CHAPTER II

LITERATURE REVIEW

1. Review of Antibiotics

The origin of the term "antibiotic" appears to have been in some dispute. Burkholder, in an excellent article on the mutual relationships of microorganisms, traces the origin of the term back to 1889 (Burkholder, 1952). However, disputing the point, Waksman lays claim to his own introduction of the word into the literature in the early 1940's (Waksman, 1953), Florey et al. , would seem to agree with Waksman, as they report that he (Waksman) proposed a definition in 1945 (Florey, 1949). Burkholder has rebutted this view, and quotes a passage in French written in 1928 that apppears to use the word " antibiotic " in discussing the phenomenon of antibiosis (Burkholder, 1953).

Further searching indicates Vullemain in 1889 (Florey, 1949) first applied the term antibiosis to an often-observed phenomenon of antagonism between living organisms. However, in so far as antibiotics are now related to microorganisms, it seems to be true that the word in its present restrictive meaning was used and interpreted originally by Waksman.

The most adequate definition for antibiotics as they discussed in this volume is "chemical compounds derived from microorganisms which have the capacity of inhibiting the growth of, and even destroying other microorganisms". This definition originated in the literature in 1942(Waksman and Woodroff, 1942). Some time later Benedict and Langlykke (Benedict and Langlykke, 1947) added the specification that these compounds be effective in low concentrations, and Waksman has since modified his original definition by adding the words "in dilute solutions" to the definition above (Waksman, 1953).

In 1877 Pasteur and Joubert (Pasteur and Joubert, 1877) noted that aerobic bacteria antagonized growth of *Bacillus anthracis*. Furthermore, they noticed that experimental anthrax in susceptible animals could be repressed by simultaneous inoculation of various non-pathogenic bacteria. Pasteur noted that this phenomenon of interaction between microbial species might ultimately find therapeutic uses.

Babes in 1885 was the first to interprete his own experiments on microbial antibiosis as being due to production of an inhibitory chemical substance by the antagonistic organisms (Babes, 1885).

Nine or ten years after Babes' work, Emmerich and Low prepared an extract of *Pseudomonas pyocyaneus* which they called pyocyanase. In very high dilution this compound was shown to be inhibitory to organisms of diphtheria, typhoid and plague, as well as to pathogenic cocci.

Some years later other bacteria were shown to have similar inhibitory effects on *Pseudomonas*. Nicolle gave one of the earliest reports of antibacterial action for *Bacillus subtilis*. Since then many reports have appeared concerning the antibiotic activity of spore-forming bacilli (Nicolle, 1907; Pringsheim, 1920; Chambers and Weidman, 1928; Weiland, 1936).

In a series of studies, Gratia and Dath extracted a lytic agent from a mold and used it in successful treatment of *staphylococcal* skin infections (Gratia and Dath , 1924; Gratia and Dath, 1926). The occurrence of antagonistic properties of molds thereby became an established fact, culminating in the discovery of penicillin by Fleming (Fleming, 1929).

Beginning in 1938 Florey and Chain, and Abraham and associates reevaluated the therapeutic possibilities of penicillin as suggested by Flemming (Chain et al., 1940; Abraham et al., 1941). These studies were carried out at the William Dunn School of Pathology, Oxford University.

In 1941, Florey and Heatley were invited to United States to hold conferences with personnel of the U. S. Department of Agriculture, of the Committee on Medical Research of the Office of Scientific Research and Development, and of the National Research Council. As a result of the conferences, private industry became interested in the commercial production of pennicillin.

During this time Dubos at the Rockefeller Institute for Medical Research, capitalized on the observation that certain pathogenic organisms disappeared from infected soils (Dubos, 1939). By careful and well-planned study, Dubos obtained the antibiotic tyrothricin from the soil bacillus, *Bacillus brevis*. The work of Dubos and associates on the study of the chemical, biological and physical properties of this antibiotic was a great contribution to what may be considered to be the beginning of the "Antibiotic Era" (Dubos and Cattanco, 1939; Dubos and Hotchkiss, 1941).

Immediately following the work of Dubos, a report emerged describing the first antibiotic to be obtained from a culture of an actinomycete. This antibiotic was called actinomycin, and was discovered by Waksman and Wooddruff (Waksman and Wooddruff, 1940). Waksman and his co-worker's contributions continued along these lines and eventually resulted in the first actinomycetes produced antibiotic which found clinical application, streptomycin (Schatz, Bugic, and Waksman, 1944).

The impact of the discovery of the streptomycin on the search for antibiotics from actinomycetes was great and soon more than search for antibiotics from actinomycetes was great and soon more than 15,000 actinomycete strains had been examined (Burkholder, 1946; Waksman *et al.*, 1946). As a result of these and more recent screening programs, a considerable number of antibiotics have been made available (Goldberg, 1959).

In 1945 bacitracin was discovered (Johnson, 1945), in 1947 polymixin (Benedic and Langhykke, 1947) was reported the same year that chloramphenicol was reported by Ehrlich et al (Ehrlich *et al.*, 1947), in 1948 chlortetracycline was found. (Dugger,1948), in

1949 Waksman contributed neomycin (Waksman and Lechevalier, 1949), and in 1950 the Pfizer group found oxytetracycline (Finlay *et al.*, 1950) in 1952 erythromycin (Mcguire, 1952) and carbomycin (Tanner, 1952) were reported.

1.1 Antibiotics from *Streptomyces*

Bacteria in the genus *Streptomyces* mostly are antibiotic producing organisms. Examples are shown in Table 1.

Table 1 Antibiotics from *Streptomyces* (Glasby, 1993)

Strain	Substance	Activity
S. albus	Abierixin	Gram positive bacteria
S. griscoviridus	Actinobolin	Broad spectrum antimicrobial
Streptomyces strain R2827	Anguinomycin B	Antitumor
Streptomyces strain DMS4769	Angucyclinon C	*Streptococcus aureus* & *Trichomonas vaginalis*
S. griscochromonogenes	Antibiotic A-221	Gram positive bacteria
S. panlus sp. NRRL 12251	Antibiotic 273	Gram positive bacteria
S. routienii sp. ATCC 39446	Antibiotic CP-61405	Gram positive bacteria
Streptomyces strain D788 OxA-4	Antibiotic DCP-2	Antitumor
S. olivaceogrisens	Antibiotic FK156	*Escherichia coli*
S. aureus	Azirinomycin	Gram-positive, Gram-negative
S. coralineus	Coralinomycin	Gram-positive, Gram-negative
S. lactamdurans	Efrotomycin	Gram-positive, Gram-negative
S. lavendulae	Ehrlichin	Influenza A,B
S. hepaticus	Elaiomycin	*Mycobacterium tuberculosis* subsp. *hominis*
S. cocoi	Fluoropolyoxin	*Escherichia coli*, *Streptococcus faecalis*

Table 1 (Continued)

Strain	Substance	Activity
S. althioticus subsp.*garlandosus* NSSI 3109	Garlandosus	Gram-positive, Gram-negative
S. carcinostaticus subsp. *neocarcinostaticus*	Neocarcinostatin	Antitumor
S. carcinostaticus subsp.FCI	Neocazirin C	Antitumor
S. lincolnensis	Rancinamycin A	Gram-positive
S. erythrochromogenes strain W-115-C	Sarkomycin A	Antitumor
S. schlerogranulatus	Schlerothericin	Gram-positive

1.2 Antifungal antibiotics from *Streptomyces*

Several strains are known to produce antifungal antibiotics, as shown in Table 2.

Table 2 Antifungal antibiotics from *Streptomyces* (Glasby, 1993)

Strain	Substance	Activity
S. abikoensis and *S. rubescens*	*Abikoviromycin*	Antifungal
S. badiocolor subsp. *abkhasus*	Abkhazomycin	Antifungal and yeast
S. enrythermus	Acrylamidine	Weakly active against Candida
Streptomyces species	Antibiotic 18-45	*Candida albicans*
S. lavendulae	Antibiotic 136	Antifungal
Streptomyces strain 1308 (NRRI 5318)	Antibiotic 1308	Antifungal
S. diastatochromogenes	Antibiotic A-661-I	Antifungal
Streptomyces strain FH317	Antibiotic H9	*Candida albicans*
S. misionensis	Antibiotic BH-890	*Candida albicans*
S. filipiensis	Filipin	Antifungal
S. levoris	Levorin	Pathogenic fungi

1.3 Antibiotics from *Stretomyces hygroscopicus*

Streptomyces hygroscopicus produces a number of antimicrobial agents, as summarized in Table 3.

Table 3 Antibiotics from *Streptomyces hygroscopicus* (Glasby, 1993)

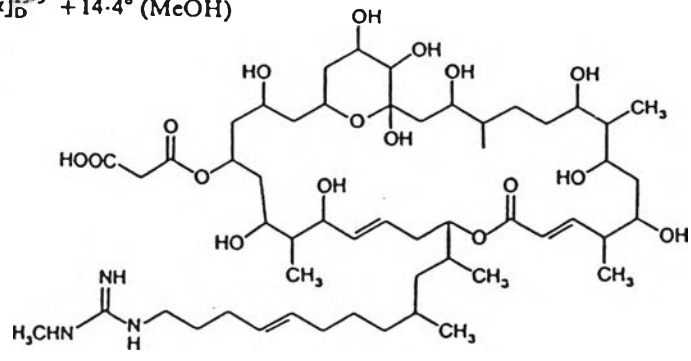
strain	substance	activity
S. hygroscopicus subsp. *aabomyceticus*	Aabomycin	*Piricularia oryzae* and *Trichophyton rubrum*
S. hygroscopicus	Angustmycin A, Angustmycin C	Gram-positive and gram-negative, antitumor
S. hygroscopicus	Azalomycin B, F	*Bacillus subtilis, Candida albicans* and gram-positive
S. hygroscopicus	Clavamycin E	Antifungal
S. hygroscopicus subsp. *crytallogenes*	Copiamycin	Fungicidal to fungi and yeasts
S. hygroscopicus	Flambamycin	Gram-positive
S. hygroscopicus sp. A-5294	Fluorovulgamycins	Gram-positive
S. hygroscopicus subsp. *crystallogenes*	Guanidolide A	Antitumor
S. hygroscopicus	Leuseramycin	Gram-positive, some phytopathogenic fungi and protozoa
S. hygroscopicus subsp. *crystallogenes* IFM 1136	Neocopiamycin B	Pathogenic fungi
S. hygroscopicus NRRL 5491	Rapamycin	Antifungal, *C. albicans*
S. hygroscopicus subsp. *nova*	Scopafungin	Gram-positive, yeast and pathogenic fungi
S. hygroscopicus	Trichostatin	Trichophytons and fungi
S. hygroscopicus	Validamycin A, B, C, D and E	Pesticide, *Pellicularia sasakii*

COPIAMYCIN

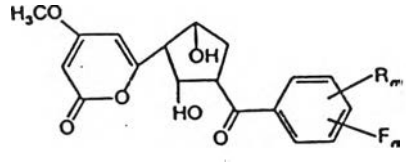
$C_{54}H_{95}O_{17}N_3$

M.p. 144°C (*dec.*)

$[\alpha]_D^{25.5}$ +14·4° (MeOH)

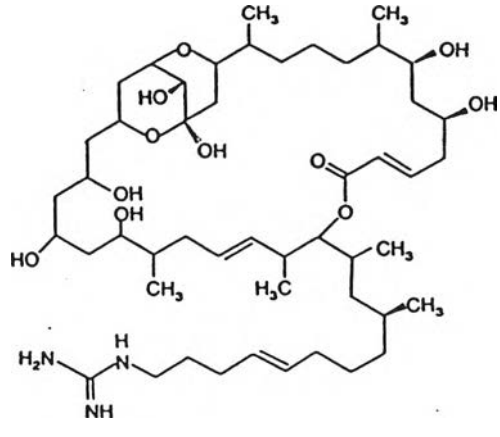


FLUOROVULGAMYCINS



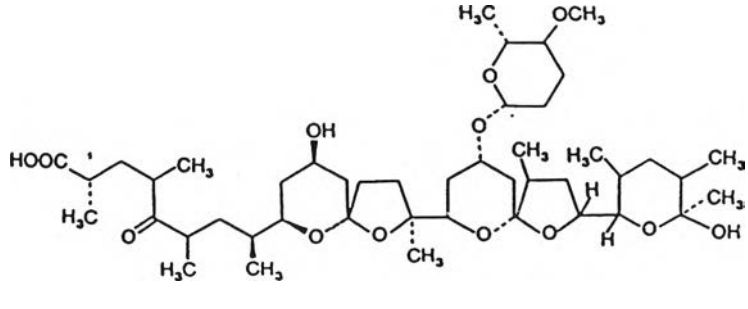
GUANIDOLIDE A

$C_{49}H_{87}O_{11}N_3$

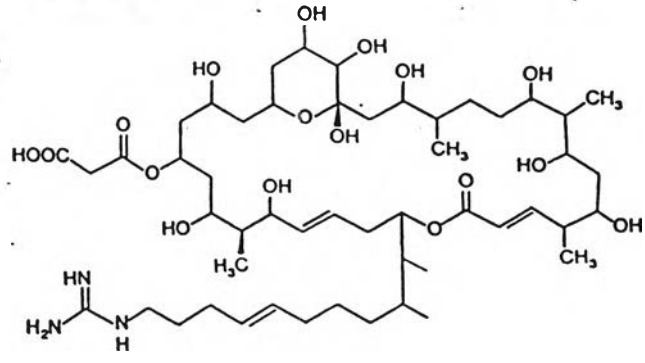


LEUSERAMYCIN

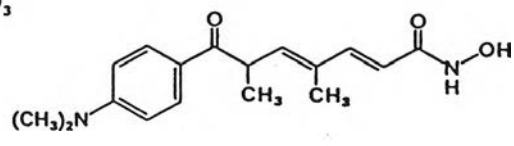
$C_{45}H_{74}O_{13}$



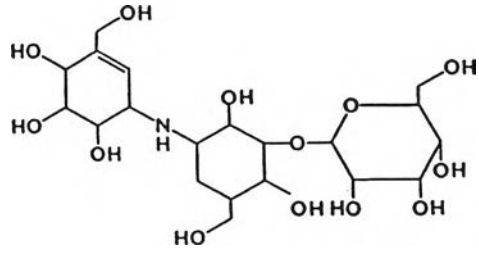
NEOCOPIAMYCIN A
$C_{53}H_{93}O_{17}N_3$



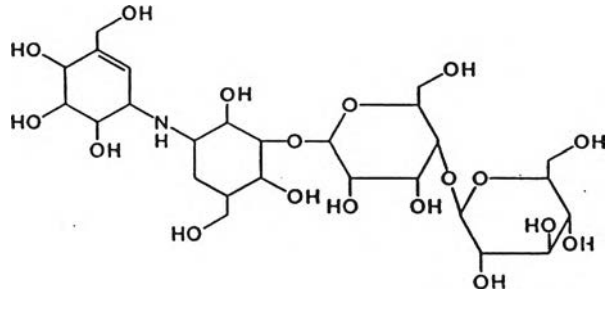
TRICHOSTATIN
$C_{17}H_{22}N_2O_3$



VALIDAMYCIN A
$C_{20}H_{35}O_{13}N$
M.p. *ca.* 135°C (*dec.*)



VALIDAMYCIN C
$C_{26}H_{45}O_{18}N$
M.p. Indefinite



2. Review of the genus *Streptomyces*

2.1 Classification of *Streptomyces*

According to Bergey' s manual of systematic bacteriology (1989) *Streptomycetaceae* is placed in the order *Actinomycetales* in the group of actinomycetes.

1) Group Nocardioforms

Genus *Caseobactor, Corynebacterium, Mycobacterium, Nocardia, Rhodococcus*

2) Group Nocardioides

Genus *Nocardioides*

3) Group Streptomycetes

Genus *Intrasporangium, Kineosporia, Sporichthya, Streptomyces, Streptoverticillium*

4) Group Thermomonosporas

Genus *Actinomadura, Actinosynnema, Microtetraspora, Nocardiopsis, Saccharothrix, Streptoalloteichus, Thermomonospora*

5) Group of other genera

Genus *Glycomyces, Kitasatosporia, Spirillospora, Thermoactinoyces*

2.2 Genus *Streptomyces*

For many years, the *Actinomycetales* were classified with the fungi, with which they share a paralelled evolution, but to which they are completely unrelated. The cells are unambiguously prokaryotic in that they have neither nuclear membrane

nor membrane bond organelles. They mainly free-living and many species are easily isolated from soil, in which they complete successfully with other microbial inhibitants (Levy, Campbell and Blackburn, 1973). Most are found in the soil, and a few cause rare types of human infections or plant infections (Gebhardt and Anderson, 1959).

Actinomycosis is an infection disease of lower animals (especially cattle) and man caused by several species of bacteria belonging to the genus *Actinomyces*. Of these, *Actinomyces bovis* (bovine actinomycosis) and Actinomyces isaelli) human actinomycosis) are the most important (Smith, 1977).

Highly significant contribution in connection with the taxonomy of actinomycetes, especially of *Streptomyces*, dates back in 1914 when Krainsky emphasized the importance of recognizing the growth characteristics of actinomycetes on synthetic media. Since then, both bacteriological and mycological media and techniques have been employed in the studies of actinomycetes because of borderline properties of these organisms (Arai, *et al.*, 1976).

There is a fundamental difference. Actinomycetes are typical prokaryotes while fungi are from a separate kingdom within the eukaryotes (Buchanan and Gibbons, 1974). In other respects, actinomycetes are much like bacteria in general, and their colonies grow at much the same rate, more slowly than those of most fungi.

Streptomyces are the streptomycin producer. Many of new and valuable antibiotics are derived from members of this genus.

Streptomyces belongs to the family *Streptomycetaceae* (plaint-fungus family). They form mycelial threads which do not break up, consisting of about 157 species distributed in 3 genera.

Streptomyces derived by spore color as white, gray, yellow, red, blue, green and violet series (Goldberg, 1959).

Streptomyces hygroscopicus is placed in Gray series (Shirling and Gottlieb, 1972) and in 1931 Jensen described *Actinomyces hygroscopicus*, n.sp. with aerial mycelium which becomes moist with dark shiny patches, and which when touched with a needle is found to be a moist, smeary spore mass (Jensen, 1931). The type culture is no longer extent according to Shirling and Gottlieb. In 1948 *Actinomyces hygroscopicus* was redefined as *Streptomyces hygroscopicus* (Shirling and Gottlieb, 1972). The "*hygroscopicus*" like cultures have been the subject of papers by Dietz and Mathews (Dietz and Mathews, 1962, 1968, 1969, 1970; Rudaya and Solov'eva, 1970; Tresner and Backus, 1956; Tresner, Davies, and Backus 1961; and Tresner, Backus, and Hayes, 1976).

Streptomyces hygroscopicus and *Streptomyces platensis* (a culture which has the characteristic "moist black patches") were lumped by Tresner and Backus in 1956 on the basis of cultural and light microscope studies. In 1967 Tresner, Backus, and Hayes , using newer techniques (especially electron microscopy), stated that the species were indeed distinguishable and therefore validly speciated.

It was proposed that "*hygroscopic*" strains with rugose spore surface be designated *Streptomyces hygroscopicus* and that those with elliptical spores (hat-shaped or crescent-shaped) be designated *Streptomyces neohygroscopicus* (Arai, 1976).

Characteristics of *Streptomyces hygroscopicus* (Jensen, 1931)

Spore-chain morphology: section Spirales: light spirals in dense cluster. Spirals may coalesce as dark, moist masses of spores. Mature spore chains generally contain 10 to 50 spores per chain. This morphology is seen on yeast-malt agar, oatmeal agar, salts-starch agar, and glycerol-asparagine agar. Spore surface: Warty, individual spores are poorly delineated.

Special morphology characteristics: Moist, black, liquefied (hygroscopic) areas are found in the aerial mycelium in 14 to 21 days. These are especially common on oatmeal agar and salts-starch agar.

Color of colony: Aerial mass color in the Gray color series (brownish gray or light grayish reddish brown) on yeast-malt agar, oatmeal agar, salts-starch agar, and glycerol-asparagine agar. Dark or medium gray areas are also reported on oatmeal agar and salts-starch agar, and moist black (hygroscopic) areas may be seen on older cultures.

Reverse side of colony: No distinctive pigments (colorless to grayish yellow, pale yellow, or light olive-brown or gray) on yeast-malt agar, oatmeal agar, salts-starch agar, and glycerol-asparagine agar, except that dark discoloration may be seen beneath hygroscopic areas.

Color in medium : Melanoid pigments are not formed in peptone-yeast-iron agar, tyrosine agar, or tryptone-yeast broth. No pigment or only a trace of yellow is found in the medium in yeast-malt agar, oatmeal agar, salts-starch agar or glycerol-asparagine agar.

Carbon utilization: D-glucose, D-mannitol, D-glucose and rhamnose are utilized for growth. Good growth is reported on the carbon-free control medium as well as on L-arabinose, D-xylose, and i-inositol, so that utilization of these carbon sources is doubtful. Sucrose and raffinose are probably not utilized (Tresner, Backus and Hayes, 1967).

2.3 Distribution of *Streptomyces*

Streptomycetes are widely distributed in terrestrial and aquatic habitats. Most are strict saprophytes, but some form parasitic associations with plants or animals. Surprisingly little is known about the role of streptomycetes in natural environments, although evidence of their occurrence and numbers in habitats is extensive. Several recent reviews on streptomycete ecology are available (Cross, 1981; Kutzner, 1981; Goodfellow and Williams, 1983; Williams *et al.*, 1984; Goodfellow and Simpson, 1987).

Soil, fodder, and composts appear to be the primary reservoirs for streptomycetes. Specific growth rates and doubling times for streptomycetes in laboratory culture are approximately intermediate between those of bacteria and fungi (Flowers and Williams, 1977a).

The survival capacity of streptomycete spores appears to be greater than that of the hyphae (Williams *et al.*, 1972). The spores usually have thicker walls than the hyphae (Sharples and Williams, 1976) and are more hydrophobic (Ruddick and Williams, 1972) owing to the outer sheath that envelopes the spore wall (Williams *et al.*, 1973). Streptomycete spores have a net negative surface charge except at low pH levels (Douglas *et al*, 1970) and a relatively low endogenous metabolism (Ensign, 1978), and are generally more resistant to heat than the corresponding hyphae (Goodfellow and Simpson, 1987). Spores are released above soil when particles are disturbed by wind or rain (Lloyd, 1969), whereas dispersal within soil is assisted by movement of water and arthropods (Ruddick and Williams, 1972). Although streptomycetes are usually considered to be strict aerobes, they can grow in sterile soil at low oxygen concentrations, but not when carbon dioxide concentrations exceed 10%. In dry soil, streptomycete counts decrease markedly at moisture tensions above pH 4.0, but their proportion to other bacteria may be higher because their spores are more resistant to desiccation than are the vegetative cells of bacteria. Optimum counts from neutral soil and optimum radial growth of streptomycetes inoculated into sterile

occur at moisture tensions between pH 1.5 and 2.5. Some streptomycetes from acid soil are able to grow on media at high osmotic potentials (Wong and Griffin, 1974). Halophilic and salt-tolerant streptomycetes have also been reported (Hunter *et al.*, 1981).

It seems unlikely that streptomycetes grow optimally in temperate soils because most strains are mesophilic under laboratory conditions. A variety of mesophilic streptomycetes are involved in the initial stages of decomposition in composts, and similar substrates, but obligate or facultatively thermophilic strains become active at temperatures above 400 °C (Lacey, 1981).

Because many soils are acidic, pH is clearly an important factor determining the distribution and activity of streptomycetes. Acidophilic and neutrotolerant streptomycetes, the latter growing between pH 3.5 and 7.5 but optimally around pH 5.5, are common in acid soils (Khan and Williams, 1975). The presence of low numbers of neutrophilic streptomycetes in acidic soils can be attributed to their ability to grow in less acidic microsites (Williams and Mayfield, 1971) and to the resistance of their spores to acidity (Flowers and Williams, 1977b).

Such colloid material can markedly affect microbial activity at the microenvironmental level. Streptomycete spores are readily absorbed to kaolin but not to montmorillonite, except at low pH (Ruddick and Williams, 1972). Addition of calcium montmorillonite or calcium humate to cultures of streptomycetes can accelerate their growth and respiration (Mara and Oragui, 1981). Further, sites of adsorption associated with humic material can lead to microsites of increased pH in acidic soils (Williams and Mayfield, 1971).

Streptomycetes have the potential to degrade other naturally occurring polymers such as chitin, hemicelluloses, keratin, pectin, and fungal cell wall material, and have been implicated in the degradation of herbicides (Percich and Lockwood, 1978), plastics (Sharpell, 1980), polyphenolic tannins (Lewis and Starkey, 1969), and

humic acids (Szegi and Gulyas, 1968). In addition, streptomycetes form melanin pigments similar to humic acids (Huntjens, 1972), which may contribute to the formation of humus (Kutzner, 1968), and are inhibited by some fungicides (Ou et al., 1978).

2.4 Criteria used for the classification and identification of *Streptomyces species* (Holt, 1989)

2.4.1 Spore Chain Morphology

This is determined by light and scanning electron microscopy of 14-days-old cultures on inorganic salts-starch agar (Shirling and Gottlieb, 1966). However, examination by light microscopy is usually sufficient to determine this character, and other media, such as oatmeal agar and glucose-yeast extract-malt extract agar (Shirling and Gottlieb, 1966), may be used. The three categories of Pridham *et al.* (1958) are employed with the modified terminology of Shirling and Gottlieb (1968).

2.4.2 Spore surface ornamentation

This is determined by scanning electron microscopy of the cultures used for the examination of spore chain morphology. Silhouettes of spores examined by transmission electron microscopy (Tresner, 1961) provide an alternative, but distinction between smooth, warty rugose surfaces is more difficult. Ornaments are assigned to the categories of Tresner *et al.* (1961) or to the rugose type recognized by Dietz Mathews (1971).

2.4.3 Other morphological features

Cultures are also examined by light and scanning electron microscopy for the fragmentation of the substrate mycelium or its production of spores. Formation of sclerotia on the colony surface was also noted.

2.4.4 Color of the spore mass

This is determined on the cultures used for the morphological examinations. Spore masses are matched against the seven color wheels of Tresner and Backus (1963) as used in the ISP (Shirling and Gottieb, 1966). Although it is sometimes possible accurately to allocate color categories without using a reference system, spore pigments are very impure (Lyons and Pridham, 1965) and hence the replication between determinations by different workers is not always satisfactory (Pridham, 1965). Variations in lighting conditions obviously contribute to this problem and therefore all color determinations by Williams *et al.* (1983), those on the substrate mycelium and diffusible pigments, are under standard lighting of illuminance C, which approximates average daylight (Dietz and Mathews, 1962).

2.4.5 Pigmentation of Substrate Mycelium (Colony Reverse)

This is determined on the cultures used for characters 2.4.1 to 2.4.4. Colors are allocated to one of five categories: yellow-brown, which included strains lacking distinctive pigmentation; red-orange; green; blue; or violet.

2.4.6 Diffusible Pigments

These are detected on glycerol / asparagine agar (Shirling and Gottlieb, 1966), which encourages pigment production, after 14 days' incubation. Colors are allocated to the five categories used for the substrate mycelium pigmentation.

The pH sensitivity of both the substrate mycelium and diffusible pigments is assessed by noting any color changes induced by the addition of acid or alkali (Shirling and Gottlieb, 1966).

2.4.7 Melanin Pigment Production

This is determined after 4 days incubation on peptone-yeast extract-iron agar and tyrosine agar (Shirling and Gottlieb, 1966). A few strains give a positive reaction on only one of these media.

2.4.8 Antimicrobial Activity

The inhibition of the growth of eight test organisms (as shown in Table 4) is detected by using on overlay technique. Spot-inoculated, 5-days-old colonies of streptomycetes on nutrient agar plates were killed by inverting the plates over 1.5 ml Chloroform for 40 minutes. After removal of excess chloroform vapor, the plates were overlaid with 5 ml sloppy agar (0.7% w/v nutrient agar) inoculated with a test organism. Zones of inhibition around the colonies are recorded after 24 hours at 30° C.

2.4.9 Enzyme Activity

Lecithinase, proteolytic and lipolytic activities are determined on egg yolk medium (Nitsch and Kutzner, 1969). The slightly modified medium contained (% w/v): bacteriological peptone (Oxoid; 1.0), glucose (0.1), NaCl (1.0), yeast extract (Oxoid; 0.5), agar (1.2), and egg yolk emulsion (Oxoid; 5.0 v/v). Proteolysis was determined after 2 days, and lecithinase and lipolysis at 2, 4 and 6 days.

2.4.10 Degradation Activity

The degradation of adenine, tyrosine (0.5% w/v), hypoxanthine, xanthine, xylan (0.4% w/v), elastin (0.3% w/v), casein (1% w/v skimmed milk), guanine (0.05% w/v), and testosterone (0.1% w/v) was detected in modified Bennett agar (Jones, 1949), with glucose replaced by glycerol after 7, 14 and 21 days.

2.4.11 Resistance to Antibiotics

Strains are tested for their ability to grow in the presence of 11 antibiotics at concentrations selected to be of diagnostic value. Filter paper disks, previously soaked in antibiotic solution and then lyophilized, were placed on the surface of modified Bennett agar plates innoculated with 0.1 ml of streptomycete spore suspension. Readings are taken at 1, 2, 3 and 7 days, the first readable result being recorded; resistance is scored as positive.

2.4.12 Growth Temperatures and pH

These are tested on modified Bennett agar. Growth at 37 °C, and 45 °C is assessed after 7 and 14 days, that at 4 °C and 10 °C after 2 and 4 weeks. Growth at pH 4.3 was determined after 7 and 14 days.

2.4.13 Growth in the presence of Chemical Inhibitors

A range of potential inhibitors at diagnostic concentrations was added to modified Bennett agar. Presence or absence of growth was noted after 7 and 14 days. Other media that support good growth of streptomycetes under optimal conditions may be employed for these and the preceding growth tests.

2.4.14 Use of Nitrogen Sources

The ability of strains to use 11 compounds is tested, each being incorporated into a basal medium containing (% w/v): D-glucose (1.0), $MgSO_4.7H_2O$ (0.05), NaCl (0.05), $FeSO_4.7H_2O$ (0.001), K_2HPO_4 (0.1) and agar (1.2). Results are determined after 15 days by comparing the growth on each source with that on the unsupplemented basal medium and on a positive control containing either L-asparagine or L-proline.

2.4.15 Use of Carbon Sources

The ability of strains to use 25 compounds (Table 4) was tested, each being incorporated into carbon utilization agar (Shirling and Gottlieb, 1966). Results are determined after 7, 14, and 21 days by comparing growth with that on unsupplemented basal medium and on a positive control containing D-glucose.

All of these 15 criteria are summarized in Table 4.

Table 4 Criteria used for the classification and identification of *Streptomyces* species

Characters	Character states
1. Spore chain morphology	*Rectiflexibiles, Retinaculiaperti,* or *Spirales.*
2. Spore surface ornamentation	Smooth, warty, spiny, hairy, or rugose.
3. Other morphological features	Fragmentation of substrate mycelium, sclerotia formation, sporulation on substrate mycelium.
4. Color of spore mass	Blue, gray, green, red, violet, white, or yellow.
5. Pigmentation of substrate mycelium (colony reverse)	Yellow-brown (no distinctive pigment), blue, green, red-orange, or violet. pH sensitivity of pigments.
6. Diffusible pigments	Yellow-brown, blue green, red-orange, or violet. pH sensitivity of pigments.
7. Melanin pigment production	On peptone-yeast extract-iron agar and tyrosine agar.
8. Antimicrobial activity	Activity against *Aspergillus niger, Bacillus subtilis, Candida albicans, Escherichia coli, Micrococcus luteus, Pseudomonas fluorescens, Saccharomyces cerevisiae* and *Streptomyces murinus.*
9. Enzyme activity	Lecithinase, lipolysis and proteolysis (on egg-yolk medium). Hydrolysis of chitin, hippurate, and pectin. Nitrate reduction. Hydrogen sulfide production. β-Lactamase and β-lactamase inhibitor production.
10. Degradation activity	Adenine, allantoin, arbutin, casein, DNA, elastin, esculin, gelatin, guanine, hypoxanthine, RNA, starch, testosterone, Tween 80, L-thyrosine, urea, xanthine and xylan.
11. Resistance to antibiotics (μg/ml)	Cephaloridine (100), dimethylchlortetracycline (500) gentamicin (100), lincomycin (100), neomycin (50), oleandomycin (100), penicillin G (10 i.u.), rifampicin (50), streptomycin (100), tobramycin (50), and vancomycin (50).
12. Growth temperatures and pH	4°,10°,37° and 45° C. pH 4.3.
13. Growth in the presence of inhibitory compounds (% w/v)	Crystal violet (0.0001), phenol (0.1), phenylethanol (0.1,0.3), potasium tellurite (0.001,0.01) , sodium azide (0.01-0.02), sodium chloride (4,7,10,13) and thallous acetate (0.001,0.01).

Table 4 (Continued)

Characters	Character states
14. Use of nitrogen sources (0.1% w/v)	DL-α-amino-*n*-butyric acid, L-arginine, L-cysteine, L-histidine, L-hydroxyproline, L-methionine, potassium nitrate, L-phenylalanine, L-serine, L-threonine, and L-valine.
15. Use of carbon sources (0.1% w/v)	Adonitol, L-arabinose, cellobiose, dextran, D-fructose, D-galactose, *meso*-inositol, inulin, D-lactose, manitol, D-mannose, D-melezitose, D-melibiose, raffinose, L-rhamnose, salicin, sucrose, trehalose, xylitol, and D-xylose, sodium acetate, sodium malonate, sodium propionate and sodium pyruvate.

3. Fermentation

Actinomycetes are usually cultivated in various natural liquid media with shaking at 180-250 rpm at 27-28 °C for the production of antibiotics (Table 5). Most media consist of carbon (e.g. glycerol, glucose, starch, 1-3%) and nitrogen (e.g. ammonium sulphate, meat extract, peptone, soybean meal, 0.5-2%) sources in combination with inorganic phosphate (5-15 mM), cations (10-50 mM) such as Ca, Mg and Na, or trace elements like Co, Cu, Fe, Mn and Zn. Since regulatory mechanisms for antibiotic production are so varied, no single medium can be chosen for the production of all types of antibiotics. It is, therefore, practical to cultivate actinomycetes strains in media with varied comparisons in order to screen for diverse antibiotics. However, certain types of media favour the production of certain groups of antibiotics. For example those containing starch and soybean meal as carbon and nitrogen source, respectively, are empirically known to favour the production of aminoglycoside antibiotics (Goodfellow, 1988).

Table 5 Media and condition for antibiotic production of *Streptomyces hygroscopicus*

Nutrient		Condition					Reference
Media	Substance	pH	Temperature (°C)	Agitation (rpm)	Time (hours)	Aeration (l/min)	
seed							
glucose, polypeptonene, beef extract, yeast extract, malt extract, fish meal, peptone, glycerol, corn starch, cotton seed meal, dried yeast, corn steep liquor, dextrin, soybean meal, meat extract, pharmamedia,	NaCl, $CaCO_3$ $MgSO_4$. 7H2O	6.5, 7.0, 7.2	27, 28, 30	200, 250	26, 72, 96	-	Takesako and Beppu, 1984 Tsunakawa *et al.*, 1985 King *et al.*, 1986 Hatanaka *et al.*,1989)
production							
soluble starch, glucose, yeast extract, malt extract, fish meal, peptone, dried yeast, gluten meal wheat germ, cotton seed meal, dextrin, D-mannose, corn steep liquor, soybean meal, pharmamedia,	NaCl $CaCO_3$ $MgSO_4$. $7H_2O$ KH_2PO_4, $FeSO_4$ Z amine type A N-acetylglucosamine Adekanol	6.8, 7.0, 7.2, 8.0-8.4	24, 27, 28, 30	48, 48-72, 90, 96-154	48, 48-72, 90, 96-154	30, 40, 96, 120	

4. Thin layer chromatography

The approach to classification and identification of antibiotics by chromatographic tecnique can be used in earlier years that a few number of antibiotics were discovered. With the thousands of antibiotics currently known, systematic chromatographic classification of this large number of compounds is extremely difficult.

Probably the greatest single influence in the systematic analysis of antibiotics was that of Betina who, in 1964, attempted to establish a systematic chromatographic separation of known antibiotics (Betina, 1964). These were distributed into five classes according to their R_f values in four principle solvent systems. Thin layer chromatography was utilized by Ikekawa *et al.* (1963) for resolving approximately 50 antibiotics utilizing seven different solvent systems. Of particular interest are several solvent systems for separation of certain groups of antibiotics. They separated the antbiotics into five groups. The groups were as follow: (1) macrolide antibiotics, (2) water-soluble basic antibiotics, (3) peptide antibiotics, (4) polyene antibiotics, (5) nucleoside antibiotics.

5. Bioautographic detection of antibiotics in preparation chromatogram

This method is used for the detection of antimicrobial agents on chromatograms. It may detect by use of suitable reagent, ultraviolet light and bioautographic detection of biologically active components (Wagman *et al*, 1969).