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Molecular Cloning of Tetracycline Resistance Genes from *Streptomyces rimosus* in *Streptomyces griseus* and Characterization of the Cloned Genes

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Two tetracycline resistance genes of *Streptomyces rimosus*, an oxytetracycline producer, were cloned in *Streptomyces griseus* by using pOA15 as a vector plasmid. Expression of the cloned genes, designated as *tetA* and *tetB*, was inducible in *S. griseus* as well as in the donor strain. The tetracycline resistance directed by *tetA* and *tetB* was characterized by examining the uptake of tetracycline and in vitro polyphenylalanine synthesis by the sensitive host and transformants with the resultant hybrid plasmids. Polyphenylalanine synthesis with crude ribosomes and the S150 fraction from *S. griseus* carrying the *tetA* plasmid was resistant to tetracycline, and, by a cross-test of ribosomes and S150 fraction coming from both the sensitive host and the resistant transformant, the resistance directed by *tetA* was revealed to reside mainly in crude ribosomes and slightly in the S150 fraction. However, the resistance in the crude ribosomes disappeared when they were washed with 1 M ammonium chloride. These results suggest that *tetA* specified the tetracycline resistance of the machinery for protein synthesis not through ribosomal subunits, but via an unidentified cytoplasmic factor. In contrast, *S. griseus* carrying the *tetB* plasmid accumulated less intracellular tetracycline than did the host, and the protein synthesis by reconstituting the ribosomes and S150 fraction was sensitive to the drug. Therefore, it is conceivable that *tetB* coded a tetracycline resistance determinant responsible for the reduced accumulation of tetracycline.

Many streptomycetes produce antibiotics that inhibit the syntheses of protein and nucleic acid of procaryotic cells. Streptomycetes are gram-positive bacteria, and the producer strains must be able to protect themselves from their own antibiotics. The mechanisms involved in antibiotic resistance of antibiotic-producing *Streptomyces* spp. are divided into three categories: alteration of target sites, detoxification by antibiotic-modifying enzymes, and reduced accumulation of antibiotics (23).

Studies with *Streptomyces azureus* and *Streptomyces erythraeus*, which produce thiostreptone and erythromycin, respectively, showed that their ribosomes (the target site) are desensitized to the antibiotics by the action of 23S rRNA methylases (4, 18). In contrast, *Streptomyces fradiae*, which produces neomycin, also an inhibitor of protein synthesis, has neomycin-sensitive ribosomes. However, this strain is endowed with neomycin phosphotransferase and neomycin acetyltransferase, both of which inactivate the antibiotic (5). Similarly, *Streptomyces vinaceus*, a viomycin producer, possesses viomycin-sensitive ribosomes and a phosphotransferase to detoxify the drug (17). All of the enzymes described above have been cloned in *Streptomyces lividans* and were confirmed to confer a self-resistance mechanism in vivo (21, 22).

On the contrary, there have been only a few studies on the tetracycline resistance of tetracycline-producing *Streptomyces* spp. Tetracycline also inhibits protein synthesis, mainly due to interference with the binding of ternary complex aminoacyl-tRNA-EFTu-GTP to the acceptor site of ribosome (14). Mikulík et al. reported that protein-synthesizing system from *Streptomyces aureofaciens* (a tetracycline producer) was much more resistant to tetracycline than was *Escherichia coli* (11). Their recent study, however, revealed that the isolated ribosomes of *S. aureofaciens* were quite sensitive to the drug (10). A factor that might have been involved in the resistance of the protein-synthesizing system remains to be examined. Moreover, efflux of the drug seems to be another mechanism of the self-resistance in *S. aureofaciens* (6).

Such a complicated situation could be deciphered by cloning tetracycline resistance genes from the producer into a heterospecific *Streptomyces* sp. strain that is sensitive to tetracycline and by characterizing the cloned resistance determinants. If tetracycline resistance genes of *Streptomyces* spp. were cloned in a plasmid, they would also be useful as selective markers in the transformation and gene expression studies in streptomycetes. The purpose of this paper is to describe the cloning of two inducible tetracycline resistance genes from *Streptomyces rimosus* (an oxytetracycline producer) in *Streptomyces griseus* and to discuss the resistance mechanisms directed by the two genes.

MATERIALS AND METHODS

Bacterial strains and plasmid. *S. rimosus* ATCC 10970, which produces oxytetracycline, was used as a donor of tetracycline resistance genes, whereas *S. griseus* ATCC 10137 was used as a host throughout.

pOA15, whose map of restriction endonuclease cleavage is shown in Fig. 1, was used as a vector plasmid. Owing to the ability of pOA15 to elicit lethal zygosis in *S. griseus*, transformants could be identified as pocks on regeneration plates (13).

