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Taxonomic Studies of *Streptomyces virginiae* Mutants Overproducing Virginiamycin M_1

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ABSTRACT. By using both the traditional *International Streptomycetes Project* methods and chemical approaches followed by a hierarchical cluster analysis, *Streptomyces virginiae* mutants A-1 and B-43 (yielding higher amounts of the M_1 component of virginiamycin complex), their wild ancestor ATCC 13161, and another virginiamycin producer, *S. pristinaespiralis* NRRL 2958, were subjected to taxonomic studies to find kinship or differences among the strains. Of the methods used, only the test of carbon utilization, investigation of spore surface and analysis of sugar constituents of cell walls proved to be reliable enough to demonstrate the species identity of *S. virginiae* strains and to distinguish them from *S. pristinaespiralis*. L,L-2,6-Diaminopimelic acid was present in all strains. Analysis of fatty acids and total proteins as well as investigations of morphology and pigmentation of agar cultures led to confusing results.

Taxonomic methods have been mainly used to classify and identify wild strains, fewer data having been published on their mutant derivatives, although microbial geneticists are often required to characterize mutant cultures for patent applications and to demonstrate how the new strain differs from its natural parent (Goodfellow and Board 1980; Goodfellow *et al.* 1985; Lechevalier 1982; Williams *et al.* 1983).

In the present paper, the suitability of fatty acid, total protein and cell wall chemotype analyses for characterizing mutant derivatives of *Streptomyces virginiae* ATCC 13161, a producer of veterinary antibiotic virginiamycin (Biot 1984), was investigated in comparison with traditional taxonomic approaches. Mutants A-3 and B-43 (resulting from five successive mutation and selection cycles) were chosen for the study as typical representatives of the "grey" and "white" lines, respectively, with regard to the aerial mass color. They differed from their wild ancestor in a substantial increase in the production of virginiamycin M_1, whereas the yields of virginiamycins S1 and M_2 were increased only moderately in strain A-3 and reduced considerably in strain B-43. The latter mutant showed also a reduced growth rate, a decreased sensitivity to carbon catabolite repression, and a wider temperature optimum for virginiamycin biosynthesis (Přikrylová *et al.* 1987). To find a possible correlation between taxonomic characters and the ability to produce the same antibiotics in different species, we included also *Streptomyces pristinaespiralis* NRRL 2958, another producer of virginiamycins M_1 and M_2 (pristiamycins IIA and IIB; Cocito 1979), as a reference strain into the experiments.

MATERIAL AND METHODS

Characterization of agar cultures. Morphology, color and physiological characteristics of *Streptomyces virginiae* ATCC 13161, of its high virginiamycin M_1-yielding mutants A-3 and B-43 (Přikrylová *et al.* 1987), and of *S. pristinaespiralis* NRRL 2958 were studied by using the media and methods of the *International Streptomyces Project* (ISP) described by Shirling and Gottlieb (1966).

Preparation of biomass for chemotaxonomic studies. Submerged cultures were grown in 500 mL conic flasks containing 60 mL of a modified Sauton medium (Mordarska *et al.* 1972) on a rotary shaker (2.7 Hz) at 28 °C. The initial pH of the medium was 7.2. The 2nd vegetative generation was inoculated with 3 mL of a 48-h culture grown under the same conditions and incubated for 5 d. Mycelium was harvested by centrifugation (2000 *g*, 10 min) and washed three times with distilled water.

Fatty acid extraction and analysis. The identification of fatty acids as methyl esters on a capillary column with SE-54 (25 m long) by using a GC-MS HP 5995 B apparatus (*Hewlett Packard,*

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USA) was described by Vančura *et al.* (1987). The fatty acids were quantified by using a capillary GLC Sigma apparatus (*Perkin Elmer*, USA) employing a SPB-5 capillary column (30 m × 0.25 mm ID × 0.25 μm) (*Supelco*, USA).

