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CGDEase, a *Pseudomonas fluorescens* protein of the PLC/APase superfamily with CDP-ethanolamine and (dihexanoyl)glycerophosphoethanolamine hydrolase activity induced by osmoprotectants under phosphate-deficient conditions

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Summary

A novel enzyme, induced by choline, ethanolamine, glycine betaine or dimethylglycine, was released at low temperature and phosphate from *Pseudomonas fluorescens* (CECT 7229) suspensions at low cell densities. It is a CDP-ethanolamine pyrophosphatase/(dihexanoyl)glycerophosphoethanolamine phosphodiesterase (CGDEase) less active on choline derivatives, and inactive on long-chain phospholipids, CDP-glycerol and other NDP-X compounds. The reaction pattern was typical of phospholipase C (PLC), as either phosphoethanolamine or phosphocholine was produced. Peptide-mass analyses, gene cloning and expression provided a molecular identity for CGDEase. Bioinformatic studies assigned it to the PLC branch of the phospholipase C/acid phosphatase (PLC/APase) superfamily, revealed an irregular phylogenetic distribution of close CGDEase relatives, and suggested their genes are not in operons or conserved contexts. A theoretical CGDEase structure was supported by mutagenesis of two predicted active-site residues, which yielded essentially inactive mutants. Biological relevance is supported by comparisons with CGDEase relatives, induction by osmoprotectants (not by osmotic stress itself) and repression by micromolar phosphate. The low bacterial density requirement was related to phosphate liberation from lysed bacteria in denser populations, rather than to a classical quorum-sensing effect. The results fit better a CGDEase role in phosphate scavenging than in osmoprotection.

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Introduction

Pseudomonas fluorescens is a psychrotrophic Gram-negative bacterium widely distributed in soil and water (Mavrodi *et al.*, 2007). The relatively large genomes of three different strains have been sequenced (Pf-5, Pf0-1 and SBW25). Its ability to grow at low temperatures, allows *P. fluorescens* to contaminate cold-stored solutions or food (Gonzalez *et al.*, 1987; Gennari and Dragotto, 1992). It occurs in laboratory environment, and tissue extracts processed in the cold room may provide adequate growing conditions for the bacterium (Crevel *et al.*, 1994; Preuss *et al.*, 2001). It is commonly found in hospitals as a contaminant of cleaning solutions and even injectable material (Namnyak *et al.*, 1999), and at times it becomes an opportunistic pathogen (Khabbaz *et al.*, 1984; Hsueh *et al.*, 1998; Chapalain *et al.*, 2008; Gershman *et al.*, 2008).

The phospholipase C/acid phosphatase (PLC/APase) protein superfamily (Stonehouse *et al.*, 2002), also named AcpA/PlcH (Felts *et al.*, 2006), comprises a group of phosphatidylcholine-preferring PLCs (PC-PLC) and another of acid phosphatases (APases). The paradigmatic members of the PLC branch are PlcH and PlcN, haemolytic and non-haemolytic enzymes of *Pseudomonas aeruginosa* (Ostroff *et al.*, 1990; Vasil, 2006), although other PLCs of this kind have been also studied, e.g. those from *Burkholderia pseudomallei* (Korbsrisate *et al.*, 2007) and *Mycobacterium tuberculosis* (Raynaud *et al.*, 2002). These enzymes are structurally unrelated to other well-known PLCs, namely the Zn-dependent

PC-PLC and the phosphatidylinositol-specific PLC (PI-PLC) (Titball, 1993; Songer, 1997). Concerning the non-PLC members of the PLC/APase superfamily, the best known is *Francisella tularensis* AcpA, whose structure has been solved (Felts *et al*., 2006).

NDP-X hydrolases are a wide group of enzymes, most of which belong to the Nudix superfamily (Bessman *et al*., 1996; Mildvan *et al*., 2005; Galperin *et al*., 2006; McLennan, 2006). Only two Nudix proteins with preference for CDP-X compounds are known: one from *Bacillus cereus*, which is rather specifically active on CDP-choline as compared with NDP-sugars (but its activity on other CDP-alcohols is unknown) (Xu *et al*., 2004); another from *Paenibacillus thiaminolyticus*, which hydrolyses several CDP-alcohols with similar activity, and other NDP-X substrates (Tirrell *et al*., 2006). Recently, we have shown that two non-Nudix proteins, belonging in fact to the metallophosphoesterase superfamily, cleave efficiently CDP-alcohols: the 5′-nucleotidase/UDP-sugar hydrolase (UshA) from *Escherichia coli* and *Yersinia intermedia*, and the Mn^{2+}-dependent ADP-ribose/CDP-alcohol pyrophosphatase (ADPRibase-Mn) from *Rattus norvegicus*. UshA shows relative preference for CDP-glycerol over CDP-ethanolamine or CDP-choline (Alves-Pereira *et al*., 2008), and ADPRibase-Mn does it for CDP-glycerol or CDP-choline over CDP-ethanolamine (Canales *et al*., 2008).

In the course of a search for CDP-alcohol hydrolases in mammalian lung, we came across such an enzyme in rat extracts kept in the cold, but it turned out to be produced by contaminant *P. fluorescens* (CECT 7229) bacteria. It was characterized as a novel, inducible hydrolase with a unique preference for CDP-ethanolamine, glycerophosphoethanolamine and dihexanoylglycerophosphoethanolamine versus their choline counterparts, while it was inactive on CDP-glycerol. Such enzyme is here named CGDEase for short. Although inactive on long-chain phospholipids, it was identified as a member of the PLC branch of the PLC/APase superfamily.

Results and discussion

Detection of an inducible CGDEase in P. fluorescens *CECT 7229 suspensions*

When kept in the cold for several days, chromatographic fractions of a partially purified rat lung extract showed a remarkable increase of several hydrolytic activities here shown to be typical of CGDEase. This was related to the presence of bacterial contamination by *P. fluorescens* CECT 7229. The time-dependent liberation of CGDEase activities was also observed when pure bacterial suspensions were incubated in the presence of the same, but sterilized, lung fraction. This response was extremely sensitive to the cell density of the bacterial suspension: optimal liberation of enzyme activity occurred in suspensions giving an A_{600} value of 0.01, while no activity was released at $A_{600} \geq 0.04$ (see Fig. S1).

The liberation of CGDEase activities by diluted *P. fluorescens* suspensions was also elicited in defined medium, with choline or ethanolamine replacing the lung fraction in a dose-dependent manner (Fig. 1A), while phosphocholine or phosphoethanolamine were ineffective (not shown). The response needed about a week to be complete and it occurred in the absence of measurable bacterial growth, as A_{600} remained constant (Fig. 1B). Enzyme liberation did not represent just secretion of stored protein, as the activity recovered from the medium was about 50-fold higher than that initially present inside the cells (not shown).

One of the CGDEase substrates tested with positive results in the first experiments was 4-nitrophenylphosphocholine (Fig. 1C), a surrogate artificial substrate used for routine assay of PLCs. This gave the first clue that CGDEase could have PLC-type activity. After the identification of CGDEase as a member of the PLC/APase superfamily (this work, below), and considering the known induction of proteins like *P. aeruginosa* PlcH by choline and related osmoprotective derivatives (Shortridge *et al*., 1992; Wargo *et al*., 2009), we found that, besides by choline, CGDEase liberation was also induced by a similar set of osmoprotectants (Fig. 1D).

As shown in Fig. 2A and D, the optimal cell density for response to choline, in terms of total CGDEase activity liberated to the suspension supernatant in a 4 or 6 day incubation, was 0.04–0.07 A_{600}, a value that remained constant for the length observed (Fig. 1B). In bacterial suspensions of increasing initial density above the optimal value, there was an abrupt decrease of the total amount of enzyme released. This amount went down to near negligible levels when expressed in relative terms with respect to the amount of cells initially suspended (Fig. 2A). Under these conditions, the A_{600} of the bacterial suspensions and the number of viable cells decreased partly during the incubation with choline, suggesting that in the denser suspensions (Fig. 2B and C), but not in the less dense ones (Fig. 2B and C inserts) some cell lysis occurred. This was fortituously confirmed by the observation that the no-substrate blanks of the assays of CGDEase activity in the supernatants of high cell density suspensions increased during the incubation with choline, reflecting the liberation of intracellular material (data not shown). Nevertheless, it should be remarked that, even after cell lysis, many more viable bacteria remained in the denser suspensions, which released little or no CGDEase, than in the less dense ones in which CGDEase liberation was optimal without appreciable cell lysis (Fig. 2C). Driven by all those observations, we decided to assay the level of inorganic phosphate in suspension supernatants obtained

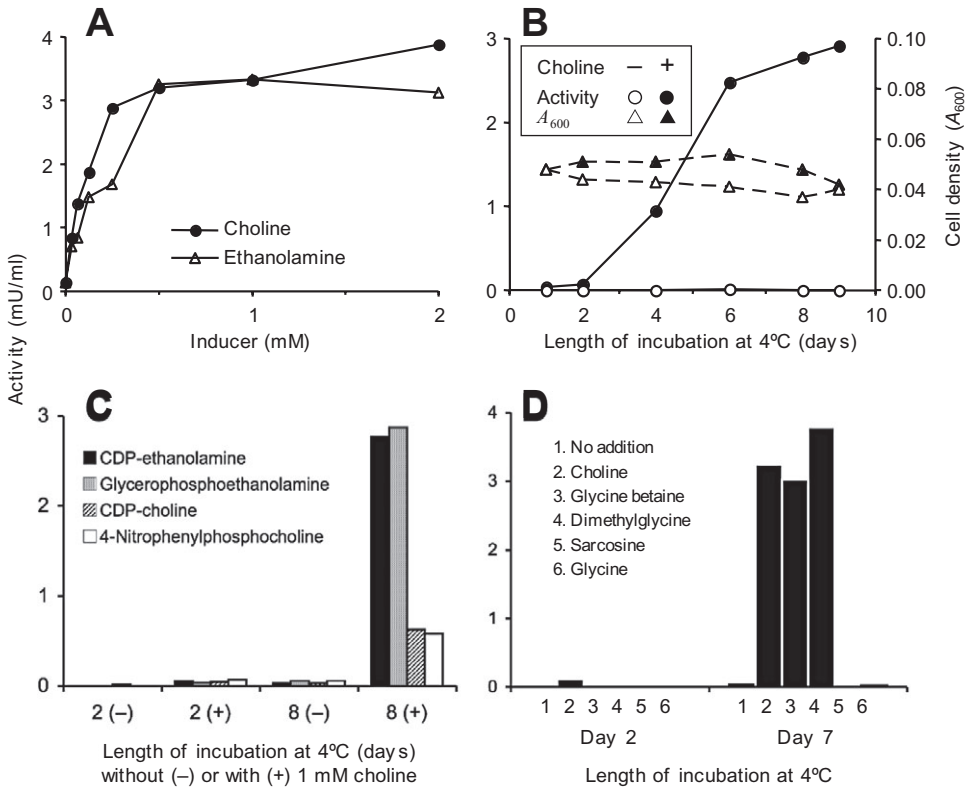

Fig. 1. Induction and release of *P. fluorescens* CGDEase. Bacterial suspensions of CECT 7229 cells prepared as described in *Experimental procedures* were used. Cell densities were estimated by measurement of A_{600} of vortexed suspensions. Aliquots were kept at 4°C without shaking for the length of the experiments. CGDEase activity was assayed in cell supernatants with CDP-ethanolamine as the substrate unless otherwise indicated.
A. Dose-dependent induction of CGDEase by choline or ethanolamine. Bacteria in suspension buffer at a density giving an A_{600} of 0.035 were kept for 11 days with the indicated additions.
B and C. Time-dependence of CGDEase induction by choline. Cells were kept in suspension buffer with 1 mM choline added as indicated.
D. CGDEase induction by choline-derivative osmoprotectants. Cells were kept in suspension buffer (at an initial A_{600} of 0.04) with 1 mM of the indicated compound.

at different population densities, at the beginning and at the end of a 4 day incubation of bacteria with choline. The results of these analyses, shown in Fig. 2D, were instrumental to understand the dependency of CGDEase release on bacterial density as a phosphate-dependent response.

