Isolation and Characterization of a *Vibrio alginolyticus* Mutant That Overproduces Extracellular Proteases

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The *Vibrio alginolyticus* prot-T₁ mutant was able to produce haloes of clearing on skim milk/peptone agar plates at 42 °C whereas proteolysis by the wild-type strain was inhibited at 37 °C. The prot-T₁ mutant overproduced the three major alkaline proteases with apparent molecular masses of approximately 28 000, 22 500 and 19 500 (proteases 1a, 2 and 3, respectively). Their synthesis was not markedly repressed by incubation at 37 °C or by non-aeration. Both treatments inhibited protease synthesis in the wild-type strain, which only produced proteases during the stationary growth phase. The prot-T₁ mutant synthesized proteases throughout the exponential and stationary growth phases in peptone medium. High protease activities were induced by glucose or glutamine in stationary phase prot-T₁ that were pre-grown in peptone medium. Glucose or glutamine had the opposite effect on protease activities in stationary phase prot-T₁ cultures that were pre-grown in minimal medium. Collagenase synthesis was not altered in the prot-T₁ mutant and was repressed by growth at 37 °C or without aeration. The independent control of collagenase synthesis supports the conclusion that there are no regulatory proteins responsible for the overall control of extracellular protease synthesis by temperature, aeration and growth phase in *V. alginolyticus*.

INTRODUCTION

The *Vibrio alginolyticus* strain isolated from hides is an aerobic, halotolerant, Gram-negative bacterium, which produces an extracellular collagenase and five extracellular alkaline serine proteases during the stationary growth phase (Welton & Woods, 1973, 1975; Reid *et al.*, 1978, 1980; Long *et al.*, 1981; Hare *et al.*, 1983). Three major alkaline proteases with apparent molecular masses of approximately 28 000, 22 500 and 19 500 (proteases 1a, 2 and 3, respectively) and two minor alkaline proteases with apparent molecular masses of approximately 15 500 and 14 500 (proteases 4 and 5, respectively) are produced.

Extracellular collagenase and alkaline protease activities in *V. alginolyticus* are subject to glucose catabolite repression and end-product repression by amino acids and ammonium ions (Reid *et al.*, 1978; Long *et al.*, 1981). The production of collagenase and alkaline protease activity is also influenced by temperature and oxygen, and enzyme synthesis is inhibited when either the growth temperature is raised from 30 to 37 °C or the rate of aeration is reduced (Hare *et al.*, 1981, 1983). The production of alkaline proteases does not involve a specific inducer and they are produced in a minimal medium (Long *et al.*, 1981), whereas collagenase production is only induced by collagen (Reid *et al.*, 1980). The production of alkaline protease activity is markedly enhanced or induced by histidine (Long *et al.*, 1981).

The regulation and production of true extracellular proteases in stationary phase by the Gram-negative *V. alginolyticus* strain is similar, in a number of aspects, to the secretion of proteases by Gram-positive *Bacillus* strains (Glenn, 1976; Priest, 1977; Reid *et al.*, 1978, 1980;

Abbreviations: CAM, Casamino acids medium; MM, minimal medium; SMM, succinate minimal medium; NTG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine.

Long *et al.*, 1981). Since there are few systems in Gram-negative bacteria for the study of the regulation of exoprotease production, we isolated and investigated the regulation of a *V. alginolyticus* protease mutant which was insensitive to repression by temperature.

METHODS

Bacterial strain and media. The proteolytic *V. alginolyticus* strain, which has been described previously (Welton & Woods, 1973, 1975; Reid *et al.*, 1980), was maintained at room temperature on peptone (2.5%, w/v) agar containing 1% (w/v) skim milk. The peptone medium, Casamino acids medium (CAM), minimal medium (MM) and succinate minimal medium (SMM) have been described previously (Long *et al.*, 1981; Hare *et al.*, 1983). All media were prepared in 0.1 M-Tris/HCl buffer (pH 7.6) containing 0.4 M-NaCl and $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (0.29 g l⁻¹). Amino acids and carbon sources were added to media to give final concentrations of 0.5 and 0.2% (w/v), respectively.

Growth conditions for enzyme studies. The growth conditions for the production of collagenase in peptone medium and alkaline proteases in SMM, by concentrated stationary phase *V. alginolyticus* cells, have been described previously (Reid *et al.*, 1980; Long *et al.*, 1981). Cultures were grown overnight in CAM or MM before resuspension in either peptone medium or MM, respectively. After incubation for 3 h the cells were harvested, washed and resuspended in SMM. Cultures were aerated on an orbital shaker at 130 r.p.m. at 30 °C. To facilitate aeration, the cultures were 5–10% of the volume of the flasks, which were covered with loose fitting aluminium foil caps.

