New Genus of the Actinomycetales: Kibdelosporangium aridum gen. nov., sp. nov.

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A new actinomycete genus, Kibdelosporangium, is described. Members of this genus have type IV cell walls (meso-diaminopimelic acid, d-glutamic acid, t-alanine, muramic acid, N-acetyl-d-glucosamine, d-galactose, and arabinose) and a type A whole-cell sugar pattern (d-galactose and arabinose) plus traces of madurose (3-O-methyl-d-galactose). No mycolic acids are present. The substrate mycelium has a tendency to fragment. The aerial mycelium bears long chains of spores, as well as sporangiumlike structures with well-defined walls. These sporangiumlike structures do not contain spores but germinate directly when they are placed on agar. The type strain of K. aridum is strain SK&F-AAD-216 (= ATCC 39323).

In the course of isolating microorganisms from unusual ecological niches for an antibiotic screening program, four unusual filamentous bacteria were isolated from desert soils collected in Pima County, Ariz. These organisms had chemotaxonomic and morphological characteristics that precluded their placement in any of the previously described genera of the Actinomycetales. They had type IV cell walls; no mycolic acids were present. The whole-cell sugar pattern was type A; traces of madurose were also usually present. Examination of the aerial mycelium showed long chains of spores and sporangiumlike structures with well-defined walls. Despite extensive manipulation and experimentation, development or release of spores from these sporangiumlike structures was not observed. When placed on agar, the sporangiumlike structures germinated directly, producing one or more germ tubes.

Subsequently, at least 10 additional organisms with the same characteristics have been isolated from soils collected in three widely scattered areas of the world. Many of these isolates produce glycopeptide antibiotics belonging to the vancomycin class. In this paper we propose a genus to accommodate these isolates and provide a detailed description of one of the isolates, strain SK&F-AAD-216 (T = type strain), which produces the novel glycopeptide antibiotics aridicins A, B, and C (45). MATERIALS AND METHODS

Bacterial strain and culture conditions. Strain SK&F-AAD-216 was isolated from a soil sample collected in a desert area in Pima County, Ariz. Stock cultures were grown on oatmeal agar (44) and thin potato-carrot agar (15). Slants and plates of these media were used for determining the growth temperature range. Morphological observations were made on plates containing oatmeal agar, thin potato-carrot agar, water agar, and soil extract agar (43). The additional media used to characterize strain SK&F-AAD-216 were inorganic salts-starch agar (44), peptone-yeast extract-iron agar (44), starch-casein-nitrate agar (22), DNF Test Agar (BBL Microbiology Systems), Bennett agar (17), tyrosine agar (44), gelatin medium 19 (49), thin Pabulum agar (31), and litmus milk medium (Difco Laboratories). All tests were performed at 28°C. For tests of growth under anaerobic conditions, the GasPak system (BBL) was used.

The sporangiumlike structures used for the germination studies were either freshly harvested or stored in sand. Sporangiumlike structures were harvested from 3- to 5-week-old oatmeal agar plates by adding 5 to 10 ml of sterile distilled water and gently dislodging the aerial mycelium with a bacteriological loop. The material harvested from 5 to 10 plates (diameter, 100 mm) was pooled and washed three times with distilled water by centrifugation at 150 × g for 10 min. At this low speed most of the hydrophobic spores remained on top of the water, while the sporangiumlike structures were pelleted. For immediate use the sporangiumlike structures were suspended in 20 to 25 ml of distilled water and filtered through two layers of sterile gauze. To prepare sand tubes, 0.5 to 1.0 ml of distilled water was used to suspend the sporangiumlike structures. This preparation was added, with gentle mixing, to approximately 1 in (2.54 cm) of sterile, washed, ignited sand in the bottom of a 16-mm screw-cap test tube. The tubes were incubated at 28°C until the sand dried (approximately 1 month) and were stored at room temperature. Sterile microspatulas were used to transfer small portions of the sand to plates containing the media used for morphological studies. The sand was spread over the surface of the medium with sterile glass petri dish spreaders. The sand tubes remained a good source of inoculum for 6 to 8 months. However, after 24 months the sporangiumlike structures stored in sand were usually not viable.