A fuller account and discussion of the induction of CGDEase, its repression by phosphate and their relationship to population density, are given under the final subheading of the *Results and discussion* section.

Purification of CGDEase from the conditioned medium of P. fluorescens *suspensions incubated with choline*

To proceed with the purification, enzyme characterization and molecular identification of the protein, 1 mM choline was chosen as the inducer. CGDEase was partially purified from *P. fluorescens* conditioned medium by chromatography on a Q-sepharose column, up to a specific activity of around 2.5 units mg^{-1} assayed with CDP-ethanolamine. The hydrolytic activities on CDP-ethanolamine, glycerophosphoethanolamine, CDP-choline and 4-nitrophenylphosphocholine copurified in this column (Fig. 3A). This preparation was stable for more than 2 weeks in the cold. However, attempts to further purify CGDEase led to strong losses of activity. So, the partially purified preparation obtained from the Q-sepharose column was used for enzyme characterization. Anyhow, further chromatography on an agarose-MP column (Fig. 3B) yielded a preparation that, even if unstable, was useful because it showed only two protein bands of 40 and 75 kDa (Fig. 3B insert), and the latter was identified as CGDEase (see below).

Enzymatic characterization of CGDEase

The enzyme did not require the addition of bivalent cations for activity (tested with CDP-ethanolamine). Thoroughly dialysed CGDEase was fully active in the absence of added bivalent cations (Fig. 4) and the reaction rate

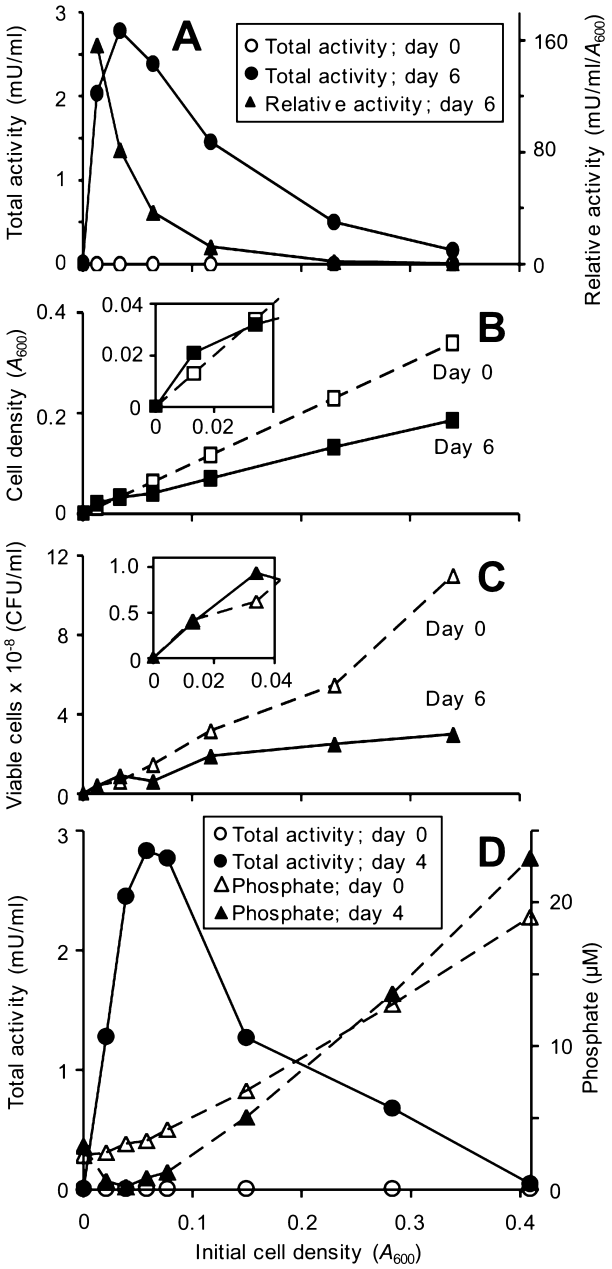

Fig. 2. Effect of bacterial population density on the induction of CGDEase by choline. Bacterial suspensions of CECT 7229 cells prepared as described in *Experimental procedures* were used and kept at 4°C in the presence of 1 mM choline, for two different experiments.
A. CGDEase activity in suspension supernatants with CDP-ethanolamine as the substrate; relative activity refers to the ratio between the total activity and the initial cell density.
B. Cell density estimated by measurement of A_{600} of vortexed suspensions. The insert shows an enlargement of the lowest part of the graph. Same experiment as in A.
C. Number of viable cells determined by plating in MacConkey agar. The insert shows an enlargement of the lowest part of the graph. Same experiment as in A.
D. CGDEase activity with CDP-ethanolamine as the substrate and phosphate concentration in suspension supernatants.

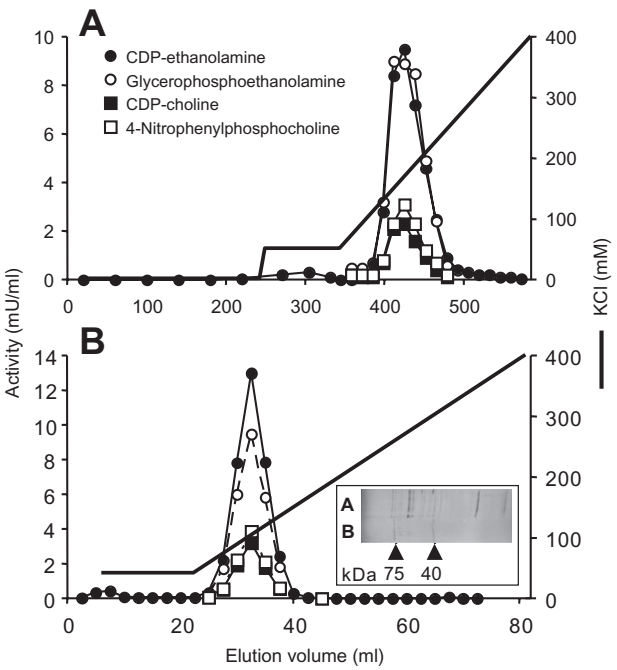

Fig. 3. Purification of CGDEase from *P. fluorescens* conditioned medium. A suspension of CECT 7229 cells (A_{600} ~0.05) prepared as described in *Experimental procedures* was kept for 9 days at 4°C in the presence of 1 mM choline.
A. After centrifugation, 250 ml of conditioned medium was applied to a Q-sepharose column. CGDEase was recovered by gradient elution with KCl and the activities on the indicated substrates were assayed.
B. The active fractions were pooled and one half of this preparation was further fractionated in an agarose-MP column, also eluted with a KCl gradient. The insert shows a silver-stained SDS-PAGE of both preparations. Further details are given in *Experimental procedures*.

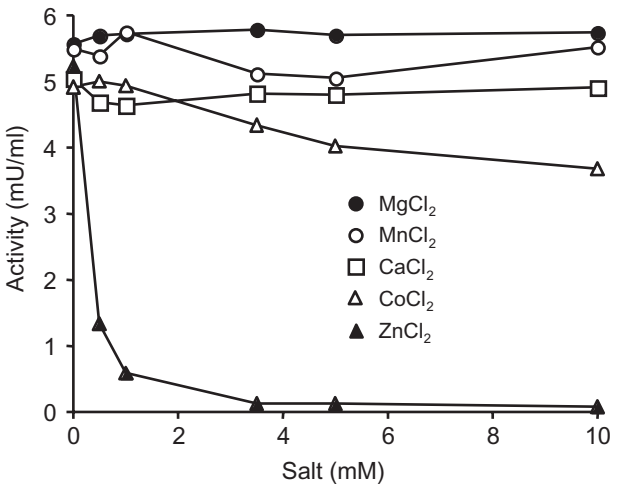

Fig. 4. Effects of bivalent cations on CGDEase activity. The enzyme was exhaustively dialysed to remove $MgCl_2$ (see *Experimental procedures*). The rate of hydrolysis of CDP-ethanolamine was then assayed under standard conditions, except that different cation concentrations were used and, when indicated, $MgCl_2$ was substituted in the reaction mixture by another metal chloride.

was less than 5% inhibited by 1 mM EDTA. The inclusion of chloride salts of Mg^{2+}, Mn^{2+} or Ca^{2+} in assay mixtures was without appreciable effect, while Zn^{2+}, and to a much lesser extent Co^{2+}, were inhibitory (Fig. 4). Despite that Mg^{2+} was not necessary for activity, as this cation was present at 5 mM concentration in the enzyme buffer, it was systematically included in assay mixtures (3.5 mM) to standardize any unexpected effect.

The substrate specificity of CGDEase was studied against a panel of 44 compounds including CDP-alcohols, glycerophosphoalcohols, NDP-sugars, dinucleoside-oligophosphates, nucleoside mono- and oligophosphates, cyclic nucleotides, related phosphorylated compounds, 4-nitrophenyl derivatives, short- and long-chain phospholipids (Table 1). The enzyme showed a strong preference for CDP-ethanolamine, glycerophosphoethanolamine and dihexanoylglycerophosphoethanolamine. Smaller activities were detected on their choline counterparts and 4-nitrophenylphosphocholine. The other compounds tested were either not substrates or only marginally hydrolysed. The specificity pattern observed with the partially purified endogenous CGDEase was confirmed with the recombinant enzyme after PCR cloning and expression (see below, and Table 1).

As the lack of activity of CGDEase on long-chain phospholipids was deemed an important aspect of its specificity, several experiments were performed to ensure that this was a genuine result. First, as Triton X-100 was included in the assays with long-chain phospholipids, proper controls were run, which proved that the CGDEase activities on the short-chain and the other soluble substrates were not affected by the detergent (Table 1). Second, the same mixed-micelle preparations used to test CGDEase activity, were tested successfully as substrates of *B. cereus* PLC under the same conditions used for CGDEase assay. As expected, this enzyme showed high activity on the phospholipidic preparations: dipalmitoylglycerophosphocholine (21 U mg^{-1}), egg phosphatidylcholine (19 U mg^{-1}), dipalmitoylglycerophosphoethanolamine (6 U mg^{-1}) and egg phosphatidylethanolamine (12 U mg^{-1}). In contrast, up to 160 ng of recombinant CGDEase (upper band of the two shown in Fig. 5) did not show any appreciable hydrolysis of those phospholipids after 90 min incubation (< 0.002 nmol min^{-1}), implying that any undetected activities of CGDEase on the long-chain phospholipids would be < 0.06%, < 0.07%, < 0.2% and < 0.1% of those shown by *B. cereus* PLC on the same substrates respectively. In addition, our choline phospholipidic preparations, but not the ethanolamine ones, were hydrolysed by *P. aeruginosa* PlcH (see below).

Kinetic studies were performed with CDP-ethanolamine, glycerophosphoethanolamine, dihexanoylglycerophosphoethanolamine, 4-nitrophenylphosphocholine, CDP-choline and dihexanoylglycerophosphocholine (see Fig. S2). In all cases, hyperbolic saturation curves were obtained, with K_m values near 0.2 mM for the three ethanolamine-containing substrates and 0.4–1.4 mM for the others. In terms of V_{max}/K_m, ethanolamine-containing compounds were similarly efficient substrates, whereas the choline-containing ones were around 10-fold less efficient (Table 1). Some saturation curves obtained with short-chain phospholipids were extended to include data above the critical micellar concentration (CMC), without showing deviation from the V_{max} plateau reached at lower concentrations: up to 10 mM dihexanoylglycerophosphoethanolamine (apparent CMC 7.0 mM; Hergenrother and Martin, 1997) or 18 mM dihexanoylglycerophosphocholine (apparent CMC 11.1 mM; El-Sayed *et al.*, 1985) (Fig. S2). The possibility of Mg^{2+} affecting importantly the kinetic parameters of CGDEase, was discounted by registering saturation curves with CDP-ethanolamine or dihexanoylglycerophosphoethanolamine as substrates both in the presence and in the absence of Mg^{2+}, which yielded kinetic parameters K_m and V_{max} agreeing within a 10–20% error (Fig. S2).

