

Aminoglycoside Antibiotic-Inactivating Enzymes in Actinomycetes Similar to Those Present in Clinical Isolates of Antibiotic-Resistant Bacteria

(streptomycetes/origin of R-factors/gentamicin-acetate)

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ABSTRACT Various species of *Streptomyces* possess aminoglycoside-modifying enzymes. *Streptomyces kanamyceticus* contains an enzyme that acetylates the 6'-amino group of kanamycin A and B, gentamicin C_{1a}, and neomycin. *Streptomyces spectabilis* produces an enzyme that acetylates the 2'-amino group of the hexose ring of gentamicin C_{1a}. These enzymes catalyze reactions identical to those catalyzed by enzymes found in gram-negative bacteria containing R(antibiotic resistance)-factors. The discovery of these enzymes suggests the possibility of an evolutionary relationship between the aminoglycoside-inactivating enzymes (produced by resistance determinants) in bacteria containing R-factors and similar enzymes found in the actinomycetes.

Resistance to antibiotics in clinical isolates of gram-negative and gram-positive bacteria is usually mediated by the presence of various enzymes that modify the antibiotic so that it can no longer interact with its target in the cell. The β -lactamases hydrolyze the penicillins and cephalosporins, chloramphenicol acetyltransferase acetylates chloramphenicol, and nine enzymes acetylate, phosphorylate, or adenylylate the aminoglycoside antibiotics (1, 2). The genetic loci coding for these enzymes are usually located on extrachromosomal elements, such as the R(antibiotic resistance)-factors in gram-negative bacteria. Since these genes are not normal chromosomal components of the resistant strains, there has been considerable speculation as to their origin. Walker and Walker (3) have suggested that some R-factors might have originated in organisms that produce antibiotics.

Molecular studies have shown that R-factors consist of two parts that are reversibly dissociable, these are the resistance transfer factor (RTF), and the r-determinants, genes that determine resistance to antibiotics (2). Watanabe (4) has suggested that the r-determinants exist somewhere in nature as chromosomal genes and that they are "picked-up" by promiscuous RTFs to form R-factors. The question is, where do the r-determinants originate?

We have initiated a search in the actinomycetes for aminoglycoside-modifying enzymes like those that have been characterized in strains carrying R-factors (R⁺) in the belief that this might represent the r-determinant gene pool. The actinomycetes are a group of organisms that have been considered to be intermediate between bacteria and fungi, but

their cellular dimensions, their cytology, and their genetics place them among the bacteria (5, 6). One of the most striking properties of the actinomycetes is the extent to which they produce antibiotics; most of the aminoglycoside antibiotics (streptomycin, neomycin, kanamycin, gentamicin, tobramycin, and lividomycin) are produced by them.

Enzymes that modify other antibiotics have been isolated from *Streptomyces* species; in studies on the biosynthesis of streptomycin in *Streptomyces bikiniensis*, Miller and Walker (3, 8) and Nimi and coworkers (9, 10) found three enzymes that phosphorylated streptomycin. In addition, Argoudelis and Coats have reported the presence of enzymes that phosphorylate lincomycin and clindamycin (11), adenylylate clindamycin (12), and acetylate chloramphenicol (13).

MATERIALS AND METHODS

Growth of Strains. Strains were started from a spore inoculum and grown with vigorous agitation on a rotary shaker at 30° for 4–6 days, until the beginning of stationary phase. *Micromonospora purpurea* was first grown at 37° for 3 days, and then a 1:20 dilution of this culture was made into fresh medium and incubated at 30°. Cultures were monitored every 12 hr for pH, mycelial dry weight, and antibiotic production, and were checked for contaminants microscopically and by streaking on appropriate media before harvesting. *S. spectabilis* (Upjohn UC 2472) and *S. kanamyceticus* (Bristol K2J; ATCC 12853) were grown in 2.5% glucose, 0.1% yeast extract, 0.1% beef extract, 0.2% casamino acids, 0.5% (NH₄)₂SO₄, 0.8% CaCO₃, 0.4% KCl, and 0.04% KH₂PO₄. *S. coelicolor* Müller (Upjohn UC 5240) was grown in 2% glucose, 0.5% casamino acids, 0.25% yeast extract, 0.3% NaNO₃, 0.1% KH₂PO₄, 0.05% KCl, and 0.5% CaCO₃. *M. purpurea* (Schering Corp.) and *S. fradiae* (Upjohn UC 2046) were grown in 0.3% beef extract, 0.5% tryptone, 0.1% glucose, 2.4% soluble starch, 0.5% yeast extract, 0.6% CaCO₃, and 0.4% KCl.

