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Genetic Analysis of Extracellular Proteins of *Serratia marcescens*

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***Serratia marcescens*, a gram-negative enteric bacterium, is capable of secreting a number of proteins extracellularly. The types of activity found in the growth media include proteases, chitinases, a nuclease, and a lipase. Genetic studies have been undertaken to investigate the mechanisms used for the extracellular secretion of these exoproteins by *S. marcescens*. Many independent mutations affecting the extracellular enzymes were isolated after chemical and transposon mutagenesis. Using indicator media, we have identified loci involved in the production or excretion of extracellular protease, nuclease, or chitinase by *S. marcescens*. None of the mutations represented general extracellular-excretion mutants; in no case was the production or excretion of multiple exoproteins affected. A variety of loci were identified, including regulatory mutations affecting nuclease and chitinase expression. A number of phenotypically different protease mutants arose. Some of them may represent different gene products required for the production and excretion of the major metalloprotease, a process more complex than that for the other *S. marcescens* exoproteins characterized to date.**

The movement of molecules from the site of synthesis to a new location is a fundamental property of biological systems. Protein export to the cell envelope has been the object of intensive study in *Escherichia coli* (4), and mutations affecting this process have been identified (18, 30). Many of these mutations are conditional, demonstrating that protein export to the cell envelope is essential to the cell. The extracellular secretion (or excretion) of some proteins into the growth medium can also be achieved by some bacteria (29, 32), although enteric bacteria as a group are not renowned for their ability to excrete proteins. In fact, *E. coli* only excretes proteins when it carries extrachromosomal elements specifying exoproteins. For some proteins, such as α-hemolysin, bacteriocins, and toxins (14, 19, 22, 26, 32), the release of the exoprotein usually requires the presence of other gene products; this implies that *E. coli* is not normally endowed with a general extracellular secretory system. Since *E. coli* is such a limited system for studying extracellular proteins, a range of other organisms has been investigated (29, 32). Usually the exoproteins these organisms excrete are either toxins or degradatory proteins like proteases and nucleases.

Some steps, such as signal sequence (31) recognition and processing, may be common to both envelope and extracellular protein translocation. Genes required for the export of both membrane-bound and extracellular proteins are essential to the cell, but genes required only for excretion appear not to be essential. By isolating mutants that are defective only in the excretion of extracellular proteins, it may be possible to separate this mechanism from envelope secretion.

Mutations in *Pseudomonas aeruginosa* strains defective in the excretion of certain extracellular proteins have been isolated (41); some of the mutations are pleiotropic in the formation of many but not all exoproteins. For example, elastase, lipase, and a stapholytic enzyme all become cell bound in certain mutants, but the alkaline protease is still excreted. These data suggest more than one pathway for excretion. Excretion (Out^-) mutants of *Erwinia chrysanthemi* are similarly defective in extracellular transport of some exoproteins; these proteins accumulate in the periplasm (1, 36). Excretion by *Aeromonas hydrophila* (16, 17) can also be blocked by mutations which prevent the excretion of protease, hemolysin, aerolysin, and other exoproteins. These proteins are found in the periplasm of the mutants and can be released by osmotic shock.

A variety of exoprotein genes have been cloned and expressed in *E. coli*. The exoproteins appear to fall into two general classes with respect to their excretion properties. First, most of the exoproteins, including pullulanase of *Klebsiella pneumoniae* (24), cellulase (13), and various *Erwinia* proteins (9, 21), are found in the periplasm of *E. coli*. These proteins include those which are blocked by the Out^- or other excretion (1, 36) mutants. Some normally excreted proteins, however, are capable of being excreted by normal *E. coli*. A streptokinase from *Streptococcus equisimilis* (23) and the *Erwinia* protease and pectase lyase have both been shown to be excreted from *E. coli* (3, 38).

