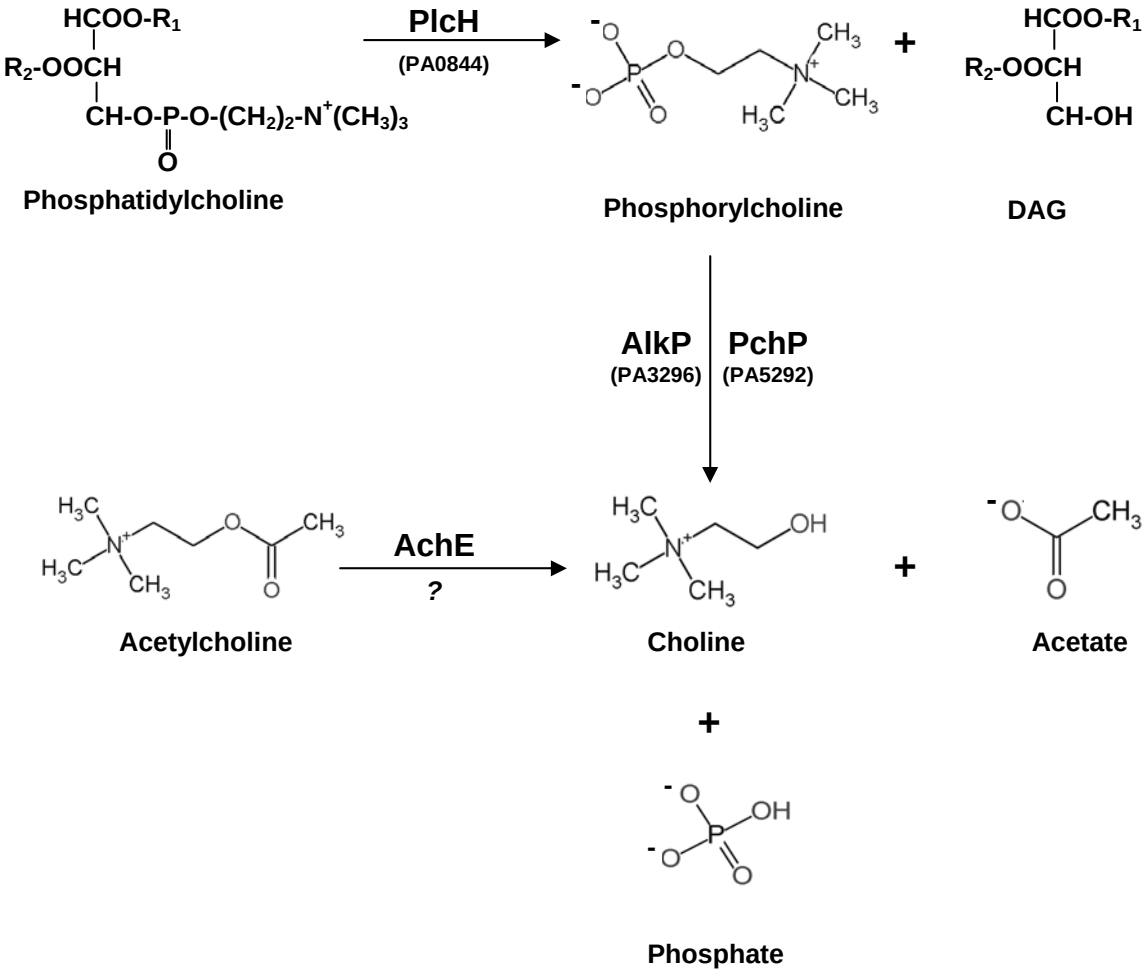

Fig. 2 Reactions catalyzed by PlcH, AlkP, PChP and AchE on choline-containing compounds. Chemical structures were taken from ChemSpider™Beta database (http://www.chemspider.com/), PubChem Database (http://pubchem.ncbi.nlm.nih.gov/) and BRENDA enzyme database (http://www.brenda.uni-koeln.de/).