Media. Glycerin-asparagine (GA) medium was described previously (16). Trypticase soy broth (TSB) was from BBL Microbiology Systems, Cockeysville, Md. ISP2M medium for maintenance and sporulation contained 15 g of malt

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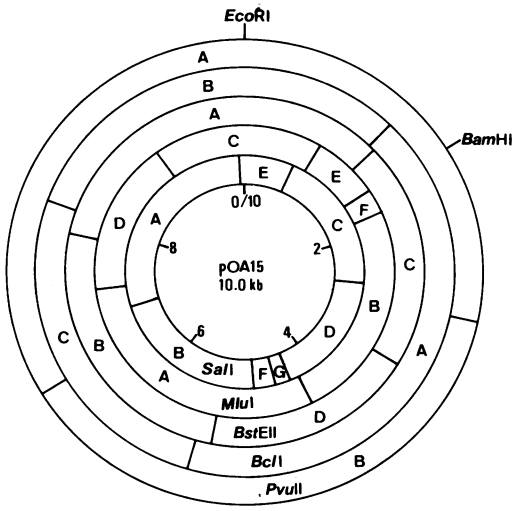

FIG. 1. Restriction endonuclease cleavage map of pOA15. pOA15 had no site for *Bgl*II, *Hin*dIII, *Hpa*I, *Kpn*I, *Pst*I, *Sac*I, *Sca*I, *Sma*I, *Xba*I, and *Xho*I.

extract, 5 g of yeast extract, 5 g of soluble starch, 3 g of calcium carbonate, and 20 g of agar per liter (pH 7.5).

Chemicals and enzymes. Polyuridylic acid [poly(U)], pyruvate kinase, phosphoenolpyruvate, and tetracycline were purchased from Sigma Chemical Co., St. Louis, Mo., and tRNA (*E. coli* MRE 600) was from Boehringher, Mannheim, Federal Republic of Germany. L-[*U*-^{14}C]phenylalanine (504 Ci/mol) and [7-^{3}H]tetracycline (635 Ci/mol) were from Radiochemical Centre, Amersham, Buckes, United Kingdom, and New England Nuclear Corp., Boston, Mass., respectively. *Bcl*I was from New England Biolabs Inc., Beverly, Mass., and *Bst*EII was from Bethesda Research Laboratories Inc., Gaithersburg, Md. The other restriction endonucleases used and T4 ligase were from Takara Shuzo Co. Ltd., Kyoto, Japan. All other reagents were from Wako Chemical Industries, Osaka, Japan, unless otherwise specified.

Preparation of plasmid and chromosomal DNA. Plasmid DNA was isolated by the rapid alkaline extraction method described previously (13). Chromosomal DNA of *S. rimosus* grown in TSB was prepared essentially as described previously (2), except that precipitation steps with polyethylene glycol and ethanol were omitted. Both plasmid and chromosomal DNAs were further purified by CsCl-ethidium bromide equilibrium density gradient centrifugation.

Cloning of tetracycline resistance genes. *S. rimosus* chromosomal DNA (10 μg) was partially digested with *Sau*3A into fragments of about 2 to 25 kilobases (kb) in length and mixed with pOA15 (1.5 μg) cleaved with *Bam*HI. After ethanol precipitation, the DNA sample was suspended in ligation buffer at a DNA concentration of 40 μg/ml and ligated with T4 ligase at 12°C overnight. The ligation buffer consisted of 61 mM Tris-hydrochloride (pH 7.6), 6.1 mM magnesium chloride, 10 mM dithiothreitol, and 1 mM ATP. The ligated DNA was introduced into 4×10^9 *S. griseus* protoplasts as described elsewhere (13). After protoplast regeneration on the modified R3 medium (13), transformants resistant to tetracycline were selected by replica plating on GA medium containing tetracycline (25 μg/ml). The host could not grow on GA medium containing 10 μg of tetracycline per ml.

Restriction enzyme mapping of plasmids. Experimental conditions used for the restriction endonucleases followed the protocol recommended by the suppliers. Agarose gel electrophoresis was carried out in a horizontal slab as described by Imanaka et al. (8). Both *Hin*dIII and *Hin*dIII-*Eco*RI digests of λ*c*I857S7 DNA were used as size standards (19). DNA of phage λ was from Takara Shuzo Co. Ltd.

Determination of the level of tetracycline resistance. Spores of *S. griseus* and *S. rimosus* were inoculated into 20 ml of TSB and incubated at 28°C on a rotary shaker for 24 h. A 1-ml sample of the preculture was transferred to 20 ml of TSB containing tetracycline of different concentrations (25 μg/ml and multiples thereof). The highest concentration that allowed visible turbidity after 24 h was designated as the maximum concentration that permitted growth.

Growth and challenge tests with tetracycline. Resistance profiles in TSB cultures were determined by a method similar to that of Clewell et al. (3). A 200-ml culture of *S. griseus* or *S. rimosus* that had been growing in the log phase (optical density at 640 nm [OD_{640}], ≈0.8) was divided into two 90-ml portions, and 5 μg of tetracycline per ml was added to one of them for induction. After further incubation for 30 min at 28°C, each division (about 90 ml) was subdivided again into four. These cultures were exposed to the challenge with different amounts of tetracycline (0, 50, 100, and 200 μg/ml). In TSB medium, *S. griseus* normally grew as a suspension of small mycelial wefts and fragments. The cell growth was monitored by OD_{640} as described by Cella and Vining (1).

