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Use of Tn*5lac* To Study Expression of Genes Required for Production of the Antibiotic TA

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The β-galactosidase activities arising from Tn*5lac* insertions in several genes required for antibiotic TA production were measured under different growth conditions. In all of the non-TA-producing mutants, the β-galactosidase specific activity was higher when the cells were grown in nutrient-limited 0.5CTS medium (0.5% Casitone plus alanine, serine, and glucose) than in rich 2CT medium (2% Casitone). One of the mutants, 420, had low β-galactosidase specific activity in both media. The other seven mutants containing inserts in genes essential for TA production had specific activities of 139 to 367 U/mg of protein in 0.5CTS medium and 11 to 48 U/mg of protein in 2CT medium. The β-galactosidase specific activities of two strains, 1030 and 420, increased during exponential growth in 0.5CTS medium. The β-galactosidase specific activities of both strains increased greatly when the cells were grown in the presence of magnesium phosphate, which traps ammonium ions. The Tn*5lac* insertions in 1030 and 420 were used to screen for mutants with increased levels of transcription. An *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine-induced mutation in 1030 that mapped 17 kb from the Ω1010 insert increased the specific activity of β-galactosidase 21 times in 2CT medium. The regulatory mutation appears to release the repression caused by 2CT medium. A UV-induced mutation in 420 increased the β-galactosidase specific activity 1.4 to 2.4 times. Medium conditions that affect the transcription of TA genes are discussed in terms of enhanced antibiotic TA production.

In recent years, it has been shown that the myxobacteria are a rich source of secondary metabolites, including antibiotics of diverse chemical structures and modes of action (10). Approximately 60% of all strains tested produce bioactive compounds. The best-studied myxobacterial antibiotic is TA, which is produced by *Myxococcus xanthus* (11, 12) and which appears to be identical to myxovirescin A, which is produced by *Myxococcus virescens* (13). The chemical structure of the antibiotic is shown in the accompanying paper (15). The production of antibiotic TA was stimulated by the addition of alanine, serine, and glycine to Casitone medium (5). On the other hand, high nutrient concentrations decreased it (14).

In the accompanying paper (15), we reported the isolation of a series of Tn*5lac* insertional mutations in genes required for TA production. Without knowledge of the enzymes involved in the biosynthetic pathway, such mutations can still be useful for obtaining data on the level and regulation of transcription of TA genes. Promoter probe Tn*5lac* was constructed to identify new developmental markers in *M. xanthus* (7). Tn*5lac* contains a promoterless *lacZ* gene inserted near one end of transposon Tn*5*. When Tn*5lac* is transposed into a transcription unit in the correct orientation, it generates a transcriptional fusion to *lacZ*, placing β-galactosidase expression under the control of the promoter for that transcriptional unit. Translation stop codons upstream of *lacZ* and in all three reading frames ensure that Tn*5lac* forms transcriptional, and not translational, fusions (7). In this study, we attempted to extend the technology developed for examining developmental genes to examine genes involved in producing antibiotic TA.

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MATERIALS AND METHODS

Bacterial strains. The *M. xanthus* strains used in this study are listed in Table 1 ("ER" is dropped as in the accompanying paper). The strains were maintained as described elsewhere (15).

Media. CT medium contained 0.2% $MgSO_4 \cdot 7H_2O$ and 2%, 1%, or 0.5% Casitone (Difco Laboratories, Detroit, Mich.) (2CT, 1CT, or 0.5CT medium, respectively). 0.5CTS medium was 0.5CT medium supplemented with 1 mg each of L-serine, L-alanine, and glycine per ml. Solid media were prepared with 1.5% Bacto Agar (Difco). Kanamycin sulfate (Sigma Chemical Co., St. Louis, Mo.) was used at 50 μg/ml.