Microscopy. Cover slip cultures used for light microscopy were prepared by a modification of the method of Kawato and Shinobu (18). Round agar plugs were cut from plates containing media by using the mouth of a sterile 16-mm test tube. The plugs were lifted onto the surface of the medium with a sterile spatula. The upper surface of the plug was then inoculated with 1 drop of a heavy cell suspension, and a sterile 22-mm cover slip was firmly pressed onto the top of the agar plug. Plates were incubated at 28°C. Cover slips were removed at intervals following 3 to 5 weeks of incubation, when sufficient growth had occurred on the cover slips as rings around the agar plugs. The cover slips were fixed for 15 min in absolute methanol and stained for 1 to 15 min in dilute, filtered crystal violet (0.1 g in 1,000 ml of distilled water). Extreme care had to be exercised in interpreting these cover slip cultures because orientation of the cover slip

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did not offer a reliable guide for distinguishing between the substrate mycelium and the aerial mycelium.

Preparations of the sporangiumlike structures for transmission electron microscopy were fixed in glutaraldehyde, embedded in Spurr resin, postfixed with uranyl acetate, and sectioned. Whole mounts of the sporophores were prepared by the method of Lechevalier and Lechevalier (29). Both preparations were viewed with a Siemens model 1A transmission electron microscope.

For scanning electron microscopy agar blocks containing numerous sporangiumlike structures were fixed in osmium tetroxide vapor for 1 week at room temperature, dehydrated, and sputter coated with gold. These preparations were viewed with a JEOL model JSM 35C scanning electron microscope.

Chemotaxonomy. Purified cell wall and whole-cell hydrolysates were analyzed by the methods of Becker et al. (2) and Lechevalier (27), respectively. Cell wall, phospholipid, and mycolic acid analyses were performed by the methods of Lechevalier et al. (28, 32). The deoxyribonucleic acid (DNA) base ratios were determined by the thermal melting method, using DNAs from Bacillus circulans ATCC 17400 as controls (guanine-plus-cytosine content = [melting point - 69.3°C]/0.41) (36).

Physiological tests. The physiological tests used to characterize strain SK&F-AAD-216T were those of Gordon (11, 12) and Gordon and Mihm (13). In the tests for acid production from carbohydrates and utilization of organic acids, all results were confirmed by subculturing the preparations onto fresh medium. The tests used to determine melanoid pigments were those of Shirling and Gottlieb (44). Phosphatase activity was determined by the methods of Kurup and Schmitt (21).

Susceptibility to antibiotics. The susceptibility of strain SK&F-AAD-216T to antibiotics and antimicrobial agents was examined by placing BBL susceptibility disks on nutrient agar plates seeded with strain SK&F-AAD-216T in an agar overlay. Plates were placed at 4°C for 1 h to permit diffusion of the antibiotics and then incubated at 28°C. The diameters of the zones of inhibition were measured after incubation for 1 week.

RESULTS

Description of Kibdelosporangium gen. nov. Kibdelosporangium (Kib. del. o. spo. ran.' gi. um. Gr. adj. kibdelos false, ambiguous; Gr. n. spora seed; Gr. n. ageion vessel; Kibdelosporangium vessel containing false seeds) strains are aerobic, gram-positive, non-acid-fast, filamentous organisms that form a mycelium differentiated into (i) a substrate mycelium that penetrates the agar and forms a compact layer on top of the agar and (ii) an aerial mycelium originating from the substrate mycelium. The well-developed substrate mycelium may exhibit varying degrees of fragmentation and usually bears specialized structures which appear to be dichotomously branched, septate hyphae radiating from a common stalk. The aerial mycelium bears long chains of spores and sporangiumlike structures. These sporangiumlike structures are surrounded by well-defined walls but do not contain spores. They germinate directly, producing one or more germ tubes when they are placed on agar. No motile elements are present in either the aerial or substrate mycelium. The cell wall is type IV (meso-diaminopimelic acid, D-glutamic acid, D-alanine, muramic acid, N-acetyl-D-glucosamine, D-galactose, and arabinose) (30); no mycolic acids are present. The whole-cell sugar pattern is type A (arabinose and D-galactose) (30); traces of madurose are usually present. The phospholipid pattern is type PI1 (phosphatidylglycerol, phosphatidylglycerol mannosides, diphostatidylglycerol, phosphatidylethanolamine; phosphatidylcholine may also be present) (28). The type species is Kibdelosporangium aridum.