Isolation of mutants. Mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) (carcinogen) was done according to Adelberg *et al.* (1965). NTG (100 µg ml⁻¹) was added to exponential phase *V. alginolyticus* cells in 0.1 M-Tris/maleic acid buffer (pH 6.0) containing 0.4 M-NaCl and $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (0.29 g l⁻¹), and the culture was incubated for 15 min at 30 °C (approximately 10% survival). Washed cell suspensions were resuspended in peptone broth and aerated for 3 h at 30 °C before plating onto skim milk/peptone agar plates. The plates were incubated at 42 °C and observed for colonies which produced zones of clearing surrounding colonies at the elevated temperature.

Enzyme assays. All enzyme assays were done at standardized cell densities. Each sample was assayed in duplicate and experiments were repeated at least three times. Collagenase was assayed by the method of Wünsch & Heidrich (1963) using the synthetic collagenase substrate phenyl-azobenzyloxycarbonyl-L-propyl-L-leucyl-glycyl-L-propyl-D-arginine (Fluka) as described previously (Reid *et al.*, 1978). Alkaline protease activity was assayed using the synthetic substrate azocasein (Sigma) at a concentration of 2% (w/v) in 0.1 M-Tris/HCl buffer (pH 9.0). The sample (1 ml) was added to 1 ml of the azocasein solution and the mixture was incubated at 37 °C for 30 min. The reaction was stopped by the addition of 10% (w/v) trichloroacetic acid (2 ml). After standing at 4 °C for 30 min the mixture was filtered and 1 ml 0.5 M-NaOH was added to 1 ml of the filtrate. One unit of alkaline protease activity is defined as the amount of enzyme that gives an increase in A_{440} of 0.1 in 30 min at 37 °C.

Gelatin-PAGE protease assay. Extracellular proteases produced by *V. alginolyticus* were characterized by PAGE in slab gels containing SDS and gelatin as a copolymerized substrate (Heussen & Dowdle, 1980) as described previously (Hare *et al.*, 1983). After PAGE the gels were washed in Triton X-100 (2.5%, v/v) for 1 h at room temperature to remove the SDS and restore enzyme activity. After incubation in 0.1 M-glycine buffer (pH 9.0) for 3 h at 37 °C bands of proteolytic activity were detected after staining with 0.2% (w/v) amido black.

RESULTS

Isolation of prot-T₁ mutant

Wild-type *V. alginolyticus* colonies produced haloes of clearing on skim milk/peptone agar plates at 30 °C but proteolysis was completely inhibited at 42 °C. The bacteria grew and formed normal sized colonies at 42 °C. After mutagenesis with NTG a single colony was isolated which formed an extensive halo at 30 and 42 °C on the skim milk/peptone plates at a frequency of 10⁻³. The mutant which had overcome protease repression by temperature was designated prot-T₁.

Azocasein protease activity

The production of azocasein alkaline protease activity was determined in liquid media. The growth rates of the wild-type and the prot-T₁ mutant were similar in peptone and glucose/peptone media at 30 °C (Fig. 1). However, a marked difference was observed in the production of alkaline protease activity. The wild-type strain produced very low alkaline protease activity in the peptone medium (Fig. 1). The prot-T₁ mutant produced high protease activity and after 7 h growth in peptone medium approximately 18-fold higher azocasein

Fig. 1. Alkaline protease activity of the wild-type and prot-T₁ mutant of *V. alginolyticus* in peptone medium at 30 °C. Overnight cultures were resuspended in peptone medium with (open symbols) and without (filled symbols) the addition of glucose (0.4%, w/v) at 115 min (arrowed). Growth of the wild-type with (○) and without (●) glucose and alkaline protease activity with (□) and without (■) glucose. Growth of the prot-T₁ mutant with (△) and without (▲) glucose and alkaline protease activity with (▽) and without (▼) glucose. SEMs were from 5 to 10% of reported values.

protease activity than the wild-type strain was obtained. The addition of 0.4% glucose after 2 h growth in peptone broth did not affect the production of protease activity by the wild-type and prot-T₁ mutant (Fig. 1).