Preparation of Cell-Free Extracts. Cells were harvested by centrifugation at 15,000 × g for 15 min, washed with distilled water, suspended in one volume of 0.01 M Tris·HCl (pH 7.8), 0.01 M MgCl₂, 0.01 M NH₄Cl, 5 mM 2-mercaptoethanol, and disrupted by two passages through a French press. The resulting suspension was centrifuged at 30,000 × g for 30 min, 4 μg of DNase I per ml was added, and the suspension was centrifuged at 105,000 × g for 4 hr to pellet the ribosomes. The supernatant was dialyzed against the above buffer.

Enzymatic Assays. The reaction mixture for acetylation

Abbreviations: R-factor, antibiotic resistance factor; R⁺, containing R-factor.

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TABLE 1. Aminoglycoside-modifying enzymes

Enzyme	Bacterial source	Modification
Kanamycin acetyl* transferase	(KAeT) R ⁺ <i>E. coli</i> <i>P. aeruginosa</i>	6-Amino group of an amino hexose is acetylated
Gentamicin acetyl transferase I	(GAeT I) <i>P. aeruginosa</i> <i>K. pneumoniae</i> <i>E. coli</i>	3-Amino group of 2-deoxystreptamine is acetylated
Gentamicin acetyl† transferase II	(GAeT II) <i>Providencia</i>	2-Amino group of an amino hexose is acetylated
Streptomycin-spectinomycin adenylyl transferase	(SAeT) R ⁺ <i>E. coli</i>	Hydroxyl group of a D-threo methylamino alcohol moiety is adenylylated
Gentamicin adenylyl transferase	(GAeT) R ⁺ <i>E. coli</i> R ⁺ <i>K. pneumoniae</i>	2-Hydroxyl group of amino hexose (III, see Figs. 1 and 2) is adenylylated
Streptomycin phosphotransferase	(SPT) R ⁺ <i>E. coli</i> <i>Staph. aureus</i>	3-Hydroxyl group of N-methyl L-glucosamine is phosphorylated
Neomycin-kanamycin phosphotransferase	(NPT) I R ⁺ <i>E. coli</i> II <i>P. aeruginosa</i> <i>Staph. aureus</i>	3-Hydroxyl group of an amino hexose (I, see Fig. 1) is phosphorylated
Lividomycin phosphotransferase	(LvPT) <i>P. aeruginosa</i> R ⁺ <i>E. coli</i>	5-Hydroxyl group of D-ribose is phosphorylated

* Present also in *Streptomyces kanamyceticus* (this paper).† Found in *Streptomyces spectabilis* (this paper).

with a cell-free extract of *S. spectabilis* contained extract, 10.2 nmol of [¹⁴C]acetyl coenzyme A (6 Ci/mol), 5–10 nmol of antibiotic, 6 μmol of Tris·maleate (pH 5.3), 0.6 μmol of MgCl₂, and 10 μmol of 2-mercaptoethanol in a total volume of 55 μl. For acetylation by the *S. kanamyceticus* extract, the Tris·maleate (pH 5.3) was replaced by 6 μmol of Tris·HCl (pH 7.4 at 30°). Incubation was at 30°; at various times, aliquots of the reaction mixture were pipetted onto squares of phosphocellulose paper which were washed, dried, and counted as described (17). The assay for the phosphorylation of aminoglycoside antibiotics contained the appropriate cell-free extract, 150 nmol of [³²P]ATP (1–4 Ci/mol), 10–30 nmol of aminoglycoside antibiotic, 1.0 μmol of Tris·HCl (pH 8.1 at 30°), 0.4 μmol of MgCl₂, and 50 nmol of dithiothreitol in a total volume of 55 μl. The assay for the adenylylation of aminoglycosides was the same except that [³²P]ATP was replaced by [¹⁴C]ATP.

