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Isolation and characterization of genes encoding two chitinase enzymes from *Serratia marcescens*

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Analysis of clones isolated from a cosmid DNA library indicates that the *Serratia marcescens* chromosome contains at least two genes, *chiA* and *chiB*, which encode distinct secreted forms of the enzyme chitinase. These genes have been characterized by inspection of chitinase activity and secreted proteins in *Escherichia coli* strains containing subclones of these cosmids. The two chitinase genes show no detectable homology to each other. DNA sequence analysis of one of the genes predicts an amino acid sequence with an N-terminal signal peptide typical of genes encoding secreted bacterial proteins. This gene was mutagenized by cloning a neomycin phosphotransferase gene within its coding region, and the insertion mutation was recombined into the parental *S. marcescens* strain. The resulting *chiA* mutant transconjugant showed reduced chitinase production, reduced inhibition of fungal spore germination and reduced biological control of a fungal plant pathogen.

Key words: biological control/catabolite repression/chitinase/protein secretion/*Serratia*

Introduction

Chitin, a β 1,4-linked polymer of N-acetyl glucosamine, is a structural component of the arthropod exoskeleton and of the cell walls of all fungi except the oomycetes (Monreal and Reese, 1969). Many organisms, including bacteria (Monreal and Reese, 1969), fungi (Elango *et al.*, 1982) and higher plants (Abeles *et al.*, 1970; Pegg and Vassey, 1973; Boller *et al.*, 1983) produce enzymes that hydrolyze chitin to chitodextrins. *Serratia marcescens* QMB1466, a Gram-negative enteric soil bacterium, secretes high levels of endochitinase activity (Roberts and Cabib, 1982). This enzyme activity is regulated by carbon source availability and is inducible by chitodextrins (Monreal and Reese, 1969).

The production of chitinase by higher plants may be part of a natural defense mechanism against fungal pathogens (Boller *et al.*, 1983). Application of chitin to soil can reduce the incidence of plant disease caused by soil fungi, perhaps because organisms which secrete chitinase proliferate under these conditions (Buxton *et al.*, 1965).

We show here that *S. marcescens* QMB1466 gives rise to an extracellular activity which can retard fungal growth. In order to investigate the role played by chitinase in this anti-fungal activity, we have isolated and characterized two chitinase genes from this organism. When one of these genes is mutated in the parental strain, the mutant derivative has a reduced capacity to inhibit the growth of the fungal plant pathogen, *Fusarium oxysporum* f.sp. *pisi* (Fop).

Results

The S. marcescens genome encodes two distinct chitinase genes

When colonies of *S. marcescens* QMB1466 grow on media containing colloidal chitin, distinct clear zones form around them in which the chitin has been dissolved by chitinase action. Since *Escherichia coli* and *S. marcescens* are closely related bacteria, a strategy was adopted for cloning the *Serratia* chitinase genes which was based on the assumption that they would function in *E. coli*.

A genomic library of QMB1466 DNA was constructed in the broad host range cosmid vector pLAFR1. Transfectants (5000) were screened for their ability to confer the chitinase-producing phenotype on *E. coli* DH1. Analysis of plasmid DNA from random colonies showed that ~50% of them contained the cloning vector with no insert DNA (data not shown). The remainder contained 20–35 kb inserts of *S. marcescens* DNA. Twelve colonies became surrounded by clear zones 7–10 days after plating of the library. Plasmid DNA was isolated from these clones and analyzed by restriction enzyme digestion with *Eco*RI, *Bam*HI and *Pst*I. This analysis divided the 12 clones into two classes. Three clones were of the class typified by pCHIT12, which contains a 29.4-kb DNA insert with *Eco*RI fragment sizes of ~3.5, 9.4 and 16.5 kb. The other nine clones, exemplified by pCHIT3, contained a 30-kb *Eco*RI fragment.

The chitinase-encoding DNA sequences from pCHIT12 and pCHIT3 were subcloned into pUC8. DNA from each cosmid clone was digested to completion with *Eco*RI and partially digested with *Pst*I and then ligated into *Eco*RI and *Pst*I-digested pUC8 DNA. Ampicillin-resistant colonies resulting from transformation of JM83 with these ligations were screened for white color on X-gal plates (Vieira and Messing, 1982) and for chitinase activity on chitin agar. The chitinase-positive subclones of pCHIT12 and pCHIT3, which were used in subsequent experiments, were named pCHIT1251 (2.51-kb insert) and pCHIT310 (3.28-kb insert), respectively, and they both confer a stronger chitin-clearing phenotype than their parental cosmid clones. Restriction maps of the two chitinase subclones are shown in Figure 1A.