Growth and measurement of β-galactosidase activity. Side-arm flasks containing medium at two-fifths of the flask volume were inoculated with an exponential culture to yield an initial turbidity of 5 to 10 Klett units (KU), as determined with a Klett-Summerson spectrophotometer with filter 54. One hundred Klett units is equivalent to an optical density at 620 nm of 0.6. All experiments were carried out at 30°C in a New Brunswick model G-53 gyratory shaker at 150 rpm. At timed intervals, samples were removed for the determination of protein and β-galactosidase activity. The samples (usually 1 ml) were centrifuged, and the cell pellets were resuspended in 1 ml of TPM buffer (10 mM Tris-HCl [pH 7.5], 1 mM KH_2PO_4, 8 mM $MgSO_4$). Suspensions were sonicated in a model B-12 sonifier (Branson Sonic Power Co.) for 20 s at 70 W (with a 30-s interval after every 5 s of sonication) or in a Microson ultrasonic cell disruptor (Heat Systems Inc.) for 40 s at 75% output (with a 30-s interval after every 10 s of sonication) with ice water cooling the samples. All samples were microcentrifuged for 4 min to remove debris. Supernatants were assayed for β-galactosidase activity (9) by the addition of 50 to 100 μl of supernatant to 450 to 400 μl of buffer containing 0.1 M Na_2HPO_4-NaH_2PO_4 (pH 7.0), 0.01 M KCl, 0.001 M $MgSO_4$, 0.05 M β-mercaptoethanol, and 1 mg of *o*-nitrophenyl-β-galactoside (Sigma) per ml. The A_{420}

TABLE 1. Tn*5lac* insertion strains used in this study

Strain[a]	Insert	Derivation
ER409	Ω409	P1::Tn*5lac* × ER15
ER419	Ω419	P1::Tn*5lac* × ER15
ER420	Ω419	Mx4 (ER419) × ER15
ER421	Ω419	UV mutagenesis of ER420
ER2425	Ω2425	P1::Tn*5lac* × ER15
ER1010	Ω1010	P1::Tn*5lac* × ER15
ER1020	Ω1010	Mx8 (ER1010) × DK1622
ER1030	Ω1010	Mx8 (ER1010) × ER15
ER1031	Ω1010	NG mutagenesis of ER1030
ER1033	Ω1010	Mx4 (ER1031) × ER15
ER1311	Ω1311	P1::Tn*5lac* × ER15
ER2513	Ω2513	P1::Tn*5lac* × ER15
ER3110	Ω3110	P1::Tn*5lac* × ER15
ER7512	Ω7512	P1::Tn*5lac* × ER15
ER5242	Ω5242	P1::Tn*5lac* × ER15
ER5244	Ω5242	Mx4 (ER5242) × ER15
ER1500	Ω1500	P1::Tn*V* × ER15
ER1503	Ω1500	Mx4 (ER1500) × ER15

[a] All the strains were TA$^-$, except for 1500, 1503, 5242, and 5244, which overproduced TA. The selection of strains 421, 1031, 5244, and 1033 is described here. The selection of the remaining strains is described in the accompanying paper (15).

was determined after 20 min to 3 h of incubation at 37°C. Protein was measured (3) by the addition of 0.8 ml of a diluted supernatant sample to 0.2 ml of a reagent from Bio-Rad Laboratories. The A_{595} (versus a blank prepared with 0.8 ml of TPM buffer) was read after 5 to 60 min. Bovine immunoglobulin G (Sigma) was used as the protein standard. β-Galactosidase specific activity is reported as nanomoles of *o*-nitrophenol (ONP) produced per minute per milligram of protein.

DNA-RNA hybridization. Total cellular RNA was extracted as follows. Cells suspended in 10 mM Tris buffer (pH 7.0) containing 100 mM NaCl and 1 mM EDTA were treated twice with an equal volume of hot phenol (65°C), once with phenol-chloroform (1:1 [vol/vol]), and three times with ether and precipitated with cold ethanol. The RNA was denatured at 60°C for 15 min, and aliquots of 1.25 and 2.5 μg were applied to Nytran membranes (Schleicher & Schuell). Hybridization was performed as described by Maniatis et al. (8). The DNA probe was prepared as described in the accompanying paper (15).

