ANTIBIOTICS FROM MARINE MICROORGANISMS WITH REFERENCE TO PLASMID INVOLVEMENT[1]

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The discovery of penicillin and its tremendous success in the chemotherapy of infectious diseases opened the door for the antibiotic era. The continuous demand for new agents for the chemotherapy of diseases has resulted in the isolation of nearly 4,000 antibiotics. These were obtained not only from natural sources, but also more and more by manipulation of microorganisms grown in culture in the laboratory. In many ways microorganisms are the most domesticated organisms in the history of mankind. These antibiotics included many novel chemical structures which had never before been synthesized or even conceived of by chemists. The chemotherapeutic effect is intrinsically based on the selective action of these chemicals on certain targets in living systems, and this selective action is dependent upon the relationship between chemical structure and biological activity. This holds true no matter whether the target organisms are bacteria or, in more recent work, viruses or tumor cells. Accordingly, the efforts in this field have been directed not only at obtaining active compounds, but also at elucidating their chemical structures. In this connection, the achievements in the fields of physics and chemistry which led to the development of new analytical techniques and instrumentation or to new purification methods have played a major role. The work on these antibiotics thus represents a great contribution not only to practical chemotherapy, but also to basic sciences such as organic chemistry and biochemistry. For example, the cepham-penam antibiotics contain as a parent nucleus a β-lactam ring which is essential for the activity and the selective toxicity of these compounds. This chemical structure is thus the basis for their use as an ideal chemotherapeutic agent; at the same time, these compounds are also important analytical tools in biochemistry. Compounds like streptomycin and chloramphenicol exhibit selective toxicity by different modes of action based on their different chemical structures. It is expected that more antibiotics with diverse chemical structures exhibiting selective toxicity by different modes of action will be obtained from natural sources. This expectation is based on the fact that we have so far been able to investigate only a small percent of the living organisms; it would thus seem worthwhile to continue our efforts to obtain new and novel chemical agents from unexplored living organisms. In the past we have in these efforts relied mostly on trial and error experiments, and we may have to continue with this approach for some time to come because the mechanisms of production of such diverse chemical structures in living cells are not well understood. At the same time, though, it would be important to carry out more intensive basic research on the biosynthesis of such complex chemical entities in nature.

On the other hand, approaches have also been devised in antibiotics research which are based on fundamental chemical, biochemical, and biological knowledge. One such approach deals with the problem of overcoming the resistance which many target organisms gradually develop against known antibiotics. For example, new

penicillins and cephalosporins were devised partly on the basis of such an approach by chemically or biologically modifying the penicillin or cephalosporin nucleus. These modifications protected the β-lactam ring against destruction by inactivating enzymes such as penicillinases or cephalosporinases of resistant organisms. However, these modifications in the β-lactam structures were again made more or less on the basis of trial and error. In contrast, a more rational approach was used in the field of aminoglycoside antibiotics. For example, kanamycin is an aminoglycoside antibiotic which is effective against a wide range of bacteria; the frequent use of this antibiotic has produced resistant strains. Studies in *E. coli* on the mechanism of resistance to kanamycin revealed that the resistant organisms produce enzymes which inactivate the antibiotic by acetylation, phosphorylation, or adenylation at specific sites in the kanamycin molecule. Based on this finding, the sites in the kanamycin molecule which could be esterified by the inactivating enzymes were altered to remove the oxygen function, thus preventing the reaction which leads to inactivation of the antibiotic. The deoxy derivative of kanamycin thus obtained was found to be effective against the resistant bacteria (1). Thus, modification devised in a rational way has opened a new direction of antibiotic research leading to useful advances in chemotherapy.

Another new direction of current antibiotic research is the attempt to gain a better understanding of antibiotic production on a molecular and genetic basis. In the early stages of antibiotics research, improvements in the production yields were achieved mainly by varying culture conditions and by making mutants of the producing organisms using uv or gamma radiation or chemicals as mutagens. Some of the mutants obtained in this way gave increased yields and were thus useful for antibiotic production and for biosynthetic studies. Conjugation, heterokaryosis, or phage transduction were also explored in attempts to obtain high yielding clones, but most of these attempts were not successful. All this work dealt with alterations in phenotypic expression, i.e., higher antibiotic production, but provided little insight into its molecular and genetic basis. Hybrimycin represents the first success in creating a new antibiotic by a genetic approach. In this case the precursors of two different antibiotics were combined to give a new entity. With a mutant which was dependent upon added aminocyclitol for the production of neomycin, the addition of heterologous aminocyclitols to the culture medium yielded the new antibiotics hybrimycin A and B (2), which contained the modified aminocyclitols in place of the normal aminocyclitol moiety of neomycin.

