MYCOLOGY

***Saccharopolyspora rectivirgula* from Quebec dairy barns: application of simplified criteria for the identification of an agent responsible for farmer's lung disease**

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Saccharopolyspora rectivirgula (*Micropolyspora faeni*) is one of the major agents responsible for farmer's lung disease, a form of hypersensitivity pneumonitis. It is frequently isolated from the air of contaminated barns. The identification of this actinomycete is difficult because most of its phenotypic characteristics are variable and classical tests are not easy to perform on actinomycetes. Fatty acid analysis is very useful for the identification of these strains, but is not available except in some research or reference laboratories. Morphological (microscopic and macroscopic observations), physiological and biochemical tests (growth properties; macromolecules degraded; citrate utilisation and acid production from carbohydrates; resistance to antibiotics, lysozyme and heat), cell wall and fatty acid analyses and IgG analyses with serum from patients with farmer's lung were performed on 12 environmental isolates presumed to be *S. rectivirgula* and two control strains of *S. rectivirgula*. From this, a simple and rapid scheme for the identification of this actinomycete is proposed: optimal growth temperature (55°C); colony appearance based on morphology (filamentous) and colour (beige to orange-brown); microscopic morphology (chains of spores on both aerial and substrate mycelium); growth on NaCl 10%; cell-wall analysis (type IV); and the verification of antibody response with serum from a patient with farmer's lung. This last criterion is important to confirm the immunogenicity of the strains identified as *S. rectivirgula*. This scheme provides an accurate and efficient way of identifying *S. rectivirgula* strains and evaluating exposure to this bacterium. The study shows the limited value and the lack of reproducibility of some classical biochemical tests.

Introduction

Thermophilic actinomycetes are important components of the microflora found in dairy barns. They contribute to the deterioration of hay, grain and straw stored at high moisture levels. Long recognised by microbiologists, these actinomycetes are frequently responsible for a common form of hypersensitivity pneumonitis called 'farmer's lung' [1]. *Saccharopolyspora rectivirgula* (*Micropolyspora faeni*) is the species most frequently responsible for this condition in Eastern Canada [2]. *S. rectivirgula* has also been associated with a respiratory

disease in horses known as heaves [3]. Environmental studies are frequently performed to measure *S. rectivirgula* exposure [4, 5].

The identification of actinomycetes is difficult as most of their differential characteristics are structural and identification requires fastidious techniques such as electron microscopy or slide culture. *S. rectivirgula* can be identified by a combination of tests. An identification scheme based on biochemical properties and microscopic and macroscopic observations has been proposed [6]. The re-assignment of the organism to the genus *Saccharopolyspora* from the genus *Faenia* and major characteristics associated with the species were described by Korn-Wensisch *et al.* [7].

When environmental samples are analysed, problems

Received 3 March 1998; revised version accepted 17 June 1998.

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with the identification of *S. rectivirgula* include variability in biochemical reactions, colony morphology and protein profiles. These parameters vary with age of the culture (personal observations) and with the different techniques used to perform biochemical and morphological analyses.

New techniques, such as polymerase chain reaction (PCR) with specific probes or monoclonal antibodies used for identifying bacteria are available for only some strains or species. Until these become widely available, classical phenotypic characterisation will continue to be used for species and strain identification.

The present report proposes a simple scheme to assist microbiologists in the identification of *S. rectivirgula* and gives the limits and usefulness of the classical identification tests for this thermophilic actinomycete.

