

A REVIEW OF PROTEASE SECRETION INSTABILITY IN *Proteus mirabilis*

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ABSTRACT

Proteus mirabilis secretes a single protease, an alkaline metallo-enzyme. Some physiological factors affecting the pattern of protease secretion in isolated clones were reported. More than hundred different clinical isolates have been tested for the ability to secrete protease. Some strains inherited the protease determinant as a stable trait (called stable strains). Other strains exhibited a quite unstable protease phenotype (designated unstable strains) with a loss of the protease production of up to 10% of the progeny colonies. Reversion of the protease-minus phenotype was never observed. Introduction of R-factors into protease-producing cells reduced the amount of protease-activity to about 50% suggesting an interference with the expression and/or secretion of the protease. Although several lines of data seems to indicate that the protease locus is extrachromosomally located, attempts to demonstrate a plasmid in several unstable strains has been failed. A model is presented which can explain the various findings.

The *Proteus* group consists of facultatively aerobic Gram-negative, rod-shaped motile bacteria, commonly found in soil, sewage and manure. They are found with some frequency in normal human faeces, but often in greatly increased numbers in individuals receiving antibiotic therapy or during diarrheal diseases due to other organisms. *Proteus* spp. are frequent causes of urinary tract infection and are also involved in other, often serious infections (Sonnenwirth, 1980). They rapidly digest and liquefy gelatin by the secretion of "gelatinase", a proteolytic exoenzyme which acts on the peptide bonds of gelatin. Since gelatinase has also been shown to be effective in degrading other proteins such as casein and albumin, it has been designated as protease.

Since bacteria may or may not digest and liquefy gelatin, gelatin stab cultures give information in the form of growth along the line of inoculation in the indigested gelatin or the degree of liquefaction extending from the line of the stab. This morphological property is remarkably constant in each species of bacterium and readily allows recognition of typical forms of growth (Kelly and Hite, 1955).

Proteases of some bacteria and molds are used for bating hides in leather manufacturing, a process in which organic tissue is removed from the skin yielding a finer texture and grain in the product. Other proteases are used as liquid glues, laundry pre-soaks, meat tenderizers, and as spot removers. Still others are employed to clean dead tissue fragments from wounds, to clarify beer, and to degum silk before processing (Alcama, 1983). The economic and ecological significance, the "state of art" for increasing yields of extracellular enzymes and the significance of advances in microbial genetics, molecular biology and biotechnology have been reviewed (Wodzinsky, 1979; Eveleigh and Montencourt, 1979; Taylor and Richardson, 1979; Ramaley, 1979; Workman *et al.*, 1986).

Protease of P. mirabilis

P. mirabilis secretes only one protease (Mills and Wilkins, 1958), an alkaline metallo-enzyme (Hampson *et al.*, 1963). Even though extracellular proteases have been studied in several bacterial species, especially in the *Bacillus* group (Priest, 1977; Volesky and Luong, 1985), little has been done with *P. mirabilis*.

Some factors affecting the patterns of protease secretion in isolated clones from *P. mirabilis* during growth or in buffer have been reported (Bonato *et al.*, 1982). Clonal isolates grown in complete medium secrete protease exclusively at the transition from the exponential to early stationary phase of growth. Washed cell suspensions harvested at this point rapidly synthesize and secrete the enzyme. No intracellular proteolytic activity was detected in extracts of actively secreting cells. There is a large body of evidence suggesting that exoenzyme production involves *de novo* protein synthesis rather than activation of pre-existing precursors (Glen, 1976). In fact, by using transcription and protein synthesis inhibitors in *P. mirabilis* cultures. Bonato *et al.* (1982) observed that such precursors do not accumulate inside the cell, suggesting that extracellular enzymatic activity is dependent on *de novo* protein synthesis, and that no mRNA pool is established. Furthermore, protease synthesis is regulated by catabolic repression as was demonstrated by the fact that the addition of cAMP reversed glucose repression of protease synthesis. The effect of rifampicin on protease synthesis has reinforced the idea that glucose or some of its metabolites are acting at the level of transcriptional control. Gelatin peptides slightly stimulated protease production in this organism, and the levels of proteolytic activity in the supernatant were higher when amino acids were present in the medium (Bonato *et al.*, 1982).

Although the mechanisms that control exoenzyme secretion in most bacterial systems are not fully understood, repression of these enzymes in the exponential phase is often observed (Pollock, 1962; Glen, 1976). Glucose repression of extracellular enzyme production has been described in some organisms, e.g. in *Aeromonas* (Litchfield and Prescott, 1970), *Arthrobacter* (Hofstein and Tjeder, 1965), *Bacillus* spp. (Heineken and O'Connor, 1972) and *Pseudomonas* (Stinson and Merrick, 1974; Boethling, 1975). Amino acids may also repress protease secretion in some organisms (Hofstein and Tjeder 1965; May and Elliot, 1968; Heineken and O'Connor, 1972), whereas amino acids or peptides seem to induce protease secretion in other species or genera (e.g. Din and Chaloupka, 1969; Murakami *et al.*, 1969).

Instability of protease secretion in P. mirabilis

Divergence between strains originating from the same pure culture is often expressed by variation in biochemical properties. As Edwards and Ewing (1955) pointed out in their biochemical analysis of principal groups of enteric bacteria, some cultures of *Proteus* liquefied gelatin and some others not. According to Topley *et al.* (1953) some strains of *P. mirabilis* maintained in the laboratory for some time liquefied gelatin more slowly, with weeks needed for the process to be completed, or, in some cases, completely lost this ability.

Until recently, the nature of this old phenomenon had not called attention of the microbiologists. Various aspects of this instability characterized by spontaneous and induced loss of protease production by individual clones of *P. mirabilis* strains have been now understood in our laboratory. Some newly obtained clinical isolates of *P. mirabilis* displayed the ability to secrete protease as a relatively stable character, where all or most individuals secreted protease (P^+ colonies). However, some cultures secreted protease as an unstable character, e.g. a large number of individuals in the population do not secrete any enzyme (P^-). Thus, it is possible to postulate the existence of stable and unstable *P. mirabilis* strains in terms of protease secretion (Costa *et al.*, 1982a).

This instability does not occur with other proteolytic bacterial species, such as *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Bacillus cereus* and *Staphylococcus aureus*. This instability has not been observed with respect to other biochemical features found in *P. mirabilis*, such as urease synthesis and hydrogen sulfide production. The occurrence of P^- cells in unstable populations of *P. mirabilis* can be greatly increased by prolonged cultivation on either nutrient agar or in nutrient broth and through successive transfers of overnight cultures in nutrient broth. Some P^+ colonies newly isolated from unstable strains may have P^- cells in their populations. P^+ colonies, isolated from stable strains and stored for several months, may show some degree of instability; however, P^+ colonies, isolated under the same conditions from unstable strains, exhibited increased instability. Nevertheless, P^+ individuals en-

dowed with great stability may be found in both stable and unstable strains (Costa *et al.*, 1982a).

Some strains of *P. vulgaris* also displayed the ability to secrete protease as an unstable character. It is possible that in both *P. vulgaris* and *P. mirabilis*, the instability of protease secretion relies on a similar mechanism (Bianco *et al.*, 1985).

Even though P⁺ and P⁻ cells displayed comparable generation times during a short cultured period in media with or without gelatin, a large outgrowth of P⁻ from P⁺ cells was observed when unstable *P. mirabilis* strains were submitted to successive daily cultures in gelatin-free nutrient broth (Costa *et al.*, 1982b). It is possible that, in a protein habitat, unstable populations will selectively maintain the protease trait, and that this enzyme could provide *P. mirabilis* with some kind of selective advantage. In fact, P⁺ and P⁻ cells which originated from the same population behave differently when intraperitoneally inoculated into mice. The protease-secreting cells turned out to be more efficient in lowering the survival rate of inoculated mice as compared to the non-secreting cells (Costa *et al.*, 1981). These data may eventually explain those of Topley *et al.* (1953). According to these authors, highly virulent *P. mirabilis* cultures exist which determine the animal's death during the first few hours, and less virulent ones which cause death only after one week or more after inoculation. Thus, stable or slightly unstable cultures, consisting almost exclusively of protease-secreting cells, would be expected to be more virulent than the unstable ones, whose populations, for the most part, or even totally, would be expected to be non-protease-secreting cells.

Influence of R plasmids on protease secretion

There are data suggesting the influence of plasmids on extracellular protease production in *P. mirabilis*. Hesselwood and Smith (1974) described that plasmid R-1818 (also known as R6K) increased swarming and abolished proteolytic enzyme secretion by *P. mirabilis* strain E-67, and that plasmid R-TEM (also known as R46) reduced swarming, increased proteolytic enzyme secretion by this organism and rendered it susceptible to sodium deoxycholate.

In contrast, it has been observed that isolated P⁺ clones from *P. mirabilis* strain L68-79 harbouring an uncharacterized R plasmid conferring a chloramphenicol (Cm), tetracycline (Tc), kanamycin (Km), ampicillin (Ap), streptomycin (Sm), trimethoprim (Tp), and mercury bichloride (Hg) resistance pattern showed only 50% of the proteolytic activity found in *P. mirabilis* L68-73 P⁺ clonal isolates without any R plasmid (Bonato *et al.*, 1982).

The reduced protease activity found in the *P. mirabilis* L-68-79 P⁺ clones, harbouring the R plasmid was confirmed for another *P. mirabilis* strain; N17G P⁺ (Costa *et al.*, 1985). This strain received the RP4 plasmid (*IncP* group; resistance to Km, Ap, and Tc) from *E. coli* strain K53 and was named N17G-22-P⁺ (RP4). The

protease trait could be mobilized by RP4 from the donor strain N17G-22-P⁺ to the recipient strain N17G-P⁻ resistant to rifampicin (Rif) and (Sm) giving transconjugants designated N17-G-P⁺ T.

The extracellular proteolytic activities of strains N17G-P⁺, N17G-22-P⁺ (RP4) and N17G-P⁺ T were compared. Upon transfer of the RP4 plasmid, the level of proteolytic activity detected in the supernatant of N17G-22-P⁺ (RP4) was reduced to 54% compared to the parental strain N17G-P⁺. The transconjugant N17G-P⁺ T, obtained by conjugation between strains N17G-22-P⁺ (RP4) and N17G-P⁻ Rif, Sm exhibited only 56% of the proteolytic activity of the parental strain N17G-22-P⁺ (RP4).

The data obtained with cells of the N17G strains agree with those obtained with *P. mirabilis* L68 suggesting that reduced levels of proteolytic activity might be a phenomenon correlated with the presence of R plasmids in *P. mirabilis*. The precise mechanism underlying the interference of R plasmids with proteolytic activity remains to be elucidated. The results suggest a mechanism whereby the plasmids interfere with the synthesis and/or secretion of the protease. The latter possibility is substantiated by the observation that the presence of R plasmids confer a greater sensitivity to sodium deoxycholate to all *P. mirabilis* strains irrespective whether they are P⁺ or P⁻. This suggests that some alteration in the bacterial envelope might be involved (Costa *et al.*, 1984a).

Possible mechanism of genetic instability

What might be the molecular basis of genetic instability of protease production in *P. mirabilis*? *P. mirabilis* P⁺ and P⁻ clones when lysogenized by bacteriophages have their protease production pattern unmodified. Therefore, it is unlikely that phages are involved in protease instability (unpublished data from our laboratory).

Some results seem to indicate that the genetic information for protease production is extrachromosomally located. In fact, P⁻ populations constituted by individuals that lost the ability to secrete protease, were not capable of reverting to the original phenotype (P⁺), even under the action of chemical and physical mutagens (Costa *et al.*, 1982a). Furthermore, some well known curing agents, such as ethidium bromide, acriflavine and mitomycin C, caused increased occurrence of P⁻ cells in the unstable *P. mirabilis* populations, but not in stable ones (Costa *et al.*, 1984b). The same occurred when subinhibitory concentrations of sodium dodecyl sulfate (SDS) were used although SDS seemed to select pre-existing non-secreting cells because the secreting cells were more sensitive to it (Costa *et al.*, 1984c).

DNA from P⁺ and P⁻ cells was analyzed by electrophoresis in agarose gels. We could not detect any plasmid DNA band correlated with protease secretion. The molecular nature of *P. mirabilis* protease instability remains to be elucidated.

To explain these results we suggest the following mechanism. The protease gene is part of a chromosomal DNA sequence which can be excised from the chromosome forming a plasmid-like structure unable to replicate. There are three different possibilities for the further fate of the excised DNA. (1) it reintegrates at its original site; (2) it integrates at another site within the chromosome or within a plasmid present in the cell; (3) it fails to reintegrate and is lost from the cell since it cannot be replicated. Several lines of data support this model. First, when unstable P^+ cells are treated with a curing agent, the protease determinant is lost at a much greater frequency. Second, P^+ unstable cells give rise to derivatives which have acquired a new auxotrophic mutation. These mutations could originate from the insertion of the protease determinant at another site within the bacterial chromosome. To verify this model, experiments are under progress to clone the protease gene. Then, part of the gene will be used as a probe to determine the location of the coding sequence within the different stable and unstable P^+ and within P^- cells.

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RESUMO

Proteus mirabilis secreta uma única protease que é uma metalo-enzima alcalina. Descrevemos aqui alguns fatores fisiológicos que afetam a secreção de protease em clones isolados. Mais de cem diferentes isolados clínicos foram examinados quanto a capacidade de secretar protease. Verificou-se que algumas linhagens herdaram o determinante de protease como um caráter estável (são denominadas linhagens estáveis); outras linhagens exigem um fenótipo muito instável (denominadas linhagens instáveis) com 10% das colônias-filhas perdendo o caráter de produção de protease.

Nunca foi observada a reversão do fenótipo protease negativa. Por outro lado, a introdução de fatores R em células produtoras de protease reduziu a atividade proteásica em cerca de 50%, o que sugere uma interferência na expressão e/ou secreção de protease. Embora haja evidências de que um locus da protease tenha localização extracromossômica, não foi demonstrada a presença de plasmídios nas linhagens instáveis. Um modelo que pode explicar os vários dados é apresentado.

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