Growth of the bacteria in CAM and peptone media or MM before washing, concentration and resuspension in SMM markedly affected the response of the cells to the addition of glucose or glutamine. After pre-growth in MM the prot-T₁ mutant produced higher protease activity than the wild-type and under these conditions protease activity in the prot-T₁ mutant was repressed by glucose and glutamine added at 55 min (Fig. 2). Since the protease activities produced by the wild-type strain under these conditions were so low, it was not possible to determine whether protease production was subject to repression by glucose and glutamine. The addition of glucose or glutamine to concentrated stationary phase cells in SMM, after pre-growth in peptone medium, resulted in very high protease activities in the prot-T₁ cultures (Fig. 3). The addition of glucose or glutamine to wild-type cultures also increased protease production but the final protease activities were approximately fourfold lower than in prot-T₁ cultures. Glucose or glutamine did not stimulate the growth of the concentrated stationary phase cultures (Fig. 3). Long *et al.* (1981) reported the stimulation of protease activity by histidine in *V. alginolyticus* wild-type cells. Similar results were observed in this study with both the wild-type and prot-T₁ strains after pre-growth in peptone medium but the protease activities in the prot-T₁ mutant obtained with histidine were markedly less than those obtained with glucose or glutamine (Fig. 3).

The production of azocasein protease activity by the wild-type in SMM after pre-growth in MM was reduced by approximately 60% by incubation at 37 °C or approximately 80% by incubation without aeration by shaking (Table 1). The high protease activities of the prot-T₁ strain were slightly reduced by these treatments.

Gelatin-PAGE protease assay

Determination of alkaline protease activity by the azocasein assay gave an indication of total protease activity but, as the *V. alginolyticus* strain can produce five different alkaline serine proteases (Hare *et al.*, 1983), it was important to distinguish which proteases were affected in the prot-T₁ mutant. The activities of specific alkaline proteases in SMM after pre-growth in MM were determined by gelatin-PAGE.

At 30 °C the wild-type strain produced the same three major protease bands as reported by Hare *et al.* (1983) (bands 1a, 2 and 3) (Fig. 4). Hare *et al.* (1983) reported that the activities of the

Fig. 2

Fig. 3

Fig. 2. Alkaline protease activity of the wild-type and prot- T_1 mutant of *V. alginolyticus* in SMM after pre-growth in MM. Concentrated stationary phase cultures grown in MM were resuspended in SMM with and without the addition of glucose (0.2%, w/v) or glutamine (0.5%, w/v) at 55 min (arrowed). Optical density of the wild-type and prot- T_1 cultures with (○) and without (●) glucose or glutamine. Alkaline protease activity of the wild-type with glucose (□) or glutamine (▼) and without additions (■). Alkaline protease activity of the prot- T_1 mutant with glucose (△) or glutamine (▽) and without additions (▲). SEMs were from 5 to 10% of reported values.

Fig. 3. Alkaline protease activity of the wild-type and prot- T_1 mutant of *V. alginolyticus* in SMM after pre-growth in peptone broth. Concentrated stationary phase cultures grown in peptone broth were resuspended in SMM with and without the addition of glucose (0.2%, w/v), glutamine (0.5%, w/v), histidine (0.5%, w/v) and $(\text{NH}_4)_2\text{SO}_4$ (100 mM) at 55 min (arrowed). Optical density of the wild-type and prot- T_1 cultures with (○) and without (●) glucose, glutamine, histidine or $(\text{NH}_4)_2\text{SO}_4$. Alkaline protease activity of the wild-type (solid line) and prot- T_1 mutant (dashed line) with glucose (△), glutamine (▽), histidine (□), $(\text{NH}_4)_2\text{SO}_4$ (■) and without additions (▲). SEMs were from 5 to 10% of reported values.

Table 1. *Effect of temperature and aeration on alkaline protease activity in cultures of the wild-type and prot- T_1 mutant of V. alginolyticus*

Bacteria were pre-grown in MM before resuspension in SMM and alkaline azocasein protease activity was assayed 1.5 h after resuspension. Cultures were incubated at 30 or 37 °C with or without aeration by shaking. SEMs were from 5 to 10% of reported values. The real values of the controls were wild-type, 1.14 units ml⁻¹; prot- T_1 , 8.23 units ml⁻¹.