The reaction products of CDP-ethanolamine and CDP-choline hydrolysis by CGDEase were CMP and the phosphoalcohol (Table 2). The formation of CMP was detected in both cases by HPLC (Table 2), whereas CDP was neither formed as a product nor used as substrate (Table 1). In the presence of ancillary alkaline phosphatase, 1 mol of cytidine and 2 mol of P_i were measured per mole of CDP-alcohol split. In addition, with CDP-choline as the substrate, the formation of phosphocholine was inferred from the fact that no choline could be detected by enzymatic assay with choline kinase, unless the reaction products were treated with alkaline phosphatase. After this treatment, followed by heat inactivation of the phosphatase, choline was detectable in amounts equimolar to those of substrate split. Altogether, these results indicated that the phosphoanhydride linkages of CDP-alcohols were hydrolysed by CGDEase, which, with these substrates, acts as pyrophosphatase.

When acting on glycerophosphoethanolamine, CGDEase attacked on the glycerol side of the phosphodiester linkage and the reaction products were glycerol and phosphoethanolamine (Table 2). This was inferred from the detection of glycerol, assayed with glycerol kinase in the absence of P_i formation, whereas L-glycerol 3-phosphate was not detected with glycerol 3-phosphate dehydrogenase. The addition of alkaline phosphatase to the reaction mixtures led to the formation of glycerol and P_i in similar amounts.

When acting on 4-nitrophenylphosphocholine, CGDEase attacked on the nitrophenol side of the phosphodiester linkage (Table 2). This was inferred from the fact that (i) the reaction could be followed directly by the increase of A_{405} (typical of 4-nitrophenolate formation) in

Table 1. Substrate specificity of CGDEase.

Substrate	Activity at 500 μM substrate (%)		K_m[a,c] (μM)	V_{max}/K_m[a,c] (%)
	Endogenous[a]	Recombinant[b]		
CDP-ethanolamine	100	100	160	100
Glycerophosphoethanolamine	96	70	140	108
Dihexanoylglycerophosphoethanolamine	115	71	180	105
4-Nitrophenylphosphocholine	29	42	390	16
CDP-choline	23	29	570	11
Dihexanoylglycerophosphocholine	7	16	1330	2.4
Glycerophosphocholine	7	9		
Glycerophosphoinositol	< 2	N.A.		
CDP-glycerol	< 2	< 2		
CDP-glucose	< 2	N.A.		
CMP-*N*-acetylneuraminate	2.9	N.A.		
ADP-ribose	< 2	< 2		
ADP-glucose	< 2	< 2		
GDP-glucose	< 2	N.A.		
GDP-mannose	< 2	N.A.		
UDP-glucose	< 2	< 2		
UDP-galactose	< 2	N.A.		
UDP-mannose	< 2	N.A.		
UDP-*N*-acetylglucosamine	< 2	N.A.		
UDP-*N*-acetylgalactosamine	< 2	N.A.		
dTDP-glucose	< 2	N.A.		
P^1,P^2-bis-Adenosine diphosphate	< 2	N.A.		
P^1,P^3-bis-Adenosine triphosphate	< 2	N.A.		
P^1,P^4-bis-Adenosine tetraphosphate	< 2	N.A.		
NAD^+	< 2	N.A.		
NADH	< 2	N.A.		
FAD	< 2	N.A.		
4-Nitrophenyl-dTMP	< 2	N.A.		
4-Nitrophenylphosphate	< 2	N.A.		
L-Glycerol 3-phosphate	< 2	N.A.		
Phosphorylethanolamine	< 2	N.A.		
Phosphorylcholine	3.1	N.A.		
AMP	< 2	< 2		
ADP	< 2	N.A.		
ATP	< 2	N.A.		
GDP	< 2	N.A.		
GTP	< 2	N.A.		
CMP	< 2	< 2		
CDP	< 2	< 2		
CTP	< 2	N.A.		
Dipalmitoylglycerophosphoethanolamine[d]	< 0.1	< 0.1		
Dipalmitoylglycerophosphocholine[d]	< 0.1	< 0.1		
Egg yolk phosphatidylethanolamine[d,e]	< 0.1	< 0.1		
Egg yolk phosphatidylcholine[d,e]	< 0.1	< 0.1		

a. CGDEase from the Q-sepharose step (Fig. 2A); specific activity 2.5 U mg^{-1} total protein with CDP-ethanolamine as substrate.
b. CGDEase from pooled fractions 2–4 of Fig. 5; specific activity 12 U mg^{-1} total protein with CDP-ethanolamine as substrate, or about 26 U mg^{-1} if only the CGDEase 72 kDa band of Fig. 5 is computed.
c. Kinetic parameters were derived from initial reaction velocities at different substrate concentrations, and the data were adjusted by non-linear least squares fit to the Michaelis Menten equation.
d. Assayed in the presence of 0.5% Triton X-100, which did not affect the activity on any of the seven major substrates.
e. Assays with egg-yolk phospholipids were implemented at a concentration of 0.4 mg ml^{-1}, near 0.5 mM as estimated from the mean M_r indicated by the vendor.
N.A., not assayed.

the absence of significant liberation of P_i, (ii) the rate of A_{405} increase was not affected by an addition of alkaline phosphatase to the reaction mixtures, which led to P_i formation in the expected amount and (iii) the hydrolytic activity of CGDEase preparation on 4-nitrophenylphosphate was negligible (Table 1).

When acting on dihexanoylglycerophosphocholine, CGDEase attacked on the glycerol side of the phosphodiester linkage (Table 2). This was inferred by the fact that, similarly as described above for CDP-choline, no choline could be detected unless the reaction products were treated with alkaline phosphatase, in

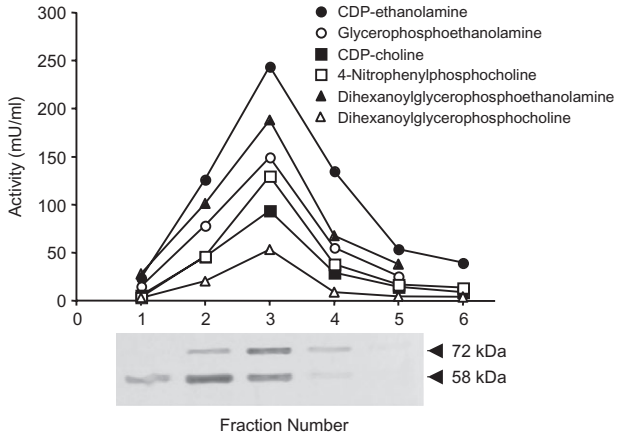

Fig. 5. Purification of recombinant CGDEase. The fusion protein GST/CGDEase was expressed in *E. coli*, and the postsonication supernatant was applied to a GSH-sepharose column. After in-column digestion with PreScission protease, the free CGDEase was eluted with 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM DTT, 150 mM NaCl. Fractions of 1.4 ml were collected, analysed for activity on the indicated substrates (all of them 500 μM, except glycerophosphoethanolamine that was 250 μM) and for protein bands by silver-stained SDS-PAGE. The 72 kDa band was identified as CGDEase (see the main text).

which case equimolar amounts of choline and P_i were measured.

Besides the coelution of activities from the Q-sepharose and agarose-MP columns (Fig. 3), evidence that all of them correspond to the same enzyme was obtained by cross-inhibition experiments. As 4-nitrophenol, the product of 4-nitrophenylphosphocholine hydrolysis by CGDEase, can be easily assayed (by measuring A_{405}) without interference by products simultaneously formed from other substrates, we investigated the inhibition of 4-nitrophenylphosphocholine hydrolysis (assayed at 0.5 mM) by CDP-ethanolamine, CDP-choline, glycerophosphoethanolamine and dihexanoylglycerophosphoethanolamine. Each one of them was included in the assay mixture at a concentration twice its K_m value (see Table 1). Competing substrates should act as competing inhibitors with K_i values similar to their K_m values as substrates (Cornish-Bowden, 1995), and an inhibition of around 44% should be observed under the conditions of the assays. The results agreed with this prediction within a 4% error.

A few inhibition experiments were also performed with a low concentration (10 μM) of nucleoside-phosphates, which revealed potent inhibition of the hydrolysis of 500 μM 4-nitrophenylphosphocholine by the diphosphate (75–95%) and the triphosphate (60–80%), but not the monophosphate derivatives of cytidine and adenine.

Inorganic phosphate was tested at various concentrations up to 10 mM and it inhibited the activity on 500 μM

Table 2. Analyses of reaction products of CGDEase with different substrates.[a]

Substrate:	CDP-ethanolamine[b]		CDP-choline[b]		Glycerophosphoethanolamine[b]		4-Nitrophenylphosphocholine[c]		Dihexanoylglycerophosphocholine[b]	
	Alkaline phosphatase treatment									
Product assayed	–	+	–	+	–	+	–	+	–	+
CDP	N.D.	N.D.	N.D.	N.D.	–	–	–	–	–	–
CMP	59 ± 1	N.D.	17 ± 1	N.D.	–	–	–	–	–	–
Cytidine	2.2 ± 0.2	62 ± 4	1.6 ± 0.1	20 ± 1	–	–	–	–	–	–
Phosphate	1.7 ± 0.4	123 ± 3	2.9 ± 1.3	45 ± 8	1.4 ± 1.4	54 ± 13	0.7 ± 0.03	33 ± 0.5	N.D.	91.8 ± 3.4
Choline	–	–	1.6 ± 1.6	21 ± 4	–	–	–	–	0.6 ± 1.1	91.1 ± 6.9
L-Glycerol 3-phosphate	–	–	–	–	N.D.	N.D.	–	–	–	–
Glycerol	–	–	–	–	67 ± 17	64 ± 18	–	–	–	–
4-Nitrophenol	–	–	–	–	–	–	36 ± 0.2	35 ± 0.2	–	–

a. Micromolar concentrations ± SD ($n = 3$) are given. Incubations were performed in reaction mixtures with 500 μM substrate, without (–) or with (+) alkaline phosphatase. Enzyme amount and incubation lengths varied depending on the substrate used, and were adjusted to obtain a partial but significant consumption of substrate as shown.
b. The incubations were terminated by 5 min on a boiling water bath, and samples were taken for the different assays.
c. The incubations were terminated with an acidic P_i reagent or with concentrated NaOH for nitrophenol assay.
N.D., not detectable.

4-nitrophenylphosphocholine with an IC50 of 700 μM. The inhibition was negligible up to 100 μM phosphate.

CGDEase, tested with CDP-ethanolamine as the substrate, was not inhibited by up to 500 μM D609, a known inhibitor of PC-PLC enzymes not belonging to the PLC/APase superfamily (Amtmann, 1996; Preuss *et al*., 2001; Stonehouse *et al*., 2002).

A bell-shaped pH-activity profile was obtained with CDP-ethanolamine as the substrate, with a maximum around 7.5, and half activity around pH 6.5 and 8.5.

The apparent native molecular weight of CGDEase was 84 700 ± 2800 ($n = 4$), estimated by sucrose gradient centrifugation in the presence of Mg^{2+}, or 86 500 ± 6100 ($n = 3$) in the absence of the cation (not shown). CGDEase molecular weight could not be measured by gel-filtration chromatography, because the use of this technique resulted in a strong loss of activity. As by SDS-PAGE two protein bands of 40 and 75 kDa were seen (Fig. 3; see above), the native enzyme could be a dimer of the 40 kDa band or a monomer of the 75 kDa band. Molecular identification (see below) indicated that the latter is the case.