Sodium dodecylsulfate polyacrylamide gel electrophoresis. SDS gel electrophoresis was carried out as described by Vančurová *et al.* (1988) by using 10 % polyacrylamide gels (90 × 180 × 1 mm) containing 0.1 % SDS. The gels were stained for protein using Coomassie Blue R-250. The color intensities of the protein bands were evaluated with a densitometer Shimadzu CS-930 (Kyoto, Japan). The molar mass of the proteins was estimated from the relative mobilities of standards: bovine serum albumin (*M*, 67 kDa), egg albumin (45), chymotrypsinogen A (25) and myoglobin (17) (*Serva*, Germany).

Paper chromatography analysis of sugars and LL-2,6-diaminopimelic acid (DAP). The cells were hydrolyzed by the procedure of Hasegawa *et al.* (1983). The products were chromatographed on Whatman 4 paper using the following solvent mixtures: 1-butanol–pyridine–water–acetic acid (60:40:30:3) for sugars, and methanol–water–HCl (10 mol/L)–pyridine (64:14:2:8) for DAP.

Statistical methods. A hierarchical cluster analysis procedure was used, with Hamming and Euclidean distances, including Ward and average distance agglomerative procedures, respectively. The computation was carried out on a IBM PC (386/33) compatible with a STATSYS program package.

RESULTS AND DISCUSSION

The characteristics of the two reference strains, *S. virginiae* ATCC 13161 and *S. pristinaespiralis* NRRL 2958, corresponded to the data published by Shirling and Gottlieb (1968*a*,*b*). The mutant strain *S. virginiae* A 3 differed from ATCC 13161 only by exhibiting a lighter shade of the aerial mass color (resembling that of *S. pristinaespiralis*) and by a substantial decrease in melanin production. The mutant strain *S. virginiae* B 43 differed from the other strains tested by both its morphology and color of the aerial mycelium (Fig. 1), but it resembled *S. pristinaespiralis* by showing full absence of melanin formation. The two above mutant strains showed the same carbon utilization pattern as the initial strain ATCC 13161 but it was different from that of *S.pristinaespiralis* (Table I).

The mutants of *S. virginiae* represented a rather simple model for comparative studies; in spite of the differences in aerial mass color, they produced the same metabolites (virginiamycin S_1, M_1 and M_2) as their initial grandparent ATCC 13161 strain, which suggested their identity with the respective species. Nevertheless, the results of the present work on two typical representatives of the *S. virginiae* mutant populations, A-3 and B-43, show that among all the taxonomic approaches used only the test of carbon utilization and the microscopic observation of spore surface proved to be reliable enough to demonstrate the species identity of the *S. virginiae* strains and to distinguish them from another virginiamycin producing organism, *S. pristinaespiralis* NRRL 2958. On the other hand, the color of the cultures and morphology of their sporophores seem to be rather irrelevant in this respect.

The qualitative and quantitative composition of fatty acids in *S. virginiae* ATCC 13161, the mutant strains A-3 and B-43 and in *S. pristinaespiralis* NRRL 2958 is shown in Table II. In contrast to the data of Saddler *et al.* (1987) and Ehret *et al.* (1987), the capillary column used in our study allowed us to separate and, subsequently by use of mass spectrometry, identify all methyl esters present. Therefore, we did not have to present unidentified peaks or even sum up to the peak areas (*e.g. ai*-15:0 and 15:1 or 17:1 and 17:0 or *i*-15:0 and *ai*-15:0), which made the cluster analysis finer and more precise.

The fatty acids identified were in the range from C_{14} to C_{18}, which was in accordance with the results published earlier in our (Vančura *et al.* 1987) and other laboratories (Batrakov and Bergelson 1978; Ballio and Barcelona 1968). All the strains produced qualitatively identical and quantitatively similar acids, typical of the genus *Streptomyces* containing *ai*-15:0, *i*-16:0 and 16:0 as the strains tested; *e.g.* in the strain B-43, an unusually high content of 18:1 was detected while only a low amount of *ai*-17:0 was observed. The differences in the content of fatty acids are summarized in Table II.

A gel visualized with Coomassie Blue is shown in Fig. 2. No pronounced similarities between the strains studied were observed from the statistical evaluation of the color intensities. The SDS electrophoresis of the total proteins present in the cell-free extract is not suitable for the taxonomic purposes but the method can serve as supplementary both in traditional taxonomic and modern chemotaxonomic studies.