A second area in which genetic approaches have proven fruitful is in the understanding of the production of secondary metabolites such as antibiotics on a genetic basis. In the work on maintenance or improvement of antibiotic production it has been frequently observed that an organism easily loses the capability for antibiotic production during successive transfers without any effect on the growth characteristics. This means that antibiotic production is not essential for the growth of the producing organism. In addition, it has been shown that many antibiotics are produced after or late in the logarithmic growth phase, the most active phase of primary metabolism. Antibiotic production is thus usually a pathway of secondary metabolism which may be viewed as an extension of primary metabolism. Growth is directly dependent on primary metabolism, which is chromosomally determined, and the loss of primary metabolism leads to cell death; loss of secondary metabolism is not related to growth. The loss of secondary metabolism is frequently paralleled by the loss of a plasmid. A plasmid is an extra-chromosomal entity which can be eliminated from a microorganism by treat-

ment with acridine dyes or exposure to increased temperature. Plasmids can be eliminated without causing cell death, but the loss or change of chromosomal entities tends to cause death of the organism.   The above parallel between the loss of a plasmid and the loss of antibiotic production frequently holds.   It is, therefore, concluded that plasmids often govern some process(es) of secondary metabolism which lead to compounds like antibiotics.   For example, the loss of production of the antibiotics kasugamycin and kanamycin or the enzyme inhibitor leupeptin coincides with the elimination of plasmids from the producing organism. The loss of leupeptin production upon acriflavine treatment can be reversed at a significant rate by conjugation with the parent strain (3).   This indicates that a plasmid governing leupeptin production is transferred to the nonproducer from the producing parent strain.   The first observation on the possibility of plasmid involvement in the biosynthesis of antibiotics was made with a streptomycete producing kasugamycin (4).   The production of chloramphenicol also was shown to be governed by extra-chromosomal genes (5), and plasmid involvement in methylenomycin production has also been demonstrated (6).

The evidence suggesting plasmid involvement in the biosynthesis of antibiotics has thus been accumulating.   However, many problems remain unsolved because of the technical difficulties, including: 1. the difficulty of plasmid preparation, particularly from actinomycetes; 2. lack of proper vectors for transformation among actinomycetes; and 3. the lack of extensive genetic maps of plasmids from actinomycetes.   To understand the role of plasmids in the formation of antibiotics, more detailed information is needed on the biosynthesis of antibiotics, including the characterization of the enzymes involved in each step and their regulatory mechanisms.   After the above problems are solved, genetic engineering and/or plasmid technology may contribute to the improvement of antibiotic production and may enable the formation of new antibiotics or other useful secondary metabolites.

Viewing the field of antibiotic research, one can define two distinguishable stages which I would like to characterize as an "expanding era" and an "interconceptual era" (figure 1).   There are two major concepts governing the antibiotics
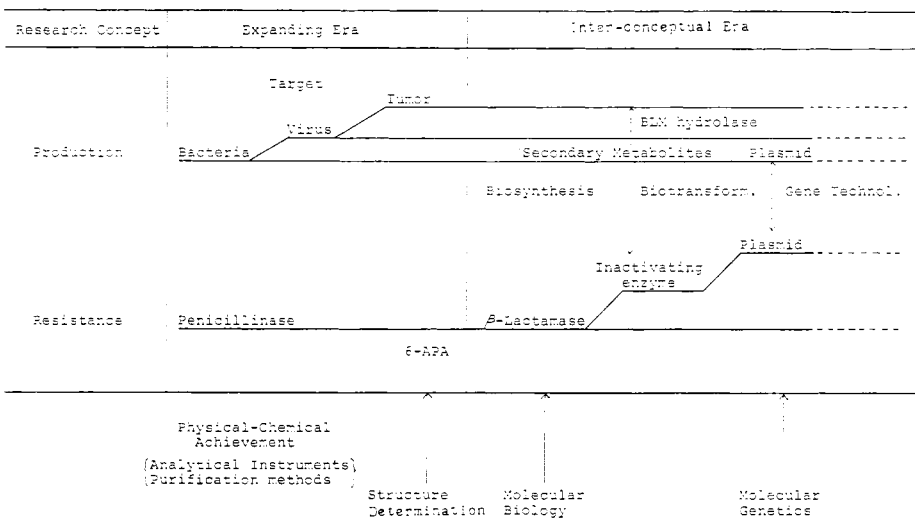

FIG. 1.   Concepts and eras in antibiotics research.

field, namely antibiotic production and antibiotic resistance. These two concepts were independent of each other in the early stages of antibiotic research, but later they have been inter-related in light of fundamental knowledge of molecular biology and molecular genetics. The "expanding era" consists of research aimed at finding new antibiotics and at improving antibiotic production. This type of research was conducted by and large on a trial and error basis. Consequently thousands of new antibiotics have been discovered by expanding the range of test organisms, e.g., from bacteria to viruses or tumor cells, and also by increasing the range of organisms screened. Likewise, efforts to achieve high yields for antibiotic production were also largely carried out by laborious trial and error experiments. When resistance to antibiotics such as penicillin or streptomycin appeared, the efforts were mainly directed towards obtaining new agents active against the resistant strains, again, essentially by trial and error. Thus this era is characterized by expanding research efforts which are more or less intra-conceptual with regard to the two major concepts above.