PlcH activity. By assuming that this was true for natural substrates, we proposed that metallic ions would not have any adverse effect on enzymes that act in a coordinated and sequential manner. In addition, we demonstrated that Al^{3+} was a powerful inhibitor of this enzyme's activity [27]. Finally, to gain a better understanding on the role of choline in the pathogenicity of *P. aeruginosa* through the action of PlcH and PchP, we focused our attention on the possibility that these bacteria may encounter different Pi concentrations or carbon and nitrogen sources in their environments. Experiments performed in this sense [24] indicated that i) even in the presence of choline, these enzyme activities were practically absent if a high Pi concentration and preferential carbon and nitrogen sources were simultaneously present. Therefore, other factors distinct to PlcH and PchP must be present to substantiate infection by *P. aeruginosa*. ii) In a low Pi medium and in the absence of choline, PlcH and AlkPase are produced. According to Liu [18], both enzymes, working sequentially, acted as a phosphate-scavenging mechanism. iii) Under Pi limitation in the environment, but in the presence of choline, the bacteria produce PlcH, PchP and AlkPase. In this case, after the action of PlcH, with both phosphatases acting on the same substrate, the bacteria increase their ability to scavenge Pi and more choline may be supplied in its environment. The increase in choline concentration prevents the repressing effect of Pi on PlcH production. In this way, a constant flow of choline may be utilized by the bacteria through its inducible [28] or constitutive uptake systems [29] to produce more enzymes that promote infection. If PlcH is a virulence factor, then choline, betaine, dimethylglycine and carnitine might be included among the factors that promote the pathogenicity of *P. aeruginosa*. By the same reasoning, PchP may also be considered as a virulence factor of *P. aeruginosa*. Therefore, under this perspective, we focused our attention on the identification of the gene responsible for its synthesis. For this purpose, more than 5000 *P. aeruginosa* Tn5-751 mutants were analyzed and only one of them was identified as $PchP^{(-)}$. This unique negative mutant, named JUF8-00, was the starting point to identify that PA5292 in the PAO1 genome was the locus responsible for the synthesis of PchP. We named the gene corresponding to PA5292 *pchP* and confirmed that it really encoded for a PchP activity through two different approaches. One of them was the restoration of PchP activity in the mutant strain JUF8-00. This experiment was performed by complementation of the JUF8-00 mutant strain with the plasmid pBB1. pBB1 was obtained by subcloning the intact *pchP* gene into pBBR1MCS-5, which was transformed in *E. coli* S17-1 and conjugated to *P. aeruginosa* JUF8-00 [30]. In the second experiment, the *pchP* gene was cloned into the plasmids pTYB12 and pUCP-Nde. They were overexpressed in *E. coli* ER2566 and in *P. aeruginosa* PAO1LAC, respectively. In both cases, protein induction with IPTG was carried out in LB medium and a PchP activity was found [30]. The identification of the PA5292 gene as the only gene responsible for encoding PchP opened a new perspective in our group. Choline seems to regulate the expression of various genes whose expression may have a significant impact on the establishment of chronic pulmonary infections, as well as corneal and urinary tract infections caused by *P. aeruginosa*. Although PchP and PlcH are involved in the breakdown of phosphatidylcholine [24,25], only the regulation of PlcH expression has been studied in detail by different laboratories. Since nothing was known about the regulation of PchP expression, we initiated studies aimed at understanding the molecular mechanisms governing the expression of the *pchP* gene. This would allow us to elucidate the environmental conditions in which these bacteria produce PchP and to get a clearer picture of the concerted mode of action and transcriptional regulation of both PchP and PlcH. The *P. aeruginosa* genome sequence indicated that *pchP* is a single gene that is localized between the convergently transcribed PA5291, which encodes a protein belonging to the choline-carnitine-betaine transporter family, and PA5293, which encodes a putative LysR family transcriptional regulator [10]. Experiments of Northern blots and RT-PCR showed that this gene is transcribed as a 1 Kb monocistronic mRNA, indicating that co-transcription with any of the surrounding genes does not occur. The conclusion of these experiments was that the transcription of *pchP* is governed by a promoter located in the PA5293-*pchP* intergenic region. Physiological studies in *P. aeruginosa* P1::LacZ, detecting *pchP* promoter activity through beta-galactosidase expression, grown under different conditions confirmed that expression was induced by choline, betaine, dimethylglycine or carnitine. The promoter activity was repressed by the addition of succinate and ammonium to the choline-containing medium. These molecular studies [Massimelli et al.,

personal communication] supported our previous physiological studies, where the AcPase activity was measured in bacteria growing in minimal media with the same choline derivatives [5,13].