We have chosen to initiate studies in *Serratia marcescens* because it is a true protein-excreting enteric bacterium. The ColE1 family of plasmid vectors can be transformed and will replicate normally in *S. marcescens* (33, 34). In this work, we show that many bacteriophages of *E. coli* can transduce *S. marcescens* DNA. Furthermore, *E. coli* mating plasmid F can conjugate into *S. marcescens* (35). As a result, *S. marcescens* provides important genetic tools which make it an excellent system for the study of extracellular protein excretion.

The extracellular proteins secreted by *Serratia* spp. include at least one nuclease, two proteases, two chitinases, and a lipase (5, 6, 12, 15, 27). In the course of this work, genes encoding exoproteins from *S. marcescens* have been cloned. The chitinases are both excreted by *E. coli* (20), as are the minor serine protease (42) and the nuclease (2, 8).

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These proteins appear to fall into the second class, i.e., self-secreting proteins. The major metalloprotease, however, is not produced in *E. coli* and presumably requires accessory genes for its production, processing, or excretion (28).

In this study, we identified mutations affecting the production of various extracellular proteins from *S. marcescens*. None of these mutations appeared to identify loci essential for extracellular secretion in general; each mutation was specific to a single exoprotein. The mutations did, however, identify a number of genes essential for the production of the exoproteins.

MATERIALS AND METHODS

Bacterial strains, plasmids, and phages. *S. marcescens* SM6 is a pigmented wild-type strain from the laboratory collection of C. Yanofsky. It was used as a source of *Sma*I restriction endonuclease and therefore is known to have that restriction system. Strain TT392 (34) is a nonpigmented restriction- and nuclease-deficient derivative of *S. marcescens* Sr41 8000 but is unrelated to SM6. It appears to have restriction and modification systems different from the *Sma*I system (M. Benedik, unpublished observations). *E. coli* Mal103 (7) was the source of Mu dII(Ap *lac*) used for Mu mutagenesis. λ *c*I857 *b*221 *c*III::Tn*5* (λTn*5*) was the donor phage for Tn*5* mutagenesis. Plasmid pTROY11 (11) is a derivative of pBR322 containing the *lamB* gene of *E. coli* expressed constitutively from an IS*3* insertion upstream. Strain K802 is an *rglAB* mutant of *E. coli* and is used as a host for the plasmid pHpa2, a derivative of pBR322 carrying the restriction and modification systems of *Haemophilus parainfluenzae* (*Hpa*II), which was obtained from Geoff Wilson of New England BioLabs, Inc.

The nuclease-expressing plasmids pNUC4 and pNUC4-R are described elsewhere (2). pNUC4 carries the nuclease gene oriented so that it is expressed from the *lac* promoter of pUC18 (37). The pNUC4-R plasmid carries the same gene in the opposite orientation; nuclease expression comes only from the nuclease promoter in the insert. The chitinase-expressing plasmids (20) are likewise inserted in opposite orientations in the pUC8 and pUC9 plasmids; pCHIT1252 expresses chitinase from the plasmid *lac* promoter of pUC9, and in the opposite orientation, pCHIT1251 has much lower expression, presumably from the chitinase promoter. Plasmid pCHIT310 carries a second different chitinase gene (20).

Media and growth conditions. Cells were routinely grown in LB medium (25). Antibiotics were added (50 μg of ampicillin or 25 μg of kanamycin per ml) for selection with *E. coli* or *S. marcescens* TT392. However, *S. marcescens* SM6 is highly resistant to many antibiotics, so a high concentration of ampicillin (1 mg/ml) or kanamycin (100 μg/ml) was used. DNase test agar with methyl green was purchased from Difco Laboratories. Milk or gelatin plates were used as indicators for protease activity. Sterile milk was prepared by the addition of 10 g of nonfat powdered milk to 100 ml of water; the mixture was autoclaved for 10 min and immediately placed on ice to prevent clotting. The milk solution was heated to 37°C and added to LB or nutrient agar cooled to 55°C. The gelatin plates were made by adding 10 g of gelatin to minimal M9 medium (25) supplemented with glycerol as a carbon source. Chitin plates were made with 5 g of nutrient agar to which was added an additional 5 g of agar and 50 g of swollen chitin per liter.

E. coli strains were normally grown at 37°C except for temperature-sensitive lysogens. *S. marcescens* was normally grown at 30°C; this allowed normal production of red pigment and increased the production of exoproteins relative to growth at 37°C.

Transformation. DNA transformation of *E. coli* was performed as described previously (10) but with 10 mM Tris (pH 8)–10 mM $MgCl_2$–60 mM $CaCl_2$. Transformation of *S. marcescens* was similar, except that the cells were first washed with cold 10 mM NaCl to remove excess nuclease and the $MgCl_2$ was left out to prevent nuclease activity. Transformation of *S. marcescens* occurred at an efficiency of about 10^{-3} of that of *E. coli*. To transform restriction-proficient *S. marcescens*, plasmid DNA was methylated in vitro with *Hpa*II methylase (New England BioLabs) according to the specifications of the manufacturer; this procedure methylates all CCGG sites, of which *Sma*I sites (CCCGGG) are a subset.

Chemical and UV mutagenesis. Nitrosoguanidine mutagenesis was done as for *E. coli* (25). For UV mutagenesis, conditions giving about 1% survival were used to generate a population of mutant cells. Appropriate dilutions were spread on indicator plates to screen mutants.

Transposon mutagenesis. Lysates of Mu dII (Ap *lac*) were prepared by temperature induction of the Mu lysogen strain MAL103 (7). Phage were added to an overnight culture of strain SM6 at a multiplicity of infection of about 10. After adsorption, the culture was diluted into fresh LB and grown for 60 min at 30°C to allow expression of antibiotic resistance; the cells were then plated on high-ampicillin LB agar. Pools were scraped and frozen at −80°C in 15% glycerol. Cultures were inoculated with at least 10^7 frozen cells, grown overnight in LB, and plated on appropriate indicator media at appropriate densities to identify mutants. No attempt to circumvent the restriction barrier of *S. marcescens* was needed for Mu mutagenesis.

Lysates of λTn*5* were prepared on K802 pHpa2 to in vivo methylate the *Sma*I sites (a subset of *Hpa*II sites) of this phage. This procedure was not essential for infection of the restriction-deficient strain TT392 but was necessary for infection of SM6. The lysate was used at a multiplicity of infection of 5 to 10 to infect *S. marcescens* cells carrying plasmid pTROY11. This plasmid supplies LamB, which is the λ receptor protein. Without this receptor, λ is unable to infect the cell. Although capable of infecting *S. marcescens* carrying pTROY11, λ is unable to form plaques on *S. marcescens* and exhibits no killing. After adsorption, expression of kanamycin resistance was allowed by incubation in LB at 30°C for 90 min. Mutants were selected by spreading the mixture on high-kanamycin LB plates. Resistant colonies arose at a frequency of about 10^{-6} to 10^{-7}. These were picked directly onto indicator media to identify mutants.

RESULTS

Isolation of strains carrying Mu insertion mutations. Stable lysogens of the defective Mu phage Mu dII were isolated after infection of wild-type *S. marcescens* SM6 with a phage lysate prepared from *E. coli* Mal103. We readily obtained lysogens, and a pool of greater than 10,000 colonies was scraped from plates and stored at −70°C. The frozen stock was used as an inoculum to grow a fresh culture (inoculum size, $>10^7$) to saturation in LB medium. Appropriate dilutions were plated to obtain 100 to 500 colonies per plate on milk, chitin, or DNase indicator plates. DNase and protease activities could be detected after 18 to 24 h at 30°C. Chitinase

activity could be detected only after incubation for 3 to 5 days.