Molecular identification, cloning and expression of CGDEase

To investigate the molecular identity of CGDEase, the two protein bands of 40 and 75 kDa, observed in the most purified enzyme preparation (see Fig. 3B) were processed to obtain their tryptic peptide-mass figerprints (PMFs) by MALDI-TOF mass spectrometry. These data were used to run Mascot searches (http://www.matrixscience.com) against the NCBInr database. Globally, neither PMF pointed to significant candidates, but peptide subfragmentation gave most useful information. On the one hand, the tandem mass spectra of several tryptic peptides of the 40 kDa protein pointed clearly to *P. fluorescens* Pf0-1 ketol-acid reductoisomerase, which is unrelated to CGDEase. In turn, the PMF of the 75 kDa protein contained a peptide-mass signal of 1284.700 Da, which, upon subfragmentation, pointed with a significant score to VPMLVLSPWSR, a theoretical tryptic peptide of a *P. fluorescens* Pf-5 hypothetical protein of 76 kDa (Accession No. YP_260232). This protein is annotated in NCBI databases as PLC by homology, and it is encoded by a gene labelled *plcB* (gene ID 3477297). As it seemed a good candidate, its coding sequence was used to design primers for PCR amplification from *P. fluorescens* CECT 7229 genomic DNA. Successful amplification ensued and, after TA cloning and subcloning in pGEX-6P-3 (to construct plasmid pGEX-6P-3-CGDEase), the amplicon was sequenced and found to contain an ORF of the same length (2085 nt; submitted to GenBank where it has been given Accession Number GU937796) but only 76% identical to the ORF of the Pf-5 hypothetical protein. Their conceptual translations were also 76% identical (see below, and Fig. S3). The above-mentioned PMF of the 75 kDa protein released by *P. fluorescens* CECT 7229 (Fig. 3) corresponded very well with the conceptual translation of the PCR-amplicon ORF as it covered at least 48% of its peptide sequence, including the diagnostic tryptic peptide VPMLVLSPWSR of the Pf-5 protein.

Plasmid pGEX-6P-3-CGDEase allowed the expression of a GST/CGDEase fusion in *E. coli* BL21 cells. The fusion protein was adsorbed to a GSH-sepharose column, from where the CGDEase moiety (with an expected 8-aminoacid N-terminal extension, GPLGSPNS) was recovered by in-column proteolysis with PreScission protease. The fractions collected from the GSH-sepharose column after this treatment were analysed for protein bands by SDS-PAGE and for CGDEase activities. Unexpectedly, two protein bands of 72 and 58 kDa were observed (Fig. 5). Both of them were analysed by MALDI-TOF mass spectrometry to obtain their tryptic PMFs. As expected, that of the 72 kDa band fit very well with the translation of the pGEX-6P-3-CGDEase insert, covering at least 79% of its sequence and including the GPLGSPNS N-terminal extension. On the other hand, the 58 kDa protein was identified as *E. coli* GroEL, a chaperone that sometimes copurifies with GST fusion proteins (Rohman and Harrison-Lavoie, 2000; Thain *et al*., 1996). The hydrolytic activities on CDP-ethanolamine, glycerophosphoethanolamine, CDP-choline and 4-nitrophenylphosphocholine, were found in the same proportions exhibited by CGDEase (Table 1), coeluting rather precisely with the 72 kDa band, not with the 58 kDa GroEL band (Fig. 5).

The results identified conclusively the protein encoded by the pGEX-6P-3-CGDEase insert as an enzyme with the same substrate specificity as the CGDEase present in *P. fluorescens* CECT 7229-conditioned medium (Table 1). However, there might be some unnoticed difference between the CGDEase purified from CECT 7229 supernatants and the recombinant enzyme expressed as a GST fusion. The former would likely be recovered after passing through the disulfide-favouring periplasmic environment, while the second would be recovered in *E. coli* lysates directly from the cytoplasm in a reducing buffer. Nevertheless, the two Cys residues that CGDEase contains are not properly positioned to form a disulfide bond in a theoretical 3D model built as explained below.

Classification of CGDEase within the PLC branch of the PLC/APase superfamily as defined by a set of conserved domains that point to a division into three different protein groups

Previous work on PLC/APase proteins supports the division of the superfamily into PLC and APase branches

(Stonehouse *et al*., 2002; Felts *et al*., 2006). A systematic study of this question was initially out of the scope of our work, but while analysing the relationship of CGDEase with other proteins, besides noticing that it belongs to the PLC branch, we observed that the PLC/APase superfamily may in fact include at least three main protein groups or branches (Table 3).

A BlastP search against the NCBInr database showed that the closest relatives of *P. fluorescens* CECT 7229 CGDEase were two hypothetical proteins labelled as PLCs by homology: the one from *P. fluorescens* Pf-5 encoded by the *plcB* gene (ID 3477297) used here as a model to design successful CGDEase PCR primers, and another from *Serratia proteamaculans*. Less closely, but also clearly related to CGDEase were several biochemically studied PLCs, for instance PlcN and PlcH from *P. aeruginosa* (Ostroff *et al*., 1990; Vasil, 2006), PlcA-D from *M. tuberculosis* (Raynaud *et al*., 2002), and Plc-1 and Plc-2 from *B. pseudomallei* (Korbsrisate *et al*., 2007) (see Table 3). This is the group of proteins that form the PLC branch proposed on the first reports on the PLC/APase superfamily (Stonehouse *et al*., 2002; Felts *et al*., 2006). To systematize the classification of CGDEase and its close, hypothetical-protein relatives within the PLC branch, we collected data from the Conserved Domain Database (CDD). Table 3 shows that all of these proteins contain conserved domains PC_PLC (aa 1–692 of the 694-aa CGDEase), PlcC (aa 1–553) and phosphoesterase (aa 23–436) with very low *E*-values. In addition, the conserved domain Acid_phos_Burk is present but with a high *E*-value. The same result was obtained for 65 representative proteins studied out of about 400 recorded in NCBInr database with BlastP scores higher than 150 with respect to CGDEase (not shown).

Table 3 shows also examples of CGDEase relatives with BlastP scores lower than 100, which contain also the four domains mentioned above but with different degrees of conservation. These proteins, rather than forming a single APase branch, form actually two groups as dissimilar between them as they are with respect to the PLC branch members. The two non-PLC groups can be distinguished from each other by CDD data. In the first one, the Acid_phos_Burk conserved domain shows very low *E*-values; this group includes a *P. fluorescens* Pf-5 protein named 'phosphoesterase family protein', encoded by gene ID 3479632 (unfortunately also named *plcB* like gene ID 3477295; see above). In the second group, only the phosphoesterase domain is conserved with low *E*-value; this group includes the prototypic *F. tularensis* acid phosphatase AcpA (Felts *et al*., 2006).

It is worth mentioning that CGDEase and many other proteins of the PLC branch (but not all) contain also two copies of a so-called DUF756 domain (aa 509–594 and 599–678 of CGDEase). These domains are absent in proteins from *Mycobacterium* and some other Actinobacteria, and in the non-PLC proteins.

For comparison, Table 3 contains also CDD data for known PLCs unrelated to the PLC/APase superfamily, including the Zn-dependent phosphatidylcholine-preferring PLCs, such as *B. cereus* PC-PLC, *C. perfringens* α-toxin, or *Listeria monocytogenes* PlcB, which contain the Zn_dep_PLPC domain; and the phosphatidylinositol-specific PLCs, such as *B. cereus* PI-PLC or *L. monocytogenes* PlcA, which instead contain the PI-PLC-X domain. Finally, another *P. fluorescens* Pf-5 hypothetical protein is shown, which does not fit any domain recorded in CDD; as discussed below, it seems to be the orthologue of biochemically studied PLCs from other *P. fluorescens* strains (Crevel *et al*., 1994; Preuss *et al*., 2001; Rossignol *et al*., 2008).

In summary, according to data in Table 3, within the PLC/APase superfamily, all the proteins display a rather conserved phosphoesterase domain (pfam04185). The hallmark of the PLC branch is a strong fit to the PC_PLC domain (TIGR03396), and that of the non-PLC group 1 is a strong fit to Acid_phos_Burk domain (TIGR03397), while the non-PLC group 2 displays a low conservation of these two domains.

CGDEase as a secretable protein

CGDEase is an inducible protein released to the medium (Fig. 1). Proteins of the PLC branch of the PLC/APase superfamily, like *P. aeruginosa* PlcN and PlcH, and *M. tuberculosis* PLCs, contain TAT signal peptides for secretion through the inner membrane via the twin arginine translocase pathway and are known to be secreted by this mechanism (Voulhoux *et al*., 2001; De Buck *et al*., 2008; Natale *et al*., 2008). The annotated translation (YP_260232) of *P. fluorescens* Pf-5 gene ID 3477297, which was the reference used to design PCR primers to clone CGDEase, does not include a TAT signal sequence (see Pf5_PLC in the protein alignment of Fig. S3), but the bioinformatic analysis (http://compbio.ornl.gov/prodigal) (Hyatt *et al*., 2010) of upstream genomic sequence revealed that the 63 nt immediately preceding its TTG start codon can be part of a longer ORF encoding a 715 amino acid protein with a MPSLTRRKLLQAAAIGTFFSS*L* N-terminal sequence (the italicized L replaces the starting M of Pf-5_PLC in Fig. S3) that contains the RR signature. Therefore, *P. fluorescens* CECT 7229 CGDEase might be also synthesized as a precursor and secreted via the TAT pathway.

Structural comparison of CGDEase to the acid phosphatase AcpA from F. tularensis

In Fig. S3, a multiple alignment of the PLC/APase superfamily proteins is shown grouped as in Table 3. The only

Table 3. Conserved Domains present in CGDEase and some related proteins: occurrence of three groups of PLC/APases.

Protein[a]			Conserved domain (Accession No.)[b]						
			PC_PLC	PlcC	Phosphoesterase	Acid_phos_Burk	DUF756	Zn_dep_PLPC	PI_PLC-X
GenBank name, *species*	Accession No.	Score[c]	(TIGR03396)	(COG3511)	(pfam04185)	(TIGR03397)	(pfam05506)	(pfam00822)	(pfam00388)
PLC proteins of the PLC/APase superfamily									
CGDEase, *P. fluorescens* CECT 7229	ADF42354	1429	< 1e-180	6e-126	4e-98	4e-18	5e-28; 4e-11	–	–
Phospholipase C, *P. fluorescens* Pf-5	YP_260232	1095	< 1e-180	8e-124	2e-94	2e-18	3e-27; 8e-18	–	–
Phospholipase C, *S. proteamaculans*	YP_001480420	1092	< 1e-180	7e-126	2e-92	7e-16	6e-26; 9e-18	–	–
Phospholipase C (Plc-3), *B. pseudomallei*	YP_110092	818	< 1e-180	8e-129	4e-99	5e-18	4e-29; 6e-22	–	–
Phospholipase C (Plc-1), *B. pseudomallei*	YP_108995	496	< 1e-180	2e-145	6e-110	4e-18	3e-25; 8e-25	–	–
Phospholipase C (Plc-2), *B. pseudomallei*	YP_106965	464	< 1e-180	2e-137	2e-96	2e-31	5e-23; 3e-18	–	–
Non-hemolytic phospholipase C (PlcN), *P. aeruginosa*	NP_252009	639	< 1e-180	1e-172	4e-115	6e-17	2e-25; 1e-19	–	–
Hemolytic phospholipase C (PlcH), *P. aeruginosa*	NP_249535	422	< 1e-180	2e-175	3e-110	4e-15	1e-16; 3e-13	–	–
Phospholipase C (PlcA), *M. tuberculosis*	NP_336895	256	6e-114	8e-170	4e-113	5e-13	–	–	–
Non-PLC proteins of the PLC/APase superfamily (first group)									
Phosphoesterase family protein, Pf-5	YP_262308	67	2e-19	9e-73	2e-74	< 1e-180	–	–	–
Phosphoesterase, *B. vietnamiensis*	YP_001117416	64	3e-13	3e-74	2e-56	< 1e-180	–	–	–
Acid phosphatase, *B. pseudomallei*	YP_109929	60	2e-11	1e-74	1e-61	< 1e-180	–	–	–
Non-PLC proteins of the PLC/APase superfamily (second group)									
Acid phosphatase, *F. tularensis*	YP_897755	99	9e-6	6e-17	1e-98	8e-23	–	–	–
Phosphoesterase family protein, *B. mallei*	YP_105371	95	8e-20	1e-26	8e-60	2e-34	–	–	–
Phospholipase C, *B. vietnamiensis*	YP_001117300	99	2e-29	1e-14	9e-65	1e-35	–	–	–
Non-PLC/APase proteins with PLC activity									
Phospholipase C, *B. cereus*	NP_977069	N.S.	–	–	–	–	–	2e-102	–
Phospholipase C (α-toxin), *C. perfringens*	NP_560952	N.S.	–	–	–	–	–	3e-71	–
Phospholipase C (PlcB), *L. monocytogenes*	NP_463736	N.S.	–	–	–	–	–	4e-98	–
Glycosylphosphatidylinositol diacylglycerol-lyase,[d] *B. cereus*	YP_085095	N.S.	–	–	–	–	–	–	3e-41
Phosphatidylinositol-specific phospholipase C (PlcA), *L. monocytogenes*	NP_463732	N.S.	–	–	–	–	–	–	5e-42
Phosphatidylcholine hydrolysing PLC, Pf-5	YP_258019	N.S.	–	–	–	–	–	–	–