In vitro poly(U)-directed polyphenylalanine synthesis. *S. griseus* and *S. rimosus* strains precultured in TSB were transferred to fresh TSB without or with tetracycline (10 μg/ml) for induction and incubated at 28°C to the late log phase (OD_{640}, ≈1.0). The S150 fraction and ribosomes were prepared by alumina grinding as described by Yamamoto et al. (24). The "crude" and "tight" ribosomes that will appear below refer to the ribosome fractions before and after the washing of ribosomes with 1 M ammonium chloride, respectively. Poly(U)-directed polyphenylalanine synthesis was done at 28°C in a 300-μl reaction mixture containing 50 mM Tris-hydrochloride (pH 7.8), 60 mM ammonium chloride, 7.5 mM magnesium acetate, 1 mM ATP, 5 mM phosphoenolpyruvate (pH 7.0), 0.4 mM spermidine, 25 μM GTP, 20 μM [^{14}C]phenylalanine (25 Ci/mol), 9 μg of pyruvate kinase, 180

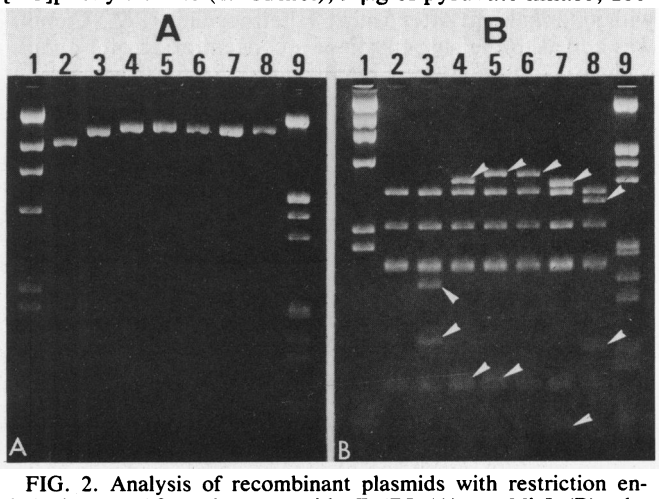

FIG. 2. Analysis of recombinant plasmids with restriction endonucleases. After cleavage with *Eco*RI (A) or *Mlu*I (B), the fragments were electrophoretically separated on a 0.7 or 1.5% gel, respectively. Lanes: 1, DNA of phage λ cleaved with *Hin*dIII as a size standard; 2, pOA15; 3, pOA151; 4, pOA152; 5, pOA153; 6, pOA154; 7, pOA155; 8, pOA156; 9, λ phage cleaved with *Eco*RI-*Hin*dIII as a size standard. The arrow heads in gel B indicate *Mlu*I fragments from the cloned DNA.

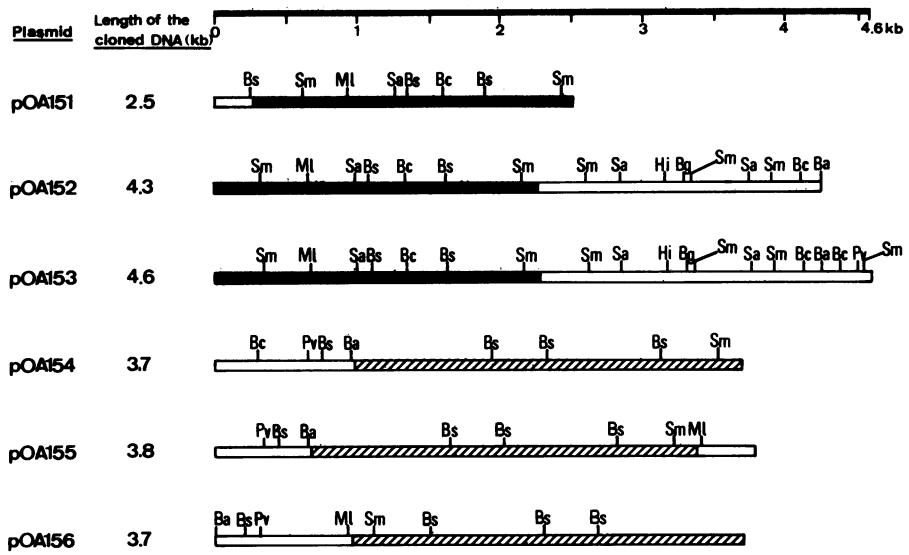

FIG. 3. Restriction endonuclease cleavage maps of the cloned DNAs. The DNA fragments inserted into the *Bam*HI site of pOA15 are shown by boxes, whose left ends are 1.6 kb apart from the *Eco*RI site of the vector plasmid. A region (■) of 2.3 kb was apparently shared by pOA151, pOA152, and pOA153, whereas another region (▨) of 2.7 kb was shared by pOA154, pOA155, and pOA156. Tetracycline resistance genes on those solid and shaded regions were designated as *tetA* and *tetB*, respectively. Abbreviations: Ba, *Bam*HI; Bc, *Bcl*I; Bg, *Bgl*II; Bs, *Bst*EII; Hi, *Hin*dIII; Ml, *Mlu*I; Pv, *Pvu*II; Sa, *Sal*I; Sm, *Sma*I.