Mutagenesis and screening for β-galactosidase-overproducing mutants. Single colonies of the non-TA-producing (TA-negative [TA$^-$]) mutants (Kan^r *lac*$^+$ TA$^-$) were inoculated into 1CT medium and grown to a density of 5×10^8 cells per ml (100 to 150 KU). Cells were centrifuged and washed once in TM buffer (8 mM $MgSO_4$, 10 mM Tris-HCl [pH 7.6]). Cells were treated with 100 μg of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NG; Aldrich Chemical Co.) per ml, freshly prepared in 0.1 M citrate buffer at pH 5.5. After being shaken for 30 min, cells were sedimented and washed with TM buffer. UV light mutagenesis was carried out by exposing 10 ml of the washed cell suspension in a 10-cm-diameter petri dish to irradiation from a mercury lamp for 1 min at a distance of 35 cm. Survival after NG treatment was about 1%, and that after UV light irradiation was about 10%. After mutagenesis, cells were diluted 1:10 into 1CT medium, incubated at 30°C for 20 h, and plated on 1CT agar containing 50 μg of kanamycin sulfate and 40 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal; Sigma or United States Biochemical Corp., Cleveland, Ohio) per ml. After 7 days of incubation at 30°C, single colonies that seemed to be more blue than the parent strain were isolated and checked quantitatively for β-galactosidase production.

Myxophage transduction. Mapping of the NG-induced mutations was performed as described in the accompanying paper (15) with myxophage Mx4-LA27 (1).

TABLE 2. Specific activities of β-galactosidase in Tn*5lac* insertion mutants

Strain[a]	β-Galactosidase sp act in the following medium[b]:		Ratio[c]
	0.5CTS	2CT	
409	367	25	14.7
420	2.4	1.5	1.6
2425	142	11	12.9
1030	250	20	12.5
1311	307	23	13.3
2513	139	23	6.0
3110	294	19	15.5
7512	191	48	4.0
5244	35	21	1.6

[a] Strain 5244 overproduced TA; the remaining strains were TA$^-$.
[b] Determined in cells grown for 48 h. β-Galactosidase specific activity is reported as nanomoles of ONP produced per minute per milligram of protein.
[c] Ratio of specific activities in 0.5CTS and 2CT media.

RESULTS

Expression of β-galactosidase from Tn*5lac* insertions in TA genes. Nine strains that contained Tn*5lac* insertions in genes involved in antibiotic TA production were grown to the early stationary phase in 0.5CTS and 2CT media and assayed for β-galactosidase activities (Table 2). These media were chosen because 0.5CTS yields a high level of antibiotic TA and 2CT supports optimal growth but reduces antibiotic synthesis about 5- to 10-fold. The insertion of Tn*5lac* had no measurable influence on the growth rate or the growth yield for all of the strains used in this study. The only TA overproducer examined, 5244, produced relatively low levels of β-galactosidase in both 0.5CTS and 2CT media. One of the non-TA-producing mutants, 420, had very low specific activities of the enzyme, 2.4 and 1.5 U/mg of protein, in 0.5CTS and 2CT media, respectively. The specific activities of β-galactosidase in the remaining strains varied from 139 to 367 U/mg of protein in 0.5CTS medium and 11 to 48 U/mg of protein in 2CT medium. For all of the mutants, expression was higher in 0.5CTS medium than in 2CT medium, varying from 1.6- to 15.5-fold. In general, strains that showed the highest specific activities of the reporter enzyme also showed the highest level of inhibition with 2CT medium.

