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Cholinesterase, Acid Phosphatase, and Phospholipase C of *Pseudomonas aeruginosa* Under Hyperosmotic Conditions in a High-Phosphate Medium

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Abstract. The presence of low choline or betaine concentrations in a culture medium containing succinate, NH_4Cl, and inorganic phosphate (Pi) as the carbon, nitrogen, and phosphate sources, respectively, permits the growth of *Pseudomonas aeruginosa* in a hyperosmolar medium. Dimethylglycine, acetylcholine, and phosphorylcholine were less effective as osmoprotectants than choline or betaine. Other alkylammonium compounds tested were virtually ineffective in this capacity. Bacterial growth was also observed in a hyperosmolar medium when choline was the sole carbon and nitrogen source. Choline could act as an osmoprotectant under all the conditions tested. However, the production of cholinesterase (ChE), acid phosphatase (Ac.Pase) and phospholipase C (PLC) took place only when choline was the carbon and nitrogen source. This fact confirms that the synthesis of PLC may occur even in the presence of a high Pi concentration in the medium. Inasmuch as in a high-P_i medium the synthesis of PLC and Ac.Pase (phosphorylcholine phosphatase) is dependent only on choline metabolism, it is postulated that both enzymes are involved in a set of reactions coordinated to produce the breakdown of the membrane phospholipids of the host cell in a hyperosmotic medium.

In previous papers [7, 17, 18, 21] we reported that *Pseudomonas aeruginosa*, grown on a medium containing choline, was capable of producing ChE [7, 17], Ac.Pase [17, 18, 21], and PLC [21]. Earlier results related to the production of ChE and Ac.Pase were obtained when the bacteria were grown in a basal salt medium containing a high Pi concentration (HP_i-BSM). The above occurred in the presence of choline or its metabolic derivatives—betaine, dimethylglycine, and sarcosine—as the carbon source [17]. The nonmethylated choline derivatives, ethanolamine or glycine, were poor substrates in reference to the support of bacterial growth and the increased production of both enzymes. Methylaminoethanol and dimethylaminoethanol were not utilized by *P. aeruginosa* as the carbon source [17]. The above series of choline derivatives was utilized in our laboratory to observe their individual effects upon the kinetic properties of the Ac.Pase [11] and ChE [10]; the choline uptake by *P. aeruginosa* [23]; and the increase of Ac.Pase in *Rhizobium meliloti* [22], which proved to be different from the *P. aeruginosa* enzyme.

Our studies on the properties of *P. aeruginosa* ChE and Ac.Pase led us to postulate that the first may be considered an acetylcholinesterase [8, 10] and the second, a phosphorylcholine phosphatase [12, 22]. These results led to the discovery that, when *P. aeruginosa* utilized choline or betaine as the sole carbon and nitrogen source in HP_i-BSM, a PLC activity was produced in addition to the Ac.Pase, but not an alkaline phosphatase activity [20, 21].

Considering that PLC and phosphorylcholine phosphatase were produced in the presence of a high Pi concentration, the role postulated for PLC and alkaline phosphatase as phosphate scavenging mechanisms [19] could not be adopted. Therefore, it was proposed that through the coordinated action of these two enzymes the bacteria may break down the choline-containing phospholipids of the host cell

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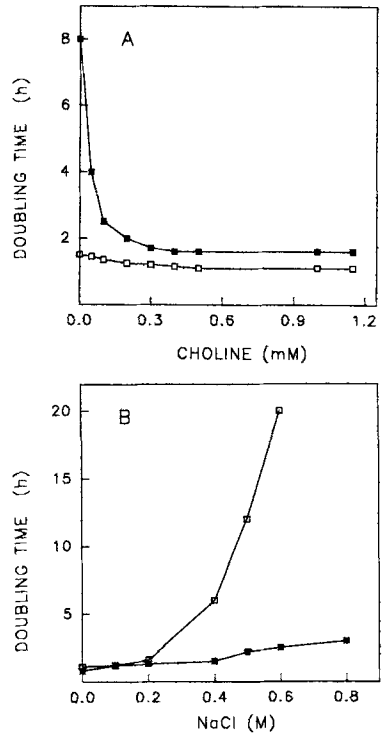