Although, in our laboratory, we have purified PchP from *P. aeruginosa* grown in choline or betaine media to study some biochemical properties [23], we focused our attention on obtaining the recombinant enzymes. With this approach, we expected to obtain enzyme in enough quantity to perform experiments related to aspects of the structure and function of this enzyme. We were able to have different preparations of recombinant enzymes in homologous and heterologous hosts [30,31]. The first results indicated that, like the native PchP, the recombinant enzymes were capable of catalyzing the hydrolysis of Pch, phosphorylethanolamine and *p*-NPP in an identical range of pHs. Phosphoserine was not a substrate of PchP. Saturation curves of PchP with Pch revealed similar catalytic behavior to that obtained with the native enzyme. The two recombinant enzymes, like the native PchP, contained the high and low affinity sites for Pch and their activities were inhibited by high substrate concentration [30]. K_{M1} values were identical for the three enzymes, but an increased value of K_{M2} for the enzyme expressed in *E. coli* was found. The increased value in the K_{M2}, plus the changes in the relationship K_{M2}/K_{M1}, were explained by the presence of the signal peptide present in the N-terminus of the recombinant enzyme. To confirm this explanation, PchP was cloned without the signal peptide, predicted by the Signal P server [32]. In this way, an enzyme equivalent (contained an extra N-terminal methionyl residue) to the mature enzyme when it is produced by *P. aeruginosa* grown with choline in the culture medium was obtained. Kinetic experiments performed with enzyme with or without the 22 amino acid residues indicated that the signal peptide was the fundamental factor responsible for increasing the K_{M2} values of PchP for Pch [31,33]. From these data, we concluded that the importance of the secretion pathway from the cytosol to the periplasmic space is to produce a mature enzyme capable of notably increasing its catalytic efficiency for the natural substrate Pch. In order to characterize this PchP in silico, some bioinformatics tools were utilized. Sequence and structure alignments of PchP allowe d us to identify the three conserved motifs that are characteristic of the haloacid dehalogenase hydrolase super family. As it is published in databases, the *pchP* gene (PA5292, gi|9951606|) encodes a protein with 349 amino acids. Therefore, the motifs I, II and III were found in ^{53}DMDNT^{57}, ^{188}S and ^{283}GDTPDSD^{289} [30]. After secretion to the periplasmic space, PchP has lost 22 amino acids and therefore the mature protein contains 327 aminoacyl residues. For that reason, motifs I, II and III were located in positions ^{31}DMDNT^{35}, ^{166}S and K$^{242/261}$GDTPDSD^{267}. These motifs are conserved in orthologous proteins of the *Pseudomonas* genus and other pathogenic organisms [31]. By utilizing the atomic coordinates of *Methanoccus janaschi* phosphoserine phosphatase [34], we obtained a structural model for PchP [31] (Fig. 3). After site-directed mutagenesis of specific residues in these motifs, the most important residues for catalysis were identified. The amino acid residues involved in the active site of PchP in interaction with Mg^{2+} and the phosphate moiety from PCh were D31, D33, T35, S166, K242, D262, and D267. D31, D33, D262 and D267 were the most important residues for catalysis. D265 and D267 could be involved in the stabilization of motif III or might contribute to substrate specificity. The substitution of T35 by S35 resulted in an enzyme with a low PchP activity, but conserved both catalytic sites involved in the hydrolysis of Pch (K_{M1} 0.03 mM, K_{M2} 0.5 mM) and *p*-NPP (K_M 2.1 mM). Mutations either on S166 or K242 revealed that these residues are also important for hydrolysis of both substrates. At position 242, the substitution of lysine by arginine or glutamine showed the importance of the positively charged group, since K242R was a functional enzyme and K242Q was completely inactive. With respect to the binding site for the alkylammonium moiety, at the moment we can only say that the choline binding domain found in gram positive bacteria or in higher organisms is not present in PchP. The mutants studied up to the present time indicated that the serine residue S166 from motif II would also be involved in the recognition of some part of the trimethylammonium moiety. This is supported by the fact that the mutant S166T was not inhibited by choline or betaine [33]. As expected, Mg^{2+}, Zn^{2+} and Cu^{2+} were activators of the purified recombinant enzyme. One striking result was observed with low (0.4 mM) or high (40 mM) Mg^{2+} concentration and Pch as a substrate. At low Mg^{2+}, the PchP activity was inhibited by high Pch concentration; with high Mg^{2+}, the metal ion abrogated the inhibition produced by high substrate concentration [Otero et al., personal communication]. S166 residue might also be involved in activation by the three metallic ions. In motif III, the aspartyl residues D261 and D266 are involved in