The second era, the "inter-conceptual" one, is characterized by work based on inter-relating concepts of antibiotic production and antibiotic resistance. This inter-relationship has been dependent upon discoveries in the field of molecular biology and molecular genetics which have led to an understanding of the phenomenon of resistance. Specifically, resistance was found to be due to inactivating enzymes that are coded for by R-plasmids. The studies on plasmid involvement in resistance have been related to the studies on plasmid involvement in the production of secondary metabolites such as antibiotics. Research in this second era has thus led to rational approaches to the modification of known antibiotics to protect them from inactivating enzymes. The development of deoxykanamycin B is an example of such an approach. Of course, even during this second era, trial and error approaches continued to be used to find new chemical compounds.

This paper surveys some of our current studies on antibiotics, particularly on antibiotics or other bioactive substances produced by marine microorganisms, with a view towards plasmid involvement. My interest in this field may serve as an example of research in the second era. The majority of antibiotics hitherto discovered are the products of microorganisms isolated from terrestrial soil. More antibiotics can be expected to be obtained from terrestrial microorganisms because many microorganisms in soil still remain to be investigated. However, the marine area is more than two times larger than the land area on the surface of our globe; it has huge dimensions in both the horizontal and vertical directions. It consists of vast amounts of water containing salts and nutrients. The deeper marine environment provides higher hydrostatic pressure and less sunlight. In contrast to the deep sea, the coastal sea is influenced greatly by land and is changeable with high and low tides. Such varied environmental conditions are likely to lead to considerable variety in the microorganisms it sustains. The marine environment is clearly different from the terrestrial environment and may thus contain different organisms than are found in terrestrial areas. Rain or irrigation water can wash out microorganisms from the soil which ultimately flow into the coastal sea area where they may adapt to the marine environment (7).

For the last seven years we have isolated microorganisms from the shallow sea, and we have observed (8) that the closer to the land we collect samples the more microorganisms of terrestrial orgin we find. Among the isolates from the sea, some were dependent upon seawater (9); more of the actinomycetes isolated from the sea were tolerant to high sodium chloride concentration than isolates from ter-

restrial soil (10).   Accordingly, we can expect a difference between the sea and terrestrial soil profiles of microbial flora.   In particular, we expected the possibility of isolating more microorganisms from the sea that are rare in terrestial soils because of the selective pressure during the migration of such terrestrial microorganisms from the soil and into the marine environment.   Thus, we have been searching for new microorganisms from the sea as a source of new antibiotics.

As stated previously, the production of secondary metabolites by microorganisms is also dependent upon culture conditions.   A trace metal in a culture medium, for example, may influence dramatically the production of various secondary metabolites.   When new chemical or, for that matter, physical conditions such as agitation or temperature are employed for the cultivation of microorganisms, new antibiotics may be found.   Examples of this are shown in table 1.

TABLE 1.   Some new products formed under new culture conditions.

New Conditions	Organisms	New products	Ref.
Increased PO_4.	*Psedomonas* sp. *Streptomyces tateyamensis* *S. sapporonensis* *Nocardia uniformis*	Pyrrolnitrin Thiopeptin Bicyclomycin Nocardicin	11 12 13 14

Lower Incub. Temp.
  (27°–12°). . . . .
  (27°–15°). . . . .
Increased O_2 supply. . . . . .
	S. griseus
	S. griseus
	S. lavendulae
		Cryomycin
		Holomycin
		Mimosamycin
		Chlorocarcin
			15
			16
			17

Accordingly, we have searched for antimicrobial activity in marine isolates cultured in medium resembling a sea environment.   As a first result, we obtained (18) an actinomycete belonging to the genus *Chainia* which exhibited antimicrobial activity when grown in a culture medium containing seaweed extract (Kobu-cha *Laminarium*) but not when cultured on ordinary media.   The active principle was isolated and purified and shown to be a new antibiotic, SS–228Y (figure 2). This antibiotic has inhibitory activity against gram-positive bacteria, Ehrlich carcinoma in mice and an enzyme, dopamine β-hydroxylase.   It is easily converted by heat or light into a stable form SS–228R (19) which shows diminished inhibitory activity against bacteria and tumor cells, but retains the activity towards dopamine β-hydroxylase.   This finding, as well as the previous isolation of unique antibiotics such as a bromopyrrole (figure 3) from a marine *Pseudomonas* by Burkholder (20) and leptosphaerin (figure 4) from marine fungi by Clardy (21) or the initial isolation of cephalosporin from a fungus obtained from coastal sewage, stimulated us to continue this line of research.

A marine actinomycete, identified as *Streptomyces griseus*, a very common soil species, exhibited antimicrobial activity only when fermented in a medium containing very dilute nutrients and increased sodium chloride concentrations, but not when cultured on ordinary media (22).   The active principle was isolated and purified.   The substance obtained as a crystalline sodium complex is an ionophoric polyether and has a symmetric structure containing a boron atom in the center. The structure and configuration were elucidated by X-ray crystallography of the silver salt (figure 5) (23).   It can hold monovalent metal ions such as Na^+ or K^- in the cavity of the molecule, and it can transport these metal ions from one solvent to another in the Pressman system (24).   The compound has inhibitory
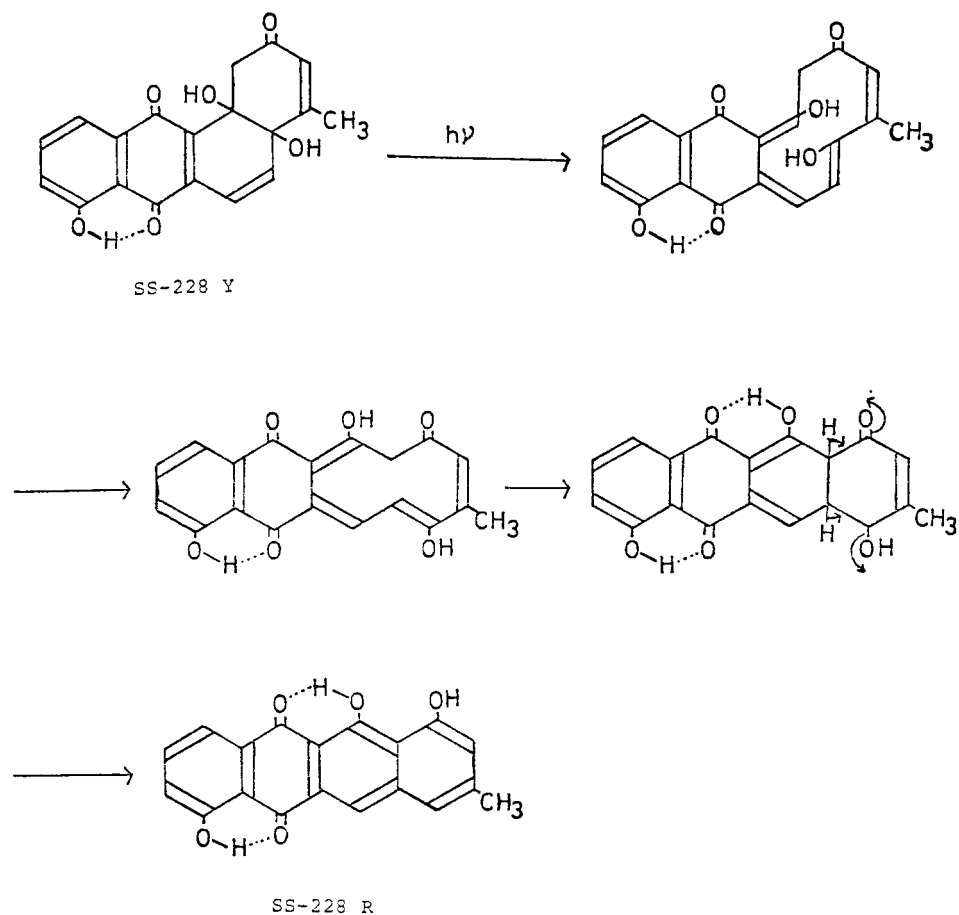
FIG. 2. Structures of antibiotic SS–228Y and its conversion products.

activity against gram-positive bacteria and malarial plasmodium; it was named aplasmomycin. Preliminary experiments showed that the ability to produce aplasmomycin was lost when the producing organism was treated with acriflavine.
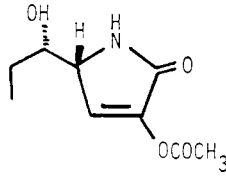
As mentioned previously, when we treated a kanamycin-producing organism with acriflavine or exposed it to increased temperature, the progeny frequently showed loss of kanamycin production. Many of the nonproducers thus obtained
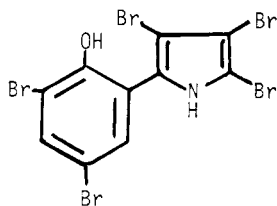
Bromopyrrole from
P. bromoutilis

FIG. 3

Leptosphaerin from

Leptosphaeria oraemaris

FIG. 4

produced kanamycin upon addition of deoxystreptamine to the culture medium (25).   Deoxystreptamine is a part of the kanamycin molecule as well as of many other aminoglycoside antibiotics.   The loss of kanamycin production following procedures which lead to elimination of plasmids suggested to us the possibility of plasmid involvement in deoxystreptamine biosynthesis.   We, therefore, tried to demonstrate the existence of a plasmid in the cells of the producing organism. However, the presence of a plasmid was difficult to confirm except for a slight indication of a satellite band of plasmid DNA shown by cesium chloride density gradient centrifugation.

Thus, it was necessary to find an actinomycete capable of producing amino-glycoside antibiotics in which the plasmid DNA in the cell could be distinguished. From our experience with marine microorganisms, we knew that they may exhibit
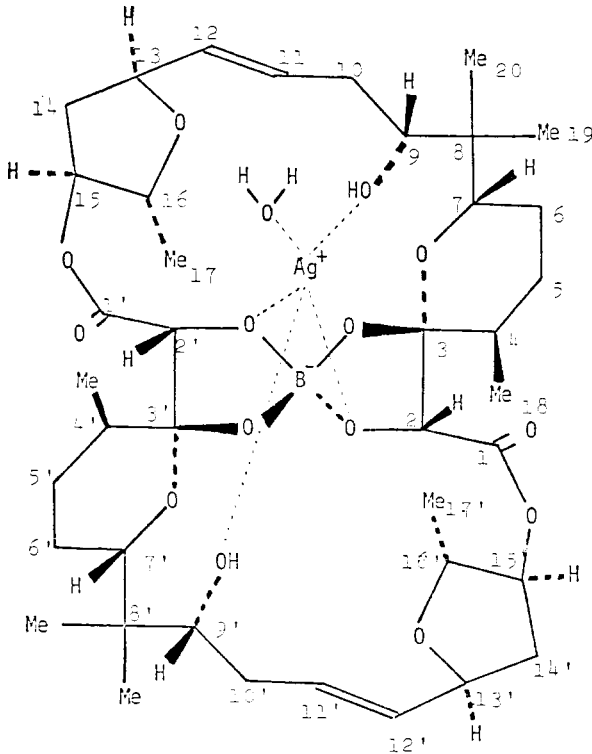

FIG. 5.   Structure of aplasmomycin (silver salt).

slightly different features from terrestrial ones, so we started to look for amiro-glycoside antibiotic producing actinomycetes from sea samples. It was hoped that these would have plasmids that are distinguishable by ultracentrifugation or gel electrophoresis. We found actinomycete SS-939, which grew on kanamycin