Fig. 1. Effect of choline (A) and NaCl (B) concentrations on the growth rate of *Pseudomonas aeruginosa*. (A) cultures were grown in HP_i-BSM containing 20 mM succinate, 187 mM NH_4Cl, and choline as indicated. The growth rate (doubling time in h) is plotted against the choline concentration in the presence (■) or the absence (□) of 0.45 M NaCl. (B) The doubling time in h was calculated from growth curves generated at 37°C in HP_i-BSM containing 20 mM succinate, 18.7 mM NH_4Cl, supplemented (■) or not (□) with 1 mM choline and the NaCl concentrations indicated. In the absence of choline the lag period increased from about 1 h at 0.2 M NaCl to 7 h at 0.6 M NaCl; at 0.8 M NaCl there was no growth after 72 h. With the addition of choline, the lag period was about 3 or 4 h at 0.6 M NaCl and 0.8 M NaCl respectively. Values indicated are averages from three independent experiments.

[12, 20, 21]. In addition, we postulated that the *P. aeruginosa* ChE, in conjunction with PLC and phosphorylcholine phosphatase, could participate in the corneal infections produced by this bacterium [8].

Our findings related to the production of PLC by *P. aeruginosa* in high P_i medium containing choline and betaine were recently confirmed by other authors. In addition, they demonstrated the osmoprotective effect of choline and PLC induction in a low P_i medium [24].

We have extended those observations in the studies presented here, using the osmolyte NaCl in HP_i-BSM. Results described below show that choline or betaine, and to a lesser extent acetylcholine, phosphorylcholine, or dimethylglycine, conferred osmoprotection upon the cells. When *P. aeruginosa* utilized choline as the sole carbon and nitrogen source in a hyperosmotic medium and in the presence of a high P_i concentration, it was capable of producing ChE, Ac.Pase, and PLC. Hence, the proposed role of these enzymes in infection [8, 21] may also be applicable in a hyperosmotic medium, even in the presence of a high P_i concentration.

Materials and Methods

Organism and growth conditions. *Pseudomonas aeruginosa* (NCTC, Fildes III, 1924 U.K.) was grown aerobically at 37°C in a high P_i medium (HP_i-BSM) described in [21]. The experiments were performed under two different culture conditions: (i) The HP_i-BSM contained 20 mM succinate and 18.7 mM or 187 mM NH_4Cl. In these media the following three series of compounds were tested as osmoprotectants: (a) choline, dimethylethanolamine, methylethanolamine, and ethanolamine; (b) betaine, dimethylglycine, sarcosine, and glycine; (c) acetylcholine, phosphorylcholine, and chlorocholine (a choline derivative containing chlorine instead of the hydroxyl group); (d) trimethylamine, tetra-methylammonium, tetraethylammonium, and trimethylphenylamine. (ii) The HP_i-BSM contained 20 mM choline as the sole carbon and nitrogen source. Hyperosmolarity in both medium was obtained by the addition of NaCl as described in the figure legends. Growth was monitored by the absorbance of the culture at 660 nm.

Enzyme preparation and assays. ChE and Ac.Pase activities were measured in washed whole bacteria with acetylthiocholine or the sodium salt of *p*-nitrophenylphosphate respectively [8, 12]. One unit of ChE activity is defined as the amount of enzyme that catalyzes the formation of 1 nmol of thiocholine/min at 37°C. One unit of Ac.Pase represents the formation of 1 nmol of *p*-nitrophenol/min at 37°C. The PLC activity was measured in culture supernatants as in [3, 16], in 100 mM Tris-HCl, pH 7.4, with 36% glycerol and 20 mM of the artificial substrate *p*-nitrophenylphosphorylcholine. One unit of PLC is defined as the amount of enzyme releasing one nmol of *p*-nitrophenol from *p*-nitrophenylphosphorylcholine per min at 37°C.