The data presented in Table 2 were obtained with Tn*5lac* inserted exclusively in ER15 backgrounds. To compare backgrounds, we compared the same Tn*5lac* insertion (Ω1010) in a DK1622 background (strain 1020) and an ER15 background (strain 1030). The specific activity of β-galactosidase in 1020 in 0.5CTS medium was 73 U/mg of protein, whereas under the same conditions, the specific activity in 1030 was 175 U/mg of protein. These results are consistent with the fact that ER15 produces about fivefold-higher levels of antibiotic than DK1622 (unpublished data).

Expression of β-galactosidase during growth. The specific activities of β-galactosidase were measured during growth in 0.5CTS and 2CT media for two strains, 1030 (Fig. 1) and 420 (Fig. 2). Strain 1030 had a higher β-galactosidase specific

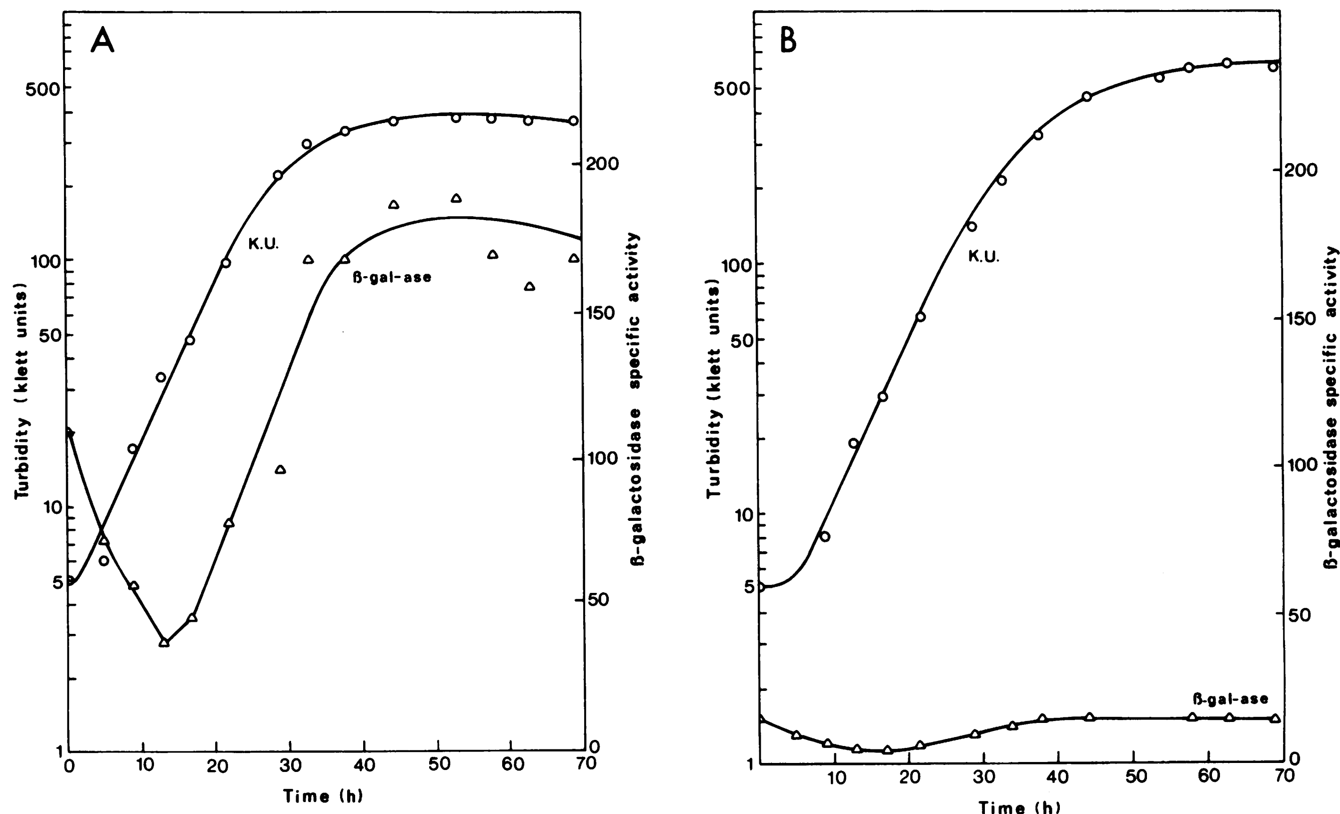

FIG. 1. Specific activities of β-galactosidase in 1030 grown in 0.5CTS (A) and 2CT (B) media. Exponentially growing cultures of 1030 in 0.5CTS and 2CT media were inoculated into the corresponding media to yield an initial turbidity of 5 KU. After incubation with shaking for various times, samples were removed and centrifuged, and protein and β-galactosidase in the disrupted cells were determined as described in Materials and Methods.

activity in 0.5CTS medium (Fig. 1A) than in 2CT medium (Fig. 1B) throughout the growth period and the stationary phase. The specific activity of the reporter enzyme in 1030 grown in 0.5CTS medium decreased during the initial 13 h of growth and then increased at a linear rate from 37 U/mg of protein at 13 h to approximately 180 U/mg of protein at the stationary phase. In 2CT medium, the specific activity of β-galactosidase remained low and relatively constant for 60 h. For strain 420, in which the insert is not linked to the major cluster of TA genes, the specific activity of β-galactosidase was very low during the growth phase (Fig. 2). In the early stationary phase, the specific activity increased slightly in both media, reaching 2.8 and 1.9 U/mg of protein in 0.5CTS and 2CT media, respectively.