μg of poly(U), the S150 fraction (250 to 400 μg of protein), and the crude or tight ribosomes (3.8 of absorbance at 280 nm is equal to approximately 250 μg of protein). Samples (50 μl each) were withdrawn, added into 0.5 ml of 5% trichloroacetic acid, and heated at 90°C for 15 min. Acid-insoluble materials were collected on Whatman GF/C filters and washed with 5 ml of cold 5% trichloroacetic acid. After drying, radioactivity was measured by liquid scintillation counting.

Uptake of [^{3}H]tetracycline. Cells that had grown to the late log phase (OD_{640}, ≈1.0) in 20 ml of TSB were harvested by centrifugation and washed three times with 0.1 M sodium sulfate and once with TSB. The washed cells were suspended in 15 ml of prewarmed TSB and incubated at 28°C with mild shaking. After 5 min [^{3}H]tetracycline (177.5 Ci/mol) was added to the suspension at a concentration of 5 μg/ml. Samples (1 ml each) were removed at 0, 5, 15, 30, and 60 min after the addition of tetracycline and filtered on membrane filters (pore size of 0.45 μm, type HA; Millipore Corp., Bedford, Mass.). The cells were rinsed with 10 ml of cold 0.1 M sodium sulfate and 20 ml of cold distilled water and then dried at 80°C for 60 min. Radioactivity was measured by liquid scintillation counting in a toluene-based scintillator. The background for the uptake was [^{3}H]tetracycline taken at 0°C in this procedure. Dry cell weight was measured after drying at 80°C overnight. The uptake was expressed as counts per min per milligram of dry cell weight.

RESULTS

Cloning of tetracycline resistance genes of *S. rimosus* in *S. griseus*. *S. griseus* protoplasts were transformed with the ligation mixture consisting of pOA15 and *S. rimosus* chromosomal DNAs and spread on 100 regeneration plates. After regeneration of protoplasts and sporulation, about 10^6 primary transformants were detected as pocks on the plates. After the pocks were replica plated to GA medium containing tetracycline (25 μg/ml), six clones appeared after 3 days at 28°C. Plasmids prepared from the tetracycline-resistant clones were used to transform *S. griseus* again. Almost all of the pocks obtained by the retransformation with each of the six plasmids were tetracycline resistant. Therefore, tetracycline resistance genes of *S. rimosus* were considered to have been cloned into these six plasmids, which were named as pOA151, pOA152, pOA153, pOA154, pOA155, and pOA156, respectively. *S. griseus* strains harboring either pOA151, pOA152, or pOA153 could grow on GA medium containing 50 μg of tetracycline per ml, but not on GA medium containing 100 μg of tetracycline per ml. On the other hand, transformants with pOA154, pOA155, or pOA156 were resistant to 100 μg of tetracycline per ml.

Agarose gel electrophoresis of pOA15 and the tetracycline resistance hybrid plasmids cleaved with *Eco*RI and *Mlu*I is shown in Fig. 2. pOA15 had a single *Eco*RI site and six *Mlu*I cleavage sites (Fig. 1). Cleavage with *Eco*RI revealed that the hybrid plasmids also had a single site for the endonuclease and were larger than the vector plasmid. When the hybrid plasmids were digested with *Mlu*I, new fragments (indicated by arrow heads in Fig. 2) were generated in addition to those originated from the vector plasmid. These *Mlu*I fragments nearly corresponded to *S. rimosus* DNAs inserted into *Bam*HI cloning site, since the *Bam*HI site of pOA15 was on a 0.2-kb *Mlu*I fragment that was beyond recognition in agarose gel electrophoresis (Fig. 1). pOA151, pOA152, pOA153, pOA154, pOA155, and pOA156 had DNA inserts of 2.5, 4.3, 4.6, 3.7, 3.8, and 3.7 kb, respectively.

Restriction endonuclease mapping of the cloned fragments. The restriction endonuclease cleavage maps of the cloned fragments were obtained by digestion of the hybrid plasmids with various endonucleases, either completely or partially (Fig. 3). pOA151, pOA152, and pOA153 shared a 2.3-kb region (Fig. 3, solid box) on the cloned DNAs, whereas pOA154, pOA155, and pOA156 shared a region of 2.7 kb (shaded area). The inserted DNAs of pOA151, pOA152, and pOA153 had the same direction, but the insert of pOA156 was opposite to those of pOA154 and pOA155. Judging from those shared regions in Fig. 3, tetracycline resistance genes of *S. rimosus* might have been cloned on the specific DNAs of 2.3 and 2.7 kb, respectively. Thus, we tentatively desig-