Results

Osmoprotection by choline and some of its derivatives independent of the production of cholinesterase, acid phosphatase, or PLC in HP_i-BSM. Before starting the osmoprotection studies, we determined that little or no growth of *P. aeruginosa* occurred after the addition of 0.6 M or 0.8 M NaCl to HP_i-BSM containing succinate and NH_4Cl (see below, Fig. 1). Bacterial growth in the presence of 0.6 M NaCl was monitored for 48 h. The absorbance values of the culture measured at 12, 24, 36, and 48 h were 0.13, 0.22, 0.66, and 0.74 respectively. These values were about 50% less than those observed in the control curve, which attained the stationary phase before the first 12 h of culture. Therefore, in

Table 1. Effect of choline derivatives and other alkylammonium compounds on the growth rate of *Pseudomonas aeruginosa* in hyperosmolar media containing a high P_i concentration and preferential carbon and nitrogen sources[a]

Effector[b]	G_+/G_- ± SD[c]	DT (hours)[d]
None	[e]	9.0
Choline	10.0 ± 0.4	1.7
Dimethylethanolamine	2.2 ± 0.2	9.0
Methylethanolamine	1.2 ± 0.1	9.0
Ethanolamine	0.9 ± 0.2	9.0
Glycine-betaine	10.0 ± 0.4	1.7
Dimethylglycine	5.1 ± 0.2	4.7
Sarcosine	2.3 ± 0.2	8.5
Glycine	1.0 ± 0.1	9.0
Acetylcholine	6.5 ± 0.3	3.7
Phosphorylcholine	8.1 ± 0.4	3.6
Chlorocholine	1.2 ± 0.1	9.0
Alkylammonium compounds[f]	1.0 ± 0.1	9.0

[a] The cells were cultured at 37°C to stationary phase.
[b] Added at the beginning of the experiment.
[c] G_+/G_- is the relation of absorbance of the culture after 12 h of growth in the absence (G_-) or in the presence (G_+) of 1 mM of each compound listed, in HP_i-BSM containing 20 mM succinate and 18.7 mM NH_4Cl supplemented with 0.6 M NaCl.
[d] The doubling times (DT) were calculated from growth curves in the absence or in the presence of 1 mM of each alkylammonium compound, in HP_i-BSM containing 20 mM succinate and 187 mM NH_4Cl supplemented with 0.45 M NaCl.
[e] The absorbance of the control culture at 660 nm was 0.120 ± 0.013.
[f] The compounds tested were trimethylammonium, tetramethylammonium, tetraethylammonium, and trimethylphenylamine. Values are averages from three independent experiments.

order to determine which compounds might act as osmoprotectants, 0.6 M NaCl plus 1 mM of the choline derivatives or other alkylammonium compounds were added to an HP_i-BSM containing succinate and NH_4Cl.

Choline, betaine, dimethylglycine, acetylcholine, and phosphorylcholine all allowed significant growth of *P. aeruginosa* in the presence of 0.6 M NaCl (Table 1). After monitoring the bacterial growth up to 48 h, we found that most of the remaining compounds proved virtually ineffective as osmoprotectants. These results were confirmed with the same medium, but varying proportionally the NaCl and NH_4Cl concentrations to obtain a similar ionic strength (data not shown). Thus, the NaCl concentration was decreased from 0.6 M to 0.45 M and that of NH_4Cl was increased from 18.7 mM to 187 mM. Table 1 shows the doubling time obtained for the growth of *P. aeruginosa* under the former condition, in the presence or the absence of the same compounds. These values indicate that choline and betaine were the best osmoprotectants. Dimethylglycine and the substrates for the ChE and Ac.Pase, acetylcholine and phosphorylcholine respectively, were less effective than choline or betaine as osmoprotectants. The other alkylammonium compounds tested did not confer any degree of osmoprotection.