a. Representative examples of PLC/APase proteins belonging to three different groups, and of biochemically characterized PLCs, which are not PLC/APases.
b. Searches were done at the NCBI site against the CDD-31608 PSSMs, using default parameters (October 21, 2009). Conserved domain full names: PC_PLC, phospholipase C, phosphocholine-specific, Pseudomonas-type; PlcC, phospholipase C; Acid_phos_Burk, acid phosphatase, *Burkholderia*-type; DUF756, domain of unknown function (DUF756); Zn_dep_PLPC, Zinc-dependent phospholipase C; PI-PLC-X, phosphatidylinositol-specific phospholipase C, X domain.
c. BlastP score using CGDEase as the query sequence.
d. Also known as phosphatidylinositol-specific phospholipase C.
N.S., not significant BlastP score (arbitrarily set as score < 50; *E*-value > 1e-4, when searched against NCBInr).

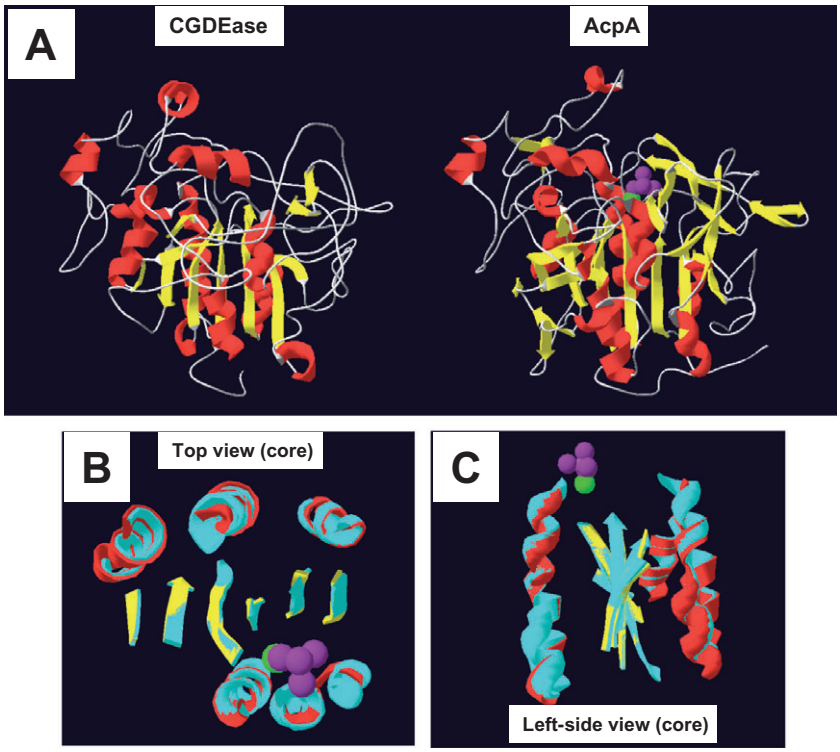

Fig. 6. Partial theoretical structure of CGDEase. The structure was built in the Swiss Model server (Arnold *et al*., 2006) by homology to *F. tularensis* AcpA crystallized in a complex with a (unidentified) metal ion and the enzyme inhibitor vanadate [2D1G; (Felts *et al*., 2006)].
A. 'Front view' of partial (aa 15–431) theoretical CGDEase structure (left), compared with AcpA subunit A (right). Helices are shown in red, sheets in yellow, the metal ion in green and vanadate in purple.
B and C. Superimposed core region of both structures (CGDEase coloured as in A; AcpA in cyan), seen from (B) the 'top' or (C) the 'right-hand side', as compared with the 'front view'.

one of them so far crystallized is *F. tularensis* AcpA, of which 10 active-centre residues have been identified (Felts *et al*., 2006). It belongs to the second group of non-PLC proteins, with the phosphoesterase domain displaying the higher conservation degree (Table 3). A direct BlastP comparison revealed a 23% of identity between the phosphoesterase domains of CGDEase (amino acids 23–436) and AcpA (amino acids 55–471). Using AcpA as template (2D1G, a dimer), a partial theoretical structure was obtained for CGDEase covering aa 15–431 (out of 694), i.e. the phosphoesterase domain. Figure 6 shows that both proteins contain near-superimposable cores formed by a highly twisted eight-stranded β-sheet (Fig. 6A, and β1–β3 and β8–β12 in Fig. S3), flanked by three α-helices on either side (Fig. 6A, and αE–αJ in Fig. S3).

The multiple alignment identified five active-site residues of AcpA identically conserved in all the proteins, including CGDEase (E32, N33, H93, H304 and D341), two active-site replacements by conservative substitutions (CGDEase T151S, and E342D), and two identically conserved salt-bridging residues (D348 and R401) (Fig. S3). The tridimensional structures of CGDEase and AcpA indicate that both proteins, not only fold with a similar general architecture, but display also a spatial conservation of residues that participate in the reaction mechanism of AcpA (Fig. 6). The roles of these residues are discussed elsewhere (Felts *et al*., 2006; Vasil *et al*., 2009).

In partial confirmation that the theoretical model derived by homology to AcpA can be biologically relevant, we prepared two CGDEase mutants (T151A and H304A) that changed conserved residues known to be involved in the AcpA active site. According to the model, T151 is part of the octahedral metal centre and may participate directly in the catalysis by providing an essential hydroxyl nucleophile. In AcpA, mutation of the equivalent amino acid to alanine (S196 in Fig. S3) completely abolishes enzyme activity (Felts *et al*., 2006). In PlcH, a similar mutation (T178A; see Fig. S3) results in a protein that retains only ~3% of activity (Vasil *et al*., 2009). Similarly, in CGDEase, our T151A mutant turned out to be essentially inactive as it displayed only traces of activity on ethanolamine-containing substrates (< 0.3% of wild-type CGDEase on a per-mg basis). As far as we know, no other residues had been previously mutated in PLC/APase proteins. The CGDEase H304A mutant could represent an additional support for the model, as amino acids in this position (H371 in AcpA; Fig. S3) are not part of the metal centre, but interact with oxygen atom(s) of a substrate phosphate group (vanadate in the crystallized complex) (Felts *et al*., 2006). We found that this CGDEase H304A mutant was also essentially inactive, displaying only traces of activity on ethanolamine-containing substrates.

Irregular phylogenetic distribution of CGDEase relatives among bacteria

BlastP searches run to help in the structural classification of CGDEase (see above) pointed clearly to irregularities

Table 4. Completed bacterial genomes with CGDEase relatives.[a]

Group (according to NCBI databases)	Completed genomes with hit(s)/all	TBlastN score of best hit median (min–max)
Proteobacteria, gamma (total)	23/218	636 (285–1095)
Pseudomonadaceae	5/18	638 (636–1095)
Enterobacteriales	1/85	1090 (–)
Xanthomonadacea	10/14	491 (285–516)
Pasteurellaceae	0/14	–
Vibrinaceae	0/12	–
Others	7/75	792 (502–795)
Proteobacteria, beta (total)	34/62	751 (437–820)
Burkholderiaceae	27/29	770 (479–820)
Bordetella	4/5	440 (437–751)
Neisseriacea	1/8	473 (–)
Others	2/20	685 (619–751)
Proteobacteria, alpha	9/104	551 (197–688)
Proteobacteria, others	0/52	–
Actinobacteria	20/61	265 (249–471)
Bacteroidetes/Chlorobi	0/26	–
Chlamydiae	0/13	–
Cyanobacteria	0/36	–
Firmicutes	0/165	–
Mollicutes	0/23	–
Spirochaetales	0/33	–
Others	2/44	231 (226–236)

a. TBlastN search was done against the NCBI genome database (837 completed genomes, on May 14, 2009), using CGDEase as query. Bacteria were grouped as in the NCBI databases. A hit was defined as a CGDEase relative with a score > 150 and an *E*-value < 1e-30. Only the best hit of each genome was considered in the right-hand side column.

in the distribution of its close relatives. To respond as firmly as possible to this question, a TBlastN search was run in the NCBI site against 837 complete bacterial genomes. This search uncovered an irregular phylogenetic pattern (Table 4). Significant hits were found only in 88 cases, in higher proportion within β-Proteobacteria (e.g. *Burkholderia* spp.) and Actinobacteria (e.g. some *Mycobacterium* spp.) than in any other group. As expected, the highest TBlastN scores were found in γ-Proteobacteria (e.g. *Pseudomonas* spp.), but in this group, two aspects should be highlighted.

The closest homologue of *P. fluorescens* Pf-5 and CECT 7229 CGDEase was a *S. proteamaculans* hypothetical protein (see Table 3), the only significant hit in Enterobacteriales, while no relatives were found in the 60 fully sequenced strains of genuses *Escherichia*, *Salmonella*, *Shigella* or *Yersinia* (Table 4).

Among Pseudomonadacea, CGDEase close relatives were found only in *P. fluorescens* Pf-5 and in the four fully sequenced *P. aeruginosa* strains. Concerning *P. fluorescens* genomes, with the aid of gMap (http://www.ncbi.nlm.nih.gov/sutils/gmap.cgi; the NCBI tool for genomic comparison that allows the definition of similarity segments between genomes), we found the gene encoding the orthologue of CGDEase (locus tag PFL_3126) in the Pf-5 genome is part of a 19 kb segment (containing loci PFL_3126 through PFL_3141) absent from the genomes of Pf0-1 and SBW25, and flanked by synteny blocks shared by the three *P. fluorescens* strains. BlastN searches revealed that the major part of this 19 kb segment (all of it except a CGDEase relative and a few other genes) is syntenically conserved in *P. putida* and *P. entomophila* genomes, and essentially absent from the rest of *Pseudomonas* spp. genomes sequenced. Concerning *P. aeruginosa* strains, two CGDEase relatives were seen in each case, corresponding to *plcH* and *plcN* (e.g. in *P. aeruginosa* PAO1, loci PA0844 and PA3319 respectively), although in genomic contexts totally different to *P. fluorescens* Pf-5. In conclusion, in *Pseudomonas* spp., CGDEase homologues, if present, are not located in specific genetic environments.

