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Mutation and Mapping of Genes Involved in Production of the Antibiotic TA in *Myxococcus xanthus*

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Transposition of Tn*V* and Tn*5lac* into *Myxococcus xanthus* yielded 8,381 kanamycin-resistant mutants that were tested for antibiotic TA production. Twenty-four of the mutants were nonproducers of TA (less than 0.4 ng/ml), and 3 produced a higher level (2.5 μg/ml) than the parent strain (1.5 μg/ml). For most of the strains, there was 100% cotransduction between kanamycin resistance and the altered TA phenotype. Southern blot analysis of restriction digests of the mutant DNA indicated that the transposons were inserted at different sites on the *M. xanthus* chromosome. The TA genes were mapped by cotransduction between pairs of mutants following replacement of the initial insert of one of the pair with the tetracycline resistance transposon Tn*5*-132. Nine of the 13 nonproducers tested were linked over a 36-kb stretch of the chromosome. There was no linkage between one of the overproducers and any of the nonproducers tested.

Myxococcus xanthus produces a broad-spectrum, bactericidal antibiotic when grown under nutritionally limiting conditions (18, 25). The antibiotic, referred to as TA, inhibits cell wall synthesis by interfering with polymerization of the lipid-disaccharide-pentapeptide (27). Antibiotic TA ($C_{34}H_{57}O_9N$) has been crystallized, and its chemical properties have been reported (16, 19). Gerth and coworkers (6) reported that *Myxococcus virescens* MxV48 produces a family of closely related macrocyclic antibiotics. The chemical structure of one of them, myxovirescin A, was elucidated (24). It appears to be identical to antibiotic TA (Fig. 1).

The interesting feature of antibiotic TA is that it adheres avidly to a variety of surfaces (17). When administered orally or intravenously to animals, the antibiotic concentrates in an active form close to the site of administration. Tissues treated with antibiotic TA and then washed continuously to remove unbound antibiotic retain bactericidal activity. To examine the potential medical applicability of adhesive antibiotic TA, we performed a clinical trial with eight human volunteers suffering from moderate to severe gingivitis (12). The data indicated that low doses of TA (0.1 mg) applied to the dentogingival junction caused a rapid reduction in plaque, as well as clinicopathological signs, such as those measured by the gingival and bleeding indices.

Further development of this potentially useful antibiotic depends on obtaining enhanced productivity. Direct manipulation of antibiotic biosynthetic genes is currently being explored as a means of strain improvement. We report here the isolation of TA mutants and the mapping of the TA genes on the *M. xanthus* chromosome by transduction. Several procedures recently were developed with *M. xanthus* for generating mutants and mapping and manipulating genes (e.g., see reference 8). Especially relevant is the demonstration by Sodergren et al. (22) that genetic mapping by cotransduction with different insertions of Tn*5* (e.g., one carrying resistance to kanamycin and the other carrying resistance to tetracycline) yields results that closely match physical mapping results. The goal of this study was to apply these procedures, with minor modifications, to better understand gene arrangement and subsequent regulation of the pathway leading to the production of antibiotic TA.

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MATERIALS AND METHODS

Bacterial strains. *M. xanthus* ER15 is a red-pigmented mutant of *M. xanthus* TA (ATCC 31046) that produces relatively high levels of antibiotic TA, up to 3 μg/ml. *M. xanthus* DK1622 was obtained from D. Kaiser. See Tables 3 and 4 for the remaining *M. xanthus* strains used in this study. *Escherichia coli* C600 was used as a host for bacteriophage P1. *E. coli* ER Kan^r, used for assaying antibiotic TA, was derived from *E. coli* ESS Cap^r (5) by transduction with P1::Tn*V* (Kan^r).

M. xanthus strains were maintained on 1CT agar (see below) at 16 to 18°C. At these temperatures, the cultures needed to be transferred only every 4 to 6 weeks.

Media. 1CT medium contained 10 g of Casitone (Difco Laboratories, Detroit, Mich.) and 2 g of $MgSO_4 \cdot 7H_2O$ per liter. The medium was supplemented with 1.5% agar for plates or 0.7% agar for soft agar overlays. The medium was supplemented with kanamycin sulfate (Sigma Chemical Co., St. Louis, Mo.) at 20 μg/ml (CTK20) or 50 μg/ml (CTK50).

