Regulation of extracellular proteolytic enzyme synthesis in vibrio SA1.

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SUMMARY

In Nature mineralization of plant and animal remains is initiated by hydrolysis of polymeric organic compounds into monomers, a process that is mainly brought about by bacteria and fungi. Many of these microorganisms are able to synthesize extracellular enzymes which can hydrolyse biopolymers and although various regulatory mechanisms are known to be involved in the control of the synthesis of these enzymes, only a few detailed studies on their regulation have been reported (see chapter II).

In this thesis the regulation of the synthesis of two extracellular proteolytic enzymes, namely an endopeptidase and an aminopeptidase, by a marine bacterium (strain SA1) are described. The results of the characterization of strain SA1 are presented in chapter III. The bacterium, which was isolated from the gills of a plaice, is usually rod-shaped, though curved cells were observed under certain conditions. Strain SA1 is Gram-negative, motile by means of one polar flagellum and facultatively anaerobic. It is sensitive to the vibriostatic compound 0/129. On the basis of its morphological and physiological characteristics, together with the low G+C content of its DNA, strain SA1 was assigned to the genus *Vibrio*. The organism could not be identified as a typical representative of one of the known *Vibrio* species. It has characteristics intermediate between those of *Vibrio parahaemolyticus*, (biotype 1), *Vibrio anquillarum* and *Vibrio fischeri* and in view of the current discussion with respect to the taxonomy of the genus, it did not seem appropriate to suggest classification of *Vibrio* SA1 as a new species.

In chapter IV results are described which were obtained in studies on the regulation of the production of the two proteases during growth of *Vibrio* SA1 in batch culture. When the organism was grown in peptone media, a rapid accumulation of both enzymes was observed in the late exponential and early stationary growth phase. During growth in mineral media, with lactate as the only carbon- and energy source, protease production was only observed when amino acids were present in the growth medium. These compounds induced protease production when added in low concentrations (below 5 mM), but progressive inhibition was observed at higher concentrations. Also, repression of protease production by lactate, succinate and glucose was observed during growth in peptone media, indicating that catabolite repression is involved in the regulation of protease synthesis. That, in spite of the presence of lactate, protease production was observed during growth of *Vibrio* SA1 in mineral media, appeared to be a consequence of the low iron content of the medium. Growth curves of *Vibrio* SA1 on such media showed a period of linear growth, during which protease production was observed. From these results it was concluded that when a growth limitation was imposed on the organism by a low iron content of the growth medium, catabolite repression by lactate could be relieved. Similar results were obtained when the organism was grown in media in which growth was limited by a low dissolved oxygen tension. Under these conditions a high rate of protease synthesis was found, which was immediately repressed when the oxygen limitation was released. Addition of glucose to oxygen-limited cultures also caused a severe repression of protease production. The results indicated that in *Vibrio* SA1 the

nthesis of the two proteases is controlled independently and at an intermediate of the energy metabolism or a compound associated with it may play a role in the regulation of protease synesis. Studies on the effect of inhibitors of protein synthesis protease production indicated that *Vibrio* SA1 does not contain large pool of m-RNA coding for these enzymes.

Additional information on the induction and repression mechanms involved in the regulation of protease production by *Vibrio* 1 was collected by means of continuous culture experiments, the sults of which are described in chapter V. During growth of *brio* SA1 in a lactate-limited chemostat in the presence of 2 mM enylalanine as inducer, the rate of production of the two prolytic enzymes was dependent upon the dilution rate. An optim in the rate of synthesis of both proteases was observed at a lution rate of 0.23 h^{-1}, and enzyme production only occurred tween dilution rates of 0.06 and 0.45 h^{-1}. Without inducer a w aminopeptidase production was found with an optimum at 0.18 $^{-1}$, but only an insignificant amount of endopeptidase activity s detectable at dilution rates between 0.05 h^{-1} and 0.18 h^{-1}. idence was obtained that the increase in enzyme production with creasing dilution rates over the range 0.06 to 0.23 h^{-1} may be plained by an increase of the degree of saturation of inducer tes by the inducer. At higher dilution rates (above 0.23 h^{-1}) e observed progressive decrease in the rate of protease production was ascribed to an increasing effect of catabolite repression the increasing concentration of the growth substrate. In these udies it was established that addition of cyclic AMP could not elieve catabolite repression caused by glucose or lactate. Repression of protease production was also found in the presence of gher concentrations (above 5 mM) phenylalanine and other amino cids, and by ammonium ions. In these experiments additional evience was obtained which suggested that the energy-status of the ell may play an important role in the regulation of protease ynthesis by *Vibrio* SA1.

