Regulation of Hut Enzymes and Extracellular Protease Activities in *Vibrio alginolyticus* hut Mutants

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(Received 24 November 1981; revised 26 January 1982)

The production of alkaline protease, collagenase and histidine utilization (Hut) enzymes by *Vibrio alginolyticus* wild-type, *hutH1* and *hutU1* strains was investigated. Alkaline protease synthesis was stimulated by histidine and urocanic acid in the wild-type and *hutU1* strains. In the *hutH1* mutant alkaline protease production was stimulated by urocanic acid and not by histidine. The Hut enzymes in the wild-type strain were coordinately induced by histidine. Urocanase and formimino-hydrolase were induced by histidine in the *hutH1* mutant which lacked histidase and was not able to convert histidine to urocanic acid. Collagenase production in peptone medium was inhibited in the *hut* mutants. It is concluded that in *V. alginolyticus* urocanic acid regulates alkaline protease synthesis but that the Hut enzymes are induced by histidine. The involvement of the Hut genetic system in the regulation of alkaline protease and collagenase synthesis is discussed.

INTRODUCTION

Vibrio alginolyticus is an aerobic, halotolerant, Gram-negative bacterium which produces an extracellular collagenase and extracellular alkaline protease enzymes during the stationary growth phase (Welton & Woods, 1973, 1975; Reid *et al.*, 1978, 1980; Long *et al.*, 1981). Common features in the control of collagenase and alkaline protease enzymes by *V. alginolyticus* include end-product and catabolite repression (Reid *et al.*, 1978; Long *et al.*, 1981) and specific regulation by temperature and oxygen (Hare *et al.*, 1981). However, these two enzyme systems differ in that alkaline protease production does not require a specific inducer. Furthermore, alkaline protease production is stimulated by histidine and urocanic acid, whereas collagenase production is inhibited by these substances (Long *et al.*, 1981).

The stimulation of alkaline protease production by histidine and urocanic acid suggests that the histidine utilization (Hut) pathway may be involved in the regulation of the alkaline proteases. In the Hut pathway histidine is converted to glutamic acid in four steps via the following intermediates: urocanic acid plus ammonia, 4-imidazolone-5-propionate and *N*-formimino-glutamate (Smith & Magasanik, 1971). The enzymes involved are histidase, urocanase, amidohydrolase and formimino-hydrolase. In the Gram-negative bacteria *Escherichia coli* and *Salmonella typhimurium*, the Hut enzymes are induced by urocanic acid and histidine which is an inducer by virtue of its conversion to urocanic acid (Smith & Magasanik, 1971; Smith *et al.*, 1971; Hagen & Magasanik, 1973). We have investigated the nature of the involvement of the Hut pathway in the control of alkaline protease production in *V. alginolyticus* by studying the regulation of the Hut enzymes.

METHODS

Bacterial strain, media and chemicals. The collagenolytic strain previously isolated and classified as *Achromobacter iophagus* by Welton & Woods (1973) but recently reclassified as a *V. alginolyticus* strain was used (Reid *et al.*, 1980). The bacterium was maintained on the complex medium of Welton & Woods (1973). The peptone medium,

minimal medium (MM) and succinate minimal medium (succinate MM) have been described previously (Reid *et al.*, 1980; Long *et al.*, 1981). The intermediates of the Hut pathway, L-histidine, urocanic acid, formimino-L-glutamic acid and L-glutamic acid were obtained from Sigma. For the characterization of the *hut* mutants the $(\text{NH}_4)_2\text{SO}_4$ in the MM was replaced by each of the intermediates (0.25%, w/v) of the Hut pathway to give the following media: histidine MM (his-MM), urocanic acid MM (uro-MM), formimino-L-glutamic acid MM (FGA-MM) and the glutamic acid MM (glu-MM). The only nitrogen source in these different media was the Hut pathway intermediate.

Isolation of hut mutants. Hut mutants were obtained by treating exponential broth cultures with 100 μg *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine ml^{-1} for 15 min at 30 °C (Adelberg *et al.*, 1965) in 0.1 M-Tris/maleic acid buffer (pH 6.0) containing 0.4 M-NaCl, 0.4 mM-MgSO₄, 7.5 mM- $(\text{NH}_4)_2\text{SO}_4$ and 2 mM-sodium acetate. The treated cultures were incubated in MM at 30 °C for 24 h and the proportion of *hut* mutants was increased by resuspending washed cells in his-MM containing ampicillin (20 μg ml^{-1}). The surviving cells were plated on to MM and the *hut* mutants identified by replica plating on to his-MM, uro-MM, FGA-MM and glu-MM plates.