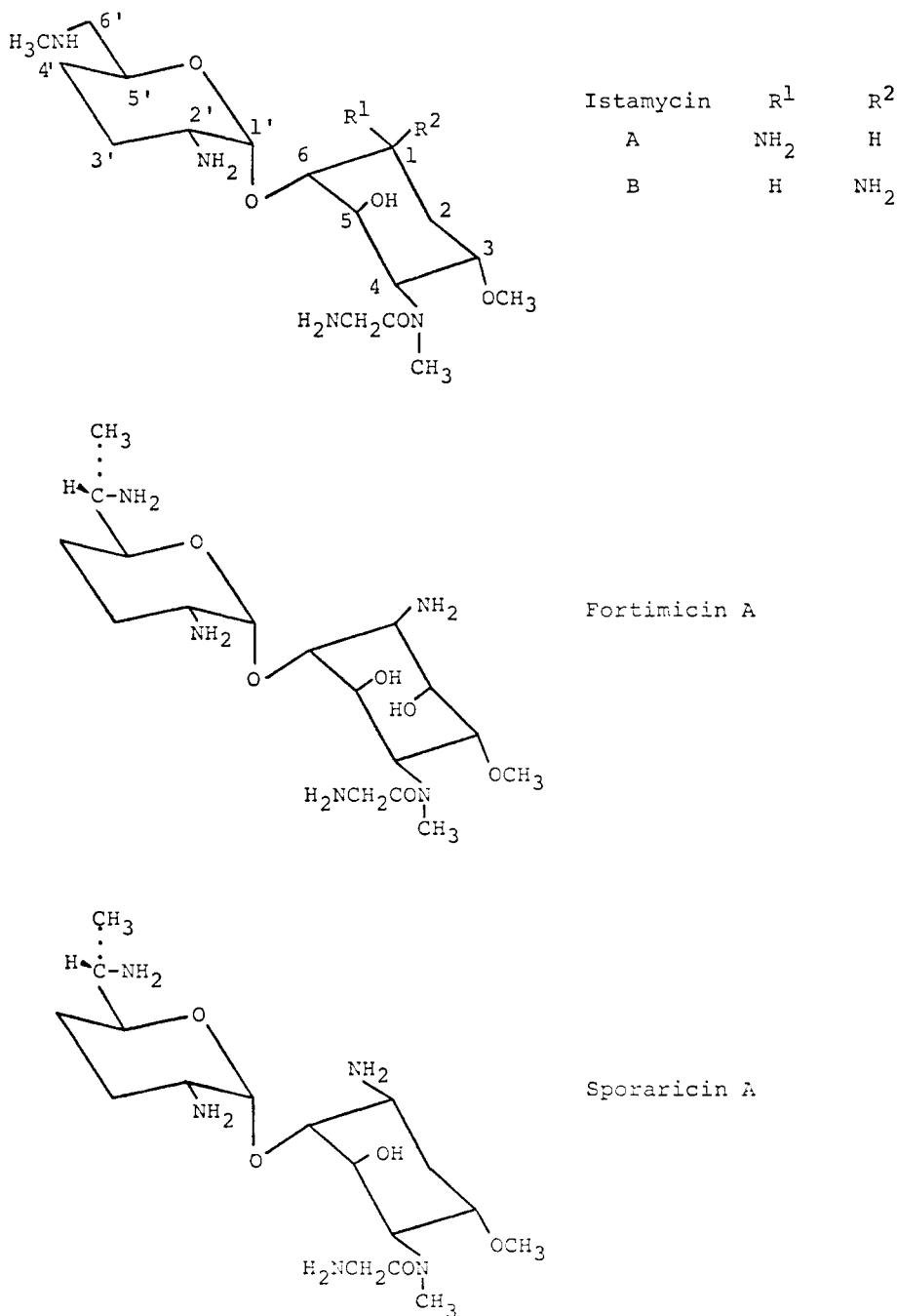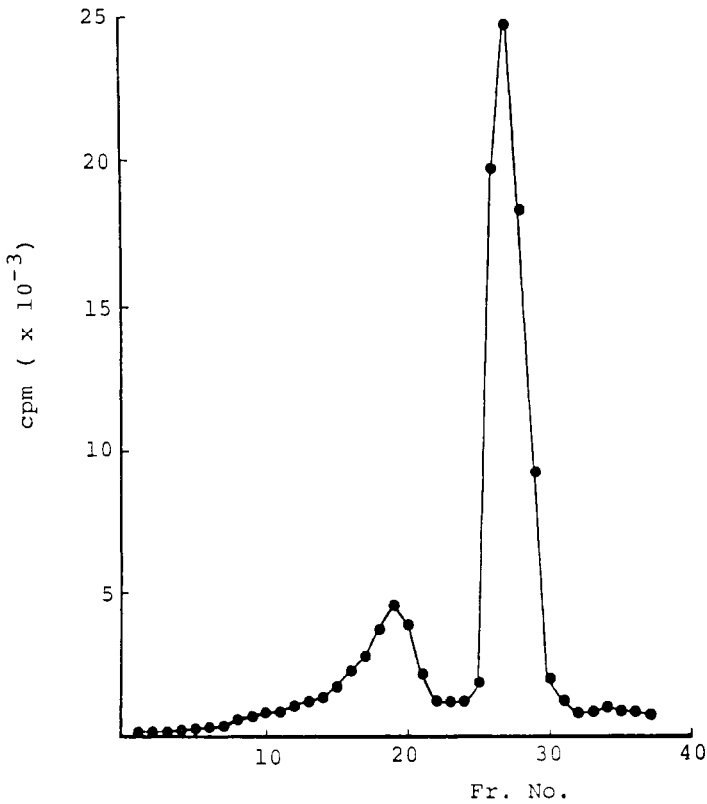| Istamycin	R¹	R²
A	NH_2	H
B	H	NH_2

Fortimicin A

Sporaricin A

FIG. 6.   Structures of the istamycins, fortimicin and sporaricin.

Ethidium Bromide -Cesium Chloride Density Gradient
Centrifugation of a Cleared Lysate from Streptomyces
tenjimariensis SS-939

FIG. 7a.

agar medium at 27°.   This organism isolated from marine mud taken from Tenjin Island in Sagami Bay exhibited broad antibacterial activity against various test organisms, including resistant bacteria.   The active principle (26) was isolated and found to be an aminoglycoside of the fortimicin-sporaricin group (figure 6). It was named istamycin.   While fortimicin and sporaricin are produced by terrestrial actinomycetes belonging to the genera *Micromonospora* and *Saccharopoly-*

Chromosomal DNA

Plasmid DNA

FIG. 7b.   Gel electrophoresis of the chromosomal and plasmid DNA bands.
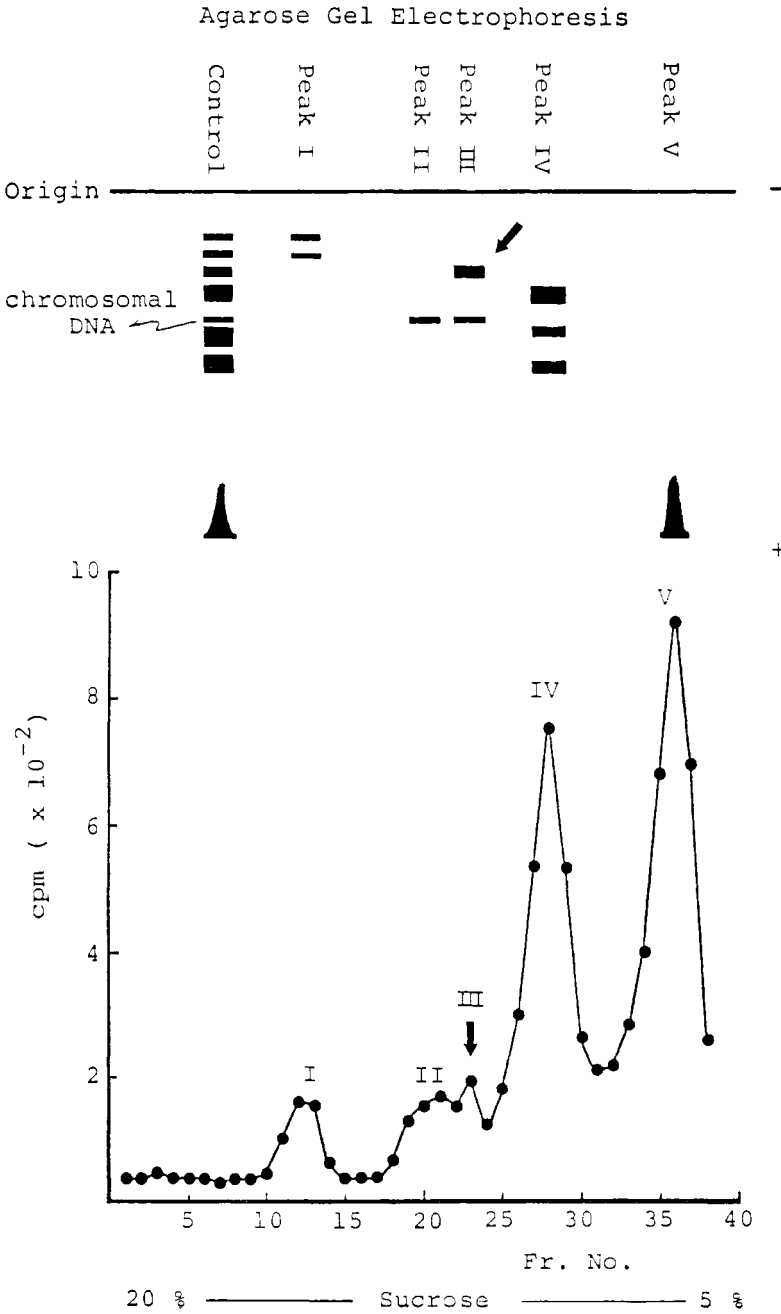
Agarose Gel Electrophoresis

FIG. 8a. Neutral sucrose density gradient centrifugation and agarose gel electro-
phoretic analysis of satellite (plasmid) DNA fractions from an istamycin-
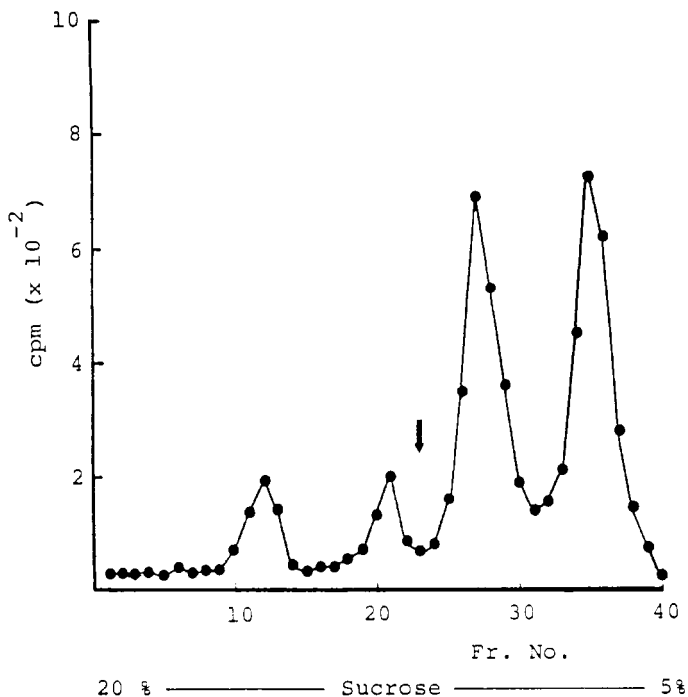producing culture of *S. tenjimariensis*.

