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Chemical Composition of Variants of Aerobic Actinomycetes

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It has been shown previously that aerobic actinomycetes can be separated into four main groups on the basis of their cell wall composition. Six representatives of aerobic actinomycetes (*Nocardia asteroides* and *Micropolyspora brevicatena*, cell wall type IV; *N. madurae*, *Microbispora rosea*, cell wall type III; *Actinoplanes* sp., cell wall type II; *Streptomyces griseus*, cell wall type I) were subjected to selecting agents which permitted the isolation of stable variants morphologically different from the parent strain. Whole cell analyses of 134 substrains from the six parents revealed no significant change in the isomeric form of diaminopimelic acid or in sugar constituents. Analyses of cell wall preparations from 52 of these did not reveal any change in the diagnostic constituents of their murein or polysaccharides.

Cummins (4) noted that anaerobic actinomycetes could be separated into two groups on the basis of cell wall composition. Yamaguchi (14), Becker et al. (2), and Lechevalier et al. (9) have reported that aerobic actinomycetes could be separated into four groups on the basis of the chemical composition of their cell wall preparations. This information is summarized in Table 1. Lechevalier and Lechevalier (7) subsequently proposed a system of classification of aerobic actinomycetes based on their morphology and chemical composition, and recently reviewed the state of the systematics of this group of microorganisms (8).

The aim of the present study was to test the value of cell wall composition as a taxonomic characteristic by submitting strains of actinomycetes of various cell wall types to the action of agents that would permit the isolation of stable variants, differing morphologically from the parents. These were chemically analyzed in an attempt to detect variation from parental types. If one considered only the major constituents which had previously been assigned diagnostic value, then no such variation was found.

MATERIALS AND METHODS

Organisms. Six parent strains were selected as sources of variants: *Nocardia asteroides* Z-16 (cell wall type IV); *N. madurae* 1136 (cell wall type III); *Micropolyspora brevicatena* 1086 (cell wall type IV); *Actinoplanes* sp. Z-20 (cell wall type II); *Microbispora rosea* L-3 (cell wall type III); and *Streptomyces griseus* IMRU 3475 (cell wall type I).

Maintenance of stock cultures. Parent strains and their variants were maintained on agar slants as follows: *N. asteroides* and its variants on yeast extract-dextrose (YD; 13); *N. madurae* and *S. griseus* and their variants on potato-carrot (PC; 3); *M. brevicatena* and some of its variants, on NZ-amine-glycerol (NZG; 10); *Actinoplanes* sp. and its variants on YD; and *M. rosea* and its variants on Pablum (Pab; 6). *M. brevicatena* and *M. rosea* were grown at 37 C; the other strains were grown at 28 C. Morphological features were observed on the following media: *N. asteroides* and *S. griseus* on PC; *N. madurae* on potato-carrot-yeast extract (PCY; PC plus 0.3% Difco yeast extract); *M. brevicatena* on PGB [Wilson peptone 851C (Wilson Co., Chicago, Ill.), 0.5%; glycerol, 70.0 ml; Amber BYF 50X (Amber Labs., Milwaukee, Wis.), 0.5%; agar, 1.5%; tap water, 1 liter, *p*H 7.5 before autoclaving]; *Actinoplanes* sp. on tap water agar (WA; 5); and *M. rosea* on Pab.

Selecting agents. Various agents were used to select morphological variants, but all were not applied to each parent strain. The agents were: irradiation with ultraviolet light (UV); exposure to various concentrations on ethyl methanesulfonate (EMS), penicillin G, bacitracin, and monosodium novobiocin; exposure to high and low *p*H values; and exposure to high temperature.

Suspensions were made by scraping growth of the parent organisms on slants of maintenance media into water. The water suspensions were shaken with glass beads and filtered through sterile Whatman no. 2 filter paper in an effort to obtain living fragments of uniform size. Thin layers of these suspensions were exposed to UV while stirring. The source of radiation was a General Electric mercury vapor lamp, FG 8144-61.

