Cloning, Expression and Release of a *Vibrio alginolyticus* SDS-resistant Ca\(^{2+}\)-dependent Exoprotease in *Escherichia coli*

By S. M. DEANE, R. MAHARAJ, F. T. ROBB AND D. R. WOODS*

Department of Microbiology, University of Cape Town, Rondebosch 7700, South Africa

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A *Vibrio alginolyticus* SDS-resistant, Ca\(^{2+}\)-dependent serine exoprotease gene, cloned on a 7.1 kb DNA fragment in the recombinant plasmid pVP100, was expressed from its own promoter in *Escherichia coli*. Although active exoprotease was produced by late stationary phase *E. coli* (pVP100) cultures after 15 h incubation in proteinaceous medium containing Ca\(^{2+}\), transcription and translation of the exoprotease occurred before 6 h, during exponential growth. The cloned exoprotease was synthesized as a pool of inactive precursor molecules during exponential growth, and released as active exoprotease 8 h later by a process which did not require protein synthesis or involve cell lysis. Release of the exoprotease by *E. coli* (pVP100) was not inhibited by o-phenanthroline, quinacrine or cerulenin. Supernatant samples from *E. coli* (pVP100) cultures contained two SDS-resistant exoproteases with apparent *M*\(_r\) values of approximately 54000 and 39000. The cloned exoprotease activity was inhibited by EDTA and a serine protease inhibitor, but was not affected by an inhibitor of trypsin-like enzymes.

INTRODUCTION

The Gram-negative *Vibrio alginolyticus* strain isolated from hides (Welton & Woods, 1973, 1975) secretes an extracellular collagenase (Reid et al., 1978, 1980), six alkaline serine exoproteases (Hare et al., 1981, 1983; Deane et al., 1986) and an SDS-resistant, Ca\(^{2+}\)-dependent, alkaline serine exoprotease (Deane et al., 1987). To characterize the secretory systems further and to determine whether they are able to function in other Gram-negative bacteria which are not usually associated with secretion of proteases, we constructed a gene bank of *V. alginolyticus* DNA in *Escherichia coli*, and here describe the cloning of a DNA fragment which contains an SDS-resistant, Ca\(^{2+}\)-dependent, alkaline serine exoprotease gene.

METHODS

*Bacterial strains and plasmids. The proteolytic V. alginolyticus strain, used as a source of DNA, was described by Welton & Woods (1973, 1975), Reid et al. (1980) and Deane et al. (1987). E. coli HB101 (Maniatis et al., 1982) and E. coli LE392 (Maniatis et al., 1982) were used as hosts. pEcoR251, a gift from M. Zabeau, Plant Genetic Systems, Ghent, Belgium, is a positive selection vector containing the *E. coli* EcorI gene under the control of the \(\lambda\) rightward promoter, the ampicillin (Ap) resistance gene, and the pBR322 origin of replication. The EcorI gene product, expressed at high levels by the \(\lambda\) promoter, is lethal unless insertionally inactivated or regulated by plasmid pC857, which contains a temperature sensitive \(\lambda\) repressor gene (Remaut et al., 1983). Plasmid pBR325 was used for subcloning.*

Abbreviations: Ap, ampicillin; LB, Luria broth; PB, peptone broth; PW, peptone water; Cm, chloramphenicol; PHMB, \(p\)-hydroxymercuribenzoate, sodium salt; PMSF, phenylmethylsulphonyl fluoride.
Media and growth conditions. \textit{V. alginolyticus} cultures were grown aerobically at 30°C in Luria broth (LB) (Miller, 1972) containing 0.4 M NaCl, or peptone broth (PB) as described by Deane et al. (1987). \textit{E. coli} strains were grown in LB (Miller, 1972), glucose minimal medium (Miller, 1972) or peptone water (PW) (2.5% w/v, peptone in distilled water) at 37°C. All bacterial strains were maintained on agar plates containing 2% (w/v) skim milk.