FIG. 8b.   Sucrose density gradient centrifugation of the plasmid DNA
fractions from a non-producing isolate.

spora, respectively, taxonomic studies of our isolate revealed characteristic fea-
tures of a strain belonging to the genus *Streptomyces*.   It shows a very limited
utilization of carbohydrates, except glucose and inositol, and is not identical with
any known species of actinomycetes.   Ultracentrifugation and gel electrophoresis,
as shown in figures 7a and 7b, as well as electron microscopy revealed the existence
of plasmids.   When the strain was cultured in the presence of acriflavine many
of the resulting cultures showed no antibiotic production.   This loss of antibiotic
formation corresponded with the loss of one plasmid band, as shown in figures 8a,
8b and 8c.   Not all, but many of the non-producers thus obtained were able to
produce antibiotic upon addition of deoxystreptamine to the culture medium.

The above results illustrate that microbial isolates from a marine environment,
particularly from the coastal sea, may often be organisms that are minor constitu-
ents of terrestrial populations and which, thus, may be difficult to isolate from
terrestrial soil.   Some of these organisms may possess slightly different features
from those of terrestrial isolates, including differences in the antibiotic constituents.
Evidently a marine isolate produced new aminoglycoside antibiotics, the ista-
mycins, which are somewhat different in structure from aminoglycosides of ter-
restrial origin such as formycin and sporaricin.   The istamycin-producing marine
isolate carries several kinds of plasmids and the loss of istamycin production cor-
responds to the loss of one of these plasmids.   This suggests a plasmid involvement
in the biosynthesis of istamycin and provides a good tool for understanding the
biosynthetic mechanism.   Since istamycin formation in the non-producer can be
restored by addition of deoxystreptamine, it is suggested that the plasmid governs
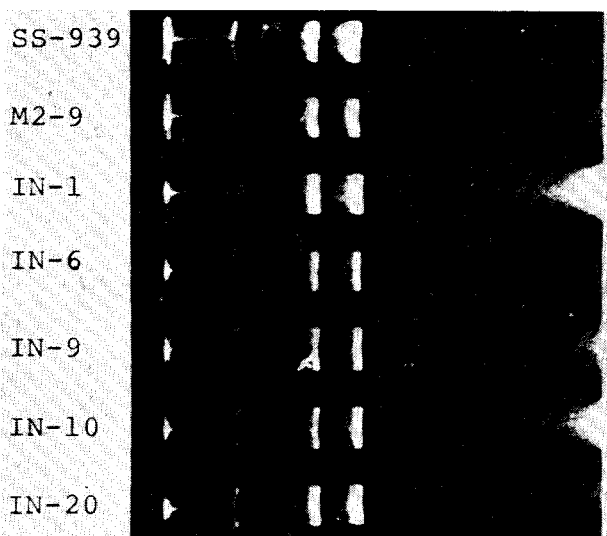
FIG. 8c. Gel electrophoretic analysis of the plasmid DNA
fraction from several non-producing isolates.

the supply of deoxystreptamine for the biosynthesis of istamycin. This plasmid
may, thus, also be related to the biosynthesis of other aminoglycoside antibiotics,
such as kanamycin, which involve the biosynthetic route of deoxystreptamine.

The derivation of kanamycin based on an understanding of the mechanism of
resistance was successful in overcoming resistance. Istamycin, which is a smaller
molecule and thus has fewer sites for potential inactivation, may be an even better
target for modification by biological or chemical means in order to arrive at an
antibiotic molecule useful against resistant organisms.

In conclusion, our results are examples illustrating research in the second era
which is characterized by inter-relating the concepts of antibiotic production and of
antibiotic resistance. We are committed to the continuation of our efforts to
uncover new antibiotic principles, particularly from marine microorganisms. We
are convinced that the marine environment is a rewarding field for the search for
new antibiotics and other biologically active substances.

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