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 R plasmid RP4. TetR repressor proteins from the Agrobacterium tet system and from RP4 interacted with the heterologous operators. While the repressible effect of the TetR protein from strain C58 (TetRC58) on the tetA gene from strain RP4 (tetA RP4) was not relieved by tetracycline, repression of tetA C58 by TetR RP4 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 Agrobacterium 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, NT7, while remaining able to mutate to tetracycline resistance, expressed the phenotype at a much lower level than did its wild-type parent. NT7 later was found to carry a Tn5 insertion in a locus encoding a protein homologous to the product of the Escherichia coli rmd gene (30). In E. coli, a mia4 mutant is unable to express Tet(M)-mediated tetracycline resistance (8). Since both the rmd and mia4 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 rmd 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 (Difeo Laboratories, Detroit, Mich.), or in A medium (33). Agrobacterium strains were grown at 28°C in LB or on nutrient agar (NA) (Difeo 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-
A. tumefaciens

AB-mannitol media, respectively. Overnight cultures were washed and diluted on the plates after 72 h of incubation at 28°C. To give tetracycline-resistant mutants was assessed by the appearance of colonies spread onto NA medium containing tetracycline at 5 or 10 μg/ml results were deposited in the GenBank database under accession no. AF090987.

Isolation of tetracycline-resistant mutants. Agrobacterium spp. were grown in LB liquid medium to saturation. A 200-μl volume of culture from each strain was spread onto NA medium containing tetracycline at 5 or 10 μg/ml. The capacity to give tetracycline-resistant mutants was assessed by the appearance of colonies on the plate after 2 h of incubation at 28°C.

Gene expression assays. E. coli and A. tumefaciens strains were grown in A and AB-mannitol media, respectively. Overnight cultures were washed and diluted 40-fold in the same respective media, and incubation was continued until the optical density at 600 nm (OD600) reached about 0.7 for E. coli and 0.3 for A. tumefaciens. When needed, cultures were divided, and inducers (arabinose or tetracycline) were added to one subculture at the concentrations specified in Results. β-Galactosidase activity was determined quantitatively as described previously (33). β-Galactosidase activity was measured as described previously (13, 24, 49). The activity of this enzyme was calculated using the Miller formula (33), with OD420 replaced by OD415. All enzymatic activities were expressed as units per 10^9 CFU.

RESULTS

Cloning a tetracycline resistance determinant from A. tumefaciens C58. Plating strain C58 onto medium containing tetracycline at concentrations ranging from 0.5 to 20 μg/ml results

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TABLE 1. Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristic(s)</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli DH5α</td>
<td>F- 8089lacZΔM15 endA1 recA1 supD77 proAB rpsL15 trpC16 thr-1 leu2-3, 112 hisD20 ade-3</td>
<td>Stratagen</td>
</tr>
<tr>
<td>pKiB/Tc</td>
<td>Km&lt;sup&gt;+&lt;/sup&gt;; tetracycline resistant cloning vector</td>
<td>Promega</td>
</tr>
<tr>
<td>pCP13/B</td>
<td>Amp&lt;sup&gt;+&lt;/sup&gt;; cloning vector</td>
<td>Promega</td>
</tr>
<tr>
<td>pRK415K</td>
<td>Km&lt;sup&gt;+&lt;/sup&gt;; broad-host-range cloning vector</td>
<td>Promega</td>
</tr>
<tr>
<td>pSSHE</td>
<td>Amp&lt;sup&gt;+&lt;/sup&gt;; source for TnHoHo1</td>
<td>Promega</td>
</tr>
<tr>
<td>pZLE4.5</td>
<td>4.5-kb EcoRI fragment containing the Tc&lt;sup&gt;+&lt;/sup&gt; trait from pZL1</td>
<td>Promega</td>
</tr>
<tr>
<td>pZLE4.5</td>
<td>4.5-kb EcoRI fragment containing the Tc&lt;sup&gt;+&lt;/sup&gt; trait in pZL1</td>
<td>Promega</td>
</tr>
<tr>
<td>pZLE4.5</td>
<td>4.5-kb EcoRI fragment containing the Tc&lt;sup&gt;+&lt;/sup&gt; trait in pZL1</td>
<td>Promega</td>
</tr>
<tr>
<td>pLE392</td>
<td>2.9-kb HindIII/EcoRI fragment containing tet&lt;sup&gt;+&lt;/sup&gt; from pZLE4.5 cloned into pBluescript SK&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Promega</td>
</tr>
<tr>
<td>pBL4</td>
<td>4.8-kb HindIII/EcoRI fragment containing tet&lt;sup&gt;+&lt;/sup&gt; from pZLE4.5 cloned into pBlueScript SK&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Promega</td>
</tr>
<tr>
<td>pBL4</td>
<td>4.8-kb HindIII/EcoRI fragment containing tet&lt;sup&gt;+&lt;/sup&gt; from pZLE4.5 cloned into pBlueScript SK&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Promega</td>
</tr>
<tr>
<td>pBetR</td>
<td>tet&lt;sup&gt;+&lt;/sup&gt;R&lt;sup&gt;+&lt;/sup&gt; cloned as a 2.7-kb NcoI fragment from pBAD22</td>
<td>Promega</td>
</tr>
<tr>
<td>pBetR</td>
<td>tet&lt;sup&gt;+&lt;/sup&gt;R&lt;sup&gt;+&lt;/sup&gt; cloned as a 2.7-kb EcoRI/BamHI fragment from pBetR into pBL4</td>
<td>Promega</td>
</tr>
<tr>
<td>pBetR</td>
<td>tet&lt;sup&gt;+&lt;/sup&gt;R&lt;sup&gt;+&lt;/sup&gt; cloned as a 2.7-kb EcoRI/BamHI fragment from pBetR into pBL4</td>
<td>Promega</td>
</tr>
<tr>
<td>pBetR</td>
<td>tet&lt;sup&gt;+&lt;/sup&gt;R&lt;sup&gt;+&lt;/sup&gt; cloned as a 2.7-kb EcoRI/BamHI fragment from pBetR into pBL4</td>
<td>Promega</td>
</tr>
</tbody>
</table>

<sup>a</sup> Amp<sup>+</sup>, ampicillin resistant; Km<sup>+</sup>, kanamycin resistant; Sp<sup>+</sup>, spectinomycin resistant; Tc<sup>+</sup>, tetracycline resistant; Str<sup>+</sup>, streptomycin resistant.
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 anti-
biotic (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 mod-
ification, we reasoned that strain C58 contains an active tetra-
cycline resistance element that functions in the rnd1 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
cosmid vector pZL1 (Table 1). This bank, in E. coli LE392,
consists of 856 clones containing cosmids with an average in-
sert 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 K84 (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-

FIG. 1. A mutation in a tRNA processing gene in NTM7 (29) affects expression of resistance in spontaneous tetracycline-resistant mutants of NT1. Approximately
10^8 cells of strains NT1 (A) and NTM7 (B) were spread on NA plates supplemented with tetracycline at 5 μg/ml. The plates were incubated for 3 (A) or 6 (B) days
at 28°C. Note that the tetracycline-resistant mutants of NTM7 arising on the plate grow considerably more slowly than do those of strain NT1 on medium containing
the antibiotic.

FIG. 2. Genetic organization of the tet locus from the tetracycline-resistant and wild-type genomes of A. tumefaciens C58. A restriction map and the locations of
the independent Tn3HoHo1 insertions, identified in clone pDLE4.5, are shown. The response of each mutant to tetracycline was assessed as described in Materials
and Methods and is expressed as resistant to the antibiotic (+) or susceptible to the antibiotic (−). The horizontal crossbar capping each insertion site indicates the
direction of transcription of the lacZ reporter of Tn3HoHo1 as determined by restriction mapping. β-Galactosidase activity was assessed on medium containing X-Gal:
the presence of blue colonies (+) or white colonies (−) is indicated. The IS426 element is indicated by the hatched portion of the bar. S, Smal; E, EcoRI; K, KpnI;
H, HindIII; Tc, tetracycline.
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 Tn3H0l insertion were subcloned and subjected to sequence analysis. BLAST searches revealed that one such EcoRI-HindIII 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 pZLE4.8 (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 C58 (tetR(C58)).

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(C58) gene product from other tet systems.

To obtain the wild-type version of the tet locus from C58, a portion of the tetA(C58) 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 Tn721 tet system (2). One we call tetA(C58) could code for a protein with an *M* of 41,458 and having 46% identity (55% similarity) with TetA from the IncP1 R plasmid RP4 (35) (Fig. 3A). The second, which could code for a protein with an *M* of 24,389 and having 46% identity (55% similarity) with TetR from RP4 (35), was named tetR(C58) (Fig. 3B). The nucleotide sequences of tetA(C58) and tetR(C58) 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(C58) (data not shown). This insertion generated a 5-bp duplication of the sequence 5′ GGGGG 3′ at the target site.

A hydropathy plot generated by the Kyte-Doolittle algorithm (26) revealed that TetA(C58) 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(C58) contains a typical helix-turn-helix domain in its N-terminal portion (data not shown). The tetA(C58) and tetR(C58) 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 tetR(C58) promoter. This IR also overlaps the potential −35 site of the tetR(C58) 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 Tn721 (2, 35). However, the promoter region of tetR(C58) lacks the second IR element present in the corresponding region of tetR(P4) and of tetR(Tn721). Furthermore, the intergenic region of tetR(C58) is considerably shorter than that of the tet elements of RP4 and Tn721 (Fig. 4B).

**Expression of tetR(C58) 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 tetR(C58) and tetR(C58) genes, was cloned into the divergent promoter detection vector pRG970b (13) to generate pZLOP1. In this construct, tetA(C58) and tetR(C58) are transcriptionally fused to the lacZ and uidA genes, respectively, of the vector. In addition, the tetR(C58) 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 tetR(C58) with arabinose. To express tetR(C58) 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 pBRRMCS2-2 (27), to construct pDBL4-tetR. Expression of tetR(C58) 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 tetR(C58) is repressed, expression levels of the tetR(C58)::lacZ and tetR(C58)::uidA fusions were lower in strain NT1(pZLOP1, pDBL4-tetR) than in strain NT1::tetR (pZLOP1, pDBL4-tetR). We attribute the lower expression levels of these fusions in NT1 to the chromosomal copy of tetR(C58). However, when the cloned copy of tetR(C58) 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(C58)::lacZ reporter on pZLOP1 was repressed by TetR(C58) 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(C58) or tetR(C58) in either host (data not shown). Although tetracycline does not induce the expression of tetA(C58), we consider the possibility that compounds similar to this antibiotic could be the ligands for TetR(C58). To test this, a total of 25 polycyclic compounds with structures similar to tetracycline were tested for their ability to induce the expression of the tetA(C58)::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(C58)::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 TetR<sub>C58</sub> and TetRR<sub>RP4</sub>. By sequence analysis, the putative 15-bp operator of tet<sub>C58</sub> is identical to those of other tet systems (Fig. 4B). Furthermore, the sequence from residues 26 to 47 of TetR<sub>C58</sub> is virtually identical to that of the N-terminal helix-turn-helix domain of several related TetR proteins, including TetR<sub>RP4</sub>, Tet<sub>R</sub><sup>R</sup>tn<sup>10</sup>, and Tet<sub>R</sub><sup>R</sup>tn<sup>1721</sup> (Fig. 3B and data not shown). Given these similarities, we determined whether TetR from A. tumefaciens and from RP4 will recognize the operators of the noncognate homologues. Strains DH5<sub>a</sub>(pRK415K, pBetR) and DH5<sub>a</sub>(pRK415K, pZLOP1) were constructed for this study. Plasmid pRK415K carries the RP4 tetracycline resistance gene unit, and expression of this determinant is inducible by tetracycline (14, 25). If TetRC<sub>C58</sub> can repress the expression of tet<sub>A</sub>RP4, upon induction of tetR<sub>C58</sub>, strain DH5<sub>a</sub>(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 μg/ml. However, when the expression of tetR<sub>C58</sub> on pBetR was induced by arabinose, this strain was unable to grow on the same medium. These results indicate that TetRC<sub>C58</sub> can repress at the tet<sub>A</sub>RP4 promoter and that this repression is not responsive to tetracycline. To determine if TetRR<sub>RP4</sub> can repress tet<sub>A</sub>C<sub>C58</sub>, the expression of the tet<sub>A</sub>C<sub>C58</sub>:<sub>lacZ</sub> reporter was examined in strain DH5<sub>a</sub>(pRK415K, pZLOP1). Results from this test indicated that TetRR<sub>RP4</sub> from pRK415K indeed repressed tet<sub>A</sub>C<sub>C58</sub> expression, and like the repressive effect on its cognate tet<sub>A</sub>, the repression was responsive to tetracycline (Table 3). Thus, TetRR<sub>RP4</sub> can recognize and repress expression from the promoter of the A. tumefaciens tet system. Furthermore, unlike the tet<sub>C58</sub> system, this repression of tet<sub>A</sub>C<sub>C58</sub> by TetR<sub>RP4</sub> is relieved by tetracycline.

Nature of the tetracycline-resistant mutants. Our data indicate that a tet unit in the genome of strain C58 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 IS26 into tet<sub>R</sub>C<sub>C58</sub>. Since tetracycline does not induce the expression of tet<sub>A</sub>C<sub>C58</sub>, 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 prepared and used in the following experiments.
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).

Surveying for the presence of the tet locus in the genomes of Agrobacterium spp. We examined a collection of wild-type isolates of Agrobacterium spp. for the tet locus by Southern analysis using a probe containing part of tetA and most of tetR. We also examined some commonly used derivatives of strain C58. Of those not derived from C58, only strain T37 contains the tetA locus from strain C58 (Table 4). Moreover, the probe hybridized to an EcoRI fragment of the same size in the two strains (data not shown).

<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>No ara</td>
<td>With ara</td>
<td>No ara</td>
</tr>
<tr>
<td>NT1TcR1(pZLOP1, pDLB4)</td>
<td>None</td>
<td>77</td>
<td>53</td>
</tr>
<tr>
<td>NT1TcR1(pZLOP1, pDLB4-tetR)</td>
<td>pDLB4-tetR</td>
<td>65</td>
<td>18</td>
</tr>
<tr>
<td>NT1(pZLOP1, pDLB4)</td>
<td>Chromosome</td>
<td>19</td>
<td>14</td>
</tr>
<tr>
<td>NT1(pZLOP1, pDLB4-tetR)</td>
<td>Chromosome/pDLB4-tetR</td>
<td>25</td>
<td>14</td>
</tr>
<tr>
<td>DHS5(pZLOP1, pDLB4)</td>
<td>None</td>
<td>2,213</td>
<td>NT&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>DHS5(pZLOP1, pDLB4-tetR)</td>
<td>pDLB4-tetR</td>
<td>1,678</td>
<td>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 DHS5 has an endogenous β-glucuronidase activity).

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 tet<sub>C58</sub> system confers resistance to tetracycline and derivatives such as chlorotetracycline and oxytetracycline but not to minocycline. This pattern of resistance suggests that tet<sub>C58</sub> 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, tet<sub>C58</sub> is organized as two genes, a regulatory gene and a gene coding for an efflux pump, transcribed divergently from an intergenic region. Potential promotor 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 promotor region of tet<sub>C58</sub> contains only one repeat while the promotor regions of the tet determinants...
of RP4 and Tn7271 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 TetR_{RP4} 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 μg/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 tetRP4::uidA reporter fusions were expressed constitutively in cells lacking TetR_{RP4}, 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 TetR_{C58} is not relieved by tetracycline. On the other hand, repression of tetC58 by TetR_{RP4} 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 TetR_{C58} 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 TetR_{C58}. 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 TetR_{RP4} 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 TetR_{C58} is partially dominant over TetR_{RP4}. Thus, since TetR_{C58} 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

<table>
<thead>
<tr>
<th>Strain</th>
<th>β-Galactosidase activitya</th>
<th>Growth on TC mediumb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No TC</td>
<td>With TC</td>
</tr>
<tr>
<td>DH5 (pRK415K, pBetR)</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>DH5 (pRK415K, pBAD22)</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>DH5 (pZLOP1)</td>
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<td>2,211</td>
</tr>
<tr>
<td>DH5 (pZLOP1, pRK415K)</td>
<td>387</td>
<td>2,154</td>
</tr>
</tbody>
</table>

TABLE 3. TetR_{C58} and TetR_{RP4} Recognize noncognate tet operators

TABLE 4. Tetracycline resistance characteristics of Agrobacterium spp.
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 NT1tC1R1 may have arisen from IS426 transposition, those with a pattern similar to that of the wild type could represent point mutations or very small deletions or insertions in the tetR_CSS gene. They also could represent operator-constitutive mutations that no longer allow TetR_CSS 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-resistant 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 tumors. Both strains contain a nopaline/agrocinopine-type Ti plasmid, but RFLP analysis indicates that these two elements are not identical. Both strains contain a nopaline/agrocinopine-type Ti plasmid, but RFLP analysis indicates that these two elements are not identical.

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