The cell wall composition was studied by paper chromatography; LL-2,6-diaminopimelic acid, the usual component of the cell wall of streptomycetes (Cummins 1982), was detected in all four strains

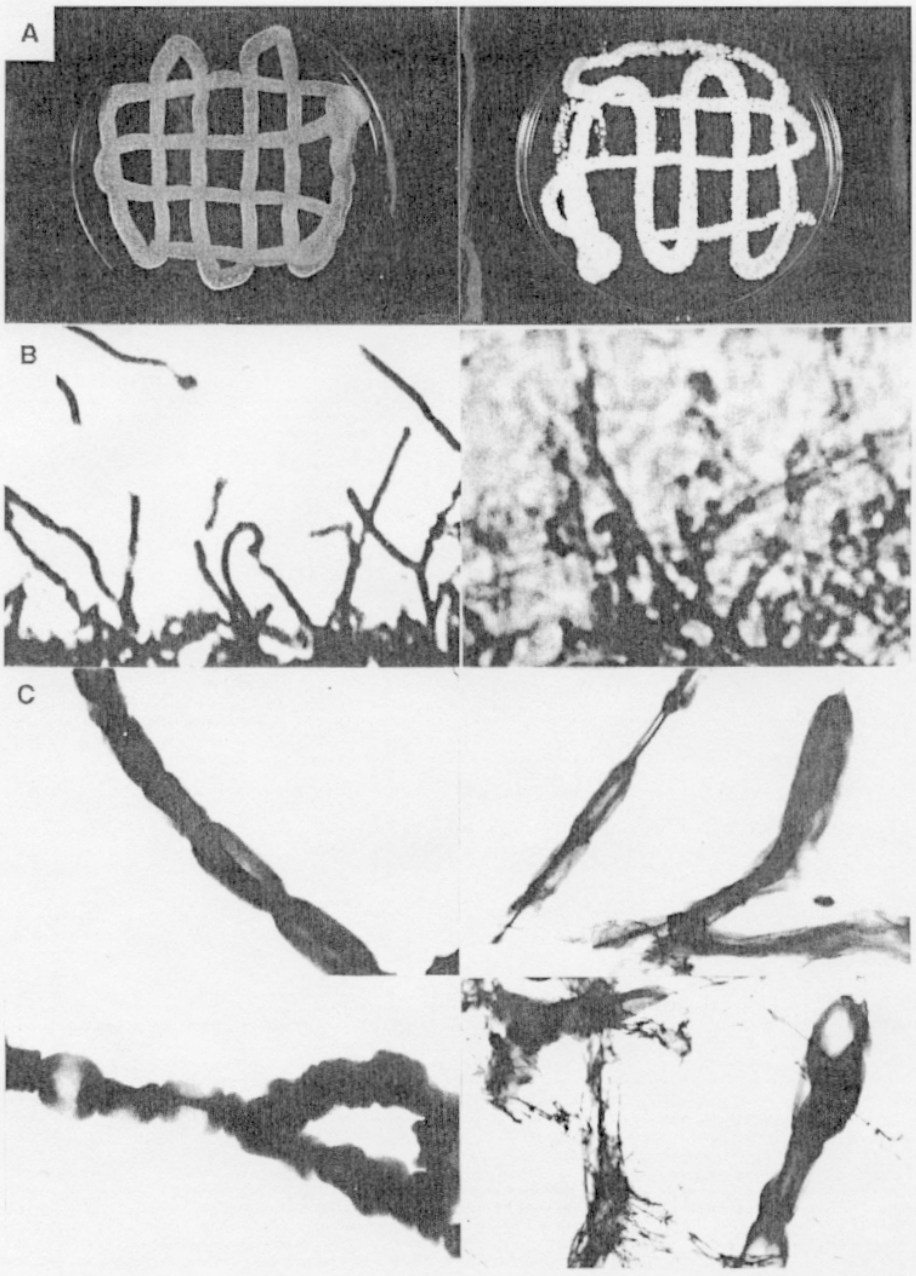

Fig. 1. Aerial growth of the initial *S. virginiae* strain ATCC 13161 (*left*) and its mutant derivative B-43 (*right*). Yeast extract-malt extract agar, 14 days, 28 °C (A); aerial mass colour: optical microscopy (B) and electron microscopy (C). Mycelium of the strain B-43 (covered by a slimy exudation) consists of irregularly thickened hyphae showing incomplete fragmentation into spores.