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TABLE 1. *Major constituents present in cell wall preparations of representative actinomycetes (2, 4, 14)*[a]

Cell wall type	Lysine	Ornithine	Aspartic acid	Glycine	L-DAP[b]	*meso*-DAP	Arabinose	Galactose	Oxygen requirement
I *Streptomyces*				+	+				Mainly, aerobic
II *Actinoplanes*				+		+			
III *Microbispora*						+			
Nocardia madurae									
IV *Micropolyspora*									
Nocardia asteroides						+	+	+	
Mycobacterium									
V *Actinomyces israelii*	+	+							Anaerobic, microaerophilic to facultative aerobic
VI *Actinomyces bovis*	+		+						

[a] All preparations contained major amounts of glucosamine, muramic acid, alanine, and glutamic acid.

[b] DAP = 2,6-diaminopimelic acid.

EMS was emulsified in the aqueous cellular suspensions at 28 C, and was then plated out on maintenance media (12).

Aqueous solutions of the antibiotics were incorporated into plates of maintenance media at different concentrations. These plates were seeded with microbial suspensions prepared as for UV irradiation.

Exposure to high and low *p*H values was carried out in YD broth which had been adjusted to the desired values with sodium hydroxide or sulfuric acid. The liquid cultures were incubated on rotary shakers.

Exposure to high temperature was carried out by incubating YD broth cultures at 42 to 47 C, or PC agar cultures at 42 C.

Selection of variants. After having exposed a population of a parent to a given selecting agent, colonies of the survivors were examined microscopically on the plates. Those differing morphologically from the parents were transferred to duplicate maintenance agar slants. After incubation and growth, one of the slants was used to inoculate agar plates on which, after proper incubation, isolated colonies were examined microscopically. If the variant morphology held true and did not revert to parental type, the second slant was used to inoculate liquid media. In some cases, survivors of the action of a selecting agent were submitted to chemical analysis even though morphologically similar to the parent.

In the case of survivors growing in presence of high and low *p*H values or at maximal temperatures, all the survivors were used as inocula for liquid cultures without any attempt to select morphological variants.

Preparation of organisms for chemical analysis. Aqueous scrapings from agar slants were transferred to YD broth. Two flasks (50 ml in 250-ml Erlenmeyer) per slant were inoculated and incubated on a rotary shaker at the proper temperature. After maximal growth was obtained, the cultures were checked microscopically for contamination and were transferred (5 to 10% inoculum) to six to ten flasks identical to those used for the primary inoculum. NZG broth was used for *M. brevicatena*. After proper incubation to obtain maximal growth, a sample of the organism was streaked on plates of the proper medium before the cellular mass was harvested for hydrolysis and chemical analysis. These plates were examined microscopically after incubation, as a last check of the morphological stability of the variant.

Determination of amino acids and sugars. Cell wall preparations were made by using a procedure previously described (2), except that extraction with 0.5% ethanolic potassium hydroxide was employed as a final step rather than at the beginning of the procedure.

Whole cell analyses for diaminopimelic acid (DAP) were carried out according to Becker et al. (1), except that development of the chromatograms was at 4 C for 24 to 48 hr. Whole cell analyses for sugars were as follows: 50 mg of air-dried, washed, whole cells grown in shaken culture were hydrolyzed in 1 N H_2SO_4 over a steam bath for 2 hr in open tubes (13 × 100 mm). The tubes were indented at their midpoint; the indentation served as a condenser during hydrolysis. The hydrolysates were neutralized to *p*H 5.0 to 5.5 with saturated BaOH and the clear supernatant fluid which remained after centrifugation was dried in a 42 C oven, with a $CHCl_3$ layer to prevent microbial contamination. Drying could also be carried out in vacuo. The dry residues were dissolved in 0.3 ml of distilled water and 20 μliters was spotted on Whatman no. 1 paper in concentric circles of decreasing size to prevent buildup of the sticky residue in one area. This was necessary in many cases to ensure the proper development of the chromatograms. Development was carried out at 25 to 28 C for 48 hr in a butanol-pyridine-water-toluene (5:3:3:4) system, which effects the separation of galactose, glucose, mannose, arabinose, xylose, ribose, and rhamnose. Spots were visualized as in the analysis of cell wall preparations.

Unless otherwise specified, "chemical analysis" refers to analyses of whole cell hydrolysates.