In a similar way, genes that code for the closest structural relatives of CGDEase, i.e. *P. fluorescens* Pf-5 phospholipase C (locus tag PFL_3126), *S. proteamaculans* 568 phospholipase C (locus Spro_4198) and *Burkholderia* spp. proteins such as *B. pseudomallei* K96243 Plc-3 (locus tag BPSS0067; chromosome 2), are all present in completely different genetic contexts. This lack of relation between CGDEase homologues and their surrounding genes was further stressed by operon predictions, done at both MicrobesOnline (http://www.microbesonline.org) (Dehal *et al*., 2010) and DOOR (http://csbl1.bmb.uga.edu/OperonDB/DOOR.php) (Mao *et al*., 2009) sites, which showed that none of these genes seems to be part of an operon. The same is true for *P. aeruginosa plcN*, but not for *plcH*, which is part of a two-gene, well-known operon (Ostroff *et al*., 1989; Stonehouse *et al*., 2002; Liffourrena *et al*., 2007).

The irregular distribution of CGDEase relatives within *Pseudomonas* spp. can be related to the high degree of ecological, metabolic and genomic diversity of the genus. Actually, only ~45% of the proteome (2468 genes) are shared by strains representing four different species (*P. aeruginosa* PAO1, *P. fluorescens* Pf-5, *P. syringae* DC3000 and *P. putida* KT2440) and, even at the species level, only 3668 genes (less then 65% of the proteome) are conserved among *P. fluorescens* strains Pf-5, Pf0-1 and SBW25 (Mavrodi *et al*., 2007).

Enzymatic significance of CGDEase

In our opinion, CGDEase represents a novel kind of enzyme. The results of Tables 1 and 2 reveal CGDEase as a three-faceted enzyme that acts as NDP-X pyrophosphatase, glycerophosphodiesterase and short-chain phospholipase C, all contained in a single protein and possibly in a single active site. In every case, the hydrolytic reaction generates a phosphoaminoalcohol as product, a PLC-type splitting pattern in terms of phospholipidic substrates,

and displays a seldom seen ethanolamine-preferring specificity.

Among NDP-X hydrolases, and particularly among CDP-X hydrolases (Xu *et al*., 2004; Tirrell *et al*., 2006; Alves-Pereira *et al*., 2008; Canales *et al*., 2008), CGDEase is unique in that it shows a strong preference for CDP-ethanolamine, which was ninefold more efficient as a substrate than CDP-choline, the only other NDP-X hydrolysed. From the structural point of view, it is also interesting that CGDEase, which belongs to the PLC branch of the PLC/APase superfamily, is the first NDP-X (X = sugar or alcohol) hydrolase with a restricted specificity pattern that does not belong to the Nudix or the metallophosphoesterase superfamilies.

Among glycerophosphodiesterases (Larson and van Loo-Bhattacharya, 1988; Yanaka, 2007; Corda *et al*., 2009), CGDEase is unique in that it hydrolyses on the glycerol side of the phosphodiester and in that it shows a strong preference for glycerophosphoethanolamine over glycerophosphocholine (a much weaker substrate) and glycerophosphoinositol (not a substrate).

Among PLCs (Titball, 1993; Songer, 1997; Stonehouse *et al*., 2002), CGDEase is almost unique in its strong preference for ethanolamine versus choline phospholipids, albeit this refers only to dihexanoylglycerophospholipids, not to long-chain phospholipids on which CGDEase is not active. The only reports of PLCs displaying some preference for ethanolamine versus choline phospholipids refer also to enzymes purified from *P. fluorescens* isolates (Doi and Nojima, 1971; Crevel *et al*., 1994). These enzymes have not been molecularly identified, but they differ from CGDEase in that they require metals and are active on long-chain substrates. In addition, the sequence of the 11 N-terminal amino acids of one of them (Crevel *et al*., 1994) points to a relationship with PLCs cloned from *P. fluorescens* isolates (Preuss *et al*., 2001; Rossignol *et al*., 2008) and to a hypothetical 'Phosphatidylcholine hydrolyzing PLC' from the Pf-5 strain (GenBank Accession No. YP_258019), all of which are clearly different to CGDEase (see Table 3). To our knowledge, this is also the first report of PLC enzymes active on nucleotidic substrates. In this concern, we would like to mention that *B. cereus* phospholipase C (used here as a positive control for activity on our long-chain phospholipid preparations; see above) was tested and found inactive on CDP-choline or CDP-ethanolamine (< 0.1% of its activity on choline phospholipids). Anyhow, one should not rule out the possibility that other PLCs have NDP-X hydrolase activity (see below). For instance, there is at least one known case of a mammalian lysophospholipase D, named NPP2 or autotaxin, which belongs to a group of wide-specificity nucleotide pyrophosphatases (Stefan *et al*., 2005; Yuelling and Fuss, 2008).

From the structural point of view, as stated above, CGDEase belongs to the PLC/APase superfamily (Table 3). However, none of its closest structural relatives has been enzymically studied. This is the case of *P. fluorescens* Pf-5 phospholipase C, *S. proteamaculans* phospholipase C or *B. pseudomallei* Plc-3 (Table 3). Therefore, it remains to be seen whether these proteins are authentic CGDEases too, authentic PLCs or both. On the other hand, PLC/APase proteins that have been characterized as active PLCs, i.e. *B. pseudomallei* Plc-1 and Plc-2 (Korbsrisate *et al*., 2007), *M. tuberculosis* Plc A-D (Raynaud *et al*., 2002) and *P. aeruginosa* PlcH and PlcN (Ostroff *et al*., 1990; Vasil, 2006), have not been probably tested for activity on all CGDEase substrates. As a preliminary approach to address this question, we obtained PlcH, the haemolytic phospholipase C from *P. aeruginosa* (Ostroff *et al*., 1990; Vasil, 2006), and assayed it for activity on several substrates at 0.5 mM concentration. In our hands, besides its known activities on the long-chain ('100%' activity) or short-chain (40%) phospholipids of choline (but not of ethanolamine), and on 4-nitrophenylphosphocholine (46%), PlcH showed lower activities on CDP-choline (23%) and glycerophosphocholine (4%). Ethanolamine phospholipids, CDP-ethanolamine or glycerophosphoethanolamine were hydrolysed only at marginal or even null rates (< 2%).

The comparison between CGDEase and PlcH suggests that PLC enzymes from the PLC/APase superfamily are active on a range of phosphodiester substrates with a phosphoaminoalcohol moiety. Within this range, two specificity variables are envisaged. On the one hand, some enzymes (e.g. CGDEase) may show preference for small, hydrophilic compounds, such as CDP-alcohol and glycerophosphoalcohol (i.e. deacylated-phospholipids), while others (e.g. PlcH or PlcN) show preference for substrates with a highly hydrophobic moiety, such as authentic phospholipids (and also related lipids, as plasmalogens and sphingomyelin, in the case of PlcH; Stonehouse *et al*., 2002). On the other hand, the enzyme specificities may show an inclination either towards ethanolamine (CGDEase) or towards choline (PlcH, PlcN) substrates. More structural and enzymatic work is needed to test this hypothesis and to shed light on the relationships between these enzymes and the members of the other PLC/APase superfamily branches.

CGDEase induction by osmoprotectants, repression by phosphate, and their relationships to the effect of bacterial population density on CGDEase induction, and to the biological relevance of the enzyme

Several biochemically proven PLC enzymes structurally unrelated to CGDEase have been found produced by *P. fluorescens* bacteria contaminating mammalian-tissue

crude extracts (Crevel *et al*., 1994; Preuss *et al*., 2001). In contrast, CGDEase was first found in our laboratory associated to a rat lung fraction that had been already partially purified by two chromatographic steps. This fraction was contaminated by *P. fluorescens* CECT 7229, and a time- and bacteria-dependent increase of CGDEase activities was observed, perhaps implying that the conditions of the purified fraction were needed for CGDEase to be induced. This included also the observation that a low density of bacterial population was required, with an optimum around 0.01 A_{600} (Fig. S1). This behaviour was essentially reproduced when choline substituted for the lung fraction, although in this case the optimal population density was several fold higher (Fig. 2).

Phosphate availability and osmoprotectants, including choline, glycine betaine and dimethylglycine, are known to regulate the expression of the PLC/APases PlcH and PlcN from *P. aeruginosa*: PlcH is induced by osmoprotective compounds independently of phosphate concentrations, while induction of PlcN requires phosphate-limiting conditions. Choline is believed to act, upon its conversion to glycine betaine (trimethylglycine) and dimethylglycine, through Gbdr transcription factor; dimethylglycine is further catabolized to sarcosine (monomethylglycine) and glycine, which are neither osmoprotectant nor PlcH inducers (Shortridge *et al*., 1992; Sage *et al*., 1997; Wargo *et al*., 2009). In addition, mammalian glycerophosphodiesterases, which to some extent can be considered as enzymatic relatives of CGDEase, are also related to the osmoprotectant role of glycerophosphocholine in the renal medullary cells (Zablocki *et al*., 1991; Gallazzini *et al*., 2008).

As we have already mentioned, once it was clear that CGDEase is a structural relative of *P. aeruginosa* PLCs, we found that the same osmoprotectant set acted as CGDEase inducers at low density (Fig. 1D). In addition, CGDEase was induced by ethanolamine (Fig. 1A), which is not an osmoprotectant in *P. aeruginosa* (Lisa *et al*., 1994) but is a known precursor of choline in eukaryotes (Kewitz and Pleul, 1976; Summers and Weretilnyk, 1993), and perhaps in some bacteria (Nyyssola and Leisola, 2001). To our knowledge, this has not been studied in *P. fluorescens*, and given the ethanolamine effect similar to choline in our experiments (Fig. 1A), it remains to investigate whether or not the effect of ethanolamine is mediated by its conversion to choline and related osmoprotectants.

To further explore the behaviour of *P. fluorescens* cells in suspension, concerning CGDEase induction and its possible biological functions, we have tested several factors, namely haemolytic activity, osmotic stress, phosphate availability, quorum-sensing lactones and cell viability.

Either APase-related or not, PLCs are key virulence factors of several pathogens (Ostroff *et al*., 1989; Smith *et al*., 1995; Raynaud *et al*., 2002; Gründling *et al*., 2003; Flores-Díaz *et al*., 2004; Urbina *et al*., 2009; Vasil *et al*., 2009). *F. tularensis* AcpA, a non-PLC member of the PLC/APase superfamily, is expressed at higher levels in virulent strains compared with the non-virulent vaccine strain (Hernychová *et al*., 2001), although its contribution to pathogenesis has been recently disputed (Child *et al*., 2010). Some PLCs, including the PLC/APase PlcH from *P. aeruginosa* (Ostroff *et al*., 1989) and an unrelated PLC produced by a clinical isolate of *P. fluorescens* (Rossignol *et al*., 2008), are involved in the haemolytic activity of the bacteria. The CGDEase-producing *P. fluorescens* CECT 7229 cells tested positive for haemolytic activity on blood agar plates (results not shown), but due to the lack of long-chain PLC activity of CGDEase it is unlikely that its enzymatic action is involved in haemolysis. Actually, when the enzyme was dropped on the surface of blood agar at the highest concentration available, haemolysis was not observed after a 48 h incubation at 37°C.

Despite the responsiveness of CGDEase to known osmoprotectants, it does not seem that osmotic stress could directly induce CGDEase liberation. In fact, sucrose up to 0.6 M did not induce CGDEase secretion in absence of choline and it had not any effect on the induction by choline (Fig. S4). This is in contrast with the previous observation that, in *P. aeruginosa*, 0.7 M sucrose strongly potentiates the inducer effect of choline on PLC secretion, although it is not clear whether PlcH, PlcN or both were affected (Shortridge *et al*., 1992). The addition of 1 M sucrose to cell suspensions almost abolished CGDEase liberation (Fig. S4), what could be related to an effect of this high sucrose concentration on cell viability. Anyway, it should be stressed that, in order to work in conditions similar to those in which the CGDEase was first seen, our cells were suspended in a very poor medium. Therefore, a detailed study on the effect of osmotic stress in CGDEase induction and on the role of CGDEase under osmotic stress conditions remains to be done. Such a study should include experiments in both poor and rich media, explore other enzyme activities eventually cosecreted with CGDEase, and analyse the behaviour of CGDEase-defective mutants in osmotic stress conditions, all of which was out of the scope of this work.

