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Secretion of alkaline phosphatase and phsopholipase C in *Pseudomonas aeruginosa* is specific and does not involve an increase in outer membrane permeability

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1. INTRODUCTION

Under conditions of inorganic phosphate limitation, *Pseudomonas aeruginosa* synthesizes an inducible alkaline phosphatase [1] and phospholipase C [2,3]. Both enzymes are released into the medium during growth [3,4], with phospholipase C existing mostly in the medium [3], whereas the distribution of alkaline phosphatase between the cell and the medium is variable depending upon culture conditions [5].

Although the mechanism of secretion of these enzymes is as yet unknown, two possibilities exist to account for their extracellular presence. One possibility is that their release is facilitated by a breakdown in the outer membrane permeability barrier, freeing them from a periplasmic location. Alkaline phosphatase is a periplasmic marker in many Gram-negative bacteria and in *Pseudomonas aeruginosa* some alkaline phosphatase activity is always detectable in the periplasm [as Tris(hydroxymethyl) aminomethane (Tris)$MgCl_2$-released enzyme] [5]. A breakdown in the outer membrane would thus be one mechanism to account for the release of these enzymes. The second possibility is that a mechanism of specific secretion across the outer membrane is responsible for their release, in the absence of any gross permeability changes.

In the present study we examined (a) outer membrane permeability as a function of alkaline phosphatase and phosphalipase C secretion (to define any changes in outer membrane permeability which might be associated with their secretion), and (b) the specificity of the release of alkaline phosphatase and phospholipase C, in an attempt to better define the nature of that release. We now report that secretion of the enzymes is indeed specific and does not involve increased outer membrane permeability.

2. MATERIALS AND METHODS

Pseudomonas aeruginosa PAO1 strain H103 [6] containing an RP1 plasmid was grown under the phosphate-deficient conditions described previously [7] using a medium buffered with sodium *N*-2-hydroxymethyl piperazine-*N'*-2-ethane sulfonate (HEPES). Cells were prepared for growth in phosphate-deficient medium by first harvesting by centrifugation overnight cultures grown in phosphate-sufficient medium [7] containing 100 μg/ml tetracycline (to maintain the plasmid), washing the cells twice in phosphate-sufficient medium to remove the tetracycline, and resuspending them in phosphate-sufficient medium at an A_{600} of 0.05. The cells were then grown at 37°C to mid log phase ($A_{600} = 0.60$) and subsequently harvested by centrifugation, washed three times in phosphate-deficient medium and resuspended in

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phosphate-deficient medium at an absorbance at 600 nm of 0.20.

Alkaline phosphate [8], phospholipase C [9] and β-lactamase [10] activities were assayed as described previously using *p*-nitrophenyl phosphate (pNPP), *p*-nitrophenyl phosphorylcholine (NPPC) and nitrocefin [11] as the respective chromogenic substrates. To assay periplasmic fractions, the method of Cheng et al. [5] as modified by Hoshino and Kageyama [12] was used to facilitate the release of periplasmic components. This modification resulted in consistently better release of periplasmic components without observable cell damage or release of cytoplasmic proteins. Intact cell alkaline phosphatase activity was measured using cells which had been centrifuged at $3000 \times g$ for 10 min and resuspended in 50 mM HEPES buffer pH 8.5.