Table I. Comparison of cultural features of *S. virginiae* mutant strains A-3 and B-43 with those of *S. virginiae* ATCC 13161 and *S. pristinaespiralis* NRRL 2958[a]

Characteristic	ATCC 13161[b]	A-3	B-43	NRRL 2958[c]
Morphology of aerial hyphae	Retinaculum-Apertum Rectus-Flexibilis	Retinaculum-Apertum	Rectus-Flexibilis	Spira to Retinaculum-Apertum
Spore surface	Smooth	Smooth	Smooth	Warty
Number of spores per chain	10–50	10–50	10[d]	10–50
Aerial mass color (series)	Red or Gray	Gray[e]	White	Gray
Reverse side of colony	Gray-yellow or yellow-brown	Light gray-yellow	Colorless to light yellow	Colorless to light yellow or light yellowish brown
Melanoid pigment	Formed	Formed[f]	Absent	Absent
Pigment other than melanoid	Absent	Absent	Absent	Absent (or traces of yellow)
Carbon utilization: D-glucose, D-fructose	+	+	+	+
L-arabinose, D-xylose *myo*-inositol, D-mannitol L-rhamnose, sucrose, raffinose	–	–	–	+

[a]By using the ISP taxonomy methods (Shirling and Gottlieb 1966).
[b]See also Shirling and Gottlieb (1968*a*).
[c]See also Shirling and Gottlieb (1968*b*).
[d]Due to a defect in morphological development the spores are not clearly separated (see Fig. 1).
[e]Lighter shade than ATCC 13161.
[f]In low quantity only.

Table II. Fatty acid composition of mutant strains of *Streptomyces*

Fatty acid	ATCC 13161	A-3	B-43	NRRL 2958
i-14:0	4.2	3.1	1.8	3.6
14:0	0.9	1.5	1.3	1.0
i-15:0	5.2	7.0	2.7	11.9
ai-15:0	23.2	31.2	14.2	28.3
15:0	2.5	2.8	1.8	3.5
i-16:0	24.5	20.5	19.0	19.9
16:1	10.1	0.5	3.8	0.3
16:0	10.6	11.8	33.4	11.8
i-17:0	0.9	2.8	7.9	4.3
ai-17:0	15.5	15.4	0.5	12.9
18:2	0.4	0.7	3.2	0
18:1[a]	0.6	0.8	2.8	0.4
18:1[a]	0.6	0	1.3	0.4
18:0	0.1	1.1	3.6	0.8

[a]Positional isomers of octadecenoic acids.

tested. Apart from that, galactose and mannose were found to be present in all strains except for the NRRL 2958 strain, where the only hexose identified was glucose (absent in the other strains). On the other hand, pentoses, *i.e.* arabinose and xylose, were not detected: ribose was present everywhere except strain A-3. Paper chromatography analysis suggests a changing content of saccharides in the cell wall both in the original strains and in mutants. Based on statistical data processing, *i.e.* the formation of distance matrix (Hamming and Euclid distance) and calculation of agglomerative procedures (Ward and average distance), the dendrograms shown in Fig. 3 were obtained. The strains appear to be related (Fig. 3, *left* and *middle*) irrespective of whether the data were obtained by using the GC-MS method of estimating fatty acid methyl esters or

SDS-gel electrophoresis of proteins. The relationship of the strains is also not influenced by the mathematical data processing. In contrast, Fig. 3 (*right*) proves the kinship of the strains resulting from improvement procedures; hovewer, even in this case a heterogeneity could not be observed that would have originated from the mathematical data processing.