Materials and methods

Environmental sampling and colony selection

Air samples were obtained with a six-stage Andersen sampler and with All-glass impingers (AGI-30) from 37 Quebec dairy barns. All sampling was done in the winter months when stored hay is fed to the animals and outside air intake is minimal to conserve heat. AGI samples were diluted in triplicate and plated on Tryptic Soy Agar (TSA; Difco). Andersen samplers were loaded with the same medium. Plates were incubated at 52°C for 5 days. Fifty-six colonies with beige to orange-brown colour and an actinomycete-like morphology (filamentous) were isolated [7]. Identical isolates were grouped according to colony morphology. This resulted in 12 groups showing little difference in colony colour and morphology. The following identification steps were performed on these 12 strains (see Table 1 for the designated numbers of the strains) and two *S. rectivirgula* reference strains, ATCC 15347 (type strain) and T-150 (provided by W. P. Kurup in 1983 before deposition to ATCC as the type strain). All identification steps were performed on freshly cultured strains started from the original strain kept at 4°C and, unless specified, plates were incubated on TSA at 52°C. The 12 strains came from 11 barns.

Morphology

Cultures were incubated for 7 days then colony morphology and aerial mycelium were described. Plates were observed directly by microscopy at $\times 650$ for spore chains, aerial and substrate mycelium.

Physiological and growth properties

Optimal growth temperature. TSA plates were inoculated with fresh cultures and incubated at 20°, 30°, 35°, 45°, 52°, 60° and 63°C. Growth intensity was evaluated after 7 days and quantified: – (no growth) to +++ (optimal growth).

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Growth in the presence of NaCl. TSA supplemented with NaCl 5% or 10% was inoculated at one point in the centre with fresh cultures. After 7 days, growth was observed (–, no growth to +++, optimal growth) and the presence (w) or absence (0) of white aerial mycelium was observed.

Adenine, xanthine, hypoxanthine and tyrosine hydrolysis. The tests were performed according to Gordon *et al.* [8] except that TSA was used as the basal medium [6]. Plates were incubated for 28 days and observed on days 7, 14, 21 and 28. The presence (+) or absence (0) of a zone of hydrolysis surrounding the growth was noted.

Starch hydrolysis. This test was performed according to Gordon and Mihm [9] except that TSA was used as the basal medium [6]. After incubation for 7, 14, 21 and 28 days, plates were flooded with Gram's iodine solution [6]. A clear zone (+) surrounding the growth indicated starch hydrolysis.

Tributylin hydrolysis. Tributyrin agar (Oxoid) was used to determine this property. Plates were incubated for 28 days. The presence (+) or absence (0) of a zone of hydrolysis surrounding the growth was noted.

Casein hydrolysis. Methods described by Gordon *et al.* [8, 10] were used for the casein hydrolysis test. Plates were inoculated with an abundant inoculum and incubated for 28 days. The presence (+) or absence (0) of a zone of hydrolysis surrounding the growth was noted.

Gelatin hydrolysis. TSA supplemented with gelatin 1% [7] was used. Plates were streaked once with abundant inoculum. After incubation for 7, 14 and 21 days, plates were flooded with Frazier reagent [9]. The presence (+) or absence (0) of a zone of hydrolysis surrounding the growth was noted.

Aesculin and arbutin degradation. For aesculin and arbutin degradation, 1 g of aesculin or arbutin and 0.5 g of ferric citrate were added to 1 L of TSA [6]. The inoculated plates were incubated for 15–21 days. A black-brown zone (+) appearing around the growth indicated degradation.

DNAase activity. DNAase Test Agar (Difco) was used to demonstrate DNAase activity. After incubation for 7 and 14 days at 52°C, plates were flooded with 1 N HCl. This acid forms a precipitate with nucleic acids. If DNA was hydrolysed, a clear zone appeared (+) around the growth.

Citrate utilisation. The citrate utilisation test was performed as described by Simmons [11] and tubes

were incubated for 7 days. Utilisation of citrate resulted in alkalinisation of the medium and the appearance of a blue colour (+).

Acid production and utilisation of carbohydrates as sole carbon source

Acid production and utilisation of carbohydrates as sole carbon source was investigated according to Gordon and Smith [10]. Growth accompanied by acid production was determined.