Growth conditions for enzyme studies. The growth conditions for the production of collagenase and alkaline protease enzymes by stationary phase *V. alginolyticus* cells in succinate MM have been described previously (Reid *et al.*, 1980; Long *et al.*, 1981). The production of the Hut enzymes in stationary phase cells was investigated using the same growth conditions. Regulation of the Hut enzymes was also investigated in exponential MM cultures. The culture supernatants were assayed for collagenase or alkaline protease activities and the cells assayed for Hut enzymes.

Enzyme assays. All enzyme assays were performed at standardized cell densities. Each sample was assayed in duplicate and experiments were repeated at least three times. Collagenase was assayed using the synthetic collagenase substrate phenyl-azobenzyloxycarbonyl-L-propyl-L-leucyl-glycyl-L-propyl-D-arginine (Fluka, Buchs, Switzerland) as previously described (Wünsch & Heidrich, 1963; Reid *et al.*, 1978). Collagenase activity was expressed as nkat ml^{-1} where 1 kat is the amount of enzyme that converts 1 mol substrate s^{-1} (Florkin & Stotz, 1973). Alkaline protease activities were assayed using the synthetic substrate azocasein (Sigma) (Long *et al.*, 1981). One unit of alkaline 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 40 °C.

Histidase, urocanase and formimino-hydrolase were assayed by the methods of Lund & Magasanik (1965) and Chasin & Magasanik (1968). Extracts (50 μl) of toluenized cells (0.5 ml culture added to 0.5 ml toluene) were assayed for histidase at 37 °C as described by Chasin & Magasanik (1968). Urocanase and formimino-hydrolase were assayed in sonicated extracts of cultures. Samples (40 ml) of cultures were sedimented by centrifugation, washed with 40 ml 0.05 M cold (4 °C) potassium phosphate buffer (pH 7.4), resuspended in 2 ml of the same buffer and sonicated for 2 min at 20 kHz on ice. The resulting suspension was clarified by centrifugation in a microfuge (Beckman) at 11000 rev. min^{-1} for 1 min. Samples (0.1 ml) of the supernatants were assayed for urocanase as described by Chasin & Magasanik (1968) and formimino-hydrolase by the method of Lund & Magasanik (1965) as modified by Chasin & Magasanik (1968). One unit of histidase or urocanase activity is defined as the amount of enzyme that gives an increase or decrease, respectively, in absorbance of 0.1 at 277 nm in 15 min at 37 °C. One unit of formimino-hydrolase activity is defined as the amount of enzyme that gives a decrease in absorbance of 0.1 at 485 nm in 30 min at 37 °C.

RESULTS

Isolation of hut mutants

Two groups of *hut* mutants were isolated. The first group were histidase mutants (*hutH*) and were characterized by no growth on his-MM but they were able to grow on uro-MM, FGA-MM and glu-MM. The second group were urocanase mutants (*hutU*) which were unable to grow on uro-MM but grew on his-MM, FGA-MM and glu-MM. Growth on his-MM was sparse and was presumably due to the utilization of ammonia which was produced with urocanic acid from histidine by histidase (Smith & Magasanik, 1971). A *hutH* mutant (*hutH1*) and a *hutU* mutant (*hutU1*) were chosen for further study. The Hut enzyme activities of these two mutants were determined and they were found to lack histidase and urocanase enzyme activities, respectively (Table 1).

Effect of Hut intermediates on the induction of Hut enzymes

The effect of intermediates in the Hut pathway on the production of histidase, urocanase and formimino-hydrolase by *V. alginolyticus* wild-type cells was investigated in stationary phase cultures (conditions for protease production) and in exponential phase cultures. In stationary

Table 1. Production of histidase, urocanase and formimino-hydrolase enzymes by *V. alginolyticus* *hut* mutants

Stationary phase cultures were assayed for histidase, urocanase and formimino-hydrolase enzymes 4 h after resuspension in MM and his-MM at standardized cell densities.

Strain	Medium	Enzyme activity (units ml ⁻¹)		
		Histidase	Urocanase	Formimino-hydrolase
Wild-type	MM	0.30	0.20	0.03
Wild-type	His-MM	3.23	2.57	0.46
<i>HutH1</i>	His-MM	0.10	2.71	0.40
<i>HutU1</i>	His-MM	1.97	0.12	0.28

Table 2. *Hut* mutants and the production of protease and *Hut* enzymes

Protease and *Hut* enzyme activities were determined in stationary phase cultures 4 h after resuspension at standardized cell densities in MM, his-MM and uro-MM. Protease activities were expressed as a percentage of the wild-type activity in MM and *Hut* enzyme activities as a percentage of wild-type activity in his-MM.