Description of Kibdelosporangium aridum sp. nov. (i) Substrate mycelium. K. aridum (arid. um. Lat. adj. aridum dry, arid) substrate mycelium is well developed, with moderately branching, septate hyphae that are approximately 0.4 to 1.0 μm in diameter. The vegetative hyphae do not fragment into rods and cocci during smear preparation. However, fragmentation without hyphal displacement occurs in plate cultures (Fig. 1). Fragmentation in fermentors and shake flasks also occurs occasionally.

FIG. 1. Micrograph of substrate mycelium exhibiting fragmentation (arrows) without hyphal displacement (22-day-old culture on soil extract agar). Bar = 10 μm.

FIG. 2. Micrograph of naked sporangiumlike structure (24-day-old cover slip culture from soil extract agar). Bar = 10 μm.
Present on the vegetative hyphae are specialized structures which consist of dichotomously branched, septate hyphae radiating from a common stalk (Fig. 2). These specialized structures may be produced either deep in the agar or just below the surface of the agar and appear to be "naked" sporangiumlike structures analogous to the conidial structures which Couch (4) observed on the substrate mycelium of members of the Actinoplanaceae. On many media K. aridum produces characteristic crystals in the agar (Fig. 3).

(ii) Aerial mycelium. The aerial mycelium is white and tends to be thin. When viewed perpendicularly with a light microscope, the surface of a sporulating colony is covered with straight to irregularly curved chains of rod-shaped spores. These spore chains are usually very long, with more than 50 spores per chain, but chains of 10 spores or less are also present. The spore chains are usually borne terminally on branched or unbranched hyphae; occasionally they are borne terminally on lateral branches of the main hyphal threads. The spores are smooth walled (Fig. 4) and irregular in length (approximately 0.4 μm wide by 0.8 to 2.8 μm long). When placed on agar, these spores germinate, producing one or more germ tubes. When a microscope is focused on the surface of the agar, sporangiumlike structures are observed beneath the long spore chains (Fig. 5). These sporangiumlike structures are borne apically on branched or unbranched hyphae, as well as terminally on short lateral branches of the main hyphal threads (Fig. 6). Sporangiumlike structures and chains of spores are frequently produced on the same aerial hyphae. These sporangiumlike structures begin as small round swellings at the tips of the hyphae. These swellings continue to enlarge, often going through a stage where they appear irregular in shape. At maturity, these sporangiumlike structures are usually round (approximately 9 to 22 μm in diameter). However, sporangiumlike structures slightly flattened along one axis are also present. Very irregular, sausage-shaped sporangiumlike structures are rarely observed. Mature sporangiumlike structures are surrounded by well-defined walls and contain septate, branched hyphae embedded in an amorphous matrix (Fig. 7 through 10). Despite extensive experimentation and observation of these sporangiumlike structures, the development or release of any type of spore has not been observed. When placed on a nutrient medium, these sporangiumlike structures germinate directly, producing one or more germ tubes (Fig. 11).

(iii) Chemotaxonomy. Purified cell wall preparations of K. aridum contain meso-diaminopimelic acid, D-glutamic acid, D,L-alanine, muramic acid, N-acetyl-D-glucosamine, D-galactose, and a minor amount of arabinose. Whole-cell hydrolysates contain D-galactose, D-glucose, D-mannose, D-ribose, L-rhamnose, and arabinose; traces of madurose are also usually present. The phospholipids present are phosphatidylethanolamine, phosphatidylmethylethanolamine, diphosphatidylglycerol, phosphatidylinositol, and phosphatidylinositol mannosides. Thus, K. aridum has type IV cell walls (30), a type A sugar pattern (30) with traces of madurose, and a type PI1 phospholipid composition (28). No mycolic acids of any type are found in the cell extracts. The DNA guanine-plus-cytosine content is 66 mol%.

(iv) Appearance on various media. All plates were incubated at 28°C in closed petri dish cans and observed at intervals for up to 21 days. The common names for the

FIG. 3. Micrograph of crystals in agar (23-day-old culture on soil extract agar). Bar = 28 μm.

FIG. 4. Transmission electron micrograph of spore chains (19-day-old culture on Bennett agar). Bar = 1 μm.
colors were chosen after comparison with color chips from either the ISCC-NBS Centroid Color Charts (19, 37) or A Dictionary of Color (35).