RNA-DNA blot hybridization. To confirm that the β-galactosidase produced by the Tn*5lac* mutants acted as a reporter protein for transcriptional control, as has been shown by others (7), we observed the pattern of RNA synthesis. Accordingly, RNA was prepared from strain ER15 grown for 48 h in 0.5CTS and 2CT media under the same conditions as those used for the experiment shown in Table 2. The RNA was probed with a chromosomal DNA fragment containing Tn*V* insert Ω1310 (7.5 kb of *M. xanthus* DNA [15]) from the TA gene cluster. Hybridization was performed by dot blotting, the blot was radioautographed, and the intensity was determined by densitometry. The amount of TA DNA hybridizing with RNA of cells grown in 0.5CTS medium was three times higher than that hybridizing with RNA of cells grown in 2CT medium, consistent with the higher levels of β-galactosidase found in cells grown in 0.5CTS medium than in 2CT medium.

Isolation and characterization of a β-galactosidase-overproducing mutant of 1030. Treatment of 1030 with NG for 30 min as described in Materials and Methods led to 99% killing of the cells. Screening of the survivors on 1CT agar containing X-Gal yielded 2 small dark blue colonies (*lac*$^{++}$) from 11,000 colonies (*lac*$^{+}$) observed. One of the *lac*$^{++}$ mutants grew poorly in liquid medium and was not studied further. The other *lac*$^{++}$ mutant, referred to as 1031, was studied further by mutation mapping. The distance between the mutation induced by NG and the Tn*5lac* insertion was measured by cotransduction with myxophage Mx4. A cross between 1031 as the donor strain (Kan^{r} *lac*$^{++}$ TA^{-}) and ER15 as the recipient strain (Kan^{s} *lac*$^{-}$ TA^{+}) was performed, and the kanamycin-resistant colonies were isolated and checked for β-galactosidase activity. The closer the point mutation is to the Tn*5lac* insertion site, the higher will be the frequency of β-galactosidase-overproducing transductants. If it is far away, most of the transductants will produce β-galactosidase at the level of the parent strain, 1030. Of the 141 Kan^{r} transductants examined, 40 were *lac*$^{++}$ and 101 were *lac*$^{+}$. Therefore, the cotransduction frequency was 28% (40 of 141), corresponding to a recombination distance of 17 kb (17). One of the transductants that overproduced β-galactosidase, 1033, was used to check the effect of different media on β-galactosidase activity in comparison with the activity of the parent strain (Table 3). The mutation had no significant effect on growth in the four media tested but