Strain	Protease activity (%)			Histidase activity (%)		Urocanase activity (%)		Formimino-hydrolase activity (%)	
	MM	His-MM	Uro-MM	His-MM	Uro-MM	His-MM	Uro-MM	His-MM	Uro-MM
Wild-type	100	165	148	100	3	100	8	100	15
<i>HutH1</i>	4	7	100	3	4	105	7	87	13
<i>HutU1</i>	13	170	148	61	3	5	8	61	11

phase cells the *Hut* enzymes were coordinately induced by histidine only. Urocanic acid and formimino-L-glutamic acid did not induce the *Hut* enzymes. Levels of activity of histidase, urocanase and formimino-hydrolase in the presence of histidine were 0.99, 0.74 and 0.67 units ml⁻¹ h⁻¹, respectively, measured over 4 h. In uninduced control cultures in the absence of histidine or in the presence of either urocanic acid or formimino-L-glutamic acid, the levels of activity for histidase, urocanase and formimino-hydrolase varied between 0.07–0.22, 0.14–0.18 and 0.08–0.28 units ml⁻¹ h⁻¹, respectively, measured over 4 h. Similar results were obtained with exponential phase cultures.

Hut mutants and the production of alkaline protease and *Hut* enzymes

The production of alkaline protease and *Hut* enzymes by stationary phase *V. alginolyticus* wild-type, *hutH1* and *hutU1* strains was determined in MM, his-MM and uro-MM (Table 2). The alkaline protease activities of the wild-type strain were stimulated by histidine and urocanic acid. This was in agreement with our previous results (Long *et al.*, 1981). The *hutH1* mutant produced very low levels of alkaline protease activity in MM and his-MM but showed significant alkaline protease activities in uro-MM. Although the *hutH1* mutant lacked histidase activity, the urocanase and formimino-hydrolase enzymes were induced by histidine. The *Hut* enzymes were not induced by urocanic acid in the *hutH1* mutant. The alkaline protease activities of the *hutU1* mutant were stimulated by histidine and urocanic acid and similar levels of activity as the wild-type strain under the same conditions were obtained. In the absence of histidine and urocanic acid the *hutU1* mutant produced low levels of protease activity. The *hutU1* mutant lacked urocanase activity and histidase and formimino-hydrolase enzymes were induced by histidine but not by urocanic acid.

Hut mutants and collagenase production

The production of collagenase by the *V. alginolyticus* wild-type strain and the *hutH1* and *hutU1* mutants was determined at the same cell densities 4 h after resuspension in peptone medium. The wild-type cells produced 3.84 nkat ml⁻¹ of collagenase. The *hutH1* and *hutU1*

mutants produced 0.65 and 0.54 nkat ml⁻¹ of collagenase, respectively (17 and 14% of the wild-type collagenase activity, respectively).

DISCUSSION

The stimulation of alkaline protease synthesis by urocanic acid and analogy with the control of the Hut operon in *E. coli* and *S. typhimurium* (Smith & Magasanik, 1971; Hagen & Magasanik, 1973) suggested that urocanic acid is the inducer of alkaline protease synthesis. Histidine is an inducer by virtue of its conversion to urocanic acid. Studies with the *hutH1* and *hutU1* mutants confirmed these suggestions since alkaline protease production in the *hutH1* mutant was stimulated by urocanic acid but not by histidine. As would be expected, alkaline protease activity in the *hutU1* mutant was stimulated by both histidine and urocanic acid. However, a surprising discovery was that in the *V. alginolyticus* strain histidine (and not urocanic acid) is the inducer of the Hut enzymes. This can be concluded from the results of induction experiments with Hut intermediates and from the induction of urocanase and formimino-hydrolase by histidine in the *hutH1* mutant which cannot convert histidine to urocanic acid. The production of amidohydrolase which is the third enzyme in the Hut pathway, was not investigated because of the non-availability and instability of 4-imidazolone-5-propionate (Smith *et al.*, 1971).

Although *V. alginolyticus* is a Gram-negative bacterium, regulation of the Hut pathway is not similar to that in *E. coli* and *S. typhimurium* (Smith & Magasanik, 1971; Hagen & Magasanik, 1973). However, it is similar to the Gram-positive bacterium *Bacillus subtilis* in that in both bacteria histidine is the inducer of the Hut enzymes (Chasin & Magasanik, 1968). The *V. alginolyticus* strain and *Bacillus* strains are also similar in other respects: they produce true extracellular proteases during the stationary growth phase (Priest, 1977); protease production is rifampin-insensitive (Both *et al.*, 1972; O'Connor *et al.*, 1978; Reid *et al.*, 1980) and it is subject to end-product repression and catabolite repression which is not relieved by cAMP (Glenn, 1976; Priest, 1977; Reid *et al.*, 1978; Long *et al.*, 1981).