Results and Discussion

N. asteroides. The parent strain of *N. asteroides* used in these studies formed pink colonies with a hairy surface. Aerial hyphae were short with side branches that occasionally terminated in a chain of poorly formed spores. Substrate mycelium was formed of long hyphae on which chains of well-formed spores were always borne, both laterally and terminally.

Exposure to UV was from 30 sec to 17.5 min, the second figure corresponding to 3,530 ergs per mm^2 per sec. Under the latter conditions, 0.17% of the cells survived. Eighteen stable variants were selected representing three main morphological groups: (I) white colonies with a smooth surface, having nonsporulating, usually long, aerial hyphae without side branches and a sterile substrate mycelium; (II) colonies having a rough surface with humps, short aerial mycelium with spores, and bright orange sterile substrate mycelium formed of short hyphae; and (III) same as II, but sterile substrate mycelium formed of long hyphae.

An examination of the survivors from various doses of UV light showed that the occurrence of the different types of morphological variants was independent of the dose. Analyses carried out on whole-cell hydrolysates revealed that 15 of the 18 variants did not exhibit any differences in major chemical constituents from the parental type, but three had less arabinose. Cell wall preparations from these three strains were made and analyzed. Again, no difference was found except for some reduction in the relative amount of arabinose which was, however, still present as a major constituent.

One of the variants of type I (white, long, aerial mycelium, smooth surface, Z-16-14) was reirradiated and nine strains representing stable subtypes were isolated. Subtype I was like the parent, but the aerial mycelium was reduced to mere stumps.

Subtype II was like the parent, but the substrate hyphae were extremely fine and granular and the aerial mycelium formed many hyphae with terminal brushlike structures. Whole cell hydrolysates of these nine strains were prepared and analyzed. No change was observed.

Shaking cellular suspensions of *N. asteroides* for 1 hr in presence of 0.05 ml of EMS per milliliter before plating out, yielded, after incubation, colonies very different morphologically from the parent. These were all unstable and readily reverted to parent morphology. Similar results were obtained with penicillin at the concentration of 14 units/ml (4% survival).

Novobiocin (130 μg/ml) allowed the survival of 1.5% of the exposed population. The survivors were all of parental morphology.

Bacitracin, at 18 units/ml, permitted a survival of 0.8% of the exposed population of *N. asteroides*, whereas 0.4% of the population survived 20 units/ml. No survivors were observed at 25 units/ml. Twelve variants were isolated which were morphologically different from the parent. Five of these were very similar to strain Z-16-14, previously mentioned. The other seven strains were slightly pink with short aerial mycelium bearing two to three spores, and long sterile substrate hyphae. Whole cell hydrolysates of ten of these variants were chemically analyzed. Five showed a slight increase in galactose. No other variation was observed.

Only 5% of a population of *N. asteroides* grew at 42 C on solid maintenance medium. Colonial morphology of these survivors was very similar to that of the parent strain. Colonies so selected did not grow in liquid shake culture at 42 C.

Cellular material, harvested after growth on a medium with an initial *p*H of 5.0, was analyzed chemically and found to contain slightly less mannose than the parent. A cell wall preparation showed a diminution of the quantity of DAP in relation to the other amino acids. Cells that grew at an initial *p*H of 8.0 had the same chemical composition as the parent.

N. madurae. *N. madurae* formed white aerial mycelium with short chains of spores which were sometimes curved or even tightly coiled. The substrate mycelium was formed of long, very ramified, thin, sterile hyphae. Soluble pigment was not formed.

Exposure to UV light for 12 min (2,490 ergs per mm^2 per sec) permitted the survival of 0.12% of the population. Twenty-five stable variants were selected. They represented two main morphological types. The first was identical with the parent, but sterile. These strains formed side branches, often curved or coiled, but did not form any spores. In the second type, a few long, sterile, aerial hyphae were noted occasionally, but the aerial mycelium was usually rudimentary or absent. The substrate mycelium was much like that of the parent, but not as well developed. Coremia-like structures were formed by strains of these two types more often than by the parent. Examination of plate cultures of cellular suspensions that had been exposed to various amounts of UV revealed that the longer the exposure to the radiation, the less aerial mycelium was formed by the survivors. Eighteen of the 25 variants were analyzed chemically. Five of the variants showed a slight reduction in galactose content as compared to the parent. Two of these five had traces of 2,6-diamino-

3-hydroxypimelic acid (11) which might have been present in the parent in even smaller amounts. No other changes were found.