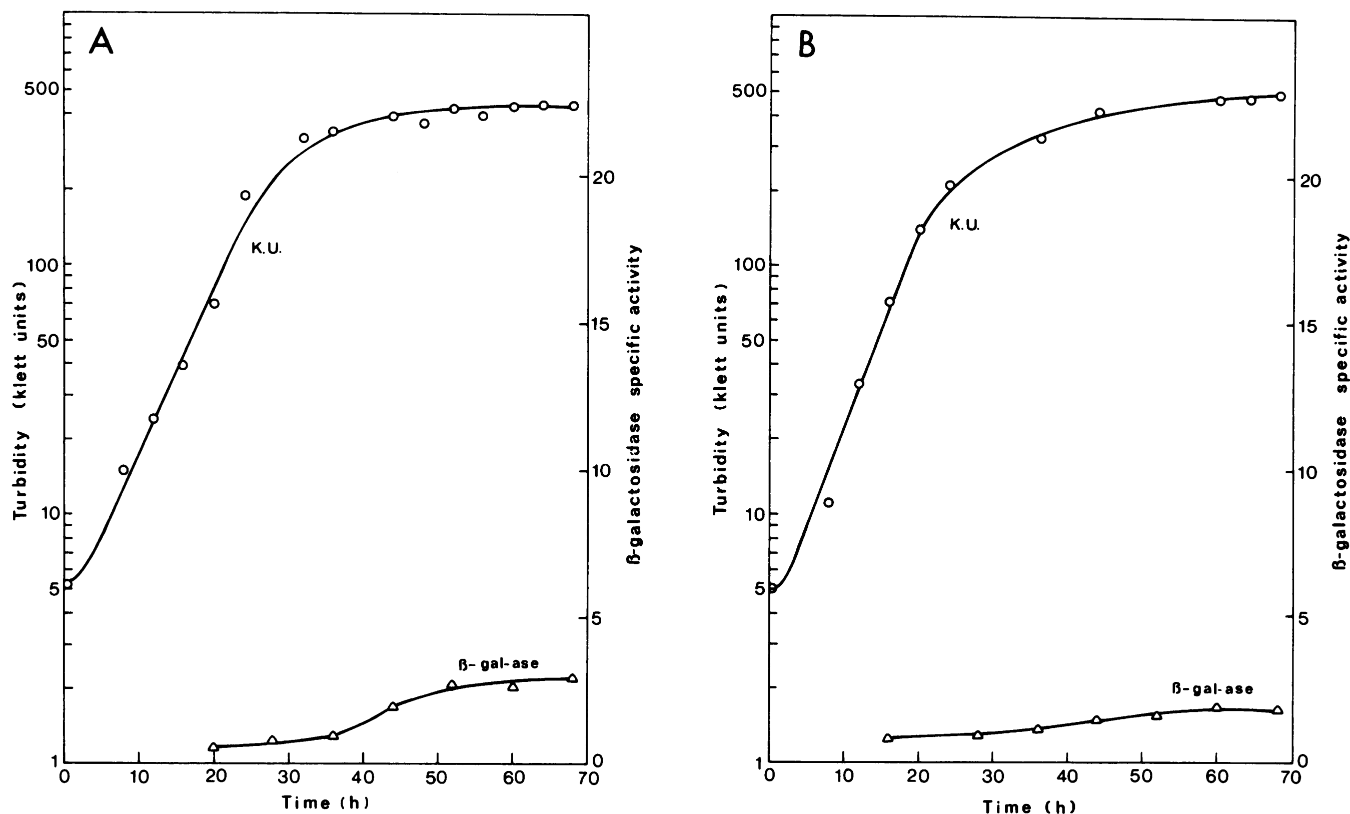

FIG. 2. Specific activities of β-galactosidase in 420 grown in 0.5CTS (A) and 2CT (B) media. The experiment was performed as described in the legend to Fig. 1.

increased 2.1- to 10.8-fold the specific activity of β-galactosidase. The strong inhibition of β-galactosidase production by increasing concentrations of Casitone was largely abolished in NG-induced mutant 1033.