Considering these results, choline and betaine were selected to determine the minimum concentration necessary for their action as osmoprotectants and their effects on the doubling time when the bacteria were grown in the presence of increased NaCl concentrations (Fig. 1A and B). Figure 1A shows that in the presence of 0.45 M NaCl and 187 mM NH_4Cl, the addition of choline at concentrations greater than 0.2 mM permitted bacterial growth with a doubling time of around 1.8 h. Similar results were obtained when the NH_4Cl concentration was decreased from 187 mM to 18.7 mM or when betaine was utilized in place of choline (not shown). Stimulation of the growth rate of *P. aeruginosa* by choline in the presence of increasing NaCl concentrations is shown in Fig. 1B. A progressive increase in the salt concentration adversely affected the lag period and the doubling time, both in the presence and in the absence of choline. However, and especially in the case of NaCl concentrations greater than 0.4 M, the doubling time was almost restored to control levels in the choline-supplemented medium. As stated previously, there was no bacterial growth with the addition of 0.8 M NaCl. Growth did occur, however, when 1 mM choline was added to the culture medium. The addition of betaine produced similar effects (not shown). The differences in the doubling times observed in Fig. 1A and B, either for the control or for a similar NaCl concentration, may be attributed to the varying NH_4Cl concentrations utilized in each experiment.

Under all the above conditions there was no production of ChE, Ac.Pase, or PLC. Similar results were obtained when the choline concentration was increased from 1 mM to 20 mM (not shown).

Osmoprotection by choline and production of cholinesterase, acid phosphatase, or PLC in HP_i-BSM. The stimulation of the growth rate and the production of these enzymes occurred when choline was added to HP_i-BSM instead of succinate. Table 2 shows the levels of activity for each enzyme when choline was utilized as carbon and nitrogen source. Under such conditions, the bacteria produced ChE,

Table 2. Cholinesterase, acid phosphatase, and phospholipase C activities of *Pseudomonas aeruginosa* grown on choline as the sole carbon and nitrogen source in the presence of different NaCl concentrations

		U/ml ± SD		
NaCl(M)[a]	Growth[b]	ChE[c]	Ac.Pase[c]	PLC[d]
None	1.0 ± 0.1	62.5 ± 16.4	37.3 ± 2.1	173 ± 38.2
0.2	1.1 ± 0.1	49.9 ± 11.6	22.4 ± 3.7	139 ± 33.6
0.4	1.1 ± 0.2	29.2 ± 10.5	20.1 ± 1.7	66 ± 6.2
0.6	1.1 ± 0.3	19.1 ± 13.2	21.6 ± 3.7	27 ± 16.9
0.8	0.8 ± 0.2	19.0 ± 12.3	23.2 ± 3.8	15 ± 5.1

[a] The cells were cultured at 37°C to stationary phase in HP_i-BSM containing 20 mM choline supplemented with NaCl at concentrations indicated in the first column.
[b] Growth is expressed as A_{660nm} ± SD.
[c] Cholinesterase and acid phosphatase were assayed in whole cells.
[d] Phospholipase C was assayed in supernatants.
Values are averages from at least three independent experiments.

Ac.Pase, and PLC, even at 0.8 M NaCl. The increase of osmolarity decreased, but did not inhibit totally, the production of ChE, Ac.Pase, and PLC. It should be noted that increasing concentrations of the osmolyte had no effect on the assay for PLC; the PLC activity in iso-osmotic supernatants was reduced by only 16% when assayed in the presence of 0.8 M NaCl. The effect of high salt on the assays for ChE and Ac.Pase activities was not considered, because the assays were done on washed whole cells suspended in isotonic medium.

Discussion

The osmoprotection studies in *P. aeruginosa* were performed in a basal salt medium containing a high P_i concentration (39 mM). This medium was selected because of our previous observations on the production of ChE, Ac.Pase, and PLC by *P. aeruginosa* [7, 17, 18, 21]. Briefly, the simultaneous presence of choline or betaine and the preferred carbon and nitrogen sources, succinate and NH_4Cl, did not permit production of ChE, Ac.Pase, and PLC. It did occur, however, when choline or betaine were utilized as the carbon or carbon and nitrogen sources. With these data in mind, the experiments were designed to observe the osmoprotective effect of the choline derivatives apart from that on enzyme production.