Resistance to antibiotics, lysozyme and heat

Novobiocin. The novobiocin resistance test was performed according to Kurup *et al.* [6]; 300 μ l (for 30 μ g/ml concentration) or 1 ml (for 100 μ g/ml concentration) of a filter-sterilised (0.22 μ m) aqueous solution of novobiocin (10 mg/ml) was added to 100 ml of Tryptic Soy Broth (TSB; Difco) and 5-ml volumes were inoculated with 50 μ l of a fresh (4-day) culture grown in TSB plus glycerol 7%. Growth in the presence of novobiocin was evaluated (– or +) after 14, 21 and 28 days.

Lysozyme. Lysozyme resistance was determined according to Kurup *et al.* [6], Korn-Wendish *et al.* [7] and Gordon [12]. A 5-ml volume of 0.01 N filter-sterilised (0.22 μ m) lysozyme 100 mg in 100 ml of 0.01 N HCl solution was added to 100 ml of TSB plus glycerol 7%. These 5-ml volumes of broth were inoculated with 50 μ l of a fresh (4-day) culture grown in TSB plus glycerol 7%. Growth in the presence of lysozyme was observed or not (– or +) after 14, 21 and 28 days.

Sensitivity to ampicillin, chloramphenicol, gentamicin, tobramycin and penicillin G. Sensitivity to ampicillin (10 μ g/disk), chloramphenicol (30), gentamicin (10), tobramycin (10) and penicillin G (10 units) was determined by the Kirby-Bauer method [13]. Mueller-Hinton medium was inoculated with a broth culture (MacFarland standard 0.5). Growth inhibition zones around antibiotic disks were measured after 24 and 48 h.

Heat resistance. To measure heat resistance, TSA plates were inoculated with liquid cultures (TSB) that had been boiled for 10 or 20 min. Plates were incubated for 10 days and growth was observed or not (– or +).

Cell-wall analysis

Sugars and diaminopimelic acid (DAP) isomers were identified by thin-layer chromatography by the method described by Stanek *et al.* [14].

Cellular fatty acid (CFA) composition analysis

Fatty acid analysis was performed according to the method described previously [15]. Fatty acids were quantified, identified and data were integrated, calculated, stored, reported and organised by Microbial Identification System (MIS) LGS software (Hewlett-Packard, Microbial Id). Sheep blood agar was used as the growth medium and cultures were harvested after incubation at 50°C for 48 h. *Saccharomonospora viridis* (ATCC 15386), *Saccharomonospora cyanea* (ATCC 43724), *Saccharothrix australiensis* (ATCC 31497), *Saccharothrix* sp. (house strain), *Saccharopolyspora erythrea* (ATCC 11635) and *Saccharopolyspora* sp. (house strain) were used as comparative control strains for fatty acids analysis.

Specific IgG measurements

S. rectivirgula strains were grown in TSB for 5 days with agitation; 5 ml of the cell suspension were centrifuged (3000 g) and the pellet was washed with sterile water. Cell extract was prepared as described by Moore *et al.* [16]: 150 μ l of Tris-chloride buffer (0.5 M, pH 7.0) and 0.15 g of 170–180- μ m glass beads were added to the cell pellet that was resuspended and then frozen (24 h at –20°C). Samples were then put on ice until thawed and agitated by vortex mixing (maximal speed) for 5 min and kept on ice. Homogenates were centrifuged (3000 g) and the supernates were kept on ice. Protein contents of glass bead extracts were measured by Bradford protein assay (BioRad). For each of the 12 environmental strains and two reference strains, 100 μ l of 25, 50 or 100 μ g protein/ml extracts were deposited in ELISA plate wells. Plates were saturated with bovine serum albumin (BSA). Sera from a patient with farmer's lung and from an asymptomatic farmer (normal farmer with positive antibodies against *S. rectivirgula*) and serum from a normal unexposed volunteer were used for analysis of antigenic response. Serum dilutions (1 in 250, 1 in 500 and 1 in 1000) were incubated in protein-coated wells. Horseradish peroxidase coupled to anti-human IgG was used to measure the antibody recognition of the strains. OPD (Sigma) was used as the substrate and optical densities were read at 490 nm.