Outer membrane permeability was determined using a modification of the method of Angus et al. [10]. Briefly, intact cell β-lactamase activity was measured on growing cultures by first taking aliquots of cells and dividing them in two. One fraction was taken up into a syringe and slowly squeezed through a millipore filter of 0.22 μm pore size to obtain a culture supernatant, while the other fraction was left unfiltered. Equal volumes of each fraction were transferred to separate cuvettes. The cuvette containing the culture supernatant was placed in the reference beam of a Perkin-Elmer (Norwalk, CT, U.S.A.) Lambda 3 dual beam spectrophotometer. The other cuvette containing intact cells and supernatant was placed in the sample beam. Nitrocefin [11], a chromogenic β-lactam, was added to each cuvette to a final concentration of 0.06 mg/ml and the differential rate of conversion of nitrocefin to nitrocefoic acid was recorded at an absorbance of 540 nm using a coupled Perkin-Elmer model 561 chart recorder. The recorded β-lactamase activity was a direct measure of intact cell activity. Because β-lactamase has been shown to be periplasmic [10,13], the activity of intact cells at a given substrate concentration is limited by the diffusion of the β-lactam, in this case nitrocefin, across the outer membrane rather than by the amount of enzyme. From theory [14], the steady state rate of hydrolysis of β-lactam in intact cells (V_{Int}) equals the rate of β-lactam diffusion across the outer membrane (V_D) and hence provides a measure of outer membrane permeability. Permeability parameters (C) were calculated using the formula $V_{Int} = V_D = C(S_{out} - S_{in})$ according to Zimmermann and Rosselet [14], where C = permeability parameter; S_{out} = concentration of substrate (nitrocefin) outside the cell and S_{in} = concentration of substrate inside the cell (which is $\ll S_{out}$, and thus negligible).

3. RESULTS

When phosphate became limiting for growth as indicated by a decline in the growth rate (Figs. 1A and 2A) both alkaline phosphatase and phospholipase C were induced (Fig. 1B and C) as were the M_r 37000 periplasmic protein and outer membrane protein P described previously [7] (data not shown). The RP1-encoded β-lactamase activity, on the other hand, remained fairly constant throughout growth and was not induced in response to phosphate depletion. In addition to their supernatant activities (Fig. 1B), alkaline phosphatase and phosphalipase C exhibited cell-associated activity which was released by a cold-shock Tris–$MgCl_2$ treatment (Fig. 1C) which does not alter the integrity of the cytoplasmic membrane [15]. In the case of alkaline phosphatase, the released activity represented, for the most part, periplasmic enzyme as indicated by the relative enzyme activities in the Tris–$MgCl_2$ wash as compared to intact cell activity. Thus the ratio of alkaline phosphatase activity in the Tris–$MgCl_2$ wash to alkaline phosphatase activity in intact cells was determined to be 3.80 ± 1.00 for cells resuspended in 10 mM Tris buffer pH 8.5 and 3.55 ± 0.26 for cells resuspended in 50 mM HEPES buffer pH 8.5.

To test the specificity of alkaline phosphatase and phospholipase C release into the supernatant, we examined the distribution of two other proteins normally found in the periplasm, the constitutive RP1-encoded β-lactamase and the M_r 37000 protein, at the time of alkaline phosphatase and phospholipase C induction and secretion. Fig. 2 indicates that throughout growth in phosphate-defi-

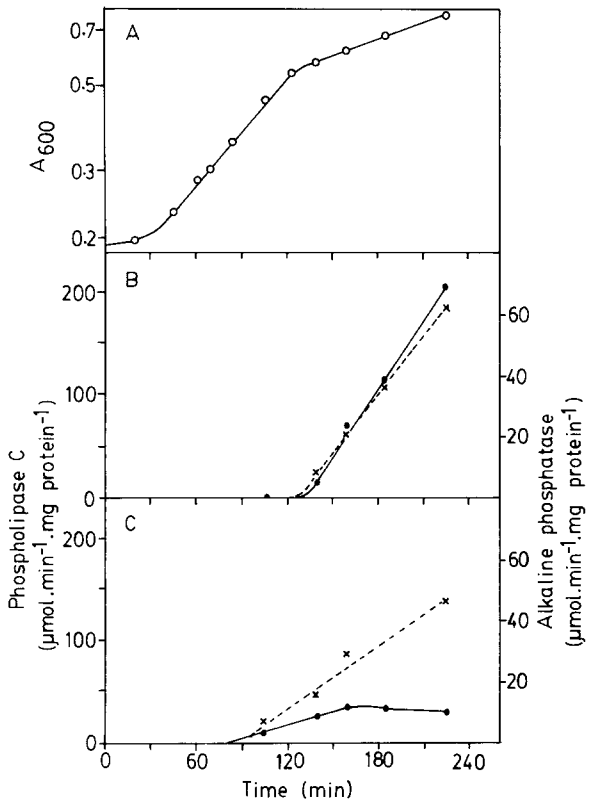

Fig. 1. Growth (A) and alkaline phosphatase (×- - - - - -×) and phospholipase C (●———●) activities in the supernatant (B) and periplasm (C) of cells growing in phosphate-deficient medium. Logarithmic-phase cells in phosphate-sufficient medium (1 mM P_i) were washed and resuspended in phosphate-deficient (0.2 mM P_i) medium at time zero. The measurements in panels B and C were representative data (of 5 separate experiments) obtained from a single culture of strain (H103) (RP1). The periplasmic enzyme data represents total activity released from cells by the Tris–$MgCl_2$ cold shock procedure of Hoshino and Kageyama [12].

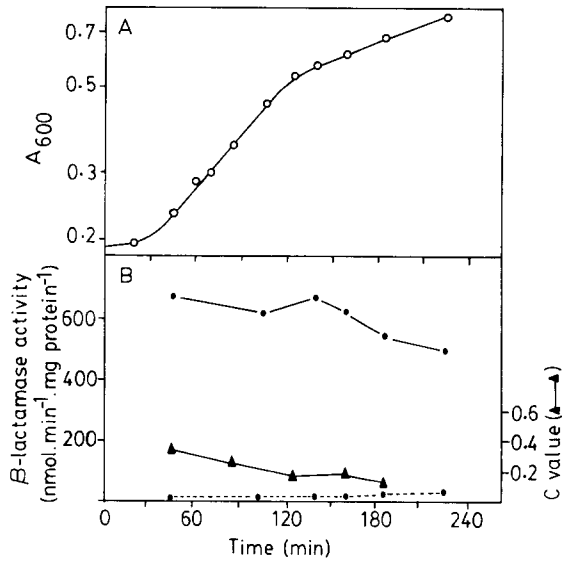

Fig. 2. Outer membrane permeability during growth on phosphate-deficient medium. Panel A shows growth after transfer to phosphate-deficient medium at time zero as described in the legend to Fig. 1. Panel B shows β-lactamase in the supernatant (●- - - - -●) and periplasm (●———●) and the outer-membrane permeability coefficient C (▲———▲) (expressed in s/μg whole cell protein) calculated as described in MATERIALS AND METHODS.

cient media, including the period when alkaline phosphatase and phospholipase C were being released, the β-lactamase activity remained essentially periplasmic (as Tris–$MgCl_2$-releasable enzyme) with only approx. 6% of the total activity present in the supernatant 240 min after growth began. The M_r 37000 protein, although present as the major protein in the periplasm upon induction [7], as undetectable in the supernatant as determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis of 50 times concentrated supernatants. By comparison, up to 58% of the total alkaline phosphatase activity and 87% of the total phospholipase C activity was found in the supernatant (Fig. 1). These results confirmed that alkaline phosphatase and phospholipase C release is indeed specific and not explainable by an increased general leakiness of the outer membrane for periplasmic proteins. Furthermore, our inability to detect major outer membrane proteins or the lipopolysaccharide (LPS)-specific sugar 2-keto-3-deoxyoctanate (KDO) (< 6 ng per unit of alkaline phosphatase secreted) in 50 times concentrated supernatants also lent support to the absence of outer membrane breakdown during enzyme release.

The periplasmic location of the β-lactamase [10,13] and the demonstration by Angus et al. that nitrocefin is taken up by the hydrophilic pathway [10] provided a means by which outer membrane permeability could be measured, as a function of nitrocefin uptake and hydrolysis. Furthermore,

treatment of cells with EDTA, an agent known to break down the outer membrane thus increasing permeability [16], is associated with a ten-fold increase in nitrocefin hydrolysis [13]. The outer membrane permeability parameter C did not increase (Fig. 2), as a function of growth in phosphate deficient media, even during the period of active alkaline phosphatase and phospholipase C release. The only alteration in outer membrane permeability observed on growth in phosphate-deficient media was a gradual 2.8-fold decrease in permeability over the 2.5 h of the experiments.