Considering the effect of the choline derivatives and other alkylammonium compounds, it may be concluded that the N-quaternary or N-tertiary moiety was not the only requisite for their action as osmoprotectants. The best example of this situation is, perhaps, the comparison between choline and chlorocholine. The former proved to be one of the best osmoprotective agents, whereas the latter was ineffective (Table 1). The quaternary ammonium moiety acquired importance when it was present as an integral part of a compound utilized by the bacteria. This was supported by the following results: first, choline, betaine, and to a lesser extent acetylcholine and phosphorylcholine, were better osmoprotectants than dimethylglycine. Second, in the series containing glycine, sarcosine, dimethylglycine, and betaine, the osmoprotective effect increased with the number of N-methyl groups.

Considering the results obtained with choline or betaine, it may be concluded that the response of *P. aeruginosa* to a hyperosmolar medium is similar to that described for many other bacteria [1, 4–6, 15, 25]. Accordingly, in the absence of choline or betaine, growth was inhibited or notably diminished by high NaCl concentrations. In other words, the presence of low choline or betaine concentrations in a culture medium containing preferred carbon and nitrogen sources permits the growth of *P. aeruginosa* in a hyperosmolar medium. Under these conditions the choline uptake is functionally active, but the bacteria are not necessarily compelled to utilize choline as either the carbon or nitrogen source. Consequently, ChE, Ac.Pase, and PLC are not produced. The synthesis of these enzymes occurred, however, under any of the culture conditions with high or low salt concentrations in which choline must be metabolized to provide the cells with the necessary carbon or carbon and nitrogen.

The present data, along with our previous findings [8, 9, 12, 20, 21], may be extrapolated to an in vivo situation in which a high P_i concentration in a hyperosmotic medium is the initial condition. Under conditions of hyperosmolarity, choline, as the carbon or carbon and nitrogen source or added in the presence of the preferred carbon source, may be taken in by the bacteria. This uptake may involve one or both components of the transport system which have Km values near 3 μM and 400 μM [23]. *Pseudomonas aeruginosa* may subsequently metabolize choline, forming one or more osmoprotectant metabolites that eventually attain a concentration permitting growth.

If choline acted only as an osmoprotectant, the increase in the PLC, Ac.Pase, and ChE activities would not occur. In this case, the pathogenicity of *P. aeruginosa* may not be explained by the sole or combined action of each of these enzymes and suggests that lung infection caused by *P. aeruginosa* probably involves the action of other pathogenic factors [27].

It is possible, however, that *P. aeruginosa* encounters a complex environment containing a low P_i concentration. Under such conditions, PLC and alkaline phosphatase are produced [2, 13, 14, 21, 26 and references cited therein]. In this case, the bacteria may use both enzymes as a P_i-scavenging mechanism [19]. The adaptation of this mechanism to an infectious process implies that the membrane phospholipid is degraded under the coordinated action of PLC and alkaline phosphatase. Apparently, this model may be extended to a situation of hyperosmolarity, because PLC was produced when a low-P_i peptone medium containing NaCl as the osmolyte was supplemented with choline [24].

It is possible, however, to opt for a second mechanism, previously postulated in [8, 9, 12, 20, 21], on condition that choline is the carbon or carbon and nitrogen source and the high osmolarity is produced by NaCl. In this case, the synthesis of PLC, Ac.Pase (phosphorylcholine phosphatase [12, 22]), and ChE would be independent of the P_i concentration in the medium and dependent only upon the choline metabolism of the bacteria. Also, in this case, each enzyme acting either separately or in a set of coordinated reactions may break down compounds containing the choline moiety to give high levels of free choline in a hyperosmotic medium.

The mechanism proposed above, involving the osmoprotective effect of choline plus the action of PLC against phosphatidylcholine and Ac.Pase against phosphorylcholine, may be useful in the explanation of the chronic lung infections in patients with cystic fibrosis. In addition, it could apply to urinary tract infections in which *P. aeruginosa* may encounter a hyperosmotic environment, accompanied by high P_i and low choline concentrations.

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