On oatmeal agar growth is good, the substrate mycelium is off-white to yellowish brown, the aerial mycelium is sparse and white, spore chains and sporangiumlike structures are present, no soluble pigment is produced, and crystals are present in the agar.

On inorganic salts-starch agar growth is good, the substrate mycelium is off-white to yellowish brown, the aerial mycelium is sparse and white, spore chains and sporangiumlike structures are present, no soluble pigment is produced, and crystals are present in the agar.

On peptone-yeast extract-iron agar growth is good, the substrate mycelium is gray to bronzish brown, there is no aerial mycelium, a dark brownish black soluble pigment is produced, and no crystals are detected in the agar.

On thin potato-carrot agar growth is fair, the substrate mycelium is off-white to pale yellowish brown, the aerial mycelium is sparse to moderate and white, numerous spore chains and sporangiumlike structures are present, no soluble pigment is produced, and no crystals are detected in the agar.

On starch-casein-nitrate agar growth is good, the substrate mycelium is off-white to yellowish brown, the aerial mycelium is sparse and white, spore chains and sporangiumlike structures are present, a pale yellowish brown soluble pigment is produced, and crystals are present in the agar.
pigment is variably present, and crystals are present in the agar.

On soil extract agar growth is fair, the substrate mycelium is pale yellowish brown, the aerial mycelium is sparse and white, spore chains and sporangiumlike structures are present, no soluble pigment is produced, and crystals are variably present in the agar.

On water agar growth is poor, the substrate mycelium is translucent to pale yellowish brown, the aerial mycelium is sparse and white, spore chains and sporangiumlike structures are present, no soluble pigment is produced, and no crystals are detected in the agar.

(v) Physiological and biochemical characteristics. No growth occurs under anaerobic conditions. The temperature range for growth is 15 to 42°C, with a trace of growth at 45°C. Growth at 10°C is inconsistent. Freshly inoculated slants survive exposure to 50°C for 8 h. Hydrogen sulfide and melanin are produced. Milk is peptonized. Gelatin is both hydrolyzed and liquefied. Nitrate is not reduced to nitrite. Casein, L-tyrosine, hypoxanthine, guanine, elastin, and testosterone are hydrolyzed, but starch, adenine, xanthine, and cellulose (Avicel) are not. Catalase and phosphatase are produced. Urea, esculin, and hippurate are decomposed; tests for allantoin decomposition are weakly positive. No growth occurs in lysozyme broth. Growth in the presence of 4% NaCl is consistent; growth in the presence of 5 to 7% NaCl is inconsistent, and no growth occurs in the presence of 8% NaCl. Acid is produced from L-arabinose, D-celllobiose, dextrin, dextrose, D-fructose, glycerol, glycogen, D-galactose, i-inositol, lactose, D-mannitol, D-mannose, α-methyl-D-glucoside, α-methyl-D-mannoside, maltose, melibiose, D-melezitose, raffinose, L-rhamnose, D-ribose, sucrose, trehalose, and D-xylose. Acid production from salicin is variable. No acid is produced from adonitol, dulcitol,
conidia and form sclerotia which pass through developmental stages that superficially resemble those occurring in the sporangiumlike structures of strain SK&F-AAD-216<sup>T</sup>. However, the sclerotia of Chainia are plurilocular structures that are held together by intercellular material. They are formed by repeated branching and swelling of the vegetative hyphae (33, 41), whereas the sporangiumlike structures of strain SK&F-AAD-216<sup>T</sup> are formed on the aerial mycelium and are surrounded by a well-defined wall. In addition, members of the genus Chainia have type I cell walls (30).

Chemotaxonomically, strain SK&F-AAD-216<sup>T</sup> appears to be closely related to members of the Nocardiaceae which have type IV cell walls (25) and either type PII or type PIII phospholipid patterns (28). Of the genera usually placed in this family, the cell wall characteristics of strain SK&F-AAD-216<sup>T</sup> most closely resemble those of Saccharomonospora. Whole-cell preparations of both strain SK&F-AAD-216<sup>T</sup> and Saccharomonospora contain large amounts of arabinose, while pure cell wall preparations contain only trace amounts, indicating that the arabinose is very loosely bound to the cell wall (M. P. Lechevalier, personal communication). However, these organisms are quite different morphologically, as Saccharomonospora produces single spores, either sessily or on short sporophores, on the aerial mycelium (39).