0.5CTS medium contained, per liter, 5 g of Casitone, 2 g of $MgSO_4 \cdot 7H_2O$, 1 g of serine, 1 g of alanine, and 1 g of glycine. NB medium contained 8 g of nutrient broth (Difco) and 5 g of NaCl per liter. It was supplemented with 1.8% agar (Bacto; Difco) for plates (the bottom layer) or 0.9% agar for the top layer.

Bioassay of antibiotic TA. *E. coli* ESS Cap^r or *E. coli* ER Kan^r was grown overnight in NB medium, and 0.1 ml of the culture was mixed with 2.5 ml of soft agar and spread over NB plates. The plates were stored in the cold for up to 1 week. Pieces of agar to be assayed for antibiotic TA or filter pads to which culture extracts had been applied were placed on top of the indicator bacteria, and the plates were incubated at 37°C for 16 h.

Mutagenesis. Mutants were isolated following transposition of Tn*V* (4) or Tn*5lac* (9) into the chromosome of *M. xanthus* ER15. In both cases, the transposon was introduced into the cells by use of bacteriophage P1 carrying the transposon. The recipient bacteria were grown in 1CT

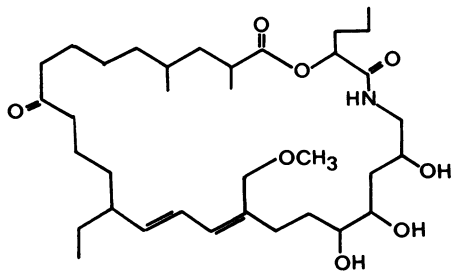

FIG. 1. Chemical structure of antibiotic TA.

medium to the early stationary phase, pelleted, and resuspended in 1CT medium plus 5 mM $CaCl_2$ to approximately 5 × 10^9 cells per ml. P1 lysates were prepared as described previously (4, 9) and used at a multiplicity of infection (MOI) of 2 to 3. After 30 min of adsorption, aliquots were plated on CTK20 plates. After 16 to 20 h at 32°C, each plate was overlaid with 1CT soft agar (0.7%) containing kanamycin to bring the concentration to 24 μg/ml (Tn*V*) or 40 μg/ml (Tn*5lac*). Colonies that appeared after 4 to 6 days were transferred with sterile toothpicks three times on CTK50 plates, after which they were examined for antibiotic production or β-galactosidase activity.

Screening of mutants for antibiotic TA production. Each kanamycin-resistant mutant was inoculated into 1.5 ml of 0.5CTS agar in a separate well of a 24-well tissue culture tray (Sterillin Ltd., Hounslow, United Kingdom). After 1 week of incubation at 30°C, standard pieces of agar (1-mm diameter) were removed from the wells and placed over lawns of indicator bacteria. The zone of growth inhibition was recorded after 16 h at 37°C. When there was no activity, the test was repeated on the next day with a larger piece of agar (6-mm diameter). Mutants that did not show any activity in the second test were assayed quantitatively as follows. Single colonies were inoculated into 2 ml of 1CT medium. After 2 days at 30°C, 0.5 ml of the starter cultures was transferred into 50-ml flasks containing 19.5 ml of 1CT medium and further incubated for another 2 days. TA was extracted and assayed as follows. Five milliliters of culture was mixed with 18.7 ml of methanol-chloroform (2:1 [vol/vol]). After 1.5 h at room temperature, the mixture was centrifuged at 10,000 rpm for 5 min. The supernatant fluid was collected in a flask and mixed with 6.25 ml of chloroform and 6.15 ml of water. The phases were separated by centrifugation, the chloroform phase was dried in vacuo, and the residue was dissolved in 100 μl of ethanol. The total sample was assayed on 6-mm-diameter filter paper pads (Schleicher & Schuell) placed on lawns of indicator bacteria. The sensitivity of the assay was 2 ng; therefore, a lack of inhibition indicated the presence of <0.4 ng of TA per ml of culture. The mutants were maintained on CF agar (7), on which they develop fruiting bodies and spores.