Isolation and characterization of a β-galactosidase-overproducing mutant of R420. UV light irradiation of a culture of 420 for 1 min led to 90% killing of the cells. One survivor from 50,000 colonies that were screened produced a distinctly more blue colony on X-Gal agar than the parent strain. This mutant, referred to as 421, was examined for β-galactosidase activity in different media (Table 4). The growth yields for 420 and 421 were similar to each other in the different media. Strain 420 had about the same low β-galactosidase specific activities in 0.5CT, 1CT, and 2CT media. Strain 421 produced larger amounts of β-galactosidase in all media. In 2CT, strain 421 had a specific activity 2.4 times as high as that of strain 420. There was only a small increase in specific activities in both strains with the addition of the precursor amino acids, alanine, serine, and glycine (0.5CTS medium).

Effect of magnesium phosphate on antibiotic TA and β-galactosidase production. Reichenbach et al. (10) reported that

TABLE 3. Growth and production of β-galactosidase by *M. xanthus* 1030 and 1033[a]

Strain	Medium	Growth (KU)	Protein (mg/ml)	β-Galactosidase		Ratio[b]
				U/ml	Sp act	
1030	0.5CT	236	0.434	54	126	
	1CT	460	0.976	56	58	
	2CT	600	2.044	42	20	
	0.5CTS	288	0.523	126	241	
1033	0.5CT	244	0.426	124	293	2.3
	1CT	460	0.842	256	304	5.2
	2CT	600	2.090	450	215	10.8
	0.5CTS	268	0.508	260	512	2.1

[a] Strains were grown for 52 h at 30°C as described in Materials and Methods. Protein concentrations and β-galactosidase activities were determined with 1-ml aliquots sonicated as described in Materials and Methods. β-Galactosidase specific activity is reported as nanomoles of ONP produced per minute per milligram of protein.

[b] Ratio of the β-galactosidase specific activity of 1033 to that of 1030 in the same medium.

TABLE 4. Growth of and β-galactosidase activity in *M. xanthus* 420 and 421[a]

Strain	Medium	Growth (KU)	Protein (mg/ml)	β-Galactosidase		Ratio
				U/ml	Sp act	
420	0.5CT	350	0.400	1.60	4.0	
	1CT	850	0.822	2.73	3.3	
	2CT	1,400	1.720	6.65	3.3	
	0.5CTS	550	0.677	2.93	4.3	
421	0.5CT	400	0.418	2.26	5.4	1.4
	1CT	900	0.732	5.25	7.2	2.2
	2CT	1,500	1.724	13.55	7.9	2.4
	0.5CTS	525	0.550	3.36	6.1	1.4

[a] The experiment was performed as described for the experiment shown in Table 3.

TABLE 5. Effect of magnesium phosphate on antibiotic TA and β-galactosidase production

Strain[a]	Magnesium phosphate[b]	Protein (mg/ml)	β-Galactosidase sp act[c]	TA (μg/ml)	Ratio[d]
1030	−	0.523	241		4.3
	+	0.342	1032		
1033	−	0.508	512		1.9
	+	0.431	968		
420	−	0.677	4.3		1.8
	+	0.626	7.8		
421	−	0.550	6.1		2.4
	+	0.454	14.8		
ER15	−			2	1.9
	+			3.7	
1503	−			2.4	1.7
	+			4.1	

[a] See Table 1. Strain 1503 is an antibiotic-overproducing strain. Cultures were grown in test tubes containing 4 ml of 0.5CTS medium for 48 to 52 h at 30°C.

[b] $Mg_2(PO_4)_2 \cdot 8H_2O$ (Johnson Matthey Alfa Products) was added at 0.2% after autoclaving.

[c] Reported as nanomoles of ONP produced per minute per milligram of protein.