N. madurae was exposed to EMS by shaking suspensions in the presence of EMS or by incorporation of this chemical into the agar. No satisfactory inhibition of the growth was obtained, and no morphological differences were found except that a few sterile strains were obtained. A cell wall preparation from one of these was of typical type III.

In the presence of 600 units of penicillin per milliliter, 0.88% of the cells of *N. madurae* survived, but no growth was observed at 1,000 units/ml. Morphological variants were of two types: one, very similar to the first type obtained by UV exposure; and the other, colonies having abundant aerial mycelium formed of straight hyphae having no side branches or spores which occasionally formed aerial coremia-like structures. Most of these were not morphologically stable, especially those of the second type. Ten stable variants were analyzed chemically and only one showed a slight diminution in galactose content over that of the parent. No other changes were found.

N. madurae was very susceptible to bacitracin; 0.88% of the population survived exposure to 3.0 units/ml. Only one variant type was observed, very similar to the second type obtained by UV irradiation. Four stable variant strains were analyzed chemically, but no changes were found.

N. madurae also showed great sensitivity to novobiocin (1% survival in presence of 5.0 units/ml). One type of variant was observed which formed abundant aerial mycelium with occasional coremia-like structures. The aerial hyphae had coiled side branches, but these were sterile. Vegetative mycelium was very thin, often of a granular structure. Four stable variant strains were chemically analyzed. No changes were found.

N. madurae grew well at 42 C with 43% of the inoculum developing. The cells obtained at 42 C were used to inoculate submerged cultures incubated at 47 C; and the harvested biomass was analyzed chemically. No changes were observed. From submerged cultures, samples were plated out on maintenance medium and incubated at 42 C. No morphological variation could be detected, but five colonies were selected at random and analyzed. Two of these had less galactose than the parent and three were of the parental chemical type. Cell wall preparations of three of the five strains were typical of type III.

M. brevicatena. The parent strain of *M. brevicatena* formed aerial mycelium with short chains of globose conidia on both aerial and vegetative hyphae.

Among the 0.7% survivors of an exposure to UV (3,240 ergs per mm² per sec), seven stable variants were selected which were mainly characterized by being sterile versions of the parent. Five of these were analyzed. No changes were found.

Exposure of *M. brevicatena* to 0.007 ml of EMS per milliliter during 1 hr killed 99.4% of the cells. Morphological variants in all cases showed a stunted growth, with an almost complete lack of sporulation, and a tendency to form coremia-like structures. Ten such stable variants were isolated, six of which were analyzed. No changes were observed in chemical composition when compared to the parental type.

In presence of 50 units of penicillin per milliliter, 1.3% of an exposed population of *M. brevicatena* grew. The colonies were nonpigmented to light cream color and very flat. Aerial mycelium was abundant with sporangia-like bodies at the tip of many hyphae. Primary mycelium was very fine, granular, and sterile. These reverted easily to parental morphology. One strain was isolated, and a cell wall preparation was analyzed, which revealed no change from the parental type IV.

Actinoplanes sp. The parent strain of *Actinoplanes* sp. produced sporangia containing motile spores on the surface of the colony. Vegetative mycelium consisted of fine, long, sterile hyphae.

Exposure to UV (1,820 ergs per mm² per sec) permitted only 0.26% of the cells to survive. Eight variants were isolated which showed complete or partial lack of formation of sporangia. All of them reverted rapidly to parental type.

The *Actinoplanes* sp. was sensitive to low concentrations of penicillin, and 0.5 units/ml allowed survival of only 4.4% of the exposed population. Two types of variants were observed: one, colonies showing complete lack of sporangia and long, fine, vegetative mycelium; and the other, colonies on which sporangia were formed less abundantly than in the parent, and in which the sporangiophores were usually longer than those of the parent. Short, rudimentary, aerial hyphae were formed and the vegetative mycelium was like that of the parent. Six stable variants were analyzed. No changes were found.