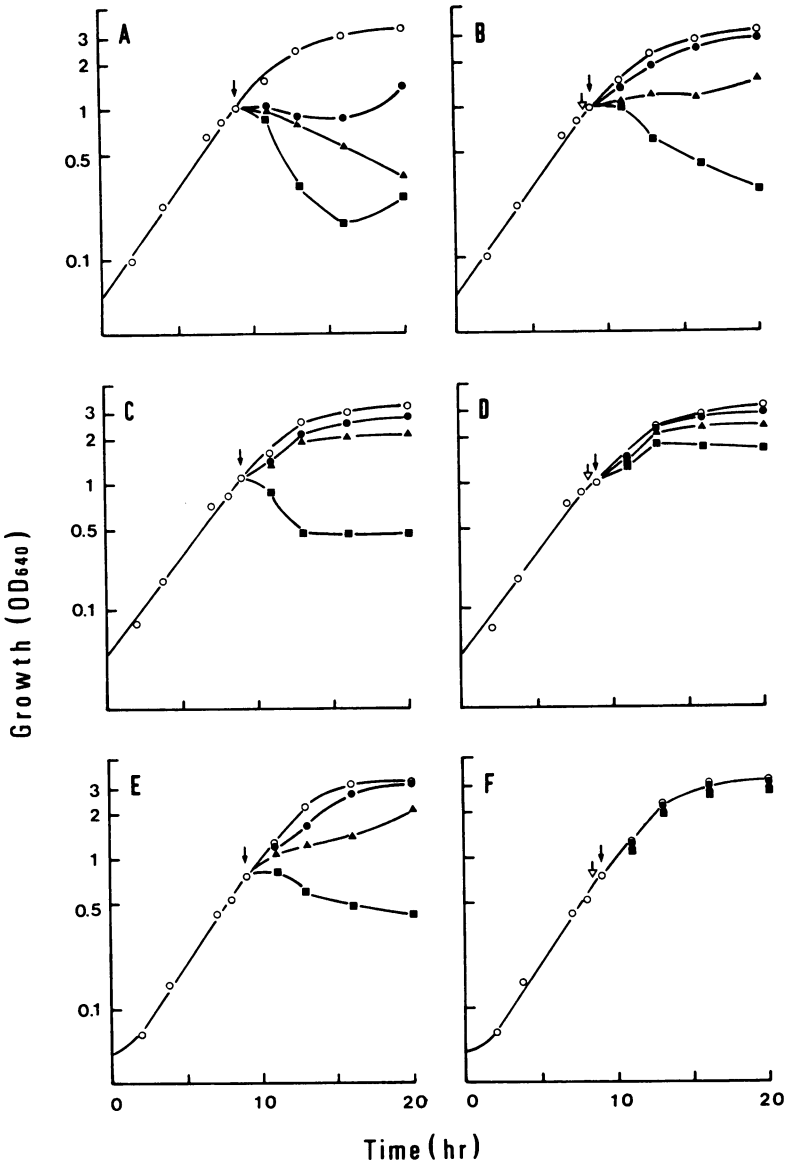

FIG. 4. Demonstration of an inducible expression of the tetracycline resistance genes. Log-phase cells of *S. griseus* (pOA151) (A, B), *S. griseus* (pOA155) (C, D), and *S. rimosus* (E, F), that had been preexposed to 5 μg of tetracycline per ml for 30 min at 28°C (B, D, F) or not preexposed (A, C, E), were challenged with 0 (○), 50 (●), 100 (▲), and 200 (■) μg of tetracycline per ml. Growth in TSB medium was recorded as increase in OD_{640} of the culture. Solid arrows indicate the challenge test initiated by adding tetracycline, whereas open arrows indicate the preexposure to the drug for induction.

nated the two tetracycline resistance genes of *S. rimosus* as *tetA* (solid area) and *tetB* (shaded area). To characterize the tetracycline resistance determinants, we selected pOA151 and pOA155 as representatives of *tetA* and *tetB*, respectively.

Determination of tetracycline resistance level. To examine whether the expression of the cloned tetracycline resistance genes was constitutive or inducible, we did challenge tests as described above (Fig. 4). When logarithmically growing cells of *S. griseus* harboring pOA151 (*tetA*) were challenged with 50 μg of tetracycline per ml, the culture growth was inhibited, whereas the preexposure to tetracycline (5 μg/ml) allowed continuation of the growth, albeit at a slower growth rate than the unchallenged control (Fig. 4A and B). The similar result was obtained for the culture of *S. griseus* harboring pOA155 (*tetB*) when challenged with 200 μg of tetracycline per ml (Fig. 4C and D). On the other hand, *S. griseus* (the host) did not respond to the preexposure (30 min) to tetracycline (data not shown). Accordingly, the expression of both *tetA* and *tetB* genes on pOA151 and pOA155 seemed to be inducible in *S. griseus* as well as in the donor strain, *S. rimosus* (Fig. 4E and F).