Screening for β-galactosidase expression. Screening for β-galactosidase expression was done by transferring Kan^r colonies to 1CT agar plates containing 40 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) (United States Biochemical Corp., Cleveland, Ohio) per ml. The plates were examined after 4 days of incubation at 32°C for blue colonies.

Myxophage transduction. The myxophages used in the transduction experiments were Mx8clp2 (9) and Mx4-LA27 (1). Stocks were prepared as described by Avery and Kaiser (1), and the phages were irradiated just before the experiment by UV light at a dose that killed approximately 90% of the population. The recipient bacteria were centrifuged and resuspended in 1CT medium to a density of 2 × 10^9/ml. Phages and bacteria were mixed at an MOI of 1 to 2 (including nonviable phage particles) for 60 min at 25°C. Aliquots were then plated (by the soft agar technique) on CTK20 plates. The plates were overlaid on the next day with a second layer of top agar, which brought the kanamycin concentration to 75 μg/ml. The plates were incubated at 30°C for 5 to 7 days. Transductants were transferred three times on CTK50 plates and assayed for antibiotic activity. Three or four transductants of each mutant were kept for further studies.

Transposon replacement. To provide an additional marker, transposon Tn*V*, carrying kanamycin resistance, was replaced in several of the strains by transposon Tn*5*-132, carrying tetracycline resistance (1). P1::Tn*5*-132, prepared as described by Avery and Kaiser (1), was used to transduce Tn*V*-containing *M. xanthus* ER15 or DK1622 grown in 1CT medium to a density of 5 × 10^8 cells per ml. The phage was added (in the presence of 5 mM $CaCl_2$) to 0.4 ml of cell suspension at an MOI of 5. After 20 min at 25°C, 2.5 ml of 1CT soft agar was added to each tube, and the mixture was plated over 1CT agar containing 2.5 μg of oxytetracycline (Sigma) per ml. The plates were incubated at 30°C for 14 to 15 h and then overlaid with an additional 4 ml of soft agar containing oxytetracycline to bring the concentration on the plates to 12.5 μg/ml. The plates were incubated for 7 to 10 days at 30°C, and colonies were transferred on 1CT agar plates supplemented with 12.5 μg of oxytetracycline per ml. After three transfers, the colonies were checked for growth on kanamycin.

DNA extraction and manipulation. DNA was prepared as described by Avery and Kaiser (1). Cloning of Tn*V*-containing fragments was done by self-ligation and transformation into *E. coli* C600 or MC1061 as described by Furuichi et al. (4). T4 ligase and restriction enzymes were obtained from International Biotechnologies Inc. and used in accordance with the instructions of the manufacturer. Southern blotting and hybridization were performed as described by Maniatis et al. (11). Plaque hybridization was done as described by Silhavy et al. (21). Plasmids were prepared by gradient centrifugation or by the use of a Qiagen plasmid kit (Qiagen Inc., Chatsworth, Calif.). They were labeled with [^{32}P]dCTP by use of the International Biotechnologies Inc. Prime Time "C" Biosystem.

TABLE 1. Mutagenesis of *M. xanthus* ER15 with transposon Tn*V*

Expt	Transduction efficiency (10^{-7})[a]	No. of Kan^r mutants examined	No. of antibiotic TA mutants that were[b]:	
			Nonproducers	Overproducers
1	5	252	0	0
2	6	300	1	0
3	2	790	0	1
4	6	1,918	3	0
5	6	2,122	2	0
Total		5,382	6	1

[a] Number of *M. xanthus* Kan^r colonies per input bacteriophage; MOI = 2.
[b] After initial screening on agar, antibiotic TA mutants were examined in liquid cultures as described in Materials and Methods. Nonproducers contained <0.4 ng/ml, the overproducer contained 2.5 mg/ml, and the parent strain contained 1.5 mg/ml.