In the presence of 5 units of bacitracin per milliliter, 8% of the inoculum of *Actinoplanes* sp. grew, but no significant morphological changes were observed.

Actinoplanes sp. did not show much sensitivity to novobiocin. Almost 100% survival was observed in the presence of 100 μg/ml of novobiocin. No morphological changes were noted.

M. rosea. The parent strain of *M. rosea* formed aerial mycelium bearing longitudinal pairs of conidia. The substrate mycelium was sterile.

Exposure to UV (3,841 ergs per mm² per sec)

TABLE 2. *Number of strains chemically analyzed for the detection of variation in major cell wall constituents*[a]

Parent	Selecting agent							
	UV	EMS	Penicillin	Bacitracin	Novobiocin	pH	Temp	Total
Nocardia asteroides	27/6			10/9		2/1		39/16
N. madurae	18/8	1/1	10/3	4/0	4/0		5/3	42/15
Micropolyspora brevicatena	5/3	6/2	1/1					12/6
Actinoplanes sp.			6/3					6/3
Microbispora rosea	13/6	3/1	3/0					19/7
Streptomyces griseus	6/2		6/1	2/1	2/1			16/5
Grand total								134/52

[a] First figure in group refers to the number of substrains of which whole cell hydrolysates were analyzed (1). Second figure in group indicates number of these substrains from which cell wall preparations were made and analyzed (2).

resulted in the survival of only 1% of the population. Two main types of variants were observed: those with bright red colonies and no soluble pigment, in which aerial mycelium was usually absent and the vegetative hyphae were long, and straight or wavy; and those exhibiting abundant, to slightly reduced, sterile aerial mycelium with brushlike structures. Eight variants were analyzed, five of the first type and three of the second. No changes were observed. In addition, five strains which survived the UV exposure, but which showed no morphological variation, were isolated and analyzed. Again, no changes were observed.

When cellular suspensions of *M. rosea* were exposed to 0.01 ml of EMS per milliliter while shaking for 180 min, 0.64% of the population survived. Five stable variants representing one type were isolated. In these, the aerial mycelium was almost absent and conidia were not formed. Three of them were analyzed. Chemically, they were of parental type.

Penicillin (50 units/ml) killed 99.8% of the population exposed to this antibiotic. No significant morphological changes were observed, but three colonies were isolated and analyzed. No chemical changes were found.

S. griseus. The parent strain of *S. griseus* formed long straight to flexuous chains of conidia on the aerial hyphae. Chains of conidia on vegetative hyphae could also be seen in old cultures.

UV doses of 3,450 ergs per mm^2 per sec killed 99.94% of *S. griseus* populations. Six strains were selected from survivors exposed to various UV doses. One of them produced a dark melanin-like pigment. Its whole cell hydrolysate contained less glucose and galactose than the parent. Another variant formed open spirals or hooks on the aerial hyphae. The cell hydrolysate showed a total lack of galactose. A third variant had no aerial mycelium and sterile vegetative mycelium. The other three strains were very similar to the parent, but they were analyzed because they had survived high UV doses. They were chemically of parental type.

None of the three antibiotics yielded morphological variants of *S. griseus*. Survival rates were as follows: 0.08% of the population survived 20 units of penicillin per milliliter, 0.95% survived 0.5 units of bacitracin per milliliter, and 0.12% survived 0.5 μg of novobiocin per milliliter. Six strains resistant to the action of penicillin and two resistant to novobiocin were analyzed chemically. They were similar to the parent. Two survivors of exposure to bacitracin were likewise analyzed. One of these lacked galactose. The other was of parental type.

Analysis of results. A total of 134 strains, obtained from the six parents, were analyzed chemically by whole cell analysis in an attempt to detect variation over parental types in certain of the major constituents of the cell wall (Table 2). Fifty-two of these were examined by cell wall analysis. No major change was noted in the constituents used to classify the organisms, as summarized in Table 1, thus indicating that cell wall composition is a stable characteristic worthy of use in the classification of aerobic actinomycetes.

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