recognition differentially for Cu^{2+} and Zn^{2+}. For example, the PchP mutant D261E was more than 200% activated by Cu^{2+} and 50% inhibited by Zn^{2+}. On the contrary, the PchP activity from the mutant D266E was near 90% inhibited by Cu^{2+} and was not affected by Zn^{2+}. In order to find plausible explanations for some of our latter findings, we focused attention on the physical properties of the enzyme. For this purpose, the enzyme was expressed in *E. coli* and purified from inclusion bodies by anion exchange chromatography under denaturing conditions. Pure urea denatured protein was refolded by dialysis against physiological buffers. Mass spectrometry of this protein indicated a molecular mass of 37105 Da,

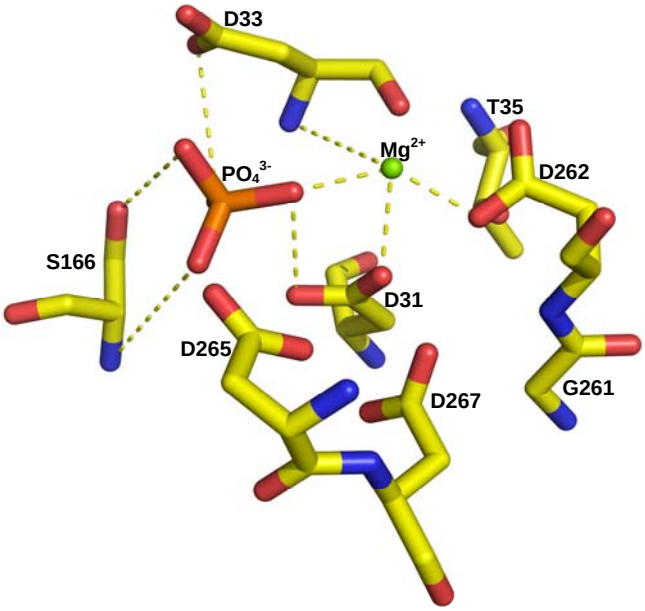

Fig. 3 Catalytic pocket of PchP. Aminoacyl residues involved in the active site plus their interaction with Mg^{2+} and the Pi moiety.

which is in a good agreement with 37089.34 Da that was obtained by calculation from the published PA5292 sequence without the signal peptide. Analysis by SEC-FPLC showed a single species with a retention time corresponding to a slightly expanded globular protein with the expected size. Fluorescence and CD analysis showed the well-preserved secondary and tertiary structures that could be modified by the interaction with choline or Mg^{2+}. Protein stability studied by thermal denaturation between 0ºC and 95ºC while monitoring ellipticity at 220 nm showed that the unfolding process was highly cooperative, with a transition temperature of 52ºC [33]. A three-dimensional model of PchP obtained by threading methodology is shown in Figure 3. Crystallization trials are in course to undertake the structural characterization of this protein at atomic resolution to complete the knowledge of the amino acid residues involved in the binding of the quaternary ammonium ions or different metal ions and to predict rational design of inhibitors for PchP. Since AChE shares many of its properties with PchP and may also be implicated in corneal infection [22,24,25], our effort is also now focused on the cloning, identification and regulation of the gene responsible for AchE synthesis when *P. aeruginosa* is found in an environment containing choline or some of its derivatives.

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