Large-Scale Acetylation of Gentamicin C_{1a} by *S. spectabilis* Extract. The reaction mixture contained, in a total volume of about 270 ml, 200 ml of a cell extract obtained by disrupting 350 g of *S. spectabilis*, an ATP-generating system consisting of 0.4 mmol of phosphoenolpyruvate and 8 mg of pyruvate kinase; 5 mmol of ATP (adjusted to pH 6); 20 μmol of yeast coenzyme A; 0.23 mmol of gentamicin C_{1a}; 0.1 M potassium Tris·maleate (pH 6.0); 6 mM NH₄Cl; 6 mM magnesium acetate; and 3 mM dithiothreitol. The incubation was done at 30° for 20 hr with gentle agitation.

Large-Scale Acetylation of Gentamicin C_{1a} by *S. kanamyceticus* Extract. The reaction mixture (100 ml) contained 80 ml of a cell extract obtained by disrupting 62 g of *S. kanamyceticus*; 0.3 mmol of phosphoenolpyruvate and 6 mg of pyruvate kinase; 1.5 mmol of ATP; 10 μmol of coenzyme A; 0.14 mmol of gentamicin C_{1a}; 0.1 M Tris·HCl (pH 7.5 at 30°); 0.01 M NH₄Cl; 0.01 M magnesium acetate; 0.01 M KCl; and 2 mM dithiothreitol. The incubation was done at 30° for 15 hr with gentle agitation.

TABLE 2. The enzymatic inactivation of aminoglycoside antibiotics

Antibiotic	Inactivating enzyme*								
	KAeT	GAeT I	GAeT II	SAeT	GAeT	SPT	NPT I	NPT II	LvPT
Kanamycin A	+	–	–	–	+	–	+	+	
B	(+)	(+)†	–	–	–	–	–	–	–
C	–	–	–	–	–	–	–	–	–
Neomycin B or C	(+)	–	–	–	–	–	–	–	+
Paromomycin	–	–	–	–	–	–	–	–	+
Lividomycin A	–	–	–	–	–	–	–	–	–
Butirosin	(+)	–	–	–	–	–	–	–	–
Ribostamycin	+	–	–	–	–	–	–	–	–
Gentamicin C _{1a}	(+)	+	+	–	+	–	–	–	–
C ₂	(+)	+	+	–	+	–	–	–	–
C ₁	–	+	+	–	+	–	–	–	–
A	–	–	(+)†	–	+	–	+	+	–
Sisomicin	(+)	+	+	–	+	–	–	–	–
Tobramycin	(+)	+	(+)†	–	+	–	–	–	–
Nebromycin factor 2	–	–	–	–	–	–	–	–	–
Streptomycin	–	–	–	+	–	+	–	–	–
Spectinomycin	–	–	–	+	–	–	–	–	–
BBK-8‡	+	–	–	–	–	–	–	–	–

* The abbreviations for the enzymes are explained in Table 1. + means an enzymatic modification inactivates the antibiotic, (+) that it is only partially inactivated, and – that it is not a substrate. Blank spaces denote reactions that have not been tested.

† These three antibiotics are modified by the enzyme, but are poor substrates and strains are essentially sensitive to them.

‡ BBK-8 is a semisynthetic kanamycin derivative.

RESULTS

Identification of Antibiotics Produced by the Actinomycetes. The aminoglycoside antibiotic-inactivating enzymes currently known to exist in antibiotic-resistant bacteria, are listed in Table 1, and the antibiotics that they modify are shown in Table 2. For a review of the properties and substrate specificities of these enzymes, see ref. 1. The actinomycetes were grown from a spore inoculum in either synthetic or complex medium as described in *Methods*. To verify the identity of the antibiotics produced by the actinomycetes, we tested supernatant medium from cultures in stationary phase as substrate for four of the aminoglycoside-inactivating enzymes: kanamycin acetyltransferase, neomycin-kanamycin phosphotransferase I, streptomycin phosphotransferase, and streptomycin-spectinomycin adenylyltransferase.

TABLE 3. Aminoglycoside acetylating, phosphorylating, and adenylylating enzymes in actinomycetes*

Actinomycete	Antibiotic produced	Gentamicin C _{1a} acetylation†	Neomycin phosphorylation	Streptomycin phosphorylation	Streptomycin or gentamicin C _{1a} adenylylation
<i>S. kanamyceticus</i>	Kanamycins	+	–	–	–
<i>M. purpurea</i>	Gentamicins	–	–	–	–
<i>S. coelicolor</i>	None	+	–	+	–
<i>S. spectabilis</i>	Spectinomycin	+	+	+	–
<i>S. fradiae</i>	Neomycins	–	+	–	–

* (+) means activity was detected with the phosphocellulose paper binding assay. (–) = no enzymatic activity detected.

† All extracts that acetylated gentamicin C_{1a} also acetylated neomycin B and paromomycin, the other antibiotics tested.

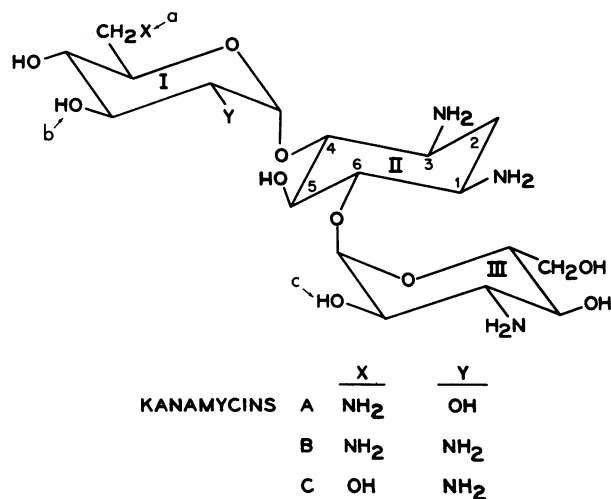


FIG. 1. The structure of kanamycins A, B, C. Arrows indicate the sites of *N*-acetylation by kanamycin acetyltransferase (*a*), the site of *O*-phosphorylation by neomycin-kanamycin phosphotransferase (*b*), and the site of *O*-adenylation by gentamicin adenylyltransferase (*c*). For the structure of neomycin and paromomycin, see ref. 17. Tobramycin is 3'-deoxykanamycin B.

Since the substrate specificities of these enzymes are known (Table 2), the identity of the antibiotics produced by the various actinomycetes could be easily verified. The supernatant from the *S. spectabilis* medium served as substrate for streptomycin-spectinomycin adenylyltransferase, and not for streptomycin phosphotransferase, as would be expected if spectinomycin were being produced. *S. kanamyceticus* produced kanamycin, a substrate for kanamycin acetyltransferase and neomycin phosphotransferase, and *M. purpurea* produced gentamicin, a substrate for kanamycin acetyltransferase. The use of gentamicin adenylyltransferase could have distinguished between neomycin and kanamycin, since only the latter is a substrate. The *S. coelicolor* supernatant contained no detectable antibiotic (no substrate activity with any of the four enzymes). Thus, the antibiotic-inactivating enzymes have analytical applications, as has been demonstrated in the use of certain enzymes for the rapid analysis of antibiotic concentration in serum (14-16).

Identification of Aminoglycoside-Acetylating, Phosphorylating, and Adenylylating Enzymes in Actinomycetes. Cell-free extracts from the various actinomycetes were tested for the presence of enzymes that would acetylate gentamicin C_{1a} or neomycin B, phosphorylate streptomycin or neomycin B, and adenylylate streptomycin or gentamicin C_{1a}. This range of substrates would reveal the presence of any of the enzymes listed in Table 1. The acetylation, phosphorylation, and adenylylation of the antibiotics was monitored as described in *Methods*. Table 3 lists the various activities that were found in the extracts from the strains.

The relationship between antibiotic production and modification among the strains that we have examined is not absolute. For example, *S. coelicolor*, which produces no detectable antibiotics, contains an enzyme that acetylates gentamicin C_{1a} and one that phosphorylates streptomycin. *M. purpurea* produces gentamicin but no gentamicin-acetylating, -phosphorylating, or -adenylylating enzymes; *S. kanamyceticus* contains an enzyme that acetylates gentamicin (and kana-

mycin); *S. spectabilis* contains neomycin- and streptomycin-phosphorylating enzymes and a gentamicin-acetylating enzyme; and *S. fradiae* contains a neomycin-phosphorylating enzyme. None of the strains possessed an enzyme that would adenylylate streptomycin or gentamicin C_{1a}. It is possible that other modifications such as *O*- or *N*-methylation or carbamoylation occurred, but we have not assayed for these activities.

Acetylation of Aminoglycosides in Streptomyces. Since two different gentamicin C_{1a}-acetates had been previously described, 6'-*N*-acetyl gentamicin C_{1a} (17) and 3-*N*-acetyl gentamicin C_{1a} [acetylated on the 3-amino group of 2-deoxystreptamine by a gentamicin-resistant strain of *Pseudomonas*—see Fig. 2 (18)], it was decided to study the gentamicin-acetylating enzymes from *S. spectabilis* and *S. kanamyceticus* in more detail. The gentamicin-acetylating activity of *S. coelicolor* was not further studied.

Acetylation of Aminoglycosides in *S. spectabilis*. The crude *S. spectabilis* extract was found to acetylate a wide variety of aminoglycosides. To determine if this was due to the presence of more than one enzyme, the extract was fractionated by passage through a DEAE-cellulose column with a linear gradient of sodium chloride, as described (17). Only one peak of acetylating activity for all the aminoglycosides tested could be found; this activity eluted at 0.21 M NaCl. The enzyme has a pH optimum of 5.3, which is the same regardless of the substrate with which the enzyme is assayed. In general, assay conditions were identical to those of kanamycin acetyltransferase (17) in terms of such reaction parameters as the Michaelis constant for acetyl coenzyme A and for antibiotic.

A wide variety of substrates is acetylated by this enzyme, including gentamicin C_{1a}, gentamicin A, sisomicin, tobra-

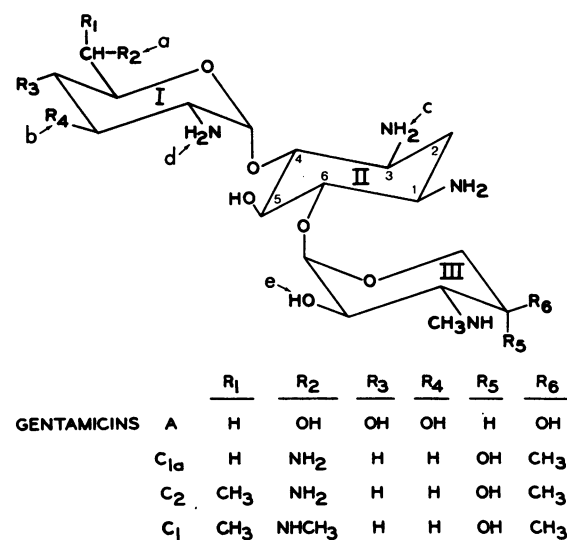


FIG. 2. The structure of the gentamicins. Ring I is purpurosamine, II is 2-deoxystreptamine, and III is gentosamine (gentamicin A) or garosamine (gentamicin Cs). Sisomicin is 4',5'-dehydrogentamicin C_{1a}. Arrows indicate where this group of antibiotics can be *N*-acetylated by kanamycin acetyltransferase (*a*), gentamicin acetyltransferase II (*d*), or gentamicin acetyltransferase I (*c*); *O*-phosphorylated by neomycin-kanamycin phosphotransferase (gentamicin A only) (*b*); *O*-adenylylated by gentamicin adenylyltransferase (*e*).

mycin, paromomycin, neomycin B, and kanamycin B and C. Kanamycin A, 3-*N*-acetyl gentamicin C_{1a} (18), nebramycin factor 2, streptomycin, and spectinomycin were not acetylated. The fact that kanamycin C is acetylated and kanamycin A is not strongly implies that the 2-amino moiety of that aminosugar ring is acetylated (Fig. 1). To verify this, we acetylated gentamicin C_{1a} in a large-scale reaction so that the product could be isolated and studied.

Large-Scale Modification of Gentamicin C_{1a} by S. spectabilis Extract. *S. spectabilis* was grown in 3.5 liters of complex medium and yielded 350 g (wet weight) of cells. The cells were disrupted by passage through a French press, and the extract was used to acetylate 100 mg of gentamicin C_{1a} in the presence of coenzyme A, magnesium acetate, and ATP.

The acetylated gentamicin C_{1a} was purified by chromatography on a Biorex 70 column (BioRad) exactly as described for the purification of 6'-*N*-acetyl gentamicin C_{1a} (17). Two peaks of radioactivity were obtained, which by analogy with previous work (17) were believed to contain mono- and diacetylated gentamicin C_{1a}, respectively. The mono-acetylated gentamicin C_{1a} was shown to be pure by the same techniques that were used for 6'-*N*-acetyl gentamicin C_{1a} (17).

Dr. Peter Daniels has characterized this mono-acetylated gentamicin C_{1a} by mass spectroscopy and has shown it to have the characteristic fragmentation pattern of 2'-*N*-acetyl-gentamicin C_{1a} (Fig. 2) (19). The diacetate has not been characterized.

Acetylation of Aminoglycosides in S. kanamyceticus. A crude *S. kanamyceticus* extract was fractionated by chromatography on DEAE-cellulose, and the acetylating activity eluted at 0.17 M NaCl. The enzyme has a pH optimum of 7.4 for the acetylation of gentamicin C_{1a}, paromomycin, and kanamycin B. The list of substrates acetylated by this extract includes gentamicin C_{1a}, gentamicin A, sisomicin, kanamycin A, B, and C, tobramycin, neomycin B, and paromomycin. Streptomycin, spectinomycin, and nebramycin factor 2 are not acetylated.

Large-Scale Modification of Gentamicin C_{1a} by the S. kanamyceticus Extract. For determination of the site of acetylation of gentamicin C_{1a}, 60 mg of this antibiotic was acetylated as described in *Methods*. A small amount of gentamicin C_{1a} was also acetylated with [¹⁴C]acetyl coenzyme A of high specific activity in order to monitor the elution of the compound during purification. When this mixture was chromatographed on Biorex 70, the same elution profile as that obtained after the *S. spectabilis* reaction was seen.

The mono-acetylated gentamicin C_{1a} has been analyzed chemically and spectrographically, and shown to be 6'-*N*-acetyl gentamicin C_{1a} (Fig. 2). Since kanamycin C and paromomycin do not contain a 6-amino moiety but are acetylated by this extract, there must be an additional acetylating enzyme in the *S. kanamyceticus* extract.

DISCUSSION

We report the presence of enzymes in actinomycetes that can modify certain of the aminoglycoside antibiotics.

Two acetylated gentamicin derivatives had already been characterized from R⁺ *Escherichia coli* and *Pseudomonas aeruginosa* (17, 18), so it was decided to study the gentamicin C_{1a}-acetylating enzymes that were discovered in *S. spectabilis* and *S. kanamyceticus*. The acetylating enzyme from *S.*

spectabilis acetylated gentamicin C_{1a} on the 2'-amino group of the purpurosamine ring, and the enzyme from *S. kanamyceticus* produced 6'-*N*-acetyl gentamicin C_{1a}.

Thus, one of the aminoglycoside-modifying enzymes discovered in *Streptomyces* appears to catalyze the same reaction as an enzyme that has previously been described in R⁺ *E. coli*, namely kanamycin acetyltransferase. Since these experiments were performed, an enzyme has been found in a clinical isolate of a *Providencia* strain that catalyzes a reaction identical to that of gentamicin acetyltransferase II discovered first in *S. spectabilis* (Chevereau, Daniels, Davies, and Le-Goffic, in preparation).

We have also detected streptomycin-phosphorylating activity in *S. griseus*, a streptomycin-producing actinomycete; this may be similar to that reported by others (3, 6-10) and has not been studied further. Studies with *S. fradiae*, which produces neomycin, have shown the presence of a phosphotransferase that appears to modify the 3'-OH of neomycin, as is the case with neomycin-phosphorylating enzymes found in gram-negative bacteria (ref. 20; Dowding and Benveniste, unpublished observations). The isolation and characterization of the inactivating enzymes in an actinomycete producing a new antibiotic might presage the mechanisms of enzymatically-mediated resistance in clinical isolates resistant to the antibiotic.

The metabolic role of the aminoglycoside-modifying enzymes in actinomycetes is not known. Miller and Walker have postulated that phosphorylated streptomycin might be important as a metabolic precursor of streptomycin or to detoxify the antibiotic (7). The enzymes might also be required for transport of these antibiotics in or out of the cell. Alternatively, they may have nothing to do with antibiotic biosynthesis and may play a role in another biosynthetic process.

Whatever the role of these enzymes, the actinomycetes are a new source of aminoglycoside-modifying enzymes that could be used to provide new antibiotic derivatives with altered biological properties. Since the aminoglycoside antibiotics have undesirable toxic effects, especially when administered in high doses for prolonged periods of time, some of these modified antibiotics might combine biological activity with reduced toxicity. Examination of other strains of bacteria (including actinomycetes) will surely result in the discovery of new modifying enzymes. *Bacillus circulans*, which produces the novel modified aminoglycoside antibiotic butirosin, may also contain some of these enzymes. Only acetylation, phosphorylation, and adenylation activities in a small number of strains have been examined here—it is possible that some modified aminoglycosides may be produced by other mechanisms. In addition, the enzymes described here were all isolated from strains in stationary phase; different enzymes may be present during the exponential growth of the organism, or under different culture conditions.

Nothing is known about the origin of R factors. The Watanabe hypothesis (4) provides a simple molecular mechanism for their origin, but we can only speculate on the environmental and evolutionary factors that play a role in their formation and maintenance. Their presence does not seem to require the extensive use of a selective antibiotic environment since Gardner *et al.* (21) have found R factors in an "antibiotic virgin population" in the Solomon Islands. These R factors, which were isolated from soil and stool specimens, mediated resistance to streptomycin and tetracycline.

The discovery of aminoglycoside-modifying enzymes in actinomycetes catalyzing the same reactions as those found in clinical isolates of other bacteria suggests an origin for resistance determinants. The fact that identical enzymatic activities are found in two separate organisms does not necessarily imply a direct transfer of genetic information from one to the other. The actinomycetes may excrete antibiotics into the soil in order to compete effectively with other soil microorganisms for nutrients, and it could be that some gram-negative or gram-positive bacteria have acquired the inactivating enzymes in order to protect themselves against these antibiotics. Hill (22) has shown that penicillin is produced in soils inoculated with a wild-type *Penicillium chrysogenum*, and that *Bacillus cereus* strains constitutive for β -lactamase production have a clear survival advantage in this soil.

At present we can only speculate as to the mechanism of the possible transfer of genetic information from actinomycetes to other bacteria. There is no evidence for a transduction or conjugation system capable of such a transfer, but a recent report of stable genetic transformation of *E. coli* by isolated R-factor DNA (23) suggests a plausible mechanism. Alternatively, genetic transfer by conjugation or transduction may have occurred through a chain of closely related organisms, even though the initial donor and final recipient may be totally unrelated bacterial species. In any case, a more detailed comparative study of the enzymatic and immunological properties of the purified enzymes from actinomycetes and other bacteria may reveal relationships between them, unless the enzymes have evolved divergently to a great extent.

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