In contrast, a clear-cut result was obtained with cells suspended in the presence of phosphate, which turned out to be a powerful repressor of enzyme liberation, with an IC50 around 3 μM (Fig. 7). This is in accordance with the data reported for other phosphate-repressible enzymes (Lamarche *et al*., 2008) and indicates that, in this regard, CGDEase ressembles *P. aeruginosa* PlcN, whose induction by osmoprotectants, as stated above, requires phosphate starvation (Shortridge *et al*., 1992). Therefore, CGDEase seems fit to play a role in phosphate scavenging. Actually, periplasmic phosphohydrolases of bacteria, with specificities partly related to CGDEase,

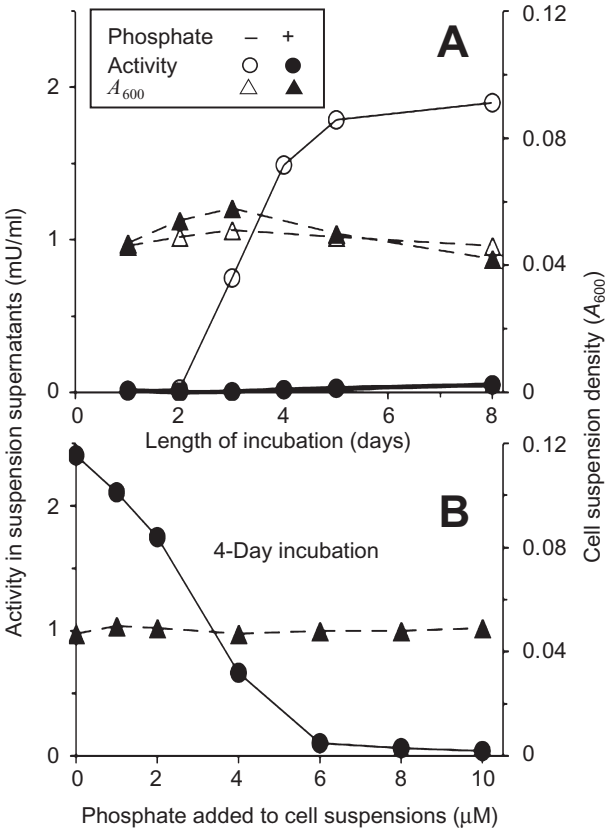

Fig. 7. Effect of inorganic phosphate on the induction of CGDEase. Bacterial suspensions of CECT 7229 cells were prepared as described in *Experimental procedures*, diluted at near 0.04 A_{600} and incubated at 4°C with 1 mM choline. Cell densities were estimated by measurement of A_{600} of vortexed suspensions.
A. Abolition of CGDEase induction by 10 μM phosphate. CGDEase activity was assayed with 1 mM 4-nitrophenylphosphocholine as the substrate.
B. Dose-dependency of the effect of phosphate. CGDEase activity was assayed with 0.5 mM CDP-ethanolamine as the substrate.

have been involved in the use of extracellular phosphorylated compounds as sources of carbon and phosphate. For instance, this is the case of the periplasmic glycerophosphodiesterase encoded by the *glpQ* gene of *E. coli* (Larson *et al.*, 1983; Tommassen *et al.*, 1991) or the 5′-nucleotidase/CDP-alcohol hydrolase/UDP-sugar hydrolase encoded by the *ushA* gene of *E. coli* and other bacteria (Yagil and Beacham, 1975; Burns and Beacham, 1986; Rittmann *et al.*, 2005; Alves-Pereira *et al.*, 2008).

The intriguing observation that CGDEase liberation from *P. fluorescens* was very sensitive to bacterial population density (Figs S1 and 2), prompted us to further investigate this phenomenon. First, we tested whether it could be due to a classical quorum-sensing effect mediated by homoserine lactones, but choline induction of CGDEase at the optimal cell density was not affected by the addition of *N*-butyryl-, *N*-(3-oxooctanoyl)- and *N*-(3-oxododecanoyl)-L-homoserine lactones (Fig. S5), known to be active in *Pseudomonas* spp. including *P. fluorescens* (Liu *et al.*, 2007; Williams *et al.*, 2007). Second, we tested whether it could be related to some kind of CGDEase toxicity towards the producer cells, but *P. fluorescens* viability was not diminished in low-density suspensions by the addition of CGDEase at concentration > 10-fold higher than that one would expect after stimulation with choline (Fig. S6). Third, we observed that, at the higher cell densities, a certain degree of cell lysis occurred during the incubation, estimated as a decrease of A_{600} and viable cell number, what did not happen at lower cell densities (Fig. 2B and C; see also Fig. 1B). Assays of inorganic phosphate indicated that the suspension buffer contained 2–3 μM phosphate as a chemical contaminant, but when the assay was performed on the supernatants of bacterial suspensions, phosphate levels were found to increase with the initial population density (Fig. 2D open triangles), possibly due to the presence of lysed bacteria. At the higher densities studied in this experiment, > 10 μM phosphate was measured, which is enough to fully abolish CGDEase induction (see Fig. 7). Interestingly, low-density bacterial suspensions, where strong CGDEase release occurred, contained initially < 5 μM phosphate, which was consumed during the incubation and decreased well below this value (< 1 μM; Fig. 2D closed triangles) down to levels clearly permissive for induction (Fig. 7). Therefore, although some alternative possibilities have not been tested, like quorum-sensing mediated by signals different from homoserine lactones, it seems that CGDEase repression in the denser cell suspensions was indeed the result of an interesting combination of phenomena: partial cell lysis, phosphate release and CGDEase repression in the remaining viable cells due to phosphate availability.

In the light of this work, CGDEase seems optimized to scavenge for phosphate under strictly deficient conditions, when hydrosoluble phosphoaminoalcohol-containing compounds are available. In this concern, short-chain phospholipids are not expected in the wild, but it is possible that glycerophosphoethanolamine (or choline), and eventually CDP-ethanolamine (or choline), could be found, e.g. in niches where cell debris are present, along with small amounts of either ethanolamine or choline. CGDEase do not render inorganic phosphate directly, but it generates phosphate monoesters that would be subsequently hydrolysed by phosphatases, which would, at the same time, generate the signal (ethanolamine or choline) required to sustain CGDEase expression as long as phosphate remains low.

In summary, although much remains to be studied before biological function(s) of CGDEase can be established, one can envisage potential roles of this novel type of enzyme, most likely not exerted by CGDEase alone but in co-ordination with other proteins. Although a role

related to osmoprotection cannot be discounted, the results support much better a CGDEase role in phosphate scavenging under phosphate-limiting conditions.

Experimental procedures

Bacteria, media and other products for bacterial cultures

The *P. fluorescens* strain used herein was isolated in our laboratory from partially purified rat lung extracts prepared in the cold. *P. fluorescens* colonies were originally isolated from cultures in Columbia agar with 5% sheep blood (COS). They were positively identified with MicroScan Combo Gram Negative 1S panels from Siemens (Madrid, Spain) and ID 32 GN galleries from Biomérieux (Madrid, Spain). The strain is deposited in the *Colección Española de Cultivos Tipo* (Burjassot, Valencia, Spain; http://www.cect.org) with Accession No. CECT 7229. For this work, we used glycerol cultures kept frozen al −80°C in our laboratory. *E. coli* JM109 and BL21 used for molecular biology procedures were purchased from Promega (Madrid, Spain) and Stratagene (Cultek, Madrid, Spain) respectively. Reagents and culture conditions were as previously described (Alves-Pereira *et al*., 2008). *P. aeruginosa* PAO1 was obtained from the *Colección Española de Cultivos Tipo* (CECT 4122).

Chemicals and biochemicals

All the inorganic salts and acids, EDTA and SDS were from Merck. Triton X-100, D609 (O-tricyclo[5.2.1.$0^{2,6}$]dec-9-yl dithiocarbonate potassium salt), *N*-butyryl-DL-homoserine lactone, *N*-(3-oxooctanoyl)-L-homoserine lactone, *N*-(3-oxododecanoyl)-L-homoserine lactone, glycine betaine, dimethylglycine, sarcosine, all the nucleotide compounds and other enzyme substrates tested were from Sigma-Aldrich. BSA for enzyme assay mixtures, glycine and Tris were from Roche. Choline chloride was from Fluka. *B. cereus* phospholipase C (phosphatidylcholine cholinephosphohydrolase) was from Sigma-Aldrich. Auxiliary enzymes were from Sigma-Aldrich (choline kinase and glycerol kinase) or from Roche (alkaline phosphatase, glycerol 3-phosphate dehydrogenase, lactate dehydrogenase and pyruvate kinase). Enzymes used for cloning included EcoRI, SalI and BamHI (Roche), T4 DNA ligase (New England Biolabs) and Advantage cDNA Polymerase Mix (Clontech). Plasmid pGEX-6P-3 was from GE Healthcare. The pGEM-T Easy Vector System was from Promega. The reagent for protein concentration assay (Bradford, 1976) was from Bio-Rad.

Short-chain (water soluble) lipids, 1,2-dihexanoyl-*sn*-glycero-3-phosphoethanolamine and 1,2-dihexanoyl-*sn*-glycero-3-phosphocholine, were from Avanti Polar Lipids. Long-chain lipids, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine, egg yolk 1,2-diacyl-*sn*-glycero-3-phosphoethanolamine, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine and egg yolk 1,2-diacyl-*sn*-glycero-3-phosphocholine, were obtained from Sigma-Aldrich. To prepare mixed micelles, long-chain phospholipids (powder) were vortexed in 20 mM Tris-HCl pH 7.5, 0.5 mM EDTA and 10 mg ml^{-1} Triton X-100, heated at 60°C, and sonicated for 3 min at 30 W. Final concentrations of lipids were about 1 mM, at a 1:16 molar ratio with Triton X-100. All the resulting phospholipid preparations were transparent at 37°C, except 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine that was transparent at 60°C but became translucid at 37°C.

Preparation of bacterial suspensions for studies of CGDEase induction and release

Bacterial cells from glycerol cultures kept at −80°C were seeded on precast 9 cm COS plates (Biomérieux) and grown for 24–36 h at 25°C. A colony was picked, resuspended in 1 ml of water, seeded in 9 cm MacConkey plates and further grown for 24–36 h at 25°C. The cells were scraped from the surfaces of the MacConkey plates (Biomérieux), suspended in a small volume of pre-cooled (4°C) suspension buffer (20 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 5 mM $MgCl_2$, 50 mM KCl, 5 mg ml^{-1} Triton X-100) to assay A_{600}, diluted as needed in the same buffer, and immediately used for experiments with the required additions. Incubations for studies of CGDEase induction and release were performed at 4°C without shaking.

The suspension buffer was the same of the partly purified rat lung fraction (see Fig. S1) in which CGDEase released from contaminant bacteria had been originally found.

Purification of CGDEase from medium conditioned by P. fluorescens *CECT 7229 cells after induction by choline*

Around 250 ml of a bacterial suspension of 0.03–0.05 A_{600} was supplemented with 1 mM choline and kept in the cold room, without shaking, for 8–12 days. Cells were sedimented by centrifugation for 10 min at 7000 *g* (4°C), and the supernatant (conditioned medium) was filtered through a 0.22 μm PES filter, and used for enzyme purification. To this end, it was chromatographed at 0.4 ml min^{-1} in a Q-sepharose column (24 cm by 1.6 cm) equilibrated in 20 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 5 mg ml^{-1} Triton X-100. After application of the sample, the column was washed at 0.4 ml min^{-1} with buffer tMg (20 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 5 mM $MgCl_2$) supplemented with 50 mM KCl, until the A_{280} of the effluent reached a value below 0.1, as an indication of Triton X-100 removal from the column, followed by a 250 ml linear gradient of 50–400 mM KCl in buffer tMg. Fractions of 5 ml were collected and assayed for enzyme activity, and those corresponding to the single peak of activity were pooled and concentrated to about 2 ml by ultrafiltration (PM10, Amicon). This was the CGDEase preparation used for characterization, except as indicated below.