Results

All isolates from dairy barns grew on TSA at 35–63°C with optimal growth at 52°C (Table 1). The colour range of the colonies was beige to yellow-brown. They grew well in the presence of NaCl 5 and 10% and, in these conditions, produced abundant white aerial mycelia. Aerial mycelia produced chains of spores (usually 5–20) as well as the substrate mycelia. Isolates were not acid-fast and were gram-positive (with some regions of the mycelium showing a gram-negative reaction).

Table 1. Growth temperatures and cell-wall analysis of 12 environmental isolates and two *S. rectivirgula* control strains

Conditions	Growth of strain no.*													
	24.1.4	24.1.4A	9.2.1	4.1.2.1.1	A.36.1	A.7.6.1	A.5.3.1	20.1.1	A.35.1	18.2.1	12.2.2	A.21.4.2	T-150	15347 ATCC
20°C	—	—	—	—	—	—	—	—	—	—	—	—	—	—
30°C	—	—	—	—	—	—	—	—	—	—	—	—	—	—
35°C	+	+	+	+	+	+	+	+	+	+	+	+	+	+
45°C	+	+	+	+	+	+	+	+	+	+	+	+	+	+
52°C	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
60°C	++	+	+	++	++	+	++	++	++	++	+	++	+++	+
63°C	+	+	+	+	+	+	—	+	+	+	+	++	+	++
NaCl 5%†	++/w	++/w	++/w	++/w	++/w	++/w	++/w	++/w	++/w	++/w	++/w	++/w	++/w	++/w
NaCl 10%	+/w	+/w	+/w	+/w	+/w	+/w	+/w	+/w	+/w	+/w	+/w	+/w	+/w	+/w
Cell wall	IV‡	IV	IV	IV	IV	IV	IV	IV	IV	IV	IV	IV	IV	IV

*Growth intensity: — to +++.

†Growth (— to +++: growth intensity)/white aerial mycelium present or absent (w or —).

‡IV, meso-DAP, arabinose and galactose.

Table 2. Physiological properties of the 12 environmental isolates and the two control *S. rectivirgula* strains

Substrate	Degradation by strain no.													
	24.1.4	24.1.4A	9.2.1	4.1.2.1.1	A.36.1	A.7.6.1	A.5.3.1	20.1.1	A.35.1	18.2.1	12.2.2	A.21.4.2	T-150	15347 ATCC
Xanthine	+	+	+	+	+	+	+	+	—	+	+	+	+	+
Hypoxanthine	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Tyrosine	—	—	+	+	—	+	+	+	+	+	+	+	+	—
Adenine	—	—	—	—	—	—	—	—	—	—	—	—	—	—
DNA	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Arbutin	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Aesculin	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Gelatin	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Starch	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Tributylin	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Nitrate reduction	—	—	—	—	—	—	—	—	—	—	—	—	+	+

+, positive reaction; —, negative reaction.

All isolates had a type IV cell wall (arabinose, galactose and *meso*-DAP) (Table 1). None of the isolates produced haemolysins nor did they hydrolyse starch or adenine. Casein, gelatin, arbutin, aesculin, xanthine, hypoxanthine and tributyrin were hydrolysed by all strains. Three strains failed to hydrolyse tyrosine. Citrate was used as sole carbon source by all strains. Nitrates were not reduced by the environmental isolates, but ATCC strains showed a variable positive reaction (Table 2). These results are consistent with those described previously by Korn-Wendisch *et al.* [7].

Utilisation and production of acid from carbohydrates gave the most inconsistent results (Table 3). These tests were performed three times and the results obtained varied. All isolates were susceptible to all antibiotics tested, to lysozyme and to heat.

According to the CFA analysis, all 14 isolates (including strains ATCC 15347 and T-150) showed a degree of variation similar to that expected for members of the same species when principle component analysis was performed. The 14 isolates clustered together and could be readily discerned from the other closely related actinomycetes used as controls. Qualitatively, the CFA composition of all isolates was like those described previously [7]. The dendrogram (Fig. 1) shows the 12 study isