Cloning and Characterization of a Tetracycline Resistance Determinant Present in Agrobacterium tumefaciens C58

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Received 14 September 1998/Accepted 7 November 1998

Agrobacterium tumefaciens C58 and its derivatives give rise to spontaneous mutants resistant to tetracycline at a high frequency. We observed that a mutation affecting a tRNA processing function significantly affected the emergence of such mutants, suggesting that C58 contained a positively acting gene conferring resistance to tetracycline. A cosmid clone conferring resistance to tetracycline in Escherichia coli and Agrobacterium was isolated from a genomic bank of one such mutant. Subcloning, transposon mutagenesis, and DNA sequence analysis revealed that this DNA fragment contained two divergently transcribed genes, tetA and tetR, encoding products that were very similar to proteins of the Tet(A) class of tetracycline resistance systems. In the clone from this mutant, tetR was disrupted by an IS426. The homologous region from wild-type NT1 contained an intact tetR gene and did not confer resistance to tetracycline. Hybridization analysis showed that of 22 members of the genus Agrobacterium surveyed, only strains C58 and T37 contained the tet determinant. Moreover, only these two strains mutated to resistance to this antibiotic. Unlike other Tet(A) systems, neither tetracycline nor a series of its derivatives induced the expression of this tet gene unit. Other polycyclic compounds, including many of plant origin, also did not induce this tet gene system. The divergent promoter region of this tet system contained a single inverted repeat element identical to one such operator repeat in the promoter region of the tet determinant from the IncP1 plasmid RP4. TetR repressor proteins from the Agrobacterium tet system and from RP4 interacted with the regulatory operators. While the repressive effect of the TetR protein from strain C58 (TetRC58) on the tetA gene from strain RP4 (tetARp4) was not relieved by tetracycline, repression of tetAc58 by TetRp4 was lifted by this antibiotic.

Genes encoding resistance to antibiotics have been identified in a wide spectrum of prokaryotic organisms, including gram-negative and gram-positive bacteria. Although most of the genes characterized to date have been isolated from clinical isolates, these determinants are believed to have originated in soil bacteria (38). More recently, genes encoding proteins that confer resistance to several different drugs, the multidrug systems, have been identified and characterized (37). Horizontal transfer of these determinants among diverse bacteria are challenging the effectiveness of antibiotics in infectious disease control (39, 48).

Genes conferring resistance to tetracycline are among the most abundant of the identified drug resistance elements (29). Although mechanisms such as target site protection and drug inactivation are known (7, 44), most tetracycline resistance determinants function through the efflux mechanism, pumping a drug-metal ion complex out of the bacterial cell by a process energized by the membrane proton gradient (37, 45).

It is a well-recognized but unpublished observation that Agrobacterium tumefaciens C58 and its derivatives give rise at a high frequency to spontaneous mutants resistant to tetracycline. Most genetic and molecular analyses concerning A. tumefaciens and its plasmids are conducted in the C58 chromosomal background. Since many broad-host-range cloning vectors encode resistance to tetracycline as the selectable marker, the propensity of this strain to mutate to tetracycline resistance poses problems to the genetic analysis of this bacterium and to its use as a plant transformation agent.

During a study of mutants affecting the induction of tra genes of pTiC58 in NT1 (51), a T1 plasmid-cured derivative of C58 (53), we observed that one mutant, NTM7, while remaining able to mutate to tetracycline resistance, expressed the phenotype at a much lower level than did its wild-type parent. NTM7 later was found to carry a Tn5 insertion in a locus encoding a protein homologous to the product of the Escherichia coli rnd gene (30). In E. coli, a miaA mutant is unable to express Tet(M)-mediated tetracycline resistance (8). Since both the rnd and miaA genes are involved in tRNA modification (12, 52), we reasoned that there may be a positively acting tetracycline resistance element in the genome of strain C58 which can function normally in the wild-type bacterium but not in the rnd mutant. A study was initiated to investigate the nature of such tetracycline-resistant mutants. We confirmed our hypothesis and report here the molecular cloning, nucleotide sequence, and genetic characterization of a tetracycline resistance gene unit from this A. tumefaciens strain.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The strains of E. coli and A. tumefaciens and the plasmids used in this study are listed in Table 1. E. coli strains were grown at 37°C in L broth (LB), on L agar plates (Difco Laboratories, Detroit, Mich.), or in A medium (33). Agrobacterium strains were grown at 28°C in LB or on nutrient agar (NA) (Difco Laboratories). AB medium (11) supplemented with 0.2% mannitol as the sole carbon source was used as the defined minimal medium for Agrobacterium strains. Biotin was added to a final concentration of 2 μg/ml for biovar 2 strains. Agar (Difco Laboratories) was added at 1.5% to make solid medium. Except when specified in the text, antibiotics were used at the following concentrations: for E. coli, kanamycin at 50 μg/ml, tetracycline at 10 μg/ml, and ampicillin at 100 μg/ml; for Agrobacterium spp., kanamycin at 50 μg/ml, carbenicillin at 50 or 100 μg/ml, and tetracycline at 2 μg/ml. X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; Sigma Chemical Co., St. Louis, Mo.) was included in the medium at 40 μg/ml to monitor the production of β-galactosidase.

DNA manipulations. Plasmids were isolated by an alkaline lysis method (40). Cloning followed standard recombinant DNA techniques (40). Restriction di-
plasmids were introduced into A. tumefaciens by using GenElute spin columns (Supelco Inc., Bellefonte, Pa.). Plasmids were introduced into E. coli by CaCl$_2$-mediated transformation (40) and into A. tumefaciens by SI7-1-mediated mating (43) or by electroporation (9).

**Southern hybridization.** Genomic DNA was prepared from overnight cultures of Agrobacterium spp. as described previously (18). After digestion with restriction endonuclease, DNA fragments were separated on agarose gels and transferred to nitrocellulose sheets by using the GenElute spin columns (Supelco Inc., Bellefonte, Pa.). Genomic DNA was digested with Sau3AI to give an average fragment size of 40 kb and ligated to pZL1 that had been digested with the cos site from pCP13 (15) as a 1.7-kb BgII fragment containing the cos site from pCP13 (15) as a 1.7-kb BgII fragment into pBluescript SK(+) and ligated to pZL1. The ligation mixture was packaged into phage M13 by using the Packagene system (Promega Corporation, Madison, Wis.) and transduced into E. coli LE392.

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in the appearance at a high frequency of mutants resistant to this antibiotic (Fig. 1A and data not shown). However, we observed that a C58 mutant with a Tn5 insertion in a gene homologous to rnd (30) yielded tetracycline-resistant mutants that grew much more slowly on medium containing the antibiotic (Fig. 1B). Since a miaA mutant of E. coli does not express Tet(M)-mediated tetracycline resistance (8) and both rnd and miaA genes encode products involved in tRNA modification, we reasoned that strain C58 contains an active tetracycline resistance element that functions in the rnd+ but not in the rnd- mutant background. To test this, a genomic library of NT1TcR1, a tetracycline-resistant mutant of the otherwise-wild-type strain NT1, was constructed in the broad-host-range cosmide vector pZL1 (Table 1). This bank, in E. coli LE392, consists of 856 clones containing cosmids with an average insert size of 42 kb. The bank was screened on LB medium containing tetracycline, and one cosmid that confers resistance to this antibiotic on its E. coli host was isolated. This clone, named pZLT1, also conferred resistance to high levels of this antibiotic on several Agrobacterium strains, including 15955, R10, and KS4 (data not shown).

The tetracycline resistance trait was subcloned from pZLT1 as a 4.5-kb EcoRI fragment into pZL1 to generate pZLE4.5 (Fig. 2). To localize the tet determinant, this DNA fragment was recloned into pDSK519 (25) to give pDLE4.5, and this plasmid was mutagenized with Tn3 HoHo1 (46). The location and orientation of the insertion in each mutant plasmid were determined, and each was tested for its ability to confer resis-
tance to tetracycline. Of six mutations mapped, the three contiguous insertions that are located in the middle of the fragment abolished resistance to this antibiotic (Fig. 2).