In chapter VI the isolation, purification and characterization f the endo- and aminopeptidase of *Vibrio* SA1 are described. The ndopeptidase was purified by ammonium-sulphate precipitation, el filtration and affinity chromatography. This protease had a olecular weight of approximately 31,000, a pH optimum at 7.8 and temperature optimum at 50°C. The enzyme was found to be thermoabile. The aminopeptidase was purified by ammonium-sulphate preipitation, gel filtration and preparative polyacrylamide gel lectrophoresis. This protease had a molecular weight of approxiately 21,000, a pH optimum at 8.6 and a temperature optimum at °C. Both the endopeptidase and the aminopeptidase were sensiive to EDTA; reactivation occurred by Ca^{2+}, Zn^{2+} and Mg^{2+} ions. oth proteases appeared to be specific for the hydrolysis of eptide bonds involving bulky hydrophobic amino acid residues, nd in this respect they showed a complementary specificity. Thus he combined action of the two enzymes on a protein molecule reults in the production of a mixture of amino acids and short eptides.

From the results presented in this thesis, it is concluded that he synthesis of the two extracellular proteolytic enzymes of *ibrio* SA1 is regulated in a rather complex way. Control mechansms such as induction, catabolite repression and endproduct re-

pression are involved, whose expression is dependent on the metabolic status of the cell which in turn is a reflection of the chemical composition of the environment. The significance of these control mechanisms for the organism may be summarized as follows: When the organism is able to grow logarithmically, both under conditions when all nutrients are available in excess and during carbon-limited growth in the absence of inducer compounds the two proteases are produced at a low rate. When protein molecules are present in the environment, small amounts of amino acids will be liberated by the action of these enzymes and in this way the organism obtains a signal, indicating the presence of potentially useful biopolymer. Under appropriate conditions, namely when growth is limited by the availability of carbon skeletons for growth and energy production, the amino acids and/or peptides produced in this way, will cause induction of enzyme synthesis. Because *Vibrio* SA1 is able to utilize a variety of amino acids as substrates, this ensures that appropriate carbon- and energy sources are supplied to the organism, thereby enabling it to continue to grow. However, when in some way or other a surplus of carbon compounds becomes available, allowing a high rate of energy generation, protease synthesis is no longer essential and is repressed. These characteristics of the regulation of the synthesis of proteolytic enzymes in *Vibrio* SA1 strongly suggest that the main strategy of the control operations involved in the production of these extracellular enzymes, is to ensure the further supply of (carbon- and) energy sources from protein substrates under conditions of (carbon- and) energy limitation.

The results obtained in this study indicated that protease synthesis in *Vibrio* SA1 is regulated in an efficient manner. Therefore it was somewhat surprising to find that during growth of *Vibrio* SA1 in continuous culture in runs lasting for many months under conditions where the enzymes were produced but were not required for growth, only in one instance, and after 3.5 months cultivation, a mutant was selected, which showed a much lower rate of protease production than the wild type. That selection of such a mutant was not observed more often, may be due to the fact that protease production represents only a very small amount of protein. On the other hand the frequency of production of protease-negative mutants may have been extremely low. In any case, the pressure to select for mutant organisms, which is known to be very effective in continuous culture, generally did not lead to selection of mutants of *Vibrio* SA1 which no longer synthesized the proteases under conditions where they were expendable. In view of this, it was indeed surprising to find that the synthesis of these enzymes is so carefully controlled in *Vibrio* SA1.

It was of interest to observe that the energy status of the cell appeared to be an important parameter in the regulation of protease synthesis by *Vibrio* SA1. Energy-limited growth could relieve catabolite repression as well as endproduct repression of protease synthesis, indicating that in the two repression mechanisms a common intermediate may function as a regulatory molecule. However, the results did not enable us to establish the nature of this intermediate in *Vibrio* SA1. Also it was not possible to describe the precise molecular mechanisms involved in the regulation of protease synthesis. However, in principle the results are compatible with the model that has been put forward to ex-

n the regulation of β-galactosidase synthesis in *Escherichia*
. Further information on the precise nature of the molecular
rol mechanisms involved in the regulation of the synthesis
he two proteases in *Vibrio* SA1 can only be expected to come
detailed genetic work with the organism.