Isolation of strains carrying Tn*5* insertion mutantions. Introduction of plasmid pTROY11, expressing a functional λ receptor, into restriction-deficient *S. marcescens* TT392 resulted in a strain which readily yielded kanamycin-resistant colonies after infection with λTn*5*. Since TT392 was heavily mutagenized during its construction and is not pigmented, we preferred to use wild-type strain SM6. The λTn*5* phage lysate was prepared from *E. coli* K802(pHpa2), which expresses the restriction and modification systems of *Hpa*II. This lysate methylated the phage genome in vivo. When this lysate was used to infect SM6(pTROY11), kanamycin-resistant colonies were obtained at a 100- to 1,000-fold-higher rate than when unmodified donor phage was used.

Kanamycin-resistant transductants were selected directly on high-kanamycin LB medium and toothpicked onto milk, chitin, or DNase indicator plates containing kanamycin. Colonies that did not show clearing of the surrounding medium were picked onto the same medium to confirm their phenotypes.

Classes of mutants isolated. A number of strains carrying mutations were isolated after transposon Tn*5*, bacteriophage Mu, or *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine mutagenesis. The most important observation was that each of the mutations affected only a single extracellular protein; no strains with pleiotropic mutations affecting multiple extracellular proteins were isolated.

Nuclease mutations. Twelve independent mutants defective only in nuclease activity were isolated after Mu and Tn*5* mutagenesis. These mutants were tested by complementation with the cloned nuclease gene from strain SM6 (2). Plasmid pNUC4 expresses the nuclease from the *lac* promotor; pNUC4-R carries the nuclease gene oriented opposite from the *lac* promoter and is expressed only from the nuclease promoter. All 11 Mu insertions were complemented by either plasmid. These insertions probably represent mutations of the nuclease structural gene which we call *nucA*. This explanation has not been confirmed by physical studies. Strain TT392 (34) is also defective in nuclease activity and is complemented by both plasmids. In contrast, a single Tn*5* insertion strain, *nuc*::Tn*5*-1, was isolated and was complemented only by pNUC4. No activity was observed when pNUC4-R was introduced.

Chitinase mutations. Eleven different chitinase-deficient mutants were isolated after Tn*5* mutagenesis. None of the mutants were deficient in nuclease or protease production. Complementation studies of these mutants were performed with pCHIT1251 and pCHIT1252. These plasmids carry the chitinase gene in vectors pUC8 and pUC9, respectively, so that chitinase expression in pCHIT1251 comes from a chitinase promoter but in pCHIT1252 comes from the *lac* promoter. The mutants fall into two classes (Table 1). One class could be complemented by either plasmid, and a second class could not be complemented by either plasmid. We confirmed that these noncomplemented mutants carried a functional chitinase plasmid by reintroducing the plasmid into *E. coli*.

To confirm that strain SM6 carries two chitinase genes, Southern blot analysis (S. Braunagel and M. J. Benedik, unpublished results) of SM6 with both pCHIT1251 and pCHIT310 (20) as probes showed that strain SM6 carried homologs of both genes which are unrelated to each other.

Protease mutations. Many different protease mutants were isolated, 14 of which are listed in Table 2. Each mutant altered in protease activity remained normal on chitinase and nuclease indicator media. There were some differences in testing for protease activity on milk versus gelatin media. Some of the mutants not indicating any change on milk medium (e.g., *prt*::Tn*5*-1) could be shown to digest gelatin. This result is most likely due to residual low-level activity of either the metalloprotease or other proteases, such as the serine protease (5). In Table 3, we present the enzymatic levels of protease found in these mutants. Some mutants clearly lost all extracellular protease activity, while some retained a reduced level of protease. In no case was the protease found intracellularly. The protease levels shown in Table 3 were due to the metalloprotease. The activities were inhibited by EDTA but insensitive to phenylmethylsulfonyl fluoride.

One observation we made was the correlation between protease expression and the pigmentation phenotype in the Mu and, to a lesser extent, the *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine mutants. We observed that a large fraction (10 to 20%) of the colonies was no longer pigmented or showed

TABLE 1. Complementation of chitinase mutants

Organism and strain	Chitinase expression[a]		
	Without plasmid	With plasmid:	
		pCHIT1251	pCHIT1252
S. marcescens SM6			
Wild type	++	ND	ND
chi::Tn*5*-1	−	−	−
chi::Tn*5*-3	−	++	ND
chi::Tn*5*-4	−	++	ND
chi::Tn*5*-5	−	++	ND
chi::Tn*5*-7	−	++	ND
chi::Tn*5*-8	−	−	−
chi::Tn*5*-9	−	−	−
chi::Tn*5*-10	−	−	−
chi::Tn*5*-11	−	++	++
E. coli JM101	−	+	++

[a] Chitinase was measured as halo size on a chitin plate. + and ++, Degree of complementation based on halo size; −, no complementation; ND, not tested.

TABLE 2. Protease mutants

S. marcescens SM6	Relative amt of protease in medium with:	
	Milk	Gelatin
Wildtype	+++	+++
prt::Tn*5*-1	−	Wk
prt::Tn*5*-2	−	Wk
prt::Tn*5*-3	−	−
prt::Tn*5*-4	+	++
prt::Tn*5*-5	−	−
prt::Tn*5*-6	+	++
prt::Tn*5*-7	+	++
prt::Tn*5*-8	+	++
prt::Tn*5*-9	−	−
prt::Mu-1	−	−
prt::Mu-2	Wk	+
prt::Mu-3	++++	++++
prt-ntg1	Wk	+
prt-ntg2	−	−

[a] Protease was measured as halo size on indicator media prepared as described in Materials and Methods. Wk, weak expression of protease; +, ++, and +++, relative amounts of protease; −, no protease.

TABLE 3. Protease activities in mutant strains[a]

S. marcescens SM6	Extracellular protease activity[b]
Wild type	2.07
prt::Tn*5*-1	<0.1
prt::Tn*5*-2	<0.1
prt::Tn*5*-3	<0.1
prt::Tn*5*-4	0.87
prt::Tn*5*-5	<0.1
prt::Tn*5*-6	1.53
prt::Tn*5*-7	1.64
prt::Tn*5*-8	0.76
prt::Tn*5*-9	<0.1

[a] Protease activity was measured as described in Materials and Methods. Samples were prepared from cultures grown overnight in LB media. Supernatants were cleared by centrifugation, and cell pellets were sonicated to release intracellular proteins. Activity was calculated by the formula: $A_{436}/(A_{600} \times t\ [h] \times 0.1\ ml)$.

[b] Intracellular protease activity was <0.1 for all mutants.

various degrees of pigmentation. The nonpigmented colonies were mostly deficient in protease production, although they were normal in chitinase or nuclease production. A few nonpigmented colonies which produced protease were found, but these were rare. The correlation between pigmentation and protease production has been observed by others (40). The rleationship between the unstable pigmentation phenotype and expression of the protease is not clear. After these observations, only pigmented colonies were chosen, and protease mutants were found at the expected frequency (10^{-3} to 10^{-4}).

DISCUSSION

The work presented here represents an essential step in developing *S. marcescens* as a model system in which to study extracellular protein secretion. Systems to generate transposon-mediated mutations have been devised to isolate mutants in wild-type *S. marcescens*. A number of mutations which identify genes required for extracellular protein production have been identified by these techniques. All of the mutations identified are specific for a single extracellular protein. Although none of these mutations identify loci for a common excretion pathway, many of them are interesting nevertheless.

Nuclease deficiency mutations appear to arise at two different loci. One group of mutants, i.e., all those isolated after Mu phage mutagenesis, and the point mutant in strain TT392 behaved as if the mutation was in the structural gene for the extracellular nuclease, which we call *nucA*. These mutants could all be complemented by the plasmid clones carrying a wild-type copy of the nuclease gene (pNUC4 and pNUC4-R). A second locus, *nucB*, was identified in a single Tn*5* mutant. This mutant could also be complemented by *nucA* expressed from the *lac* promoter of the plasmid (pNUC4). This mutant was not complemented by *nucA* expressed from its own promoter (pNUC4-R). This observation suggests that the *nucB* mutation represents a gene essential for the expression of nuclease from its own promoter. Since the mutation was not *cis* specific yet prevented expression of nuclease from plasmids using the nuclease promoter, it may represent an essential regulatory protein, a positive regulator of nuclease expression.

Mutations which increase nuclease activity have been isolated (39). These are probably regulatory mutations. Whether these mutations represent alleles of *nucB* or another regulatory gene, such as a repressor, is not known. The apparent ease with which they were isolated suggests that they are not promoter mutants, which should be rare. The complexity of nuclease regulation is somewhat surprising in that the expression of nuclease appears to be constitutive and there are no known regulatory effector molecules.

S. marcescens has two chitinase genes (20). They have both been cloned, and each gene can express chitinase activity in *E. coli*. In this work, we isolated only mutants completely deficient in chitinase expression. Mutants defective in only a single chitinase gene would have an intermediate level of chitinase (20). One class of mutants, represented by mutants *chi*::Tn*5*-3, -4, -5, -7, and -11, are complemented by plasmids carrying the CHIT12 insert in either orientation relative to the *lac* promoters of vectors pUC8 and pUC9. That the mutants are complemented by both types of plasmid suggests that the mutation is in the structural gene for chitinase. This possibility presents a paradox in that there are two structural genes. The simplest explanation is that the structural genes are linked in an operon and that the class I mutants are the result of polar mutations early in the operon. This would explain how single mutations suppress the expression of both structural genes. One fact not consistent with this explanation is that cosmid clones carrying either *chiA* (pCHIT1251) or *chiB* (pCHIT310) were isolated (20). If the genes were tightly linked, then they should be found together in the cosmid clones. However, *chiT3* pCHIT310 is found as a 30-kilobase *Eco*RI fragment which would probably always be cloned as a separate fragment in the *Eco*RI partial cosmid library used to isolate the clones.

The class II mutants, *chi*::Tn*5*-1, -8, -9, and -10, are not complemented by either plasmid, even though pCHIT1252 expresses chitinase from the *lac* promoter. The class II mutations are difficult to explain. It is unlikely that they are secretion mutations, since they have no effect on the extracellular secretion of nuclease or protease. In *E. coli*, chitinase is expressed and secreted from plasmid clones, suggesting that no chitinase-specific secretory proteins are required. The mutations may destroy a factor needed posttranscriptionally for chitinase production; this factor must either exist in *E. coli* but not be required for the production of other extracellular proteins or not be needed in *E. coli* at all. Another model consistent with the data would be that the class II mutations cause the constitutive production of a negative regulator which could act to prevent chitinase expression at either the transcriptional or posttranscriptional level.

A number of classes of protease mutants have been identified in this work. These include mutants with severely reduced protease expression and some mutants totally deficient in extracellular protease. Without the cloned protease genes, we were unable to pursue the analysis of these mutants in any detail. There are at least two distinct proteases produced by *S. marcescens*, a minor serine protease (42) and a major metalloprotease (5, 6, 28). The serine protease is expressed and secreted from plasmid clones in *E. coli*. This is similar to nuclease and chitinase. However, the metalloprotease is not produced when the gene is introduced in *E. coli* (28). This fact suggests that, unlike the situation with the other extracellular proteins of *Serratia* spp., accessory genes are required for the production, processing, or secretion of the metalloprotease. Some of the mutants in the collection described here may define these accessory genes. Complementation tests with the cloned structural gene will

be required to further analyze these mutants. The mutants showing weak protease expression on gelatin medium (*prt*::Tn*5*-1 and -2) may express only the serine protease. The protease activities shown in Table 3 were all due to the metalloprotease. Those mutants devoid of all extracellular protease would be explained most simply as regulatory mutants preventing the expresion of either protease. Future work should differentiate between these alternatives and elucidate the nature of these different classes of mutations.

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