**DNA sequence analysis.** DNA fragments corresponding to the region defined by the Tn3Holo1 insertion were subcloned and subjected to sequence analysis. BLAST searches revealed that one such EcoRI-HinIII fragment from plasmid pZLHE1.6 (Table 1) contained a portion of a tetR(A) homologue. As sequencing continued, however, this gene was abruptly disrupted by a totally different DNA sequence identical to the sequence of IS426, an IS element identified from A. tumefaciens T37 (50). Apparently, the 4.5-kb EcoRI fragment from the mutant contains an incomplete tet locus; insertion of IS426 introduced an EcoRI site which divided this region into two fragments when this enzyme was used for cloning. To obtain the complete sequence of the mutant version, the adjacent 4.9-kb EcoRI fragment was cloned into pBlueScript SK(+) from pZLT1 to generate pZLE8.4 (Table 1). Analysis of the insert identified sequences corresponding to the remainder of IS426 followed by the 3′ end of the tetR gene of strain CS8 (tetR
cs8).

Analysis of sequence data from pZLHS2.9 (Table 1), a plasmid harboring an insert from the opposite end of the region encoding the tetR(A) homologue, identified a single open reading frame of 1183 bp (Fig. 2 and data not shown). BLAST analysis indicated that this open reading frame encodes a protein very similar to the tetA(A) gene product from other tet systems.

To obtain the wild-type version of the tet locus from CS8, a portion of the tetA(A) homologue was used as a probe to screen a genomic bank of the wild-type, tetracycline-sensitive strain NT1 (16). A cosmid hybridizing strongly to the probe was identified and designated pZLT2 (Fig. 2). The hybridizing region was subcloned as an 8.5-kb EcoRI fragment into pBlueScript SK(+) to generate pZLE8.5 (Fig. 2), and the insert in this plasmid was used as the template to sequence a 2.6-kb region containing the wild-type tet locus.

Examination of the sequence indicated that this region contains two divergently transcribed open reading frames (Fig. 2 and data not shown). Database searches revealed that the two open reading frames can code for products that are very similar to the Tet(A) class of tetracycline resistance genes from E. coli transposons, with the highest similarity to that of the Tn1721 tet(A) homologue. As sequencing continued, however, this gene was abruptly disrupted by a totally different DNA sequence identical to the sequence of IS426, an IS element identified from A. tumefaciens T37 (50). Apparently, the 4.5-kb EcoRI fragment from the mutant contains an incomplete tet locus; insertion of IS426 introduced an EcoRI site which divided this region into two fragments when this enzyme was used for cloning. To obtain the complete sequence of the mutant version, the adjacent 4.9-kb EcoRI fragment was cloned into pBlueScript SK(+) from pZLT1 to generate pZLE8.4 (Table 1). Analysis of the insert identified sequences corresponding to the remainder of IS426 followed by the 3′ end of the tetR gene of strain CS8 (tetR
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The nucleotide sequences of tetA
cs8 and tetR
cs8 from the mutant and wild-type strains are identical. However, in the mutant, the sequence is interrupted by the IS426 insertion at bp 520 of tetR
cs8 (data not shown). This insertion generated a 5-bp duplication of the sequence 5′ GGGGGG 3′ at the target site.

A hypothetical path generated by the Kyte-Doolittle algorithm (28) revealed that TetA
cs8 contains 12 putative hydrophobic segments (data not shown). Such domains, which could represent transmembrane segments, are a typical feature of the major facilitator superfamily of proteins (31, 36). Sequence analysis indicated that, like other class A and class B TetR proteins, TetR
cs8 contains a typical helix-turn-helix domain in its N-terminal portion (data not shown). The tetA
cs8 and tetR
cs8 genes are separated by an 88-bp intergenic region with characteristics of regulated divergent promoters (Fig. 4A). A 15-bp inverted repeat (IR) element overlaps the putative −10 region of the tetA
cs8 promoter. This IR also overlaps the potential −35 site of the tetR
cs8 promoter. As shown in Fig. 4B, the sequence of this IR is identical to those of the operators found between the tetA and tetR genes of other class A and class B tet systems, including those of RP4 and Tn1721 (2, 35). However, the promoter region of tetA
cs8 lacks the second IR element present in the corresponding region of tetA RP4 and of tetR
tn1721. Furthermore, the intergenic region of tetA
cs8 is considerably shorter than those of the tetA elements of RP4 and Tn1721 (Fig. 4B).

**Expression of tetA
cs8 is not induced by tetracycline or related compounds.** In class A and class B tet systems, TetR represses expression of tetA and tetR, and this repression is relieved by subinhibitory levels of the antibiotic (3, 21, 23). Tetracycline does not induce the Agrobacterium tet system; as noted above, when a large number cells are spread onto medium containing this antibiotic, only a very small fraction of the cells grow to produce colonies. Moreover, of those such colonies tested, all now are constitutively resistant to tetracycline (data not shown). To study the regulation of the tetA and tetR genes of Agrobacterium spp., a 66-bp fragment of the intergenic region, which comprises sequence between the putative ribosome binding sites for the tetA
cs8 and tetR
cs8 genes, was cloned into the divergent promoter detection vector pRG9700 (13) to generate pZLOP1. In this construct, tetA
cs8 and tetR
cs8 are transcriptionally fused to the lacZ and uidA genes, respectively, of the vector. In addition, the tetR
cs8 gene was cloned as a NcoI fragment from pZLT2 into the expression vector pBAD22 (19) to generate pBetR. This construct allows us to regulate the expression of tetA
cs8 with arabinose. To express tetA
cs8 in Agrobacterium spp., this gene was excised from pBetR as an EcoRI-BamHI fragment and cloned into pDBL4 (Table 1), a broad-host-range expression vector containing the arabinose promoter and downstream transcriptional terminator from pBAD22 cloned into pBBR MCS-2 (27), to construct pDBL4-tetR. Expression of tetA
cs8 in these two constructs is inducible in A. tumefaciens and E. coli by addition of arabinose to the culture medium at a final concentration of 0.4%.

Under conditions in which tetA
cs8 is repressed, expression levels of the tetA
cs8::lacZ and tetR
cs8::uidA fusions were lower in strain NT1 (pZLOP1, pDBL4-tetR) than in strain NT1TcR1 (pZLOP1, pDBL4-tetR). We attribute the lower expression levels of these fusions in NT1 to the chromosomal copy of tetR
cs8. However, when the cloned copy of tetR
cs8 was induced by adding arabinose, expression of the reporter fusions in both strains was inhibited to similar levels (Table 2). Similarly, expression of the tetA
cs8::lacZ reporter on pZLOP1 was repressed by TetR
cs8 in an E. coli host but only upon addition of arabinose (Table 2).

Tetracycline, when added at final concentrations ranging in 10-fold increments from 0.0001 to 1 µg/ml, had no effect on the expression of tetA
cs8 or tetR
cs8 in either host (data not shown). Although tetracycline does not induce the expression of tetA
cs8, we consider the possibility that compounds similar to this antibiotic could be the ligands for TetR
cs8. To test this, a total of 25 poly cyclic compounds with structures similar to tetracycline were tested for their ability to induce the expression of the tetA
cs8::lacZ fusion. These included active derivatives of tetracycline as well as various nontoxic compounds, most of plant origin. None of these agents induced the expression of the tetA
cs8::lacZ fusion at a detectable level (data not shown). When the tetracycline-sensitive strains NT1 (pZLOP1) and NT1 (pZLOP1, pDBL4-tetR) were used as reporters, resistant mutants formed in the zones of growth inhibition caused by chlorotetracycline and oxytetracycline. However, no such mutants appeared in the zone of growth inhibition caused by minocycline (data not shown).
Cross-operator recognition by TetRₐCₕₕₜₐ and TetRRₐₚₕₜₐ. By sequence analysis, the putative 15-bp operator of tetₐₕₜₐ is identical to those of other tet systems (Fig. 4B). Furthermore, the sequence from residues 26 to 47 of TetRₐCₕₜₐ is virtually identical to that of the N-terminal helix-turn-helix domain of several related TetR proteins, including TetRₐₚₕₜₐ and TetRTn₁⁰ and TetRTn₁⁷₂₁ (Fig. 3B and data not shown). Given these similarities, we determined whether TetR from A. tumefaciens and from RPₐₜ would recognize the operators of the noncognate homologues. Strains DHₕₕₜₐ (pRK₄₁₅K, pBetR) and DHₕₕₜₐ (pRK₄₁₅K, pZLOP₁) were constructed for this study. Plasmid pRK₄₁₅K carries the RPₐₜ tetracycline resistance gene unit, and expression of this determinant is inducible by tetracycline (1₄, 2₅). If TetRCₕₜₐ can repress the expression of tetₐₘₜₜₐ upon induction of tetRₐₚₕₜₐ, strain DHₕₕₜₐ (pRK₄₁₅K, pBetR) should fail to grow on medium containing tetracycline. As shown in Table 3, this strain grows well on LB medium with tetracycline at a concentration of 10 mg/ml. However, when the expression of tetRₐₕₜₐ on pBetR was induced by arabinose, this strain was unable to grow on the same medium. These results indicate that TetRₐₕₜₐ can repress the tetₐₘₜₜₐ promoter and that this repression is not responsive to tetracycline. To determine if TetRₐₚₕₜₐ can repress tetₐₕₜₐ, the expression of the tetₐₕₜₐ::lacZ reporter was examined in strain DHₕₕₜₐ (pRK₄₁₅K, pZLOP₁). Results from this test indicated that TetRₐₚₕₜₐ from pRK₄₁₅K indeed repressed tetₐₕₜₐ expression, and like the repressive effect on its cognate tetₐₘₜₜₐ, the repression was responsive to tetracycline (Table 3). Thus, TetRₐₚₕₜₐ can recognize and repress expression from the promoter of the A. tumefaciens tet system. Furthermore, unlike the tetₐₕₜₐ system, this repression of tetₐₚₕₜₐ by TetRₐₚₕₜₐ is relieved by tetracycline.

Nature of the tetracycline-resistant mutants. Our data indicate that a tet unit in the genome of strain Cₕₜₐ accounts for the appearance of tetracycline-resistant mutants when this strain is grown in the presence of this antibiotic. In the particular mutant used in this study, the tetracycline resistance phenotype arose from the transposition of IS₄₂₆ into tetₐₕₜₐ. Since tetracycline does not induce the expression of tetₐₚₕₜₐ, we were interested in determining whether all tetracycline-resistant mutants were caused by the same type of mutation. To examine this, genomic DNA from eight independent tetracycline-resistant mutants was analyzed by restriction mapping, and the results are shown in Fig. 5. The results from all the mutants are similar, and they differ significantly from the restriction map of the wild-type strain (Table 4). The resistance phenotype was found to be due to the transposition of IS₄₂₆ into tetₐₕₜₐ.

**FIG. 3.** Relatedness of TetA and TetR from strain Cₕₜₐ to the gene products of tetₐₘₜₜₐ and tetRₐₚₕₜₐ from RPₐₜ. Alignments between the predicted amino acid sequence of TetACₕₜₐ and TetARPₐₜ (A) and of TetRCₕₜₐ and TetRRₐₚₕₜₐ (B) were performed by using the GAP algorithm from the GCG package as described in Materials and Methods. Identical amino acids are shown as white letters on a black background, and conserved amino acids are shown as black letters on a shaded background.
resistant mutants was probed with a DNA fragment containing the tetC58 genes. These eight mutants grouped into four types based on the size of the hybridizing fragment (data not shown). One class, represented by three mutants, contained a fragment indistinguishable in size from that of the wild type. In the second, two of the eight mutants gave a fragment indistinguishable in size from that of NT1TcR1, the original mutant from which the tet locus was cloned. The remaining three mutants contained two restriction fragment length polymorphism (RFLP) patterns different from that of the wild type and of NT1TcR1 (data not shown).

Comparison of the DNA sequences of operators from the tet systems of C58, RP4, and Tn1721. Inverted repeat sequences are boxed. The transcriptional directions of the tetR and tetA genes are indicated by arrows. A-10 and A-35, the −10 and −35 sites for the tetR promoters; R-10 and R-35, the −10 and −35 sites for the tetA promoters; O-2C58, putative operator of tetC58. O_L and O_R, the left and right operators, respectively, of the tet systems of Tn1721 and RP4.

DISCUSSION

We report here the characterization of a tet system from A. tumefaciens C58. This active resistance system accounts for the long-known observation that C58 and its derivatives give rise to tetracycline-resistant mutants at a high frequency.

This resistance determinant is structurally similar to that of tet systems found in plasmids and transposons from E. coli and other gram-negative bacteria, with the highest relatedness being to the class A tetracycline resistance gene system from Tn1721. The tetC58 system confers resistance to tetracycline and derivatives such as chlorotetracycline and oxytetracycline but not to minocycline. This pattern of resistance suggests that tetC58 does not belong to the Tet(S) class of tetracycline resistance genes, which confers resistance to minocycline as well as to tetracycline, chlorotetracycline, and oxytetracycline (10).

Like other class A systems, tetC58 is organized as two genes, a regulatory gene and a gene coding for an efflux pump, transcribed divergently from an intergenic region. Potential promoter elements for both genes are overlapped by a single putative operator composed of a 15-bp perfect IR element. Our genetic analysis indicates that this intergenic region contains all of the cis-acting information required for the regulated expression of these two genes. The DNA sequence of the putative operator is identical to that of operators from other tet systems. However, the promoter region of tetC58 contains only one repeat while the promoter regions of the tet determinants

**TABLE 2. tetR<sub>C58</sub> controls expression of tetA<sub>C58</sub> and tetR<sub>C58</sub>**

<table>
<thead>
<tr>
<th>Strain</th>
<th>tetR&lt;sub&gt;C58&lt;/sub&gt; source</th>
<th>β-Galactosidase activity&lt;sup&gt;a&lt;/sup&gt; (tetA&lt;sub&gt;C58&lt;/sub&gt;/lacZ)</th>
<th>β-Glucuronidase activity&lt;sup&gt;a&lt;/sup&gt; (tetR&lt;sub&gt;C58&lt;/sub&gt;/uidA)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No ara With ara</td>
<td>No ara With ara</td>
</tr>
<tr>
<td>NT1TcR1(pZLOP1, pDLB4)</td>
<td>None</td>
<td>77 85</td>
<td>53 45</td>
</tr>
<tr>
<td>NT1TcR1(pZLOP1, pDLB4-tetR)</td>
<td>pDLB4-tetR</td>
<td>65 2</td>
<td>18 6</td>
</tr>
<tr>
<td>NT1(pZLOP1, pDLB4)</td>
<td>Chromosome</td>
<td>19 14</td>
<td>14 20</td>
</tr>
<tr>
<td>NT1(pZLOP1, pDLB4-tetR)</td>
<td>Chromosome/pDLB4-tetR</td>
<td>25 2</td>
<td>14 7</td>
</tr>
<tr>
<td>DH5(pZLOP1, pDLB4)</td>
<td>None</td>
<td>2,213 2,243</td>
<td>NT&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>DH5(pZLOP1, pDLB4-tetR)</td>
<td>pDLB4-tetR</td>
<td>1,678 29</td>
<td>NT NT</td>
</tr>
</tbody>
</table>

<sup>a</sup> β-Galactosidase and β-glucuronidase activities are expressed as units per 10<sup>9</sup> CFU as described in Materials and Methods. Activities were determined in cultures with or without 0.4% arabinose (ara) added as an inducer.

<sup>b</sup> NT, not tested (E. coli DH5s has an endogenous β-glucuronidase activity).
of RP4 and Tn1721 each contain two copies of the operators (Fig. 4B).

Heterologous repressor-operator recognition occurs between different classes of tet systems, even those with dissimilar operators (26). The high degree of sequence similarity between operators from the tetC58 and tetRP4 systems suggested that TetR proteins from these two systems could recognize the noncognate operator. This proved to be the case: TetR of RP4 repressed the tet system of C58 and TetR of C58 repressed tetracycline resistance conferred by the RP4 tet system. The capacity of TetRP4 to repress expression of the tetA\_c58::lacZ fusion suggests that the 15-bp IR is the operator and indicates that a single copy of this element is sufficient for regulation of both genes.

The expression of almost all tetracycline resistance gene systems examined to date is under strict control (29, 45). Among these, repression of the Tet(B) class by TetR is the best understood (21, 45). In the absence of tetracycline, the repressors bind to operators overlapping the promoters of both the tetA and tetR genes (21). Binding of tetracycline to TetR(B) induces a conformational change that leads to release from the operators and concomitant expression of tetA (22, 34). Tetracycline-inducible TetR-mediated regulation of other tet systems is believed to function by a similar mechanism (21). Two lines of evidence indicate that tetracycline does not induce the Agrobacterium tet system. First, when cells were spread onto medium containing tetracycline at concentrations ranging from 10 to 1 \( \mu \text{g/ml} \), only a small portion of the cells grew into colonies (data not shown). Moreover, those that did grow now expressed constitutive resistance to tetracycline. If tetracycline functioned as the inducer for tetC58, virtually all of the cells should be resistant to this antibiotic. Furthermore, E. coli and Agrobacterium strains harboring pZLE8.5 (Table 1), which carries the wild-type tetC58 locus, are sensitive to tetracycline, even at concentrations to which TetRP4 confers resistance (data not shown). Second, while the tetA\_c58::lacZ and the tetR\_c58::uidA reporter fusions were expressed constitutively in cells lacking TetRP4, the expression of both fusions was depressed considerably in cells harboring tetR\_c58. Furthermore, neither reporter was inducible by tetracycline. Repression of tetA\_RP4 by TetRC58 is not relieved by tetracycline. On the other hand, repression of tetC58 by TetRP4 does respond to tetracycline. These two sets of results indicate that the failure of the tet system of C58 to respond to tetracycline is a function of TetR and not of the tet operator. Mutants of the related repressor from Tn10 that do not respond to tetracycline have been isolated and characterized. These noninducible mutants repress the expression of tetA\_c58::lacZ fusions by binding to the operators even in the presence of tetracycline (5, 20, 34). We propose that TetRC58 represents such a noninducible form of TetR. Clearly, this repressor can bind DNA. Why it no longer reacts to tetracycline remains to be determined. We considered the possibility that tetracycline is not the true ligand for TetRC58. However, none of the 25 compounds we tested induced the system. Certainly, this was not an exhaustive survey. But on balance, that the locus of strain C58 can be regulated by TetRP4, in response to tetracycline strongly suggests that this gene unit once was responsive to this antibiotic.

The interchangeability with respect to operator recognition exhibited by the two TetR proteins could explain another observation concerning tetracycline resistance in strain C58. Derivatives of this A. tumefaciens strain harboring RP4 and vectors carrying tetRP4 express only low level resistance to tetracycline. We propose that TetRC58 is partially dominant over TetRP4. Thus, since TetRC58 does not respond to tetracycline, the tetRP4 determinant is not fully expressed in its A. tumefaciens host.

Expression of tetracycline resistance in C58 apparently occurs only following a mutation affecting regulation. These mutations can be of several types; in the particular mutant used in this study, the tetracycline resistance phenotype resulted from the insertion of IS426 in tetC58. In genetic analysis carried out with C58 and its derivatives, this element is commonly associated with phenotypes in which the selection gives derepressed mutants (17). However, diverse RFLP patterns in Southern

\[ \text{TetR}_{\text{C58} \text{ and TetR}_{\text{RP4}} \text{ Recognize noncognate tet operators}} \]

<table>
<thead>
<tr>
<th>Strain</th>
<th>( \beta )-Galactosidase activity(^a)</th>
<th>Growth on TC medium(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No TC With TC No ara With ara</td>
<td></td>
</tr>
<tr>
<td>D250</td>
<td>NT NT ++ --</td>
<td></td>
</tr>
<tr>
<td>D250</td>
<td>NT NT ++ ++</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\)\( \beta \)-Galactosidase activity is expressed as units per 10\(^7\) CFU as described in Materials and Methods.

\(^b\) Growth was assessed on LB medium supplemented with tetracycline at 5 and 10 \( \mu \text{g/ml} \) (TC medium) and with or without 0.4% arabinose (ara) added as an inducer.

\[ \text{TetR C58 and TetR}_{\text{RP4}} \text{ recognize noncognate operators}} \]

<table>
<thead>
<tr>
<th>Agrobacterium species</th>
<th>Strain(^c)</th>
<th>Appearance of TC-resistant mutants on medium with TC at (5 \mu \text{g/ml})</th>
<th>Homology with tetC58(^d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. tumefaciens</td>
<td>C58 (NT1, UIA5, A281)</td>
<td>++ ++ ++</td>
<td></td>
</tr>
<tr>
<td>T37</td>
<td>++ ++ ++</td>
<td></td>
<td></td>
</tr>
<tr>
<td>K108</td>
<td>++ ++ ++</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adh5 (LBA4404)</td>
<td>++ ++ ++</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AnNC</td>
<td>++ ++ ++</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R10</td>
<td>++ ++ ++</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15955</td>
<td>++ ++ ++</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B6</td>
<td>++ ++ ++</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bc542</td>
<td>++ ++ ++</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chty5</td>
<td>++ ++ ++</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ANT4</td>
<td>++ ++ ++</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^c\) Determined by Southern analysis as described in Materials and Methods.

\(^d\) Strains shown in parentheses are derivatives of the type strain that also were tested.

\[ \text{TetR C58 and TetR}_{\text{RP4}} \text{ recognize noncognate operators}} \]
analysis of eight independent mutants indicated that other types of mutations can result in the tetracycline resistance phenotype. While mutants with a pattern indistinguishable from that of NT1TcR1 may have arisen from IS26 transposition, those with a pattern similar to that of the wild type could represent point mutations or very small deletions or insertions in the tetRCS8 gene. They also could represent operator-con- sutive mutations that no longer allow TetRCS8 to bind at the promoter.

Among the agrobacteria we examined, only strain T37 contains DNA with detectable homology to the tet locus from C58. Furthermore, only C58 and T37 give rise to tetracycline-resis- tant mutants (Table 4). These two strains were isolated from different parts of the United States (42). Moreover, although strain C58 induces unorganized tumors, strain T37 causes ter- atomas. Both strains contain a nopaline/agrocinopine-type Ti plasmid, but RFLP analysis indicates that these two elements have diverged considerably from each other (42). Our survey also shows that generation of tetracycline-resistant mutants is associated with the presence of a tetCS8 homologue in the genome of the particular isolate; those lacking this element do not yield mutants at a detectable level. The presence of the tet locus in strain UIA5, a derivative of C58 lacking both pTiC58 and pATCS8, indicates that this resistance determinant is asso- ciated with one of the two chromosomes of strain C58 (1). Interestingly, this is not the only antibiotic resistance determi- nant in strain C58. This strain, which also mates to resistance to chloramphenicol at a high frequency, contains a gene coding for an atypical chloramphenicol acetyltransferase (47). Whether this cat gene is present in strain T37 or any other isolate of Agrobacterium spp. is not known.

ACKNOWLEDGMENTS

We thank Abigail Salyers for supplying the tetracyclines and related chemicals and Stanton Gelvin and Ramon Penhaller for helpful discus- sions. We also thank Malcolm Winkler, whose seminar on RNA processing got us started on the problem.

Portions of this research were supported by grants R01 GM52465 from the NIH and NC CNTRL SOY SKF ANT'C from the North Central Soybean Association to S.K.F.

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