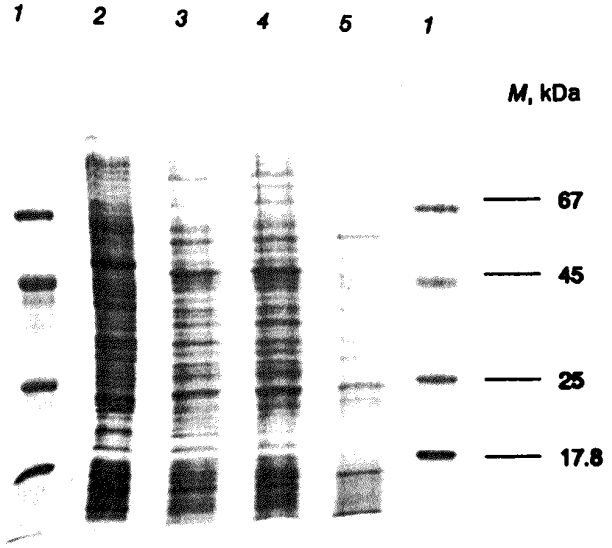

Fig. 2. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis. *Lanes 1* show marker proteins: horse myoglobin (*M* 17.8 kDa), chymotrypsinogen A (25), egg albumin (45) and bovine serum albumin (67) from *Serva*, Heidelberg (Germany). *Lane 2* = cell free extract of the sample ATCC 13161, *lane 3* = A-3, *lane 4* = B-43, *lane 5* = NRRL 2958.

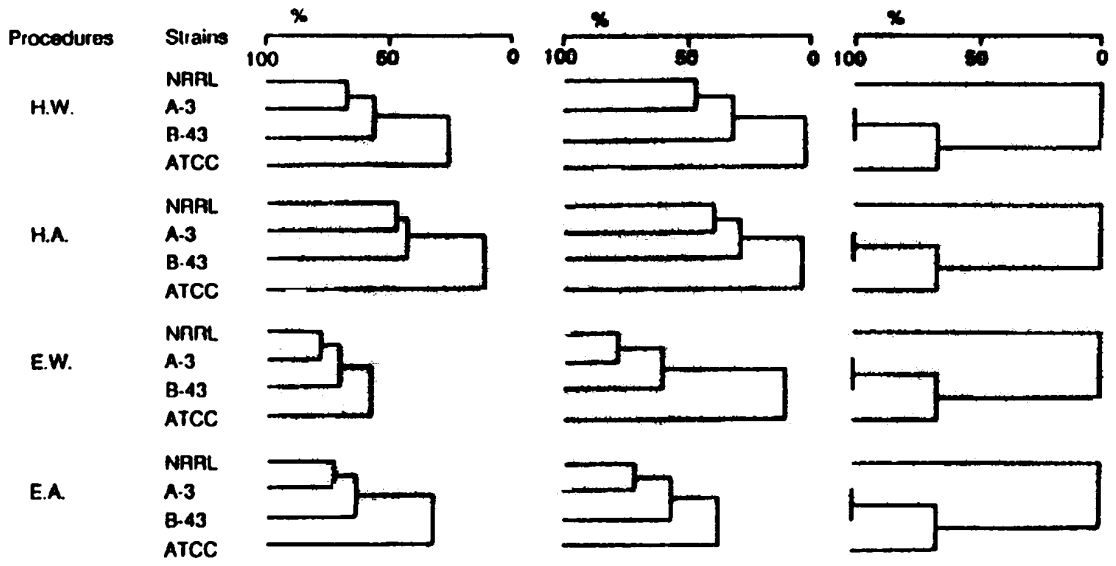

Fig. 3. Clustering (%) of *Streptomyces* strain using the computer program, dendrograms by using FAME by GC-MS (*left*), proteins by electrophoresis (*middle*) and sugars and DAP by PC (*right*). H.W.; H.A.; E.W. and E.A. are abbreviations of computer statistical methods (Hamming and Euclidean distances, Ward and Average distance agglomerative procedures).

We can conclude that by comparing the various methods used, *viz.* three chemotaxonomic ones (methyl ester determination by GC-MS, protein electrophoresis and paper chromatography of DAP and saccharides) and the classical taxonomic methods (morphology, substrate utilization, pigmentation), unequivocal results cannot be obtained and employed for the characterization of mutants.