Preparation of DNA and recombinant DNA techniques. Plasmid DNA was prepared by the alkali-lysis method of Ish-Horowicz & Burke (1981). \textit{V. alginolyticus} chromosomal DNA was isolated by a modification of the method of Marmur (1961). Overnight \textit{V. alginolyticus} cultures (100 ml) in PB were harvested by centrifugation. Cells were resuspended in 4 ml sucrose buffer (0.73 M sucrose; 10 mM-Tris/HCl, pH 8.0; 10 mM-EDTA) containing lysozyme (4 mg ml⁻¹), and incubated at 37°C for 60 min with shaking. The cell suspension was then cooled on ice for 5 min before the addition of 2 ml of 0.2 M-EDTA (pH 8.0). Cells were lysed by the addition of SDS buffer (2%, w/v, SDS; 10 mM-EDTA; 10 mM-Tris/HCl, pH 8.0) and heating at 65°C for 10 min. DNA in the cleared lysate was purified by CsCl-ethidium bromide gradient centrifugation as described by Maniatis et al. (1982).

A \textit{V. alginolyticus} genomic library was constructed using pEcoR251, as described by Maharaj et al. (1986). \textit{V. alginolyticus} DNA was partially digested with Sau3A endonuclease, and ligated with BglII endonuclease digested pEcoR251. Ap+ \textit{E. coli} HB101 transformants were pooled in lots of 1000, and plasmid DNA was extracted from each pool to constitute the genomic library.

Selection of protease clone. Competent \textit{E. coli} HB101 cells were transformed with the plasmid pools, and transformants were selected for their ability to form haloes of clearing on skim milk plates containing Ap (100 μg ml⁻¹) after 48 h at 37°C. Several colonies with haloes of various diameters were observed.

Restriction endonuclease mapping and subcloning. Standard techniques were utilized to obtain a restriction endonuclease map of pVP100. A complete PstI endonuclease digest of pVP100 was ligated to PstI endonuclease digested pBR325 and transformed in competent \textit{E. coli} HB101. Transformants which formed haloes of clearing on skim milk agar plates containing chloramphenicol (Cm) (20 μg ml⁻¹) were selected.

DNA hybridization. Chromosomal DNA from \textit{V. alginolyticus} was digested with a variety of restriction endonucleases, and the restriction digests were resolved by electrophoresis in 0.8% agarose Tris-acetate gels and transferred to Hybond-N hybridization transfer membranes (Amersham).

Plasmid pVP100 was nick-translated (Rigby et al., 1977) using [α-³²P]dCTP, and used as a hybridization probe.

Enzyme assays. Protease activity was assayed using the synthetic substrate azocasein (Sigma) (Long et al., 1981). One unit (U) of protease activity is defined as the amount of enzyme that gives an increase in absorbance of 0.1 at 440 nm in 30 min at 37°C. The effects of the following protease inhibitors on the protease activities of cell-free supernatant PW + CaCl₂ samples were determined by incubating the supernatants together with the various inhibitors at 37°C for 20 min, before assaying with azocasein: o-phenanthroline (2.5 mM), soybean trypsin inhibitor (1 mM), p-hydroxymercurobenzoate, sodium salt (PHMB) (2.5 mM), phenylmethyisulphonyl fluoride (PMSF) (10 mM) and EDTA (10 mM). Control experiments showed that the ethanol, and the organic solvent dimethyl sulphoxide (DMSO), used to dissolve the o-phenanthroline and PMSF, respectively, did not affect the protease activity. β-Galactosidase and β-lactamase activities were determined by the methods of Pardee et al. (1959) and Sykes & Nordström (1972) respectively.

Gelatin-PAGE protease assay. Cell-free supernatant samples were mixed with SDS (2.5%, w/v) and glycerol (2.0%, v/v), and incubated at 37°C for 30 min. Treated samples were then subjected to PAGE in slab gels containing SDS and gelatin as a co-polymerized substrate (Heussen & Dowdle, 1980) as described by Hare et al. (1983). After PAGE, the gels were washed in Triton X-100 (2.5%, w/v) for 1 h at room temperature to remove the SDS. After incubation in 0.1 M-glycine buffer (pH 9.0) for 3 h at 37°C, bands or zones of proteolytic activity were detected on staining with 0.2% (w/v) amido black. Alternatively, the SDS-treated samples were resolved by conventional SDS-PAGE without gelatin. Bands of protease activity were then detected by means of a gelatin gel-overlay as described by Deane et al. (1987).

Preparation of supernatant and cell lysate fractions. Overnight cultures of \textit{E. coli} HB101(pVP100) and \textit{E. coli} LE392(pVP100) were diluted (1 in 10) in LB or PW + CaCl₂ (10 mM) and isopropyl β-D-thiogalactopyranoside (IPTG) (2 mM) and incubated at 37°C with good aeration. Samples (10 ml) were removed, disintegrated in a French press, and given a clearing spin of 2 min in a microfuge before being assayed. Cells from a 10 ml sample were also harvested by centrifugation, washed twice in PW + CaCl₂ (10 mM) and disintegrated by the French press. Supernatant samples were collected after 2 min centrifugation in a microfuge.

Periplasmic enzymes were released from \textit{E. coli} LE392(pVP100) cells by a modification of the method of Willis et al. (1974). Samples (100 ml) were harvested by centrifugation, washed and resuspended in PW containing 30 mM-NaCl, 10 mM-CaCl₂ and 33 mM-Tris/HCl (pH 7.3). The cells were resuspended in 33 mM-Tris (pH 7.3) [10 ml (g wet wt)⁻¹], and an equal volume of TSE buffer [33 mM-Tris/HCl (pH 7.3), 40% w/v sucrose, 2 mM-EDTA] was added, with stirring. The cells were harvested by centrifugation, and subjected to osmotic shock by resuspension in ice-cold deionized water [20 ml (g wet wt)⁻¹], followed by the immediate addition of MgCl₂ (10 mM).
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Cloning of the V. alginolyticus protease gene

E. coli HB101 was transformed with recombinant pEcoR251 plasmid pools, and a transformant, which produced a halo of clearing on skim milk plates containing Ap, was isolated. Plasmid DNA isolated from this colony retransformed E. coli HB101 and E. coli LE392, and colonies able to form haloes of clearing on skim milk plates were obtained at the same frequency as Ap' transformants. The recombinant plasmid was designated pVP100. Restriction endonuclease mapping of pVP100 revealed a 7.1 kb DNA insert in pEcoR251 (Fig. 1). The 2.75 kb PstI fragment was subcloned in both orientations to produce plasmids pVP101 and pVP102 (Fig. 1). E. coli LE392(pVP101) and E. coli LE392(pVP102) strains both formed haloes of clearing on skim milk plates.

DNA homology

The origin of the 7.1 kb insert in pVP100 was determined by Southern blotting and DNA hybridization, with 32P-labelled pVP100 as the probe. Labelled pEcoR251 did not hybridize to V. alginolyticus DNA (data not shown). The electrophoretic mobility of the homologous DNA sequence in the EcoRI digest of the chromosomal DNA corresponded with that of the EcoRI fragment of insert DNA of pVP100.

Production of exoproteases

Exoprotease production by E. coli HB101(pVP100) and E. coli LE392(pVP100) in PW + 10 mM CaCl₂ was determined in cell-free supernatant samples by gelatin-PAGE. Staining of the gels immediately after gelatin-PAGE or after the removal of the SDS by soaking in Triton X-100 for 1 h at 20 °C revealed broad biphasic zones of protease activity at the top of the gel (Fig. 2). A more rapidly migrating zone of low protease activity was followed by a zone of high protease activity. Control E. coli culture supernatants did not produce a zone of SDS-resistant protease activity after gelatin-PAGE (data not shown). V. alginolyticus culture supernatants produced a zone of SDS-resistant exoprotease activity at the top of the gelatin-PAGE gel (protease A, apparent \( M_r \) approximately 54000; Deane et al., 1987) (Fig. 2). Gelatin-PAGE gels containing V. alginolyticus samples, which were soaked in Triton X-100 and incubated in buffer for 3 h at 37 °C revealed the SDS-sensitive exoproteases, la and lb reported previously by Deane et al. (1986) (Fig. 2).

To obtain the \( M_r \) values of the exoproteases produced by E. coli(pVP100) in PW + CaCl₂, cell-free supernatant samples were resolved by conventional SDS-PAGE without gelatin, and the proteases were detected by the gelatin overlay technique (Fig. 3). Staining of the SDS-PAGE gels revealed the \( M_r \) markers, but no bands were visible in the lanes containing protease samples. After addition of the gelatin overlay the E. coli(pVP100) cell-free supernatant sample...
Fig. 2. Analysis by gelatin-PAGE of exoproteases produced in PW + 10 mM CaCl₂ by either *E. coli* HB101 or *E. coli* LE392 (pVP100), and *V. alginolyticus*. Cell-free supernatant samples of *E. coli* (pVP100) (lanes B, D and F) and *V. alginolyticus* (lanes A, C and E) cultures were analysed. Each lane was loaded with 10 µl of a supernatant sample prepared with SDS as described in Methods. Gelatin-PAGE was carried out with slab gels containing SDS and gelatin as a co-polymerized substrate. A, B, gels stained immediately after gelatin-PAGE; C, D, gels washed with Triton X-100 for 1 h at 20 °C before staining; E, F, gels washed with Triton X-100 and then incubated in 0.1 M-glycine buffer pH 9.0 for 3 h at 37 °C before staining. The SDS-sensitive exoproteases, 1a and 1b (Deane et al., 1986), are indicated.

Fig. 3. Analysis of exoproteases produced by *E. coli* LE392 (pVP100) (lane A) and *V. alginolyticus* (lane B) in PW + 10 mM CaCl₂ cultures by the gelatin overlay technique. Cell-free supernatant samples were resolved by conventional SDS-PAGE without gelatin. Exoproteases were detected by overlaying with a gelatin gel after electrophoresis.

showed two bands of exoprotease activity with apparent *Mₗ* values of approximately 54000 and 39000. A control sample containing the *V. alginolyticus* SDS-resistant exoprotease produced three bands of exoprotease activity with apparent *Mₗ* values of approximately 54000, 41000 and 37000 as described previously by Deane et al. (1987).

Exoprotease activity of *E. coli* HB101 (pVP100) and *E. coli* LE392 (pVP100) cultures grown in PW + 10 mM-CaCl₂ was detected in late stationary phase after 15 to 18 h incubation (Fig. 4). Exoprotease production by *E. coli* HB101 (pVP100) was accompanied by the release of intracellular β-galactosidase and a decrease in turbidity. Although *E. coli* LE392 (pVP100) cells produced similar amounts of exoprotease activity, no extracellular β-galactosidase activity was detected and there was no decrease in turbidity (Fig. 4). The kinetics and amounts of exoprotease production by *E. coli* HB101 (pVP100) and *E. coli* LE392 (pVP100) were similar at 30 °C (data not shown) and 37 °C.

The production of exoprotease by *E. coli* LE392 (pVP100) was investigated in minimal and complex media. SDS-resistant exoprotease activity was detected in cultures grown in PW + 10 mM-CaCl₂ but not in cultures grown in PW without CaCl₂, or in minimal medium.
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![Graph showing kinetics of exoprotease production](image)

**Fig. 4.** Kinetics of exoprotease production by *E. coli* HB101(pVP100) (open symbols) and *E. coli* LE392(pVP100) (filled symbols). ○, ●, Growth (log OD600); △, ▲, protease activity; □, ■, supernatant β-galactosidase activity expressed as a percentage of the total β-galactosidase activity. SEMS were 5 to 10% of reported values.

![Graph showing effect of Cm on exoprotease production](image)

**Fig. 5.** Effect of Cm on exoprotease production by *E. coli* LE392(pVP100). Cm (100 μg ml⁻¹) and IPTG were added at 6 h (arrow) to exponential phase cultures (open symbols); controls were also done to which no Cm was added (closed symbols). ○, ●, Growth (OD600); △, ▲, exoprotease activity; □, ■, β-galactosidase activity. SEMS were 5 to 10% of reported values.

with or without CaCl₂. The addition of CaCl₂ to cell-free supernatant samples from 18, 20, 24, 30 and 48 h *E. coli* LE392(pVP100) cultures grown in PW did not result in the activation of exoprotease activity. No exoprotease activity was produced by *E. coli* LE392(pVP100) grown in PW containing MgCl₂, ZnCl₂, NiCl₂ or LiCl.

**Effect of Cm on exoprotease production**

Since exoprotease production by *E. coli* LE392(pVP100) was only detected in supernatant samples after 15 to 18 h, the time of synthesis of the exoprotease was determined by the addition of Cm (100 μg ml⁻¹) to exponential phase cultures (Fig. 5). The addition of Cm at 6 h inhibited the growth of *E. coli* LE392(pVP100). The simultaneous addition of Cm and IPTG at 6 h prevented the synthesis of β-galactosidase. Control cultures without Cm produced β-galactosidase. There was very little difference in the kinetics and amounts of exoprotease
production by *E. coli* LE392(pVP100) in the presence or absence of Cm added at 6 h (Fig. 5). Similar results were obtained when Cm was added at 18 h.

**Effect of o-phenanthroline, cerulenin and quinacrine**

The release of exoprotease by *E. coli* LE392(pVP100) cultures in PW + CaCl$_2$ was not affected by the addition of o-phenanthroline (50 μg ml$^{-1}$), cerulenin (1 μg ml$^{-1}$) or quinacrine (25 μg ml$^{-1}$) at the start of exoprotease release (16 h). The control culture, and cultures treated with o-phenanthroline, cerulenin or quinacrine, all produced between 3-2 and 3-6 units ml$^{-1}$ of exoprotease activity after 20 h.

**Localization of protease activity**

The experiments with Cm indicated that the exoprotease produced by pVP100 in *E. coli* LE392 was synthesized at least 6 to 9 h before it was secreted into the medium. To determine whether active exoprotease was stored in the cytoplasm or periplasm prior to secretion, *E. coli* LE392(pVP100) cells in PW + CaCl$_2$ were harvested before the release of exoprotease, at 6 h, and during the release of exoprotease, at 20 h. The washed cells were disintegrated in a French press (total cellular protease activity) or subjected to osmotic shock treatment (periplasmic protease activity). No intracellular or periplasmic protease activity was detected. Treatment of control supernatant samples, containing exoprotease activity, in the French press did not inactivate the exoprotease.

The distribution of active exoprotease, intracellular β-galactosidase and a periplasmic enzyme, β-lactamase, was determined in *E. coli* LE392(pVP100) cultures after 24 h growth in PW + CaCl$_2$ at 37 °C. The majority of the exoprotease and β-lactamase activities were extracellular (91 and 63% respectively) whereas 98.2% of the β-galactosidase activity was intracellular.

**Characterization of the cloned exoprotease**

The cloned exoprotease showed similar heat inactivation kinetics in PW + CaCl$_2$ to the SDS-resistant exoprotease A produced by *V. alginolyticus* (Deane et al., 1987). It was stable at 40 °C but lost 35 and 88% of its activity after holding for 1 h at 50 and 60 °C respectively.

Cell-free supernatant samples of the cloned exoprotease in PW + CaCl$_2$ (4-47 units ml$^{-1}$) after treatment with PMSF, PHMB, soybean trypsin inhibitor, o-phenanthroline and EDTA, contained 0-07, 3-78, 3-44, 4-14 and 0-69 units ml$^{-1}$ of protease activity respectively.

Dialysis of the cloned exoprotease in PW + CaCl$_2$ at 4 °C against distilled water or 10 mM EDTA for 18 h, resulted in the inactivation of the exoprotease. Dialysis against 10 mM-CaCl$_2$ did not result in the loss of exoprotease activity. EDTA-inactivated exoprotease activity was reactivated by dialysis against 10 mM-CaCl$_2$.

**DISCUSSION**

A 7·1 kb DNA fragment from *V. alginolyticus* was cloned on a recombinant plasmid, pVP100, in *E. coli*, and shown to produce an SDS-resistant, Ca$^{2+}$-dependent exoprotease. A 2·75 kb fragment from this DNA, subcloned in both orientations in another vector, pBR325, expressed exoprotease activity in *E. coli*. It is concluded that the cloned exoprotease gene was expressed in *E. coli* from a *V. alginolyticus* regulatory region.

The exoprotease produced by pVP100 in *E. coli* was able to digest gelatin in the presence of SDS, and broad zones of proteolytic activity were detected after staining gelatin gels which still contained SDS. When a gelatin overlay was added, after SDS-PAGE without co-polymerized gelatin, two bands of exoprotease activity, with apparent $M_r$ values of approximately 54000 and 39000 were detected. Recently, we reported that *V. alginolyticus* produced three SDS-resistant, Ca$^{2+}$-dependent exoproteases with apparent $M_r$ values of approximately 54000, 41000 and
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37000, termed exoproteases A, B and C respectively (Deane *et al.*, 1987). Dialysis against distilled water, of cell-free supernatant samples, which contained predominantly exoprotease A, resulted in the increased appearance of exoproteases B and C. It is suggested that the cloned *V. alginolyticus* DNA contains the gene for the 54000 M, SDS-resistant exoprotease. This suggestion is supported by other similarities between the two exoproteases; they are Ca²⁺-dependent, inhibited by EDTA and a serine protease inhibitor (PMSF) but not affected by inhibitors of trypsin-like enzymes, and they show similar heat inactivation kinetics.

The production of the SDS-resistant exoprotease activity by *V. alginolyticus* and *E. coli* (pVP100) cells differed in certain respects. *V. alginolyticus* synthesized and secreted active exoprotease during exponential growth in proteaceous media containing Ca²⁺ (Deane *et al.*, 1987). In the absence of Ca²⁺, *V. alginolyticus* cells synthesized and secreted inactive exoprotease molecules which could be activated by the addition of Ca²⁺ to cell-free supernatants. In contrast, exoprotease activity was only detected in very late stationary phase *E. coli* (pVP100) cultures grown in proteaceous media containing Ca²⁺. In the absence of Ca²⁺, the *E. coli* (pVP100) cells did not produce an inactive exoprotease which could be activated by Ca²⁺.

The production of the cloned exoprotease by two different *E. coli* strains is interesting. The kinetics of exoprotease production by *E. coli* HB101 (pVP100) and *E. coli* LE392 (pVP100) cells were similar. However, exoprotease activity was associated with lysis of *E. coli* HB101 (pVP100) cells and the release of an intracellular enzyme. No cell lysis or release of an intracellular enzyme was associated with exoprotease activity in *E. coli* LE392 (pVP100) cultures. However cell-free exoprotease activity in *E. coli* LE392 (pVP100) cultures was associated with the release into the medium of a periplasmic enzyme. We conclude that stationary phase *E. coli* LE392 (pVP100) cells are able to release exoprotease and β-lactamase without cell lysis.

The production of exoprotease by *E. coli* LE392 (pVP100) cells is similar to the release of a cloned penicillinase from an alkalophilic *Bacillus* strain in *E. coli* (Kudo *et al.*, 1983). Release of the cloned penicillinase occurred during late stationary phase and was accompanied by release of the periplasmic enzymes of the *E. coli* host. In contrast, release of a cloned serine protease from a *Serratia marcescens* strain occurred in parallel with growth of the host without accompanying leakage of the host periplasmic enzymes (Yanagida *et al.*, 1986).

Studies with Cm on exoprotease synthesis and activity in *E. coli* LE392 (pVP100) cells indicated that, although cell-free exoprotease activity was only detected after 15 to 18 h in late stationary phase, transcription and translation of the exoprotease occurred before 6 h, during exponential growth. Since intracellular SDS-resistant protease activity was not detected in *E. coli* LE392 (pVP100) cells, it appears that the cloned exoprotease is synthesized as a pool of inactive precursor exoprotease molecules during exponential growth. The pool of inactive exoprotease molecules is released approximately 8 h later during late stationary phase by a process which does not require protein synthesis or involve cell lysis. The release process seems to be involved in the activation of the exoprotease. Since it was not possible to detect the cellular localization of the inactive exoprotease molecules, it is not known whether the exoprotease is released from a cytoplasmic or periplasmic pool of inactive precursor molecules.

The inhibition of the production of exoproteins in Gram-positive bacteria by o-phenanthroline and quinacrine (Traficante & Lampen, 1977; Berkeley *et al.*, 1978; Fishman *et al.*, 1980) has been used to support the suggestion that an exoenzyme-release protease system is involved in the secretion of bacterial exoproteins. Since o-phenanthroline and quinacrine inhibited the secretion of the SDS-resistant exoprotease in *V. alginolyticus*, Deane *et al.* (1987) suggested that a similar exoenzyme-releasing protease system may be operating in *V. alginolyticus*. However the release of the SDS-resistant protease in *E. coli* LE392 (pVP100) cells was not affected by these inhibitors, and it is suggested that the release of the cloned SDS-resistant protease by *E. coli* LE392 (pVP100) cells involves a different mechanism from that operating in *V. alginolyticus* cells. Further evidence for this suggestion involves experiments with cerulenin, a specific inhibitor of fatty acid synthetase (Omura, 1976), which reduced exoprotease production by *V. alginolyticus* cells (Hare *et al.*, 1981), but did not affect exoprotease production by *E. coli* LE392 (pVP100) cells.
REFERENCES