4. DISCUSSION

Little is known about protein secretion into the medium of Gram-negative organisms since most of the work on extracytoplasmic proteins has been done in *Escherichia coli,* which has few, if any, extracellular enzymes [17]. In addition, Gram-positive studies are not directly relevant due to the lack of an outer membrane. In the present study, we undertook to better define the mechanism of release of extracellular proteins in a Gram-negative bacterium, *Pseudomonas aeruginosa,* by looking specifically at alkaline phosphatase and phospholipase C, two enzymes whose secretion has already been demonstrated [3,5].

Under conditions of phosphate limitation both enzymes were found to be released into the medium in addition to an observed periplasmic activity. The observed periplasmic phospholipase C activity is in contrast to results obtained by Stinson and Hayden [3] who showed no detectable $MgCl_2$-releasable phospholipase C activity. The increased efficiency of release of periplasmic proteins imparted by the cold shock step of Hoshino and Kageyama [12] used in conjunction with Tris–$MgCl_2$ may account for our ability to detect cell associated, Tris–$MgCl_2$ released phospholipase C activity. The periplasmic alkaline phosphatase and phospholipase C activities started to increase prior to the appearance of extracellular enzyme (Fig. 1). However, whether the activity in the supernatant actually represents secreted periplasmic enzyme or whether the periplasmic and subsequent activities are distinct entities is as yet undefined.

In any case, results from this study indicate that the release of alkaline phosphatase and phospholipase C is specific, being associated with no increased general leakiness of the outer membrane as shown by the maintenance of an apparent periplasmic location for both the β-lactamase and the M_r 37000 protein during active release of alkaline phosphatase and phospholipase C into the medium. Furthermore, a lack of increased outer membrane leakiness was confirmed directly by outer membrane permeability measurements which revealed that no permeability increase was concomitant with enzyme secretion; in fact, a slight decrease was observable.

One hypothesis proposed to account for the release of the alkaline phosphatase of *Pseudomonas aeruginosa* involves the secretion of alkaline phosphatase as a complex with LPS [18,19]. Ingram et al. [18] have indicated that secreted alkaline phosphatase is associated with LPS, as suggested, in part, by parallel increases in KDO and alkaline phosphatase in the supernatant during growth. They reported KDO levels in the supernatant approaching 1 μg per unit of alkaline phosphatase (one unit being defined as 1 μmol pNPP hydrolyzed or phosphate released per min). In contrast, we could detect no KDO in 50-fold concentrated supernatants of cultures actively secreting alkaline phosphatase (and phospholipase C) which indicated that < 0.0055 μg KDO per unit of alkaline phosphatase was present. These differences may well be due to technique since Ingram and colleagues [18,19] grew their cells in a Tris-based medium, and it has been demonstrated that Tris can interact with the outer membrane to permeabilize it [13,20] and release LPS (and alkaline phosphatase) [20]. In contrast, HEPES, which buffered our media, has been shown not to influence outer membrane permeability [20].

One other important implication of these results concerns the function of outer membrane protein P which is apparently coregulated with alkaline phosphatase and phospholipase C [7]. This protein was purified to homogeneity and shown to form anion-specific channels in model membranes. These channels allow the passage of small anions and were proposed to be involved in phosphate uptake into the cell. The results in Fig. 2 suggest

that nitrocefin permeation into *Pseudomonas aeruginosa* cells does not increase as protein P is induced. These results strongly suggest that protein P does not function as a channel for nitrocefin, and furthermore indicate that protein P induction is not superfluous, in that the increase in phosphate transport on phosphate deprivation cannot be explained merely on the basis of enhanced non-specific outer membrane permeability despite the active secretion of the coregulated enzymes.

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