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REFERENCES

Ballio A., Barcellona S.: Relations chimiques et immunologiques chez les actinomycetales. 1. Les acides gras de 43 souches d' actinomycétes aérobies. *Ann.Inst.Pasteur* **114**, 121–137 (1968).

Batrakov S.G., Bergelson, L.D.: Lipids of the streptomycetes. Structural investigation and biological interrelation. *Chem.Phys.Lipids* **21**, 1–29 (1978).

Biot A.M.: Virginiamycin: Properties, biosynthesis, and fermentation, pp. 695–720 in *Biotechnology of Industrial Antibiotics* (E.J. Vandamme, Ed.). Marcel Dekker, New York–Basel 1984.

Cocito C.: Antibiotics of the virginiamycin family, inhibitors which contain synergistic components. *Microbiol.Rev.* **43**, 145–198 (1979).

Cummins C.S.: Bacterial cell wall structure, pp. 197–227 in *CRC Handbook of Microbiology* (A.I. Laskin, H.A. Lechevalier, Eds). CRC Press, Boca Raton 1982.

Ehret W., Jacob N., Ruckdeschel G.: Identification of clinical and environmental isolates of *Legionella pneumophila* by analysis of outer membrane proteins, ubiquinones and fatty acids. *Zbl.Bakt.Hyg.A* **266**, 261–275 (1987).

Goodfellow M., Board R.G.: *Microbiological Classification and Identification.* Academic Press, London 1980.

Goodfellow M., Jones D., Priest F.G.: *Computer-assisted Bacterial Systematics.* Academic Press, London 1985.

Hasegawa T., Takizawa M., Tanida S.: A rapid analysis for chemical grouping of aerobic actinomycetes. *J.Gen.Appl. Microbiol.* **29**, 319–322 (1983).

Lechevalier M.P.: Lipids in bacterial taxonomy, pp. 435–541 in *CRC Handbook of Microbiology* (A.I. Laskin, H.A. Lechevalier, Eds) CRC Press, Boca Raton 1982.

Mordarska H., Mordarski M.: Chemotaxonomic characters and classification of some nocardioform bacteria. *J.Gen. Microbiol.* **71**, 77–86 (1972).

Přikrylová V., Blumauerová M., Sedmera P., Vaněk Z.: Strain development in *Streptomyces virginiae*, a producer of virginiamycin. *Biotechnol.Bioind.* (Sofia) **2**, 20–22 (1987).

Saddler G.S., O'Donnell A.G., Goodfellow M., Minnikin D.E.: SIMCA pattern recognition in the analysis of streptomycetes fatty acids. *J.Gen.Microbiol.* **133**, 1137–1147 (1987).

Shirling E.B., Gottlieb D.: Methods for characterization of *Streptomyces* species. *Int.J.Syst.Bacteriol.* **16**, 313–340 (1966).

Shirling E.B., Gottlieb D.: Cooperative description of type cultures of *Streptomyces*. II. Species descriptions from first study. *Int.J.Syst.Bacteriol.* **18**, 69–189 (1968*a*).

Shirling E.B., Gottlieb D.: Cooperative description of type cultures of *Streptomyces*. III. Additional species descriptions from first and second studies. *Int.J.Syst.Bacteriol.* **18**, 279–392 (1968*b*).

Vančura A., Řezanka T., Maršálek J., Křišťan V., Basařová G.: Fatty acids and production of tylosin-like compounds in *Streptomyces fradiae*. *J.Basic Microbiol.* **27**, 167–171 (1987).

Vančurová I., Volc J., Flieger M., Neužil J., Novotná J., Vlach J., Běhal V.: Isolation of pure anhydrotetracycline oxygenase from *Streptomyces aureofaciens*. *Biochem.J.* **253**, 263–267 (1988).

Williams S.T., Goodfellow M., Alderson G., Wellington E.M.H., Sneath P.H.A., Sackin M.J.: Numerical classification of *Streptomyces* and related genera. *J.Gen.Microbiol.* **129**, 1743–1813 (1983).