The decreased constitutive level of alkaline protease synthesis in the *hutU1* mutant in MM is interesting since elevated constitutive protease synthesis would normally have been expected. Urocanic acid would be expected to accumulate in the *hutU1* mutant since histidine made endogenously would give rise to urocanic acid which cannot be metabolized and would therefore induce alkaline protease synthesis. This suggests that the regulation of alkaline protease synthesis by the Hut operon is complex and does not only involve urocanic acid concentrations. Similarly, the involvement of the Hut operon in the regulation of collagenase synthesis is also suggested by the inhibition of collagenase synthesis in the *hut* mutants.

G. Bowden acknowledges a postgraduate research bursary from the South African Council for Scientific and Industrial Research.

REFERENCES

- ADELBERG, E. A., MANDEL, M. & CHEN, G. C. C. (1965). Optimal conditions for mutagenesis by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine in *Escherichia coli* K12. *Biochemical and Biophysical Research Communications* **18**, 788-795.
- BOTH, G. W., MCINNES, J. L., HANLON, J. E., MAY, B. K. & ELLIOTT, W. H. (1972). Evidence for an accumulation of messenger RNA specific for extracellular protease and its relevance to the mechanism of enzyme secretion in bacteria. *Journal of Molecular Biology* **67**, 199-217.
- CHASIN, L. A. & MAGASANIK, B. (1968). Induction and repression of the histidine-degrading enzymes of *Bacillus subtilis*. *Journal of Biological Chemistry* **243**, 5165-5178.
- FLORKIN, M. & STOTZ, E. M. (1973). *Comprehensive Biochemistry*, vol. 13, 3rd edn. Amsterdam: Elsevier.
- GLENN, A. R. (1976). Production of extracellular proteins by bacteria. *Annual Review of Microbiology* **30**, 41-62.
- HAGEN, D. C. & MAGASANIK, B. (1973). Isolation of the self regulated repressor protein *hut* operons of *Salmonella typhimurium*. *Proceedings of the National Academy of Sciences of the United States of America* **70**, 808-812.
- HARE, P., LONG, S., ROBB, F. T. & WOODS, D. R. (1981). Regulation of exoprotease production by temperature and oxygen in *Vibrio alginolyticus*. *Archives of Microbiology* **130**, 276-280.
- LONG, S., MOTHIBELI, M. A., ROBB, F. T. & WOODS, D. R. (1981). Regulation of alkaline protease activity by histidine in a collagenolytic *Vibrio alginolyticus* strain. *Journal of General Microbiology* **127**, 193-199.

- LUND, P. & MAGASANIK, B. (1965). *N*-formimino-L-glutarate-formimino hydrolase of *Aerobacter aerogenes*. *Journal of Biological Chemistry* **240**, 4316–4319.
- O'CONNOR, R., ELLIOT, W. H. & MAY, B. K. (1978). Modulation of an apparent mRNA pool for extracellular protease in *Bacillus amyloliquefaciens*. *Journal of Bacteriology* **136**, 24–34.
- PRIEST, F. G. (1977). Extracellular enzyme synthesis in the genus *Bacillus*. *Bacteriological Reviews* **41**, 711–753.
- REID, G. C., ROBB, F. T. & WOODS, D. R. (1978). Regulation of extracellular collagenase production in *Achromobacter iophagus*. *Journal of General Microbiology* **109**, 149–154.
- REID, G. C., WOODS, D. R. & ROBB, F. T. (1980). Peptone induction and rifampicin insensitive collagenase production by *Vibrio alginolyticus*. *Journal of Bacteriology* **142**, 447–454.
- SMITH, G. R. & MAGASANIK, B. (1971). The two operations of the histidine utilisation system in *Salmonella typhimurium*. *Journal of Biological Chemistry* **246**, 3330–3341.
- SMITH, G. R., HALPERN, J. S. & MAGASANIK, B. (1971). Genetic and metabolic control of enzymes responsible for histidine degradation in *Salmonella typhimurium*. *Journal of Biological Chemistry* **246**, 3320–3329.
- WELTON, R. L. & WOODS, D. R. (1973). Halotolerant collagenolytic activity of *Achromobacter iophagus*. *Journal of General Microbiology* **75**, 191–196.
- WELTON, R. L. & WOODS, D. R. (1975). Collagenase production by *Achromobacter iophagus*. *Biochimica et biophysica acta* **384**, 228–234.
- WÜNSCH, E. & HEIDRICH, H. G. (1963). Zur quantitativen bestimmung der kollagenase. *Hoppe-Seyler's Zeitschrift für physiologische Chemie* **333**, 149–151.