For some experiments performed to explore the requirements of divalent cations, enzyme samples were dialysed for 13 h against 100 vols of 20 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, followed by a second dialysis step for 8 h against 20 mM Tris-HCl, pH 7.5 at 4°C.

For molecular identification of protein bands potentially associated with enzyme activity, an additional purification step was performed on an agarose-MP column (6.3 cm by 0.9 cm; Roche) equilibrated in buffer tMg supplemented with 40 mM KCl, to which a sample of concentrated CGDEase (1 ml), diluted five times in buffer tMg, was applied. The column was washed, at 0.2 ml min^{-1}, with 4 vols of equilibra-

tion buffer and was eluted with a linear 60 ml gradient of 40–400 mM KCl in buffer tMg. The active fractions were pooled, concentrated against solid sucrose, dialysed against electrophoresis sample buffer and immediately submitted to SDS-PAGE. Protein bands stained with Coomassie blue were processed for PMF analysis and for selected peptide subfragmentation at the *Servicio de Proteómica* (Universidad Autónoma, Madrid, Spain).

PCR cloning of CGDEase and of P. aeruginosa *PlcH*

Genomic DNA was isolated from *P. fluorescens* CECT 7229 using the GenElute™ Bacterial Genomic DNA kit according to manufacturer's instructions (Sigma-Aldrich). The ORF of CGDEase was amplified with primers (see Table S1) designed from the sequence that in the genome of *P. fluorescens* Pf-5 encodes hypothetical protein YP_260232, the candidate pinpointed by tandem-mass spectra of the endogenous CGDEase of *P. fluorescens* CECT 7229 (see the previous paragraph and the *Results and discussion* section). A product of the expected size was obtained and cloned by T/A ligation in the pGEM-T Easy vector and transformation of JM109 competent cells. The insert was cut with EcoRI and SalI, and was subcloned into the corresponding sites of pGEX-6P-3 plasmid to obtain pGEX-6P-3-CGDEase. Both strands of the insert were sequenced in the *Servicio de Secuenciación Automática* (Instituto de Investigaciones Biomédicas Alberto Sols, CSIC-UAM, Madrid, Spain). The 2085 nt sequence was deposited in GenBank (Accession No. GU937796).

The ORF encoding the 730-aa precursor of the haemolytic phospholipase C PlcH was cloned from *P. aeruginosa* PAO1 genomic DNA (obtained with the GenElute™ Bacterial Genomic DNA kit), using the primers indicated in Table S1. The amplicon was inserted by T/A ligation in vector pGEM-T Easy. Both chains of the 2193 pb insert of the resulting pGEMTeasy-prePlcH plasmid were sequenced and found equal to GenBank Accession No. AE004091 REGION: 919258.921450 except for the single-nucleotide change C2175T, which does not change the amino acid translation (GenBank Accession No. AAG04233). For expression of the 692-aa mature PlcH as a GST-fusion protein, the first 38 codons of the 2193 pb precursor ORF were removed by a second PCR amplification (see primers in Table S1), followed by insertion in the BamHI site of pGEX-6P-3 to obtain pGEX-6P-3-PlcH plasmid. A clone with the correct orientation of the insert with respect to the plasmid GST ORF was selected by restriction analysis, and its identity confirmed by sequencing of both strands.

Site-directed mutagenesis of CGDEase

Mutated plasmids pGEX-6P-3-T151A-CGDEase and pGEX-6P-3-H304A-CGDEase were constructed from pGEX-6P-3-CGDEase according to the QuikChange® mutagenesis procedure (Stratagene), using the mutagenic oligonucleotides indicated in Table S1 and PfuUltra™ High Fidelity DNA Polymerase (Stratagene) to synthesize mutated chains, and DpnI (New England Biolabs) to degrade the methylated wild-type templates. The correct generation of mutated ORFs in the absence of undesired changes was confirmed by double-strand sequencing.

Expression and purification of recombinant proteins

To express the product of the *P. fluorescens* CECT 7229 amplified gene, BL21 cells transformed with pGEX-6P-3-CGDEase were grown overnight at 37°C in liquid Luria–Bertani (LB) medium with 0.1 mg ml^{-1} ampicillin (amp-LB). Five millilitre of the overnight culture was inoculated in 100 ml of fresh amp-LB and cultured at 27°C until the A_{600} reached 0.8; then, 0.25 mM isopropylthiogalactoside (IPTG; Roche) was added, and incubation was continued for 4 h at 17°C. Finally, cultures were centrifugated 20 min at 8000 *g* (4°C) and the pellet was resuspended in 10 ml of 20 mM Tris-HCl, 0.5 mM EDTA, 5 mM DTT, 500 mM KCl (pH 7.4), and supplemented with 0.1 ml of 20 mg ml^{-1} lysozyme. After incubation for 30 min at room temperature, solid $MgCl_2$ and Triton X-100 were added to a final concentration of 5 mM and 5 mg ml^{-1} respectively. Cells were sonicated on ice at 170 W with a duty-recovery cycle of 0.1 (0.1 s pulse per second) for 5 min (repeated three times at 1 min interval). The suspension was left overnight in the cold room with gentle shaking and then centrifuged at 30 000 r.p.m. for 30 min at 4°C in a Beckman Ti70.1 rotor to collect the supernatant that was frozen until use. When needed, it was thawed, diluted fivefold in buffer tMg and applied to a 3 ml GSH-sepharose column (GE Healthcare), equilibrated in buffer tMg with 1 mg ml^{-1} Triton X-100, to bind the GST/CGDEase fusion protein. The unbound proteins were washed out of the column with 20 ml of 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM DTT, 150 mM NaCl. After this wash, to recover CGDEase separated from the GST tag, 30 µl of PreScission® protease (Amersham) was diluted in 2.5 ml of wash buffer and applied to the column. The flow was then stopped for 12 h and the column was kept at 4°C. After incubation, the product of in-column specific proteolysis was eluted with 9 ml of wash buffer. Fractions of 1.4 ml were collected and analysed for enzyme activity and for protein bands by SDS-PAGE.

T151A- and H304A-CGDEase mutants, and mature PlcH were expressed from plasmids pGEX-6P-3-T151A-CGDEase, pGEX-6P-3-H304A-CGDEase and pGEX-6P-3-PlcH, respectively, using the same protocol described above.

Enzyme activity assays

Unless otherwise indicated, phosphohydrolytic activities were determined at 37°C by discontinuous assay of P_i liberation, either directly from substrates with terminal phosphate (chain) or coupled to alkaline phosphatase for substrates without terminal phosphates. The standard reaction mixtures contained 50 mM Tris-HCl, pH 7.5, 3.5 mM $MgCl_2$, 1 mg ml^{-1} BSA, and amount of enzyme sample within the linearity range, 0.5 mM substrate, and, when needed, 9 units ml^{-1} alkaline phosphatase. Mg^{2+} was not necessary for activity, but was included to standardize the assay, as it was present in the enzyme buffer. In some control experiments with different substrates, to check for possible effects of Triton X-100 on CGDEase activity, the detergent was included at the concentration present when phospholipidic mixed micelles were tested as substrates, i.e. 5 mg ml^{-1} Triton X-100. The incubations were stopped by the addition of one of the P_i reagents indicated below. In some assays with 4-nitrophenylphosphocholine as the sub-

strate, the formation of the yellow product 4-nitrophenol was assayed at 405 nm either continuously ($\varepsilon = 12\,900\ M^{-1}\ cm^{-1}$) or discontinuously after stopping the reaction with 7 vols of 0.2 M NaOH ($\varepsilon = 18\,500\ M^{-1}\ cm^{-1}$). Alcohol dehydrogenase was assayed spectrophotometrically at room temperature, in reaction mixtures containing 50 mM Tris-HCl, pH 8.8, 170 mM ethanol and 1.25 mM NAD; the increase of A_{340} ($\varepsilon = 6220\ M^{-1}\ cm^{-1}$) was recorded.

All the enzyme activity assays were performed under conditions of linearity with time and amount of enzyme. In all cases, blanks without enzyme sample and/or substrate were incubated and assayed in parallel. One unit is defined as the activity transforming 1 μmol of substrate per minute under the conditions of the assay.

Assay of enzyme reaction products

Inorganic phosphate was assayed colorimetrically with a dodecyl sulphate-ascorbate-molybdate reagent prepared in two different versions depending on the sensitivity required (Ribeiro *et al.*, 2001): either 0.7 ml of the standard reagent was added to 100 μl samples or 0.2 ml of the concentrated reagent was added to 0.4 ml samples. 4-Nitrophenol was assayed by measuring A_{405} after stopping the reaction with NaOH.

L-Glycerol 3-phosphate was assayed by spectrophotometric end-point assay with glycerol 3-phosphate dehydrogenase. The assay mixture contained 0.2 M hydrazine/0.5 M glycine buffer, pH 9.5, 2.5 mM EDTA, 2.2 mM NAD and 1.4 units ml^{-1} glycerol 3-phosphate dehydrogenase. Choline and glycerol were assayed by spectrophotometric end-point assays with choline kinase or glycerol kinase, both coupled to pyruvate kinase and lactate dehydrogenase to measure the stoichiometric formation of ADP. Assay mixtures contained 100 mM Tris-HCl, pH 8.8 (pH 7.5 for glycerol assay), 50 mM KCl, 8 mM $MgCl_2$, 1 mM EDTA, 0.175 mM NADH, 2 mM ATP, 1 mM phosphoenolpyruvate, 6.7 units ml^{-1} pyruvate kinase, 9 units ml^{-1} lactate dehydrogenase and to initiate the assay reaction 0.04 units ml^{-1} of choline kinase (0.2 units ml^{-1} of glycerol kinase for glycerol assay). The net change of A_{340} along the reactions was used to calculate the concentration of the product assayed ($\varepsilon = 6220\ M^{-1}\ cm^{-1}$).

Nucleoside and nucleotides were assayed by ion pair reverse-phase high-performance liquid chromatography in a Hypersil-ODS column as described (Alves-Pereira *et al.*, 2008). Chromatograms were recorded at 250 nm. Product peaks were identified by their retention times, compared with authentic standards, and integrated. In every reaction studied, the substrate and the nucleotidic products had equivalent molar extinction coefficients, so the product peak areas were converted to molar amounts using the sum of the areas of substrate and product peaks as an internal standard.

Determination of native M_r *of CGDEase by sucrose gradient centrifugation*

For M_r determination, CGDEase from the Q-sepharose step was sedimented along with M_r markers by ultracentrifugation in linear, 50–200 mg ml^{-1} sucrose gradients of 11 ml, prepared in 50 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 50 mM KCl, either supplemented or not with 5 mM $MgCl_2$. Samples of 0.4 ml, supplemented with BSA (M_r 66 000; Sigma-Aldrich Cat. No. A8531) and alcohol dehydrogenase (M_r 150 000; Sigma-Aldrich Cat. No. A8656), were applied to the top of the gradient and centrifugation was run at 38 000 r.p.m. (180 000 *g*), for 20 h at 4°C, in a Beckman SW-41Ti rotor. After centrifugation, the gradients were fractionated starting from the bottom and collecting 0.4 ml fractions. The sedimentation profile of alcohol dehydrogenase was determined by its enzyme activity and that of the monomer of BSA, which was present in excess over any other protein, according to Bradford (1976). The M_r of CGDEase (assayed by its activity on CDP-ethanolamine; see above) was calculated according to Martin and Ames (1961).

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