Next, the tetracycline resistance level conferred by pOA151 and pOA155 was determined. For induction of the tetracycline resistance genes, a culture grown in TSB was transferred to the broth containing different amounts of tetracycline, and further incubated at 28°C for 24 h. The culture, irrespective of whether uninduced or induced, was inoculated into TSB containing higher concentration of tetracycline; after 24 h, growth was examined (Table 1). *S. griseus* (pOA151) could grow in TSB containing 250 μg of tetracycline per ml when induced with 30 μg of tetracycline

TABLE 1. Determination of tetracycline resistance level of *S. griseus* and transformants with the tetracycline resistance plasmids

Strain	Tetracycline concn (μg/ml)	
	In preculture	Maximum permitting growth
S. griseus	0	10
	5	20
S. griseus (pOA151)	0	75
	5	125
	10	150
	30	250
	50	175
S. griseus (pOA155)	0	125
	5	200
	10	225
	30	300
	50	300
S. rimosus[a]	0	600
	10	800
	50	800
	100	1,200
	200	1,200

[a] *S. rimosus* was a source of tetracycline resistance genes on pOA151 and pOA155.

per ml. *S. griseus* (pOA155) could grow at 300 μg of tetracycline per ml when induced with 30 μg of tetracycline per ml. In both cases, the tetracycline resistance level increased with concentration of tetracycline for induction (up to 30 μg/ml). This tendency was remarkable in *S. rimosus*. Full expression of the tetracycline resistance (1,200 μg/ml) in *S. rimosus* required a high concentration of tetracycline (100 μg/ml) for induction.

In vitro poly(U)-directed polyphenylalanine synthesis. We examined the tetracycline resistance level in protein-synthesizing system reconstituted from either the sensitive host or the resistant transformants by using in vitro poly(U)-directed polyphenylalanine synthesis. Crude ribosomes and the S150 fraction were prepared from both induced and uninduced cultures. Under our conditions, the components from *S. griseus* continued to polymerize phenylalanine for 50 min, whereas polyphenylalanine synthesized by the crude ribosomes from *S. rimosus* reached a plateau after 25 min of incubation, regardless of the source (*S. griseus* or *S. rimosus*) of the S150 fraction (data not shown).

The progress of phenylalanine polymerized after 50 min of incubation was compared among various combinations (Table 2). When both the crude ribosomes and the S150 fraction were derived from the susceptible strain or the transformant with pOA155 regardless of induced or uninduced, the polyphenylalanine synthesis was about 70, 80, and 90% inhibited by 10, 20, and 40 μg of tetracycline per ml, respectively. In contrast, when the components were prepared from the induced culture of *S. griseus* (pOA151) or *S. rimosus*, inhibition of polyphenylalanine synthesis by 10 μg of tetracycline per ml was only 50% or less; at a higher concentration of tetracycline (20 and 40 μg/ml), the synthesis was inhibited also to an extent less than the host and *S. griseus* (pOA155). Thus, it was concluded that *S. griseus* (pOA151) and *S. rimosus* possessed the machinery for protein synthesis, whose resistance to tetracycline was induced by tetracycline.

To investigate whether the observed resistance was due to the crude ribosomes or the S150 fraction, we crossed the components either from the induced culture of *S. griseus* (pOA151) or from *S. rimosus* against those from the sensitive cells of *S. griseus* and examined the sensitivity of polyphenylalanine synthesis to tetracycline (Table 2). The resistance was reproducible whenever the crude ribosomes were derived from induced *S. griseus* (pOA151) or induced *S. rimosus*. In addition, a combined system between the S150 fraction from the resistant cells and the crude ribosomes from the sensitive host was relatively sensitive to tetracycline, although the resistance was slightly noticed in the S150 fraction from *S. rimosus* (76% inhibition by 40 μg of tetracycline per ml). The above cross-experiments revealed that most of the resistance of the protein-synthesizing system in *S. griseus* (pOA151) as well as in the donor strain resided in the crude ribosomes.

Second, the tight ribosomes, which were prepared from the crude ribosomes by washing with 1 M ammonium chloride, were used for polyphenylalanine synthesis in place of the crude ones (Table 3). Tetracycline resistance observed in Table 2 with the crude ribosomes from *S. griseus* (pOA151) and *S. rimosus* disappeared with the ammonium chloride washing, although the resistance remained slightly when the degree of inhibition with 5 μg of tetracycline per ml

TABLE 2. Effect of tetracycline on polyphenylalanine synthesis with crude ribosomes

Source of crude ribosomes	Source of S150 fraction	% Inhibition[a] of polyphenylalanine synthesis with tetracycline (μg/ml):			
		0	10	20	40
S. griseus uninduced	*S. griseus* uninduced	0 (7,845)[b]	71	83	91
S. griseus induced	*S. griseus* induced	0 (3,855)	71	78	84
S. griseus (pOA151) uninduced	*S. griseus* (pOA151) uninduced	0 (7,922)	68	78	84
S. griseus (pOA151) induced	*S. griseus* (pOA151) induced	0 (6,150)	51[c]	67[c]	77[c]
S. griseus (pOA155) uninduced	*S. griseus* (pOA155) uninduced	0 (7,360)	76	82	90
S. griseus (pOA155) induced	*S. griseus* (pOA155) induced	0 (5,140)	72	82	89
S. rimosus uninduced	*S. rimosus* uninduced	0 (8,286)	69	73	76[c]
S. rimosus induced	*S. rimosus* induced	0 (4,594)	39[c]	45[c]	63[c]
S. griseus (pOA151) induced	*S. griseus* uninduced	0 (5,527)	57[c]	69[c]	78[c]
S. griseus uninduced	*S. griseus* (pOA151) induced	0 (7,561)	66	82	86
S. rimosus induced	*S. griseus* uninduced	0 (6,211)	61[c]	66[c]	73[c]
S. griseus uninduced	*S. rimosus* induced	0 (11,512)	68	74	76[c]

[a] Percent inhibition was defined as {1 − [(phenylalanine polymerized with tetracycline)/(phenylalanine polymerized without tetracycline)]} × 100.

[b] The numbers inside parentheses refer to the radioactivities (counts per minute) of phenylalanine polymerized without tetracycline after 50 min of incubation.

[c] The degree of inhibition by tetracycline in the protein-synthesizing system was distinctively less than that (average) in crude ribosomes and the S150 fraction from *S. griseus* uninduced: 73 ± 2, 81 ± 2, and 90 ± 4% (derived from four assays) in the presence of 10, 20, and 40 μg of tetracycline per ml, respectively.

TABLE 3. Effect of tetracycline on polyphenylalanine synthesis with tight ribosomes

Source of tight ribosomes	Source of S150 fraction	% Inhibition[a] of polyphenylalanine synthesis with tetracycline (μg/ml):			
		0	5	10	20
S. griseus uninduced	*S. griseus* uninduced	0 (6,390)[b]	71	82	86
S. griseus induced	*S. griseus* induced	0 (3,096)	71	80	87
S. griseus (pOA151) induced	*S. griseus* (pOA151) induced	0 (6,026)	49[c]	82	87
S. rimosus induced	*S. rimosus* induced	0 (8,960)	44[c]	66[c]	72[c]
S. griseus (pOA151) induced	*S. griseus* uninduced	0 (5,823)	65	80	85
S. griseus uninduced	*S. griseus* (pOA151) induced	0 (5,535)	50[c]	80	83
S. rimosus induced	*S. griseus* uninduced	0 (8,485)	65	81	86
S. griseus uninduced	*S. rimosus* induced	0 (8,406)	56[c]	67[c]	74[c]

[a] See footnote *a* of Table 2.
[b] See footnote *b* of Table 2.
[c] The degree of inhibition by tetracycline in the protein-synthesizing system was distinctively less than that (average) in tight ribosomes and the S150 fraction from *S. griseus* uninduced: 71 ± 2, 84 ± 3, and 88 ± 3% (derived from three assays) in the presence of 5, 10, and 20 μg of tetracycline per ml, respectively.

was reckoned in Table 3. Another cross-examination of the tight ribosomes and the S150 fraction from the resistant and susceptible cells revealed that the slight tetracycline resistance detected was associated with S150 fraction of *S. griseus* (pOA151) and *S. rimosus* (Table 3).

The results described so far point out that *tetA* cloned on pOA151 coded a tetracycline resistance determinant rendering the machinery for protein synthesis resistant to tetracycline and that the resistance specified by *tetA* might well be mediated by a cytoplasmic factor, but not by a constituent of the ribosomes.

Uptake of [^{3}H]tetracycline. Since the reduced accumulation of tetracycline would be an alternative for the tetracycline resistance mechanism in a tetracycline-producing microorganism (6), we studied the uptake of [^{3}H]tetracycline by using *S. griseus* as well as the transformant that carried pOA151 or pOA155 (Fig. 5). It was desirable to compare the uptake of tetracycline by the susceptible host with that by the resistant transformants. Hence, the experiments were done at a low external concentration of tetracycline (5 μg/ml), which allowed the growth of the susceptible host. The results given in Fig. 5 are typical of the many experiments that have invariably shown a definite difference between the susceptible and the resistant strains.

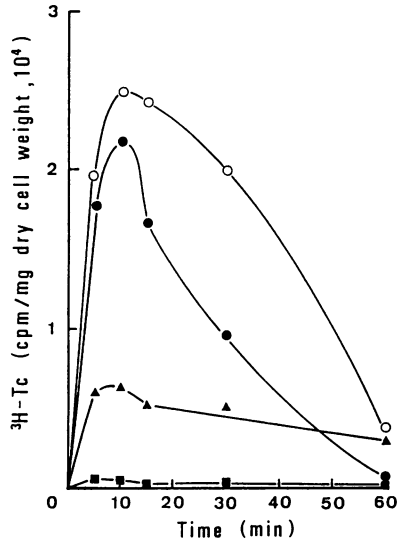

FIG. 5. Uptake of [^{3}H]tetracycline by *S. griseus* and transformants with tetracycline resistance plasmids. Symbols: ○, *S. griseus*; ●, *S. griseus* (pOA151); ▲, *S. griseus* (pOA155); ■, *S. rimosus*. Cells were suspended in TSB at an OD_{640} of ≈1.2. [^{3}H]tetracycline was added to give 5 μg/ml (0.4 μCi/μg), and uptake was measured by the filter method.

Tetracycline accumulated by the susceptible host was found to reach a maximum level at 10 min and thereafter tended to decrease gradually. *S. griseus* harboring pOA151 (*tetA*) accumulated tetracycline to an extent nearly equal to that of the susceptible cells at 10 min.

In contrast, in *S. griseus* carrying pOA155 (*tetB*), the amount of tetracycline that had accumulated at 10 min was only 25% of that in the susceptible host (Fig. 5). The reduced accumulation was also clearly observed in the donor strain, *S. rimosus*, wherein the accumulation of tetracycline was scarcely observed. Thus, *tetB*, cloned on pOA155, was most probably supposed to code the tetracycline resistance determinant responsible for the reduced accumulation of tetracycline.

DISCUSSION

We have cloned two tetracycline resistance genes (*tetA* and *tetB*) of *S. rimosus* (the oxytetracycline producer) in *S. griseus* by using a pock-forming plasmid, pOA15. By comparing the behavior of the sensitive host and the transformants with the resultant tetracycline resistance plasmids, it was possible to examine the self-resistance mechanism that protects the producer from suicidal action. As a result, *tetA* and *tetB* genes were found to encode tetracycline resistance determinants responsible for the resistance in protein synthesis and the reduced accumulation of tetracycline, respectively.

Antibiotic resistance in protein synthesis has been studied by many workers in some *Streptomyces* spp. strains that produce antibiotics (4, 12, 18, 24). According to these studies, the resistance to antibiotics has been reported to reside in tight ribosomes in all cases. Here in this study, however, a resistance factor specified by *tetA* was not a constituent of the tight ribosomes, i.e., ribosomal proteins and rRNAs. Indeed, the factor responsible for the resistance was associated most predominantly with ribosomes (crude ribosomes), and a minor portion of the factor remained free in a cytoplasmic space (S150 fraction). Judging from the localization of the tetracycline resistance studied thus far, *tetA* would have directed either modification of a cytoplasmic factor or association of an unidentified factor with ribosomes, thus giving protein synthesis the resistance to tetracycline. A similar factor might be involved in the self-resistance in another tetracycline producer. Mikulík et

al. reported that *S. aureofaciens* possessed a protein-synthesizing system resistant to tetracycline despite the sensitivity of the isolated ribosomes, leaving, however, the resistance factor unlocalized (10, 11). Identification of the resistance factor merits further study.

In the course of the experiments for tetracycline uptake, a loss of the accumulated tetracycline was observed in *S. griseus* (Fig. 5). In *E. coli*, such a loss does not occur in the presence of energy (7, 9). Exceptionally, a minicell-producing *E. coli* clearly exhibited the rapid loss of the intracellular tetracycline (20). An explanation of the loss observed here must await the further studies for the mechanisms of uptake or efflux (or both) of tetracycline in *S. griseus*.

From an aspect of DNA structure, *tetB* deserves attention. Supposing that the restriction endonuclease cleavage map around *tetB* were constructed from those of pOA154, pOA155, and pOA156 (Fig. 3), *tetB* on the chromosome of *S. rimosus* would have been flanked by a 0.3-kb direct repeat of the *Pvu*II-*Bst*EII-*Bam*HI cleavage site. In fact, when cloning of a larger DNA fragment containing *tetB* was attempted, a 9.5-kb *Bcl*I fragment was cloned into *Bam*HI site of pOA15 (data not shown). Contrary to our expectation, *tetB* on this *Bcl*I fragment was flanked by only one 0.3-kb DNA oriented as in pOA154 and pOA155. These facts and the consideration about a source of DNA segment cloned into pOA156 suggest that DNA rearrangement such as amplification might have occurred around the *tetB* gene in the donor strain. pOA156 would be most unlikely to have resulted from a casual ligation of *tetB* and the independent *Sau*3A fragment containing a 0.3-kb region indistinguishable from that of pOA154 and pOA155, although we could not rule out the possibility.

The expression of both *tetA* and *tetB* genes was inducible in *S. griseus* as well as in the donor strain. Thus, the DNA sequence required for the regulation might also have been cloned into the plasmids. It is worthwhile investigating the induction system for the antibiotic resistance of antibiotic producers, since the resistance would increase in parallel with antibiotic synthesis (15). As a corollary, the cloned tetracycline resistance genes established in this work would provide a useful means to further the study on the induction system.

Despite many reports on the shotgun cloning of *Streptomyces* spp. genes, the host used in the previous experiments was limited to *S. lividans* and *Streptomyces coelicolor*. We selected *S. griseus* as a host, since *S. griseus* produces an important antibiotic, streptomycin. As described in this paper, we developed a cloning system in *S. griseus*, and the resultant tetracycline resistance plasmids would enable us to isolate genes involved in the streptomycin biosynthesis.

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