The chitinase-encoding DNA in pCHIT1251 is contained within a *Hin*dIII/*Eco*RI fragment which on recloning into pUC9 becomes reversed in orientation relative to the pUC β-galactosidase (*lacZ*) promoter. The resulting plasmid, pCHIT1252, confers greater chitin-clearing capacity on transformed JM83 than does pCHIT1251. Cleared halos surrounding bacteria containing pCHIT1252 were visible by 2 days after plating, whereas halos were often not visible until 5 days after plating of bacteria containing pCHIT1251. This suggests that in the pCHIT1252 subclone the chitinase gene is oriented such that both the lacZ gene promoter of pUC9 and the endogenous chitinase gene promoter initiate transcription in the same direction. By contrast,

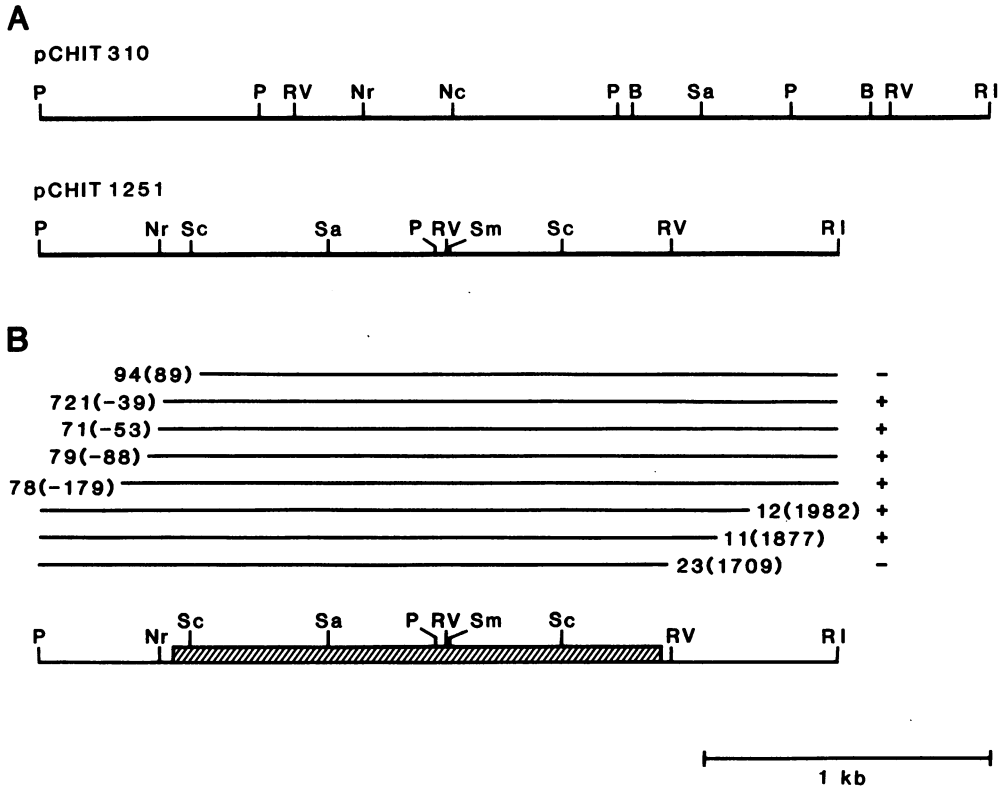

Fig. 1. Maps of chitinase-encoding DNA. (**A**) Restriction enzyme maps of two chitinase-encoding fragments of *S. marcescens* QMB1466 DNA. The map of pCHIT1251 DNA was deduced from the DNA sequence and confirmed by enzyme digestions followed by fractionation of digestion products on agarose gels (Maniatis *et al.*, 1982) and that of pCHIT310 DNA was derived from analysis of fragments produced by various digestions. B, *Bam*HI; P, *Pst*I; Nc, *Nco*I, Nr, *Nru*I; RI, *Eco*RI; RV, *Eco*RV; Sa, *Sal*I; Sc, *Sac*II; Sm, *Sma*I. (**B**) *Bal*31 nuclease-generated deletions of the pCHIT1251 subclone which lead to loss of chitinase activity. Each deletion fragment is designated by a number, and the number of nucleotides removed from each fragment is indicated in parentheses by the position of the fragment end relative to the translation start site of the chitinase gene. The 1684-bp open reading frame of the chitinase gene and the locations of the restriction sites shown in 1a are displayed below the deletions. The phenotypes of deletions 721, 94, 11 and 23 are shown in Figure 2B.

the orientation of the pCHIT310 insert with respect to the *lacZ* promoter did not detectably influence the rate of clearing of chitin.

Southern hybridization experiments (Southern, 1975) were conducted to investigate whether there was homology between the two chitinase genes. No DNA sequence homology was detected between pCHIT1251 insert DNA and pCHIT3 or pCHIT310. Likewise no homology was observed between the pCHIT310 insert DNA and pCHIT12 or pCHIT1251 (data not shown). Hybridization and wash stringencies were such that if the two genes were at least 82% homologous, cross-hybridization could have been observed (Davis *et al.*, 1980). Additionally, neither pCHIT1251 nor pCHIT310 hybridized to *E. coli* chromosomal DNA.