[d] Ratio of TA or β-galactosidase production in the two media.

the continuous removal of NH_4^+ during fermentation resulted in a large improvement in antibiotic yield for myxobacteria. The removal of NH_4^+ can be achieved by including in the growth medium magnesium phosphate, which forms with ammonia an insoluble complex, magnesium ammonium phosphate. As shown in Table 5, magnesium phosphate increased antibiotic TA production in *M. xanthus* ER15 and β-galactosidase production in the Tn*5lac* mutants carrying insertions Ω1010 and Ω419. The latter effect was observed in the original *lac*$^+$ mutants as well as in their *lac*$^{++}$ derivatives, strains 1033 and 421.

DISCUSSION

Several lines of evidence presented here suggest that transcriptional control is involved in regulating antibiotic TA biosynthesis. Using Tn*5lac* as a promoter probe for genes essential for TA production, we demonstrated that the transcription of these TA genes was inhibited by increasing concentrations of Casitone and enhanced in the presence of magnesium phosphate in the medium. These data correlate with the observation that the production of antibiotic TA was decreased by high concentrations of Casitone and increased in the presence of magnesium phosphate. One possible explanation for these effects is that growth on high concentrations of Casitone produces ammonia by deamination of amino acids and that the ammonia activates a common repressor for TA genes. Accordingly, magnesium phosphate relieves the repression by trapping the ammonium ions in an insoluble compound. Another correlation between the transcription of TA genes and antibiotic production was the observation that insertion of the same Tn*5lac* insert (Ω1010) in strains ER15 and DK1622 yielded higher levels of β-galactosidase in the ER15 background. ER15 produces about five times more antibiotic than DK1622.

A further correlation between the transcription of TA genes and antibiotic production, but one that is not easily explained by an ammonia effect, was the finding that transcription of the reporter gene in 1030 increased during the exponential growth phase. Antibiotic TA behaves as a typical secondary metabolite, being produced at higher rates at the end of exponential growth (14). It is possible that amino acids or peptides in the growth medium inhibit transcription and consequently TA production. As the cells grow, they metabolize the compound(s) and thereby derepress TA genes. Thus, the transcription of TA genes appears to be regulated by at least two metabolites, ammonia and a component in the medium.

The characterization of NG-induced mutant 1033, which overproduces β-galactosidase, is consistent with the above suggestions. The mutation in this mutant mapped 17 kbp from the insert that it affected but still within the cluster of TA genes. 1033 was not repressed by Casitone but was still derepressed by magnesium phosphate. Thus, the mutation may have caused a represser molecule to be insensitive to Casitone but still sensitive to ammonia.

The TA gene that contains insert Ω419 is interesting for several reasons. First, it is essential for antibiotic production but is transcribed at a very low level; thus, it could represent a rate-limiting step in antibiotic production. Second, its transcription is not significantly repressed by high concentrations of Casitone (Table 4). Third, it is not linked to the major cluster of TA genes (15). In fact, it is present on a completely different part of the *M. xanthus* chromosome, the L fragment (4). The situation may be similar to what was shown previously for the two clusters of oxytetracycline genes in *Streptomyces rimosus* (6). One cluster apparently contained the genes controlling the earliest steps of the biosynthetic pathway, while the other contained genes involved in the later steps of the pathway. It was recently shown that all the genes required for oxytetracycline biosynthesis are contained in one cluster. What was originally thought to be the second cluster turned out to be a gene involved in the synthesis of a necessary flavin cofactor (2).

The procedure described in this report can be extended to further study of the transcriptional regulation of TA genes. We plan to obtain and map additional regulatory mutations affecting β-galactosidase production from inserts in the TA genes. It should be possible to obtain data on the direction of transcription and size of the transcriptional units by studying double mutants that contain Tn*5* inserts both upstream and downstream from reporter genes. This approach was recently used to study the regulation of frizzy mutants of *M. xanthus* (16).

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