Cloning of the Genes of the Chitin Utilization Regulon of
Serratia liquefaciens

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The set of genes that determine the expression of the enzymes involved in chitin degradation by Serratia liquefaciens was cloned. The role of each gene was investigated, and for the first time regulatory genes were identified in this system. The chiA and chiB genes coded for separate chitinase activities. The chiC region coded for a chitobiase activity, but it was not formally separated from chiB. Transposon mutagenesis and deletion analysis identified a region, chiD, whose absence led to higher expression of chiA, chiB, and chiC. chiD may therefore be a gene that codes for a repressor. Loss of function of another adjacent region, chiE, prevented induction unless a chiE+ strain was a near neighbor, suggesting that this gene may code for a protein that is involved in the synthesis of the inducer. chiB, chiC, chiD, and chiE are closely linked, while chiA is in a separate location on the chromosome.

Chitin is a β-1,4-linked unbranched polymer of N-acetylglucosamine. It is degraded by chitinases, yielding predominantly the dimer chitobiose, which is further degraded by chitobiase to the monomer N-acetylglucosamine.

Chitin is a major cell wall component of many agronomically important pests, including insects, fungi, and nematodes (for a review, see reference 3). Plants do not contain chitin; however, chitinases are produced by many plants upon infection by fungi, bacteria, and viruses. Purified plant chitinases have been shown to inhibit fungal growth (10, 15). Since chitinases do not have any apparent function in the metabolism of plants and since chitinases can inhibit fungal growth, it is likely that they are involved in the defense mechanism of the plant. Enhancement of the activity or availability of the chitinase of a plant and provision of a foreign chitinase in plant cells constitutively or in response to infection represent possible components of strategies for the development of fungal resistance in plants.

Certain bacterial (14) and plant (3) species are a ready source of chitinase. Chitinase genes have been cloned from Serratia marcescens (8-10), Vibrio harveyi (18), Vibrio vulnificus (19), and Phaseolus vulgaris (4); chitobiase genes have been cloned from S. marcescens (9), V. harveyi (18), and V. vulnificus (19).

Here we describe the cloning of two Serratia liquefaciens chitinase (chiA and chiB) genes and a chitobiase (chiC) gene. We also describe the identification and cloning of two genes (chiD and chiE) which we show by transposon mutagenesis and deletion analysis to be involved in the regulation of expression of chitin-degradative enzymes in S. liquefaciens. Regulatory genes for chitin degradation have not been described in any other species. The available evidence indicates that chiD codes for a trans-acting repressor of chitinase and chitobiase expression, while the product of chiE may be involved in inducer synthesis.

MATERIALS AND METHODS

Chemicals and enzymes. Chitin was obtained from Sigma Chemical Co. (St. Louis, Mo.), all restriction enzymes were from Bethesda Research Laboratories (Gaithersburg, Md.), T4 DNA ligase was from New England Biolabs, Inc. (Beverly, Mass.), and calf intestinal phosphatase was from Boehringer Mannheim Biochemicals (Indianapolis, Ind.). Other chemicals and reagents were from Fisher Scientific Co. (Pittsburgh, Pa.) and were of reagent grade.

Bacterial strains and plasmids. Chitinase genes were cloned from S. liquefaciens 1-141 (J. Kloepfer, Allelix Inc.), carrying genes for ampicillin (Ap+) and rifampin (Rif+) resistance. Escherichia coli RR1 (2) was used for transformations. E. coli CSH4 carrying the appropriate plasmid (13) was used as the host in Tn5 mutagenesis. Plasmids pBR329 (5) and pRK290 (6) were used for all cloning experiments, and plasmid pKK2013 (7) was used for mobilizing pRK290. E. coli RR1 containing plasmids pMKK10 and pMKK23 (M. Kozlowski and D. Wilkinson, unpublished data) were used for Tn5 mutagenesis of the S. liquefaciens genome. Lambda b221 c1857 Pam Oam rex:Tn5 (16) was used for Tn5 mutagenesis of the cloned DNA sequences in E. coli; this bacteriophage is referred to as lambda-Tn5.

Construction of a genomic DNA library. Unless stated otherwise, all recombinant DNA techniques were performed as described by Maniatis et al. (12). EcoRI fragments of S. liquefaciens 1-141 genomic DNA of 10 to 15 kilobases (kb) were ligated into plasmid pBR329, and E. coli RR1 was transformed. Inserts were subcloned into plasmid pBR329 (Tc+) for transfer back into S. liquefaciens.

Tn5 mutagenesis of wild-type S. liquefaciens. Tn5 mutagenesis with plasmids pMKK10 or pMKK23 (M. Kozlowski and D. Wilkinson, unpublished data) was carried out as described elsewhere (16). The transconjugants (Rif+ Km+) were first tested for the loss of Tc+ and then for chitinase activity as described below.

Tn5 mutagenesis of plasmid DNA. Tn5 mutagenesis of cloned fragments in E. coli plasmids was carried out with lambda-Tn5, as described elsewhere (16).

Conjugation. Triparental matings between E. coli and S. liquefaciens 1-141 were carried out as described elsewhere (16), and transconjugants were selected on 2YT plates containing 100 μg of rifampin per ml and 20 μg of tetracycline per ml.
**Chitinase and chitobiase assays.** Chitin (Sigma) was pretreated with HCl (11). In order to determine the chitinase activity from *S. liquefaciens*, 5 µl of a culture grown overnight in 2YT was plated on minimal chitin-containing agar plates (17) and the plates were incubated at 30°C. The size of the zone of chitin clearance was measured every day.

In order to screen *E. coli* clones for chitinase activity, the assay was performed as described above, except that minimal chitin-containing agar plates containing 40 µg of leucine per ml, 40 µg of proline per ml, 5 µg of thiamine per ml, and 200 µg of mannose per ml were used and the zone of chitin clearance was measured for up to 2 weeks. The size of the clearance zone and the time required for its detection was correlated with the amount of chitinase produced.

Chitobiase assays were performed as described by Wortman et al. (19) with 4-methylumbelliferyl-β-N,N'-diacetylchitobioside (Sigma).

**RESULTS**

Cloning of chitinase (*chiA* and *chiB*) and chitobiase (*chiC*) genes. An *S. liquefaciens* EcoRI partial genomic DNA library, containing 10- to 15-kb fragments, was constructed in pBR329 and screened for chitinase activity in *E. coli*, as described above. Among the first 300 transformants, 2 showed chitinase activity. Two *E. coli* clones containing plasmids pSJ21 and pSJ12 showed a zone of chitin clearance.

The DNAs from plasmids pSJ21 and pSJ12 were characterized by digestion (1) with restriction enzymes (Fig. 1). These plasmids contained 9- and 8-kb EcoRI fragments, respectively. The restriction maps of these two *S. liquefaciens* fragments were different. The chitinase genes present on them were different on the basis of their physical maps (Fig. 1), and they did not cross-hybridize with each other (data not shown). Therefore, two different chitinase genes were cloned in this experiment.

In order to map the chitinase genes on plasmids pSJ21 and pSJ12, smaller fragments were subcloned from the original *S. liquefaciens* fragment and these subclones were tested for their chitinase activities. Plasmid pSJ21 DNA was cut with *SalI*, *ClaI*, *SphI*, or *PstI* and religated to obtain the subclones pSJ21-1, pSJ21-2, pSJ21-3, and pSJ21-4, respectively. Only *E. coli* containing plasmid pSJ21-1 showed chitinase activity, the zone of chitin clearance increased with time.

**Phenotypes**

![Physical Maps](image)

**Fig. 1.** Restriction maps of plasmid pSJ21 (A), plasmid pSJ12 (B), and their derivatives. One of the two EcoRI sites in pSJ21 and pSJ12 was arbitrarily designated 0 for restriction map construction. All other restriction enzyme sites were numbered clockwise from this coordinate. Map coordinates of all the subclones and deletion derivatives refer to the parental clones pSJ12 or pSJ21. The *chi* genes are indicated with open boxes. The *S. liquefaciens* DNA in the subclones is indicated with thin lines. The *Tn5* insertion is indicated with a closed square. The numbers above the lines represent coordinates, in kilobases. The symbols on the right represent the relative levels (+, ++, +++, and +++++) of chitinase production. This is a combination of the size (in millimeters) of the zone of chitin clearance on the plates after 10 days (direct correlation) and the time required before a zone of clearance was detectable (inversely correlated). All mutants and strains behaved consistently in this semi-quantitative assay. The data are presented as follows (symbol, size [in millimeters] of zone of chitin clearance, time [in days] to detection of a zone of clearance): +, 0.5 to 1, 7; ++, 2, 4; +++, 3, 2; and +++++, 4 to 5, 1. A minus indicates that no chitinase activity was detectable even after 2 to 3 weeks. (C) Complementation properties of the *chi* mutant of *S. liquefaciens* in the presence of plasmids pSJ12-1 and pSJ12-2. The thick dashed line represents the region of the *S. liquefaciens* chromosome which codes for the *chib*, *chic*, *chid*, and *chiE* genes.
jugants chitinase with altered expression. These pMKK1O liquefaciens plasmids were obtained by ligating plasmid pRK290. Plasmid pSJ12-2 was a derivative of plasmid pSJ12 in which the DNA between the SalI site in pBR329 and the single SalI site in the insert (Fig. 1B) was deleted. Plasmid pSJ12::Tn5-1 (see below) was digested with SmaI or SalI and self-ligated to obtain plasmids pSJ12-4 and pSJ12-5, respectively. Chitinase activity was assayed (Fig. 1B) in all these subclones. Plasmid pSJ12-3 showed less chitinase activity than plasmid pSJ12-5, and plasmids pSJ12-1, pSJ12-2, and pSJ12-4 did not show any chitinase activity. These results suggest that another chitinase gene, referred to as chiB, is present between map coordinates 1 and 2200 of plasmid pSJ12.

Chitinase activity was tested as described by Wortman et al. (19) in E. coli clones containing plasmids pSJ21 and pSJ12. This activity was found in E. coli clones containing plasmid pSJ21-5 but was absent in E. coli RR1 carrying the parental plasmid pSJ12 (Fig. 2A). No chitinase activity was detected from plasmid pSJ21 or from any of its subclones.

Cloning of regulatory genes. S. liquefaciens genomic DNA was mutagenized by using suicide vectors containing Tn5 (M. Kozlowksi and D. Wilkinson, unpublished data). The suicide plasmids pMKK10 and pMKK23 contained a Km+ gene within Tn5 and a Tc' gene outside Tn5. After S. liquefaciens Rif' and E. coli clones containing plasmid pMKK10 or pMKK23 (Km' Tc') were mated, the transconjugants were selected for their growth on 2YT plates containing rifampin and kanamycin. They were Tc', confirming that the plasmid could not replicate in S. liquefaciens. Of 3,000 transconjugants screened, 2 mutants were identified with altered chitinase and chitobiase activities: SJ101, a chitinase and chitobiase overproducer (Chi+++), and SJ1, which was chitinase and chitobiase deficient (Chi-).

In the Chi+++ mutant, the Tn5 mutation may have inactivated a gene responsible for chitinase and chitobiase repression in S. liquefaciens. In order to ascertain whether such a regulatory gene was present on plasmid pSJ21 or pSJ12, these plasmids were mutagenized with lambda-Tn5. About 300 E. coli RR1 colonies containing plasmid pSJ21::Tn5 or pSJ12::Tn5 were tested for their chitinase activities. No chitinase overproduction was seen when plasmid pSJ21 was mutagenized with Tn5. However, the clones containing plasmid pSJ12::Tn5 revealed two types of chitinase-overproducing mutants. Ten Tn5 insertion mutants, which all showed a very large zone of chitinase overproduction, were physically mapped between map coordinates 2200 and 2500 of plasmid pSJ12 (Fig. 2B). In 10 other mutants that showed a lower level of chitinase overproduction, Tn5 insertions were mapped between map coordinates 2500 and 2700. The location of the leftmost and rightmost insertions are shown in Fig. 1B. This region between coordinates 2200 and 2700 is presumed to contain a repressor gene that is designated chiD.

Since there are two chitinase genes (chiA and chiB) and one chitobiase gene (chiC), mutation in the Chi⁻ mutant (SJ1) must be in a different gene (chiE) that affects expression of all three genes. In order to map this gene, sequences flanking the Tn5 insertion in the Chi⁻ mutant were cloned as follows. Total genomic DNA from the Chi⁻ mutant was cut with EcoRI (since Tn5 has no EcoRI site). These DNA fragments were ligated into plasmid pBR329, transferred into E. coli RR1, and selected for Km' in Tn5. A comparison of the restriction map from this plasmid with those from plasmids pSJ21 and pSJ12 revealed that it was identical to plasmid pSJ12 except for a Tn5 insertion at map coordinate 2800 (Fig. 1C).

The Chi⁻ mutant of S. liquefaciens was complemented by plasmid pSJ12-2 but not by plasmid pSJ12-1 (Fig. 1C and 3). Since all the transconjugants were chitinase proficient, the result must have been due to complementation by a trans-acting element rather than recombination. The plasmid yield from S. liquefaciens was low; therefore, plasmids were transferred from the Chi⁻ mutant containing plasmid pSJ12-2 to E. coli C600 by conjugation. Plasmid DNA analysis from these E. coli transconjugants confirmed the presence of plasmid pSJ12-2.

A functional map showing the organization of the chitinase (chiB), chitobiase (chiC), and the putative regulatory genes (chiD and chiE) for plasmid pSJ12 is presented in Fig. 1.

**DISCUSSION**

Cloning and characterization of chitinase (chiA, chiB) and chitobiase (chiC) genes. Results of the cloning and characterization of chitinase (chiA, chiB) and chitobiase (chiC) genes

FIG. 2. (A) Chitobiase activity of E. coli clones containing plasmids pBR329 (spot 1), pSJ12 (spot 2), and pSJ12-5 (spot 3). (B) Chitinase activity from E. coli clones containing plasmids pBR329 (spot 1), pSJ12 (spot 2), and pSJ12::Tn5-1 (spot 3).

FIG. 3. Complementation of the Chi⁻ Tn5 insertion mutant of S. liquefaciens SJ1 by recombinant plasmids. Chitinase plate assays were for SJ1 (spots 1); wild-type S. liquefaciens (spots 2); and SJ1 carrying plasmids pSJ12-2, pSJ12-1, and pRK290 (spots 3 to 5, respectively).
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indicate that two separate genes, *chiA* and *chiB*, each of which codes for a chitinase activity, were cloned. The restriction maps of these genes showed no similarity to the maps of chitinase genes from *S. marcescens* (8–10), *V. harveyi* (18), *V. vulnificus* (19), or *P. vulgaris* (4).

Chitinase and chitobiase activities of different subclones of plasmid pSJ12 revealed that both the *chiB* and the *chiC* genes were present between map positions 1 and 2200 of plasmid pSJ12 (Fig. 1B). In all other cases reported to date (9, 18, 19), restriction maps of the plasmids containing chitinase and chitobiase genes have differed from each other, suggesting that these enzymes might be encoded by distinct genes. There is enough coding capacity for two genes in this region, but we have no direct evidence for or against the fact that these are distinct genes.

**Cloning and characterization of regulatory (*chiD*, *chiE*) genes**. The phenotype of the Tn5-induced Chi′** +** mutant of *S. liquefaciens* is most easily explained as being caused by the inactivation of a repressor. Thus, Tn5 insertions in the region of nucleotides 2200 to 2700, e.g., pSJ12::Tn5-1, of plasmid pSJ12 resulting in chitinase and chitobiase overproduction (Fig. 1B) presumably define a gene that encodes a repressor. This hypothesis is supported by the finding that the deletions of this region in pSJ12-5 also allowed increased chitinase production. When the right-hand end of this region was disrupted (in pSJ12-3 or in pSJ12::Tn5-4), a phenotype intermediate between those of pSJ12-5 and pSJ12 was observed (Fig. 1B), suggesting that in these mutants either a reduced level of repressor is made or it is only partially active.

At least two chitinase genes and one chitobiase gene were identified in *S. liquefaciens*. Since no chitinase or chitobiase expression was seen in the Chi′ Tn5 insertion mutant, the Tn5 insertion in this mutant must be in a regulatory gene (*chiE*) and not in the structural genes. This Chi′ mutant was caused by the insertion of Tn5 into that part of the plasmid that corresponds to nucleotide 2800 of pSJ12 and could be complemented in trans by plasmid pSJ12-2. The rightmost *chiD* mutation was at nucleotide 2700. This indicates that *chiE* is contained within the region from nucleotides 2800 to 4600 of pSJ12. Since the *chiD* gene was mapped between map positions 2200 and 2700 of plasmid pSJ12, the *chiE* gene would be located adjacent to it (Fig. 1).

The *chiE* gene does not encode a permease similar to that of *V. harveyi* (18) or *V. vulnificus* (19), since no permease activity could be detected by plating the *E. coli* clones onto MacConkey agar (19). Moreover, when the Chi′ mutant (mutated in the *chiE* gene) was grown near wild-type *S. liquefaciens*, an inducer released by the wild-type *S. liquefaciens* was able to induce chitinase production from the Chi′ mutant (data not shown). This suggests that in wild-type *S. liquefaciens*, the function of the *chiE* gene product is to catalyze a step in the synthesis of the active inducer from chitin. This inducer is diffusible and can be taken up by the cell to block repressor function and hence allows chitinase and chitobiase production.

**Regulation of *chiA*, *chiB*, and *chiC* gene expression**. Chitinase expression in *E. coli* harboring different subclones of plasmid pSJ12 containing chitinase, repressor, or regulatory genes and in different mutants of *S. liquefaciens* is summarized in Table 1. According to the model described in Fig. 4, in the absence of chitin, a repressor is synthesized that blocks the expression of chitinase (*chiA*, *chiB*) and chitobiase (*chiC*) genes. Indeed, no chitobiase activity could be detected from *E. coli* RR1 containing plasmid pSJ12 with an intact repressor gene, while it was clearly detectable (Fig. 2A) from *E. coli* RR1 containing plasmid pSJ12-5 with a deletion of the repressor gene. When chitin was the sole carbon source, *chiE* produced the inducer by modifying the degradation products that were present in the chitin preparation or that were provided by basal-level expression of chitinase and chitobiase. The function of the inducer is to inactivate the repressor, since no *chiE* function is required in the absence or on inactivation of the repressor. This was shown by the fact that *E. coli* RR1 containing plasmid pSJ12::Tn5-1 (repressor negative, inducer positive) showed a level of chitinase expression similar to that of *E. coli* RR1 containing plasmid pSJ12-5 (repressor and inducer negative). Once synthesized, the inducer blocked the function of the repressor and allowed high levels of chitinase and chitobiase production by the cell.

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**TABLE 1. Effects of *chiD* and *chiE* genes on chitinase expression**

| Host strain | Genotype | Chitinase activity |
|-------------|----------|-------------------|
| *S. liquefaciens*, E. coli | *chiB* | *chiD* | *chiE* |
| Wild type | pSJ12 | + | + | + | Normal |
| Chi′ | NA | + | - | - | Inhibition |
| Chi′** +** | pSJ12::Tn5-1 | - | + | + | Overproduction |
| NA | pSJ12-5 | - | - | + | Overproduction |

* E. coli RR1 containing the indicated plasmids.
NA, Not available.
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