Proteins secreted by *S. marcescens* QMB1466 and *E. coli* JM109 harboring pUC9, pCHIT12, pCHIT3, pCHIT1252 or pCHIT310 were analyzed by SDS-PAGE. Figure 2A shows that unique proteins were produced by JM109 transformed with pCHIT1252 and pCHIT310. pCHIT1252 specifies synthesis of a 58-kd protein (lane e), and pCHIT310 encodes synthesis of a 52-kd protein (lane d). Neither the 58-kd nor the 52-kd protein could be detected in supernatants obtained from JM109 harboring pCHIT12 or pCHIT3. *S. marcescens* QMB1466 produces 58-kd and 52-kd proteins (lane b), and purification of the *Serratia* chitinase activity has shown that it is associated with proteins of 52 and 58 kd in size (Roberts and Cabib, 1982). Neither protein was observed in JM109 containing pUC9 (lane c). We conclude that pCHIT1251 and pCHIT310 confer the chitinase phenotype upon *E. coli* by specifying the synthesis of 58- and 52-kd chitinase proteins, respectively. We propose that these genes be named *chiA* and *chiB*.

DNA sequence analysis of the chiA gene

The region of DNA sequence in pCHIT1251 which specifies chitinase activity and production of the 58-kd protein was further localized by deletion analysis. Subclones of pCHIT1251 with successive deletions of 50–200 bp were obtained using *Bal*31 nuclease, and these deletions were assayed for their ability to confer the chitinase phenotype and the production of a 58-kd protein on transformed JM83. These deletions were also cloned into mp10 or mp11 for dideoxy sequencing.

Figure 1B shows the limits of the *chiA* gene in pCHIT1251 as determined by this analysis. Deletions from the *Hind*III site which extend further than deletion #721 give rise to plasmids which do not confer chitinase activity. Deletions from the *Eco*RI site which extend past deletion #11 also exhibit a chitinase-minus phenotype. This defines a 1.74-kb region within which the gene must lie. Figure 2B shows that subclones containing deletions into this region no longer specify the production of the 58-kd peptide. These subclones also fail to exhibit the chitinase-clearing phenotype. No intermediate phenotypes were observed.

The sequence of 2.3 kb of DNA from the pCHIT1251 subclone was obtained by dideoxynucleotide DNA sequencing of deletions extending throughout the gene on both strands (Figure 3). It contains a 1686-bp open reading frame which encodes a 61-kd pro-

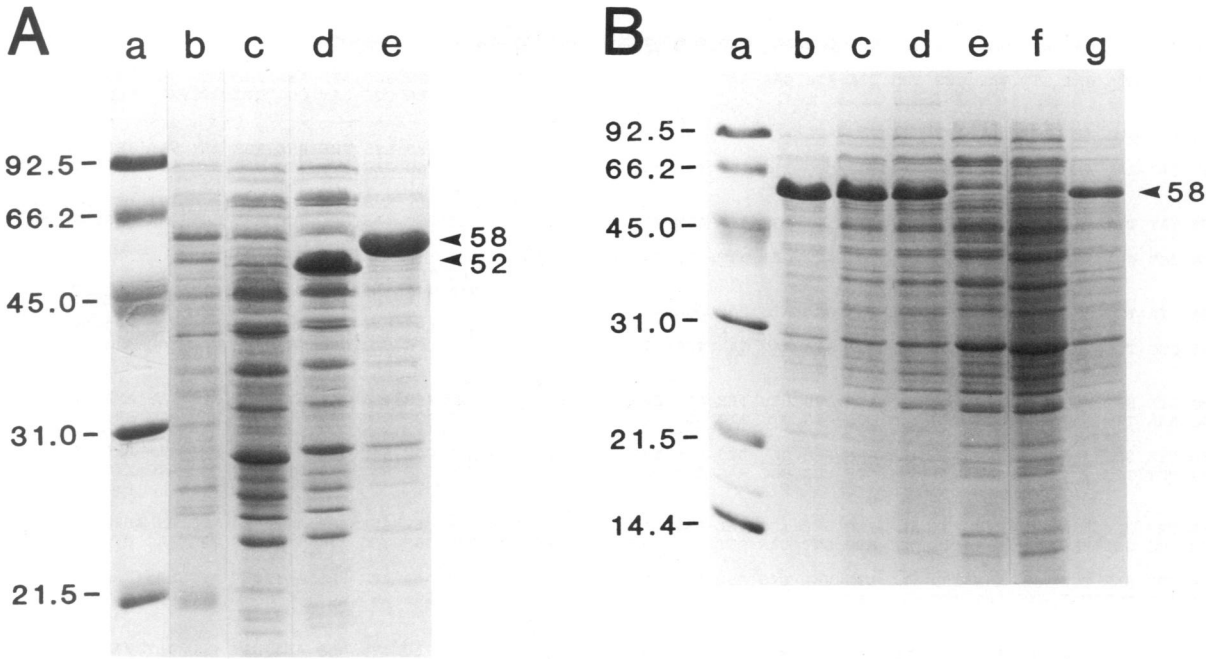

Fig. 2. Gel analysis of proteins in supernatants of various bacterial strains. **(A)** SDS-PAGE analysis of secreted proteins from *S. marcescens* QMB1466 (**lane b**), *E. coli* JM109/pUC9 (**lane c**), JM109/pCHIT310 (**lane d**) and JM109/pCHIT1252 (**lane e**). Mol. wt. markers are shown in **lane a**. The 58- and 52-kd proteins encoded by pCHIT1252 and pCHIT310, respectively, are indicated. Secreted protein from 0.5 ml (**lanes b, c, d**) or 0.05 ml (**lane e**) were loaded on each lane. **(B)** SDS-PAGE of proteins secreted from JM109 harboring selected *Bal*31 deletion mutants of pCHIT1251 and subcloned in pUC9. Mol. wt. markers (**lane a**), pCHIT1252 (**lane b**), pCHIT1252-78 (**lane c**), pCHIT1252-721 (**lane d**), pCHIT1252-94 (**lane e**), pCHIT1252-23 (**lane f**), pCHIT1252-11 (**lane g**). Deletion mutants pCHIT1252-78, pCHIT1252-721, and pCHIT1252-94 (**lanes c, d, e**) were generated by removing nucleotides from the *Hin*dIII site bordering the chitinase fragment of pCHIT1252. Deletion mutants pCHIT1252-23 and pCHIT1252-11 (**lanes f, g**) were generated by deleting nucleotides from the *Eco*RI site of pCHIT1251. The relative sizes of each deletion are illustrated in Figure 1B. The 58-kd protein encoded by pCHIT1252 is indicated.

tein with a 3-kd potential signal peptide at its N terminus. This reading frame is transcribed towards the *Eco*RI site (Figure 1B). This is consistent with the observation of a higher level of chitinase activity when the *chiA* gene is cloned in pUC9 rather than pUC8. Further evidence for this assignment of gene orientation comes from mapping the 5′ end of chitinase mRNA extracted from *S. marcescens* cultures grown on medium containing chitin. Solution hybridization and nuclease protection experiments (as determined in Jones *et al.*, 1985) and primer extension experiments using a synthetic oligonucleotide complementary to the chitinase mRNA both indicate a transcriptional start site at −158 as indicated by the asterisk in Figure 3. The assignment of the initiation site is based on experiments in which primer extension products and dideoxy DNA sequencing reactions primed by the same oligonucleotide were run in adjacent lanes on denaturing gels (data not shown).

The predicted amino acid sequence for the 1251 chitinase gene is also shown in Figure 3. Comparison of the chitinase N-terminal sequence with signal sequences of other prokaryotes (Inouye *et al.*, 1983; Watson, 1984; von Heijne, 1985) reveals a probable chitinase signal peptide. The amino-terminal region contains seven amino acids and carries a net charge of +3. It is followed immediately by a sequence of 10 hydrophobic amino acids. Based on published analysis of signal sequences, the predicted cleavage site is between the alanines at +23 and +24 (von Heijne, 1985). The N terminus of the mature form of the *chiA* chitinase protein was determined and confirms this predicted cleavage site (C.Ampe, personal communication). The DNA sequence reveals a GC-rich stem and loop structure (underlined in Figure 3) centered just prior to this proposed cleavage site.

The nucleotide sequence surrounding the coding sequence contains potential regulatory sequences. At positions −166 to −146 and −108 to −86 from the transcriptional start site (asterisk in Figure 3) sequences are observed (Figure 4) which show homology to the consensus CAP−cAMP binding site for *E. coli* genes which are catabolite repressed (de Crombugghe *et al.*, 1984). These sequences may play a role in the observed catabolite repression of chitinase activity in *Serratia* (Monreal and Reese, 1969). Computer analysis of this region using a Staden programme (Staden, 1984) reveals a plausible promoter sequence overlapping the −108 to −186 region [TTGTCA-(16 bases)-TATGAA] consistent with one model for the mechanism of CAP−cAMP repression of transcription (Malan and McClure, 1984). At −35 and −10 from the transcriptional start site the sequences TCTTGTTT-(16 bases)-TATAGT are observed in appropriate positions relative to each other and the initiation site (Hawley and McClure, 1984; Rosenberg and Court, 1979). There follows a 158-base leader sequence in which a Shine-Dalgarno sequence AGGA (underlined in Figure 3) precedes by nine bases the translational initiation positions.

At the end of the chitinase reading frame the DNA sequence shows a GC-rich inverted repeat sequence (underlined in Figure 3) beginning 16 bp beyond the translation termination codon. This structure has an 8-bp stem and a loop of 11 bases. The series of five T residues located 14 bp downstream from the centre of dyad symmetry of this inverted repeat is a common feature of trancription termination signals (Rosenberg and Court, 1979). The deletion subclone pCHIT1251-23 terminates within this postulated loop structure (Figures 1B, 2B). Interestingly, both phenotypic and protein analyses (Figure 2B) indicate that JM83

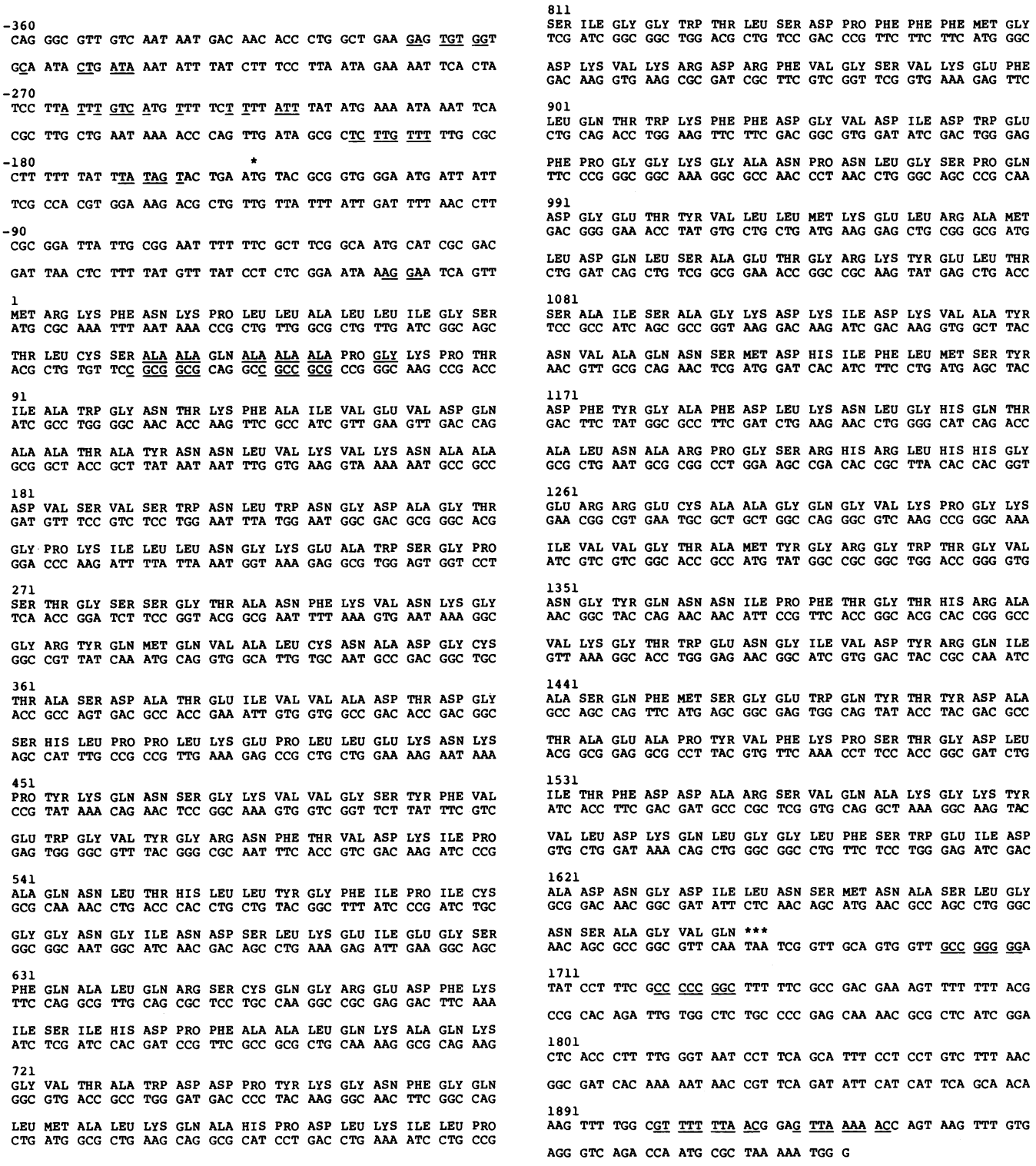

Fig. 3. DNA sequence of a 2.3-kb DNA segment from pCHIT1251, as determined by M13-dideoxynucleotide DNA sequencing of a set of *Bal*31 nuclease-generated deletion subclones which permit reading on either strand from locations which differ by 50−150 bp. The transcriptional initiation site at −158 is indicated by an asterisk and other features of the DNA sequence are underlined (see text). The predicted amino acid sequence of the *chiA* gene is given above the nucleotide sequence.

harboring this subclone do not produce the chitinase enzyme. A similar result was reported for deletion mutants generated in the tryptophan operon of *E. coli* (Bertrand *et al.*, 1977). pCHIT1251 deletion subclones that terminate beyond position 1779 show normal chitinase production. An AT-rich inverted repeat is located at positions 1901−1913.

A chiA mutant of S. marcescens shows reduced biocontrol capacity

A 1.3-kb *Sal*I DNA fragment from pUC71K (Vieira and Messing, 1982) which encodes the neomycin phosphotransferase I (NPTI) gene from Tn*903* was cloned into the unique *Sal*I site

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POSSIBLE SERRATIA CHITINASE CAP-cAMP BINDING SITES
E.COLI CONSENSUS        NNNN AA N TGTGA NN T NNNN CA N ATT NNNNN
CHITINASE -166 TO -146   AA GA G TGTGG TG C AATA CT G ATA AATAT
CHITINASE -108 TO -86    TT AT T TGTCA TG T TTTC TTTT ATT TATTATGAA
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Fig. 4. Comparison of two putative CAP−cAMP protein binding sequences upstream of the chitinase gene with the consensus *E. coli* sequence (de Crombugghe *et al.*, 1984). In the −108 to −86 region a possible RNA polymerase binding site (−35 and −10 region) is underlined.

(+525) of pCHIT1251. *E. coli* JM83 transformed with this insertion mutant failed to exhibit the chitin-clearing phenotype. This mutated chitinase clone was linearized with *Eco*RI and ligated to *Eco*RI-cut pRK747. This construction (pRK−CHITK) was then mobilized using pRK2013 (Figurski and Helinski, 1979) into an *S. marcescens* QMB1466 derivative containing pPH1 (Hirsch and Beringer, 1985), an incP plasmid conferring gentamycin resistance. Transconjugants were selected by their resistance to kanamycin (100 mg/l) and gentamycin (50 mg/l) on M9 sucrose agar. Reduced chitinase activity was observed in 40% of the transconjugants. Replacement of the wild-type 1251 chitinase gene with the insertion mutant in these exconjugants was confirmed by Southern blot analysis (data not shown) and an individual colony was designated QMB1466 *chiA* and chosen for further work.

The capacity of various bacterial strains to influence fungal growth was evaluated both on agar plates and in the greenhouse. In plate assays, bacterial culture filtrates were incorporated into the media on which fungal spores were allowed to germinate so that the effect of the filtrates on the rate of germ tube elongation could be determined (Table I). In the greenhouse experiments seedlings were dipped in fresh bacterial cultures prior to planting in soil containing the same fungal plant pathogen (Table II).

Bacterial strains producing chitinase enzymes or a single chitinase enzyme reduce the mean germ tube length of *Fusarium oxysporum* f.sp. *pisi* (Fop) *in vitro* (Table I). The filtrates should be regarded as fungistatic rather than fungicidal since they do not kill the fungus. The inhibition by *E. coli* JM83 containing pCHIT1252-78 and pCHIT1252-721, plasmids which specify high levels of production of the *chiA* enzyme, was similar to that observed for the *S. marcescens* strain from which the chitinase genes were derived. Though *E. coli* JM83 and JM83/pUC9 do not produce chitinase, extracts from these strains do slightly inhibit germ tube elongation. Similar results were observed in *in vitro* assays using other pathogenic fungi (data not shown).

Inhibition of germ tube development by crude filtrates from QMB1466 *ChiA* was consistently less than that observed with wild-type QMB1466 (Table I). The additional inhibition of germ tube elongation due to the presence of the 58-kd chitinase in the medium ranged from 11 to 13%. These differences were significant ($P = 0.05$) in all experiments.

Pea seedlings dipped in cultures of wild-type QMB1466 showed significantly reduced incidence of *Fusarium* yellows of pea in greenhouse pot trials compared with those dipped in cultures of QMB1466 *ChiA* (Table II). The frequency of recovery of *Fusarium* from plant tissue was also reduced by application of the QMB1466 strain but not the mutant strain. These data clearly indicate that the wild-type *S. marcescens* reduces or delays fungal infection resulting in a reduced incidence of disease symptoms. They also indicate that the otherwise isogenic QMB1466 *ChiA* strain is significantly impaired in its capacity to inhibit fungal activity. The relative difference between wild-type and mutant *Serratia* was greater *in vivo* than *in vitro*, perhaps because soil factors, host plant resistance and interactions with other microbes are also involved in infection and the expression of disease symptoms.

Table I. Effect of chitinase-producing bacteria on germ tube elongation of Fop[a]

Strain	Bacterial concentration (log c.f.u./ml)[b]	Mean germ tube length (μm)[c]
No bacteria	–	199 ± 8.9 (A)
JM83	8.9	173 ± 7.9 (A)
JM83/pUC9	8.9	177 ± 7.9 (A)
JM83/pCHIT1252-78	9.1	94 ± 5.8 (B)
JM83/pCHIT1252-721	9.0	98 ± 5.7 (B)
No bacteria[d]	–	178 ± 8.6 (A)
QMB1466[d]	9.2	65 ± 4.3 (C)
QMB1466 ChiA[d]	8.9	82 ± 4.1 (B)

[a]*Fusarium oxysporum* f.sp. *pisi* Race 1.
[b]Log_{10} c.f.u./ml of original culture prior to filter sterilization.
[c]Mean germ tube length for Fop macroconidia on 2.0% malt agar. Numbers followed by the same letter are not significantly different from each other at $P = 0.05$, $F = 16.6$.
[d]Values for this treatment are from a different replication of an identical experiment.

Table II. Comparison of wild-type and chitinase-deficient *Serratia* strains in disease control

Treatment	Disease incidence (%)[a]		Isolation frequency (%)[b]	
	Expt.1	Expt.2	Expt.1	Expt.2
Non-infested	26	7	5	3
+ *Fusarium*[c]	84	64	25	26
+ *Fusarium* + QMB1466	49	33	8	11
+ *Fusarium* + QMB1466 chiA	75	89	18	29
	LSD = 18	LSD = 21	LSD = 9	LSD = 12

[a]Disease incidence is the mean symptomatic leaf pairs/total pairs × 100 per replication. Replications consisted of six sets of three pots each containing three plants per pot. Randomization was in complete block design. ANOV was performed as arc sin transformation of disease incidence ($P = 0.05$).
[b]Isolation frequency of *Fusarium* (Fop) from three plants per block per treatment at 35 days after planting. Frequency is defined as infected nodal segments/total nodal segments × 100. ANOV performed as arc sin transformation of isolation frequency ($P = 0.05$).
[c]*Fusarium oxysporum* f.sp. *pisi* Race 1.

Discussion

Two chitinase genes have been isolated from a cosmid library of *S. marcescens* QMB1466 chromosomal DNA and subcloned in pUC plasmids. Both are secreted by *E. coli* and hydrolyze chitin in agar medium to form clear halos surrounding the bacterial colonies. They encode proteins that appear identical in size to those previously isolated from *S. marcescens* and shown to exhibit similar enzyme activities (Roberts and Cabib, 1982). These two genes do not share any detectable DNA sequence homology.

The DNA sequence of one chitinase gene was determined. Phenotypic analysis of deletions of DNA encoding this gene substantiates the conclusions about gene location and orientation made on the basis of sequence analysis.

Insertion of a 1.3-kb DNA sequence encoding kanamycin resistance into the *chiA* coding region eliminated the capacity of the resulting of pCHIT1251 derivatives to specify chitinase production. Recombinational exchange of the NPTI marker into the parental *S. marcescens chiA* coding sequence produced transconjugants with a reduced chitin-clearing phenotype. Since the *chiB* gene was unaffected by this procedure, complete elimination of the chitin-clearing phenotype was not anticipated.

Compared with the parental strain, *S. marcescens* QMB1466 *ChiA* exhibited reduced inhibition of fungal spore germination and dramatically reduced biological control of *Fusarium* infection of pea plants. These results show that chitinase production by the parental *S. marcescens* strain is necessary for the expression of its full anti-fungal phenotype. However, we have no evidence that chitinase alone is an effective anti-fungal agent, and we presume that it acts with a spectrum of other compounds to slow fungal growth. This perspective would suggest that adding an extra chitinase enzyme to the array of anti-fungal compounds already made in plants would enhance their disease resistance. We will be testing this theory in future experiments.

Materials and methods

Materials

Restriction endonucleases, T4 DNA ligase and *Bal*31 nuclease were purchased from New England Biolabs. DNA polymerase I (Klenow fragment) and calf intestinal alkaline phosphatase (CIAP) were purchased from Boehringer Mannheim. [α-^{32}P]dATP and [α-^{35}S]dATP (both at 600 Ci/mmol) were purchased from Amersham, and proteinase K and crab-shell chitin were obtained from Sigma. 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) was purchased from Boehringer Mannheim.

Bacterial strains and plasmids

S. marcescens QMB1466 is available from ATCC (ATCC #990) and *E. coli* strains DH1, HB101, JM83, JM101 and JM109 have been described (Maniatis *et al.*, 1982; Vieira and Messing, 1982; Messing, 1983). The following plasmids were used in this study: pLAFR1 (Friedman *et al.*, 1982), pRK2013 (Figurski and Helinski, 1979), pPH1 (Hirsch and Beringer, 1984), pRK404 (Ditta *et al.*, 1985) pUC8, pUC9 and pUC71K (Vieira and Messing, 1982) and pRK747. pRK747 is an unpublished derivative of pRK404 in which the *Eco*RI site outside the polylinker has been deleted. The single-stranded DNA bacteriophages M13mp10 and M13mp11 (Messing, 1983) were used for dideoxynucleotide DNA sequencing.

Recombinant DNA techniques

Plasmid DNA preparation, restriction enzyme digestion, ligation, CIAP treatment, cloning into M13 vectors and dideoxynucleotide sequencing were carried out using standard techniques (Maniatis *et al.*, 1982; Sanger *et al.*, 1980). Construction of deletions by *Bal*31 nuclease from DNA sequence analysis was carried out as described by Dean *et al.* (1985). Enzyme reactions were buffered as recommended by the manufacturer and performed as described (Maniatis *et al.*, 1982).

Genomic library construction

S. marcescens QMB1466 chromosomal DNA was isolated as described (Dhaese *et al.*, 1979), ethanol-precipitated and then re-dissolved in 10 mM Tris buffer, pH 7.5 and 1 mM EDTA. An *Eco*RI partial digest of the chromosomal DNA was size-fractionated on 10–30% glycerol gradients (Maniatis *et al.*, 1982). Fractions containing fragments of 20–30 kb were pooled and ligated to *Eco*RI-digested pLAFR1 DNA. Recombinant cosmids were packaged in phage lambda packaging extracts (Maniatis *et al.*, 1982) and transfected into DH1. Transfectants were selected by resistance to 10 mg/l tetracycline.

Colony screen for chitinase activity

Chitin medium was modified from that of Monreal and Reese (1969) and contained 0.1 mM FeEDTA, 0.5% Difco yeast extract, 2% colloidal chitin (Lingappa and Lockwood, 1962), M9 salts (Davis *et al.*, 1980), and 1% TC agar (KC Biologicals. Colonies of *E. coli* containing plasmids encoding the chitinase genes were identified on agar plates by the presence of surrounding clear zones in which the chitin had been dissolved by chitinase action.

Protein analysis

S. marcescens QMB1466 and *E. coli* JM109, with and without chitinase genes, were grown for 4 days in liquid chitin medium. Cells were removed by centrifugation at 10 000 *g* for 10 min. The supernatant was brought to 10% trichloroacetic acid (TCA) at 0°C, and precipitated proteins were rinsed with acetone and re-dissolved in Laemmli sample buffer (Laemmli, 1970). SDS-PAGE was performed with a 10–17.5% linear polyacrylamide gradient slab gel and a 1-cm 5% stacking gel that incorporated the buffer system of Laemmli (1970). Gels were fixed and stained for protein with Coomassie Blue.

Marker-exchange mutagenesis in Serratia

The Tn*903* NPT gene was cloned from pUC71K into the unique *Sal*I site of the *chiA* gene cloned in pCHIT1251 (see text) and the mutated gene was subcloned into pRK747 creating the plasmid pRK-CHITK. Exchange of the NPT marker into the chromosomal chitinase gene of *S. marcescens* QMB1466 was accomplished using a modification of the technique of Ruvkun and Ausubel (1981). pPH1 was first mobilized from HB101/pPH1 into QMB1466 by plate matings followed by selection on medium containing M9 salts, 0.2% sucrose and 50 mg/l gentamycin. This strain was then incubated in a three-way plate mating with HB101/pRK2013 and HB101/pRK-CHITK. Transconjugants were selected and screened for chitinase activity on medium containing M9 salts, 0.2% sucrose, 50 mg/l gentamycin and 100 mg/l kanamycin and their structure confirmed by Southern hybridization.

Fungal spore germination assay

The effect of chitinase on the germination of macroconidia of *Fusarium oxysporum* f.sp. *pisi* Race 1 (Fop) was tested by incorporating bacteria culture filtrates containing this enzyme into nutrient media. Chitin medium lacking agar was dispensed in 50-ml aliquots in 500-ml flasks and inoculated with the bacterial strains listed in Table I. All strains were grown on a rotary shaker (220 r.p.m.) for 48 h at 32°C. Cells were then pelleted by centrifugation at 2500 *g* for 10 min and the supernatant was passed through 0.45 and 0.20 μm filters. Equal volumes of culture filtrate and molten 3.2% Seaplaque LGT Agarose (FMC) containing 4.0% malt extract (Difco) were mixed quickly at 42°C, and 3-ml aliquots were dispensed into Petri dishes (35 × 10 mm), allowed to solidify and surface-dried in a laminar flow hood.

A spore suspension of Fop was prepared and adjusted to $\sim 10^3$ propagule-forming units (p.f.u.) per ml. One 33-μl drop was placed on each of two or three replicate plates. Plates were rotated to disperse spores uniformly and then incubated at 21°C for 24 h. The lengths of germ tubes in five random fields of vision were measured in a dissecting microscope at 16× magnification with a calibrated ocular micrometer.

Disease control by Serratia strains

Hesperia Fine Sandy Loam (HFSL) was infested with spores of Fop and air-dried at 21°C for 2 weeks to allow development of chlamydospores. The soil was adjusted to $\sim$1000 p.f.u./g by dilution with pasteurized HFSL. Pre-germinated seeds of *Pisum sativum* cv. Grenadier were dipped in a fresh culture of 5×10^8 colony forming units (c.f.u.)/ml of wild-type or mutant *S. marcescens* (grown as above) and plated in 13.5-cm pots. Pots were arranged in randomized complete block design in a greenhouse. Temperatures ranged from 21°C to 26°C, and supplemental lighting was used to maintain daylengths of at least 12 h.

Plants were evaluated for symptoms of wilt and systemic infection. Systemic infection by Fop was quantified by surface sterilizing nodal segments with 10% commercial bleach for 20 min and plating them on 2% agar containing 200 μg/ml streptomycin or on Nash-Snyder PCNB medium (Nash and Snyder, 1962). Six nodal segments were evaluated from each of three plants in each treatment replication.

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