Strain	Temp. (°C)	Aeration	Activity (percentage of control)
Wild-type (control)	30	+	100
Wild-type	37	+	41
	30	—	22
prot- T_1 (control)	30	+	100
prot- T_1	37	+	86
	30	—	92

two minor proteases (bands 4 and 5) varied and that they were always present in small amounts and were often not detected after gelatin-PAGE. In this study the minor protease 4 was detected in the wild-type culture at 30 °C. In comparison with the study by Hare *et al.* (1983), a new minor protease band (1b) was detected (Fig. 4). At 30 °C the protease band profile of the prot- T_1 mutant was similar to that of the wild-type except that the activities of the major proteases (1a, 2 and 3) and the minor protease 5 were enhanced.

Fig. 4. Extracellular protease activity of cultures of the wild-type (lanes *a, c, e, h, j*) and prot- T_1 mutant (lanes *b, d, f, g, i, k*) of *V. alginolyticus* in SMM after pre-growth in MM. The lanes represent supernatant samples (10 μ l) of cultures at 30 °C (*a, b, g*) and at 37 °C (*c, d*); at 30 °C without aeration (*e, f*); at 30 °C with 0.2% glucose (*h, i*); and at 30 °C with 100 mM-(NH₄)₂SO₄ (*j, k*). The arrows indicate the positions of proteases 1a, 1b, 2, 3, 4 and 5.

Although incubation of the wild-type strain at 37 °C or without aeration markedly inhibited azocasein protease activity, protease activity was detected by the gelatin-PAGE technique (Fig. 4). Lack of aeration or incubation at 37 °C caused a marked reduction in the activity of protease 1a and a reduction in the activities of proteases 1b, 2 and 3. In contrast, the activities of proteases of the prot- T_1 mutant were not affected by the lack of aeration. At 37 °C there was a reduction in the activities of the major protease 2 and the variable minor proteases 4 and 5.

The effect of the addition of glucose and (NH₄)₂SO₄ on the activities of proteases was assayed by gelatin-PAGE. The addition of glucose and (NH₄)₂SO₄ to the wild-type cultures at 30 °C resulted in a decrease in the levels of all the proteases (Fig. 4). Activities of the three major proteases 1a, 2 and 3 were still detected but the minor bands 1b, 4 and 5 were either very weak or absent. With the prot- T_1 mutant, the activities of the major proteases 1a and 2 were not affected by the addition of glucose but there was a slight reduction in the activity of protease 3. The addition of (NH₄)₂SO₄ to the prot- T_1 mutant did not affect the activity of protease 1a but decreased the levels of proteases 2, 3, 4 and 5.

Collagenase production

The production of collagenase by wild-type and prot- T_1 cells was determined in peptone medium at 30 or 37 °C, with or without aeration or glucose. The characteristics of collagenase production were identical in the wild-type and prot- T_1 strains and similar to those described previously by Welton & Woods (1973, 1975) and Reid *et al.* (1978, 1980).

DISCUSSION

The regulation of extracellular protease production in the wild-type and prot- T_1 mutant of *V. alginolyticus* differed in a number of respects. The prot- T_1 mutant overproduced the three major proteases and their synthesis was not subject to marked repression by temperature or lack of aeration. The temporal expression of protease production was also altered in the prot- T_1 mutant and in contrast to the wild-type it produced proteases throughout the exponential and stationary growth phases. The high levels of protease production by the prot- T_1 mutant will assist in attempts to clone, express and study the production and secretion of proteases in *Escherichia coli*.

An interesting difference between the two strains was the very high protease activities obtained with stationary phase prot- T_1 cultures supplemented with glucose or glutamine. The pre-growth conditions affected the glucose or glutamine response and an increase in protease activity was only observed when the stationary phase cultures were pre-grown in a rich peptone medium. The addition of glucose or glutamine to stationary phase cultures pre-grown in MM resulted in a decrease in protease activity and a normal glucose (catabolite) or end-product

repression, respectively, of protease activity as reported by Long *et al.* (1981). The reason for the opposite effects of glucose or glutamine depending on the pre-growth medium is not known. Long *et al.* (1981) reported that histidine stimulated protease production in the wild-type strain. The stimulation of protease activity by histidine was not altered in the mutant and the protease activities obtained with histidine were much lower than with glutamine.

Collagenase synthesis was not altered in the prot-T₁ mutant and was repressed by incubation at 37 °C or without aeration. The independent control of collagenase synthesis supports the suggestion that there are no regulatory proteins responsible for the overall control of extracellular protease synthesis by temperature, aeration and growth phase in *V. alginolyticus*.

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