Two genera of the Nocardiaceae, Nocardia and Rhodococcus, have type PII phospholipids but contain organisms that may be at least partially acid fast and contain mycolic acids (8, 24). Morphologically, the genus Nocardia is a diverse group of organisms. The substrate mycelia of...
these organisms often fragment into rods or coccobacillary units. An aerial mycelium is usually produced, but may range from being barely visible to being a heavy mat that completely covers the substrate mycelium. Short chains of arthrospores may be present. Members of the genus Rhodococcus tend to be pleomorphic, with the substrate mycelium fragmenting into irregular elements. No arthrospores or conidia are produced, although some strains may produce a few sterile aerial hyphae. Micropolyspora brevicatena (Nocardia brevicatena) has type PII phospholipids, contains mycolic acids, and produces short chains of spores on both aerial and substrate mycelia (9, 32). N. orientalis and N. mediterranei have been observed extensively by us. The aerial mycelia of these organisms bear straight to irregularly curved chains of spores, but sporangiumlike structures like those of strain SK&F-AAD-216 have never been observed. A literature search indicated that N. rugosa (6; Goodfellow and Lechevalier, in press) totally lacks an aerial mycelium, while N. sulphurea produces only a sterile aerial mycelium (Goodfellow and Lechevalier, in press; T. J. Oliver and A. C. Sinclair, U. S. patent 3,155,582, November 1964). Thus, these four organisms are readily differentiated from Kibdelosporangium on the basis of morphology.

A final group of organisms currently placed in the Nocardiaceae are the following organisms which lack mycolic acids and have type PIII phospholipid patterns: Nocardia autotrophica, Nocardia saturnea, Nocardia hydrocarbonoxydans, Micropolyspora (Faenia) faeni, and the genera Saccharopolyspora and Pseudonocardia (9, 34; Goodfellow and Lechevalier, in press). N. autotrophica and N. saturnea produce only chains of rectangular or long oval-shaped spores on their aerial mycelia (16; Goodfellow and Lechevalier, in press), while both the aerial and vegetative mycelia of N. hydrocarbonoxydans fragment into long squarish units (38; Goodfellow and Lechevalier, in press). The aerial mycelium of Saccharopolyspora bears chains of round to oval spores that are covered by a sheath carrying tufts of long hairs (23). M. faeni produces chains of 2 to 20 round spores on both the aerial and substrate mycelia (5). Members of the genus Pseudonocardia have a very distinctive morphology: chains of spherical to cylindrical spores are produced by acrosporal budding of the aerial hyphae (14). Therefore, these organisms are all readily differentiated from strain SK&F-AAD-216 on the basis of morphology, as well as phospholipid pattern.

Members of other actinomycete families that have type IV cell walls are Bacterinemia, Mycobacterium, Corynebacterium, and Actinopolyspora (24). The first three genera produce a transient substrate mycelium and contain characteristic mycolic acids (1, 20, 40). Actinopolyspora is an extreme halophile with type PII phospholipids (7). Therefore, we propose placing strain SK&F-AAD-216 in a new genus, Kibdelosporangium, as described above. It is extremely difficult to place this new genus in any of the currently recognized families of the order Actinomycetales. Chemotaxonomically, it is most closely related to the Nocardiaceae. However, no genus currently placed in this family produces sporangia or spore vesicles that in any way resemble the sporangiumlike structures of this new genus. Morphologically, Kibdelosporangium most closely resembles those genera of the Actinoplanaceae which form aerial mycelia. However, no genus currently placed in this family exhibits fragmentation of the substrate mycelium or has type IV cell walls. In addition, the morphological definition of the Actinoplanaceae is currently being questioned because the family already contains organisms that produce spores in at least two distinct types of sporangia (42, 50). Much further work, including DNA-DNA homology and DNA-ribosomal hybridization studies between Kibdelosporangium and representatives of other actinomycete families, will be necessary to determine the relationship of this new genus to existing actinomycete families. Meanwhile, Kibdelosporangium should be considered one of the several genera of the order Actinomycetales which are of uncertain familial placement.

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