TABLE 2. Mutagenesis of *M. xanthus* ER15 with transposon Tn*5lac*[a]

Expt	Transduction efficiency (10^{-7})	No. of Kan^r (Kan^r *lac*$^+$) mutants examined	No. of antibiotic TA mutants that were:		
			lac$^+$ TA$^-$	*lac* TA$^-$	*lac*$^+$ TA^{++}
1	3	209 (68)	1	0	0
2	3	1,536 (ND)	5	4	1
3	3	1,246 (ND)	4	4	1
Total		2,991	10	8	2

[a] The experiments were carried out as described in Table 1, footnotes *a* and *b*, except that P1 was used at an MOI of ca. 3. β-Galactosidase activity was examined on 1CT agar containing X-Gal. Antibiotic TA nonproducers (TA$^-$) and overproducers (TA^{++}) were as defined in Table 1, footnote *b*. ND, not determined.

RESULTS

Mutagenesis of *M. xanthus* ER15 with transposons Tn*V* and Tn*5lac* and isolation of antibiotic TA mutants. Transposition of Tn*V* into *M. xanthus* ER15 yielded kanamycin-resistant mutants at a frequency of 2×10^{-7} to 6×10^{-7} colony per PFU (Table 1). This frequency is higher than that reported with other *M. xanthus* strains, e.g., 6×10^{-8} (4). The frequency of Tn*5lac*-induced kanamycin-resistant mutants was also relatively high (Table 2), although it could not be accurately determined (because of the inability of P1::Tn*5lac* to form regular plaques [9]). Of 5,382 kanamycin-resistant mutants obtained with Tn*V*, 6 did not produce antibiotic TA (<0.4 ng/ml) and 1 produced a higher level (2.5 μg/ml) than the parent strain (1.5 μg/ml). The proportion of Kan^r colonies that were TA mutants was higher among the P1::Tn*5lac*-induced mutants than among the P1::Tn*V*-induced mutants: of 2,991 kanamycin-resistant mutants tested, 18 were nonproducers (10 of these were also *lac*$^+$) and 2 were overproducers of TA (both were *lac*$^+$). Approximately one-third of all the Kan^r mutants were β-galactosidase positive.

To examine whether the transposon insertions were responsible for the mutation in TA production, we examined the linkage between the transposon insertions and the genes responsible for the alteration in TA production (referred to as TA genes) by myxophage transduction into *M. xanthus* ER15 or DK1622. Except in two of the mutant strains, there was 100% cotransduction between kanamycin resistance and the TA phenotype. With strains 4637 and 2513, the cotransduction frequencies were 33 and 20%, respectively. Tables 3 and 4 list the insertional mutants and their derivatives that were used in this study. All new strains were designated by the prefix "ER" followed by an arbitrary identification number. Since "ER" was used for all the new strains mentioned in this work, it is dropped in the text.

Phenotypic complementation between pairs of mutants. Five antibiotic TA-negative (TA$^-$) mutants that contained both the tetracycline and kanamycin resistance markers (3707, 1909, 4637, 6117, and 6144) were examined for phenotypic complementation by spotting all possible mixtures of two mutants on 0.5CTS agar. Antibiotic TA was not detected in any of the combinations.

Resistance of mutants to antibiotic TA. It has been shown in several cases that genes coding for antibiotic resistance are coupled to genes involved in the biosynthesis of the same antibiotic (14, 23). Six TA$^-$ mutants from the DK1622 background (1912, 3708, 4639, 6118, 6199, and 1310) and seven TA$^-$ mutants of the ER15 background (419, 1130, 1010, 3110, 7512, 2513, and 1027) were examined for resistance to 10, 20, and 30 μg of TA per ml. Two overproducing mutants (1500 and 5242, both from an ER15 background) were also examined. Growth was estimated after 4 days at 31°C and compared with that of the two parental strains, DK1622 and ER15.

TABLE 3. *M. xanthus* strains originating from Tn*V* mutagenesis

Strain	Insert	Phenotype			Derivation
		TA[a]	Kan^r	Tet^r	
ER1309	Ω1309	−	+	−	P1::Tn*V* × ER15
ER1310	Ω1309	−	+	−	Mx8 (ER1309) × DK1622
ER1321	Ω1309	−	−	+	P1::Tn*5*-132 × ER1310
ER3707	Ω3707	−	+	−	P1::Tn*V* × ER15
ER3708	Ω3707	−	+	−	Mx8 (ER3707) × DK1622
ER3710	Ω3707	−	−	+	P1::Tn*5*-132 × ER3708
ER4637	Ω4637	−	+	−	P1::Tn*V* × ER15
ER4639	Ω4637	−	+	−	Mx8 (ER4637) × DK1622
ER6117	Ω6117	−	+	−	P1::Tn*V* × ER15
ER6118	Ω6117	−	+	−	Mx8 (ER6117) × DK1622
ER6119	Ω6117	−	−	+	P1::Tn*5*-132 × ER6118
ER6160	Ω6117	−	+	−	Mx8 (ER6117) × DK1622
ER6144	Ω6144	−	+	−	P1::Tn*V* × ER15
ER6199	Ω6144	−	+	−	Mx8 (ER6144) × DK1622
ER1909	Ω1909	−	+	−	P1::Tn*V* × ER15
ER1912	Ω1909	−	+	−	Mx8 (ER1909) × DK1622
ER1913	Ω1909	−	−	+	P1::Tn*5*-132 × ER1912
ER1923	Ω1909	−	+	−	Mx8 (ER1909) × DK1622
ER1500	Ω1500	++	+	−	P1::Tn*V* × ER15
ER1502	Ω1500	++	+	−	Mx8 (ER1500) × DK1622
ER1512	Ω1500	++	−	+	P1::Tn*5*-132 × ER1500

[a] See Table 2, footnote *a*, for explanation of symbols.

All strains grew on 10 μg of TA per ml. On 20 μg of TA per ml, ER15 and all mutants from the ER15 background grew normally, whereas DK1622 and all mutants from the DK1622 background failed to grow (except for strains 6118 and 6199, which grew, but poorly). On 30 μg of TA per ml, none of the strains from the DK1622 background grew; ER15, 419, and 1130 also did not grow on 30 μg of TA per ml. However, all other ER15 background strains grew well. In addition, strains 1010, 3110, 7512, 1027, 1500, and 5242 grew on 50 μg of TA per ml. None of the mutational events in the 13 mutants examined appeared to be accompanied by a loss of resistance to the antibiotic.

Southern blot analysis of transposon insertions in TA mutant chromosomes. Chromosomal DNA of the mutants was digested with various restriction enzymes and electrophoresed, and Southern blots were hybridized with ^{32}P-labelled pTF1 (4) as a probe. pTF1 consists of pBR322 and transposon Tn*V*. For two of the enzymes, *Sal*I and *Xho*I, the molecular sizes of the digested DNA that hybridized with pTF1 are given in Table 5. The data show that (with the possible exception of strains 6118 and 6199) each of the mutants has the insert on a different site on the bacterial chromosome. They also show that the different mutants can be used for cloning chromosomal fragments of different sizes. For example, DNA prepared from strain 4639 and cut with restriction enzyme *Sal*I should yield a clone containing 1.7 kb of *M. xanthus* DNA (the size of Tn*V* being 6.0 kb [4]), whereas DNA prepared from strain 3708 should yield the largest fragment of *M. xanthus* DNA (10.9 kb). Since transposon Tn*5lac* carries a *Sal*I restriction site (9.1 kb from one end), digestion with this enzyme should yield two fragments. However, only one of them, the upstream flanking region of the insertion, will carry the *kan-lac* region and therefore be

TABLE 4. *M. xanthus* strains originating from Tn*5lac* mutagenesis

Strain	Insert	TA phenotype[a]	Derivation
ER1311	Ω1311	−	P1::Tn*5lac* × ER15
ER1312	Ω1311	−	Mx4 (ER1311) × ER15
ER2513	Ω2513	−	P1::Tn*5lac* × ER15
ER2514	Ω2513	−	Mx4 (ER2513) × ER15
ER3110	Ω3110	−	P1::Tn*5lac* × ER15
ER3111	Ω3110	−	Mx4 (ER3110) × ER15
ER1010	Ω1010	−	P1::Tn*5lac* × ER15
ER1030	Ω1010	−	Mx8 (ER1010) × ER15
ER1020	Ω1010	−	Mx8 (ER1010) × DK1622
ER409	Ω409	−	P1::Tn*5lac* × ER15
ER410	Ω410	−	Mx4 (ER409) × ER15
ER419	Ω419	−	P1::Tn*5lac* × ER15
ER420	Ω419	−	Mx4 (ER419) × ER15
ER2425	Ω425	−	P1::Tn*5lac* × ER15
ER2426	Ω425	−	Mx4 (ER425) × ER15
ER1130	Ω1130	−	P1::Tn*5lac* × ER15
ER1131	Ω1130	−	Mx4 (ER1130) × ER15
ER1027	Ω1027	−	P1::Tn*5lac* × ER15
ER1037	Ω1027	−	Mx4 (ER1027) × ER15
ER7512	Ω7512	−	P1::Tn*5lac* × ER15
ER7513	Ω7512	−	Mx4 (ER7512) × ER15
ER4034	Ω4034	++	P1::Tn*5lac* × ER15
ER5242	Ω5242	++	P1::Tn*5lac* × ER15

[a] All were Kan[r]. See Table 2, footnote *a*, for explanation of symbols.

suitable for cloning. For strain 1020, it will be 2.9 kb, and for strain 420, it will be 5.4 kb.

Mapping of the TA genes. The Tn*V* and Tn*5lac* insertional mutations were mapped by transduction between pairs of mutants. To provide a second marker in addition to the kanamycin resistance marker, transposon Tn*V*, carrying kanamycin resistance, was replaced in several of the strains by transposon Tn*5*-132, carrying tetracycline resistance (1). The tetracycline-resistant strains were used as recipients in the transduction experiments, whereas the kanamycin-resistant strains served in most cases as donors. Table 6 shows the results of transduction experiments with the TA nonproducers and strain 1321. Of the 12 mutant strains examined, at least 8 were linked to 1321. The physical distance between each pair of genes, as determined with the data in Table 6, was calculated by Wu's equation (26), and the results are summarized in Fig. 2. This procedure has been shown to provide mapping distances that coincide with those obtained by physical methods (22). Similar map positions were obtained when the mutations were transduced into Kan[s] Tet[r] strains 3710 and 6119.

TABLE 5. Southern blot analysis of TA mutants[a]

Strain[b]	Molecular size(s) (kb) of DNA fragments produced by:	
	*Xho*I[c]	*Sal*I[d]
1310	5.0, <2, <2	12.7
3708	5.0, 3.8, <2	16.9
4639	5.0, 10.5, 2.4	7.7
6118	5.0, 2.7, <2	12.1
6199	5.0, 3.3, <2	11.9
1912	5.0, 8.3, <2	9.5
1502	5.7, 4.1, 2.6, <2	8.2
1020	2.5, 14.0, <2	12.0, 6.6
420	2.5, 11.5, 3.6	14.5, 3.2

[a] *Xho*I and *Sal*I digests of chromosomal DNA were probed with ^{32}P-labelled pTF1 (4).
[b] 1020 and 420 contained Tn*5lac* insertions; the others contained Tn*V* insertions.
[c] Transposons Tn*V* and Tn*5lac* have two restriction sites for *Xho*I. The fragment expected between the two *Xho*I restriction sites on Tn*V* is 5.0 kb (4), and that for Tn*5lac* is 2.4 kb (9a).
[d] Transposon Tn*5lac* is 12 kb long and carries a *Sal*I restriction site 9.1 kb from one end (9a). Transposon Tn*V* is 6 kb long (4) and carries no *Sal*I restriction site.

TABLE 6. Mapping of the TA genes by myxophage transduction[a]

Donor[b]	No. of Kan[r] transductants[c]			Cotransduction frequency (%)[d]
	Total	Tet[r]	Tet[s]	
3708	141	0	141	>99
4639	144	129	15	10
6199	144	27	117	81
6160	44	14	30	68
1923	144	144	0	<1
1030	48	28	20	42
3111	144	144	0	<1
7513	35	0	35	>97
1037	135	113	22	16
2514	144	142	2	1.4
1131	24	24	0	<5
420	22	22	0	<5

[a] Strain 1321 (Kan[s] Tet[r]) was used as the recipient.
[b] The donor strains were Kan[r] Tet[s]. Myxophage Mx4 or Mx8 grown on these strains was used to infect the recipient strain.
[c] Transductants were initially isolated on 1CT agar containing kanamycin and then tested for Tet[r] as described in Materials and Methods.
[d] Percentage of transductants with the donor phenotype.

The fact that mutations in 1309 and 1909 were unlinked was also shown by DNA-DNA hybridization. An *M. xanthus* ER15 DNA library was prepared in phage vector lambda D69 (13). The DNA was cut with *Sau*3A, and fragments of approximately 10 kb were packaged into the phage particles. The phages were probed with ^{32}P-labelled DNA prepared from cloned DNA of mutants 1310 (Ω1309 *Sal*I fragment, 13.5 kb, including Tn*V*) and 1912 (Ω1909 *Sal*I fragment, 9.5 kb, including Tn*V*) by the plaque hybridization technique as described by Silhavy et al. (21). Plaques reacting to each of the two clones were isolated. Clone 1912 responded in a specific way to its homologous phage particles. Clone 1310 reacted strongly with its homologous phage particles, but it also reacted, albeit weakly, with phage particles carrying clone 1912. Upon reexamination, it was

TABLE 7. Crosses between TA-overproducing strain 1512 and different non-TA-producing strains

Donor	Recipient	No. of transductants[a]	TA phenotype[b]
1512 (Tet[r])	1500 (Kan[r])	99 (Tet[r])[c]	++
1512 (Tet[r])	1310 (Kan[r])	7 (Tet[r])	+
1512 (Tet[r])	3708 (Kan[r])	6 (Tet[r])	+
1512 (Tet[r])	1010 (Kan[r])	22 (Tet[r])	±
6199 (Kan[r])	1512 (Tet[r])	83 (Kan[r])	−
6160 (Kan[r])	1512 (Tet[r])	21 (Kan[r])	−
4639 (Kan[r])	1512 (Tet[r])	39 (Kan[r])	−
1923 (Kan[r])	1512 (Tet[r])	119 (Kan[r])	−

[a] All transductants were kanamycin resistant, unless noted otherwise.
[b] ++, overproduction of antibiotic TA; +, parental level of TA production; ±, low level of TA production; −, no TA production.
[c] All 99 transductants obtained in this cross were kanamycin susceptible.

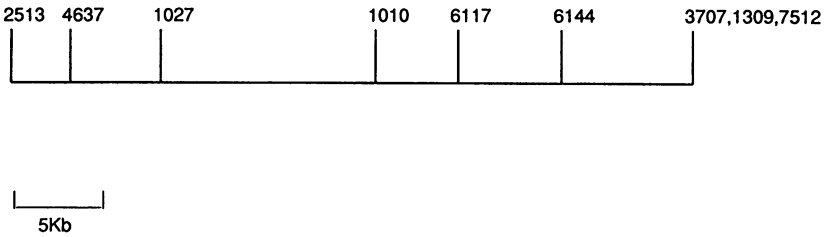

FIG. 2. Map of clustered genes involved in antibiotic TA production. Cotransduction frequencies (Table 6) were used to obtain recombination distances by the equation of Wu (26) as applied to phages Mx4 and Mx8 (8).

found that clone 1310 reacted with lambda plaques, even those not carrying any fragments of *M. xanthus* DNA.

Table 7 shows the results of experiments in which the overproducer strain carrying Tn*V* insert Ω1500 was used either as a donor or as a recipient in crosses with different nonproducers whose genes mapped in the cluster shown in Fig. 2. There was no linkage between the overproducer and any of these nonproducers. One of the noncluster mutants, 1909, was also checked and found to be unlinked. Interestingly, the overproducer restored the antibiotic activity of the mutants whose genes mapped to the right terminus of the cluster.

DISCUSSION

When *M. xanthus* is infected by bacteriophage P1 carrying transposon Tn*5* or Tn*5lac*, the transposon moves from P1 to many different sites on the *M. xanthus* chromosome (9, 10). Analysis of many transposon-induced mutations suggested that Tn*5* is inserted anywhere in the *M. xanthus* chromosome (8, 9). The fact that the mutants blocked in TA production were found to be different from each other by Southern blot analysis is consistent with this conclusion.

Of the 8,381 kanamycin-resistant mutants screened in this study, 24 (0.29%) were blocked in TA production. The level of detection of the antibiotic indicated that these mutants produced <0.03% of the amount produced by the parent strain. The genome size of *M. xanthus* is 9,454 kbp (2). On the basis of the above-described data, it is possible to estimate roughly that about 27 genes are essential for TA production. Since no information is available on the biosynthetic pathway for TA production, we cannot estimate how many enzymes are involved. Some of the mutants blocked in TA production may have defects in amino acid or vitamin synthesis that could indirectly prevent antibiotic synthesis.

Transduction experiments indicated that 9 of the 13-non-TA-producing mutant genes examined were linked. Transducing particles of bacteriophage Mx8 carry 56 kb of DNA. Thus, any linkage between two markers indicates that they are <56 kb apart. The frequency of spurious cotransduction of two unlinked markers is <0.02% (8). Besides simply grouping the TA mutations into a cluster on the chromosome, the frequency of cotransduction has provided the first map of genes essential for TA production (Fig. 2). The estimations should be interpreted with caution, because the number of transductants examined was relatively low in some cases and the estimations did not take into account the effects of finite marker size or gene conversion (20). The nine clustered genes that were studied extended over a 36-kb segment. Assuming that inserts Ω1309 and Ω3707 are at the right terminus of the linkage group, insert Ω2513 appears to be close to the left terminus. Inserts Ω1909, Ω419, Ω1130, and Ω3110 are not linked to the right-terminal markers of the linkage group, but they may be linked to the left-terminal markers. For technical reasons, an experiment testing the last suggestion could not be performed.

Recently, a physical map of the *M. xanthus* chromosome was determined by pulsed-field gel electrophoresis of *Ase*I-digested DNA (3). Sixteen restriction fragments were obtained. The locations of seven of the antibiotic TA mutations described here were determined (3; unpublished data). Four mutations that mapped in the cluster (1010, 3707, 1309, and 6117) were found on the same large (ca. 1,800-kb) restriction fragment, P. Two mutations that were not linked to the cluster by the transduction experiments, 419 and 1500, were found on different fragments, L and O, respectively. One mutation, 1909, was found to be present on the large P fragment, but the transduction experiments reported here failed to show any linkage between 1909 and the TA cluster. The physical mapping of 1909 indicated that it was ca. 320 kb from one end of the P fragment, whereas 1010 (located within the cluster by transduction) was 256 kb from that end. Thus, it is possible that 1909 is close to the cluster of TA genes but is just beyond the maximum distance (ca. 56 kb) that bacteriophage Mx8 can be used for in measuring linkage. In general, the above-described data are consistent with the results of the transduction experiments presented here.

The only insert studied that led to the overproduction of antibiotic TA, Ω1500, was not linked to any of the nonproducing genes. However, it did restore antibiotic production to mutants with mutations mapping to the right terminus of the linkage group. These data suggest that there are bypass mechanisms for the production of antibiotic TA. For this explanation to be valid, under the growth conditions used in this study, the alternative genes would not be operative, thereby allowing for the isolation of nonproducers by a single insertional mutation in a TA gene. The Ω1500 insertion would lead to the expression of certain alternative TA genes, causing overproduction in the parent strain and a recovery of TA production in certain nonproducing mutants. It should be mentioned that there is genetic evidence for two alternative pathways for part of the developmental cycle in *M. xanthus* (15).

The mutations isolated and mapped in this investigation provide useful data for a rational approach to the study of the regulation of the biosynthesis of the secondary metabolite antibiotic TA and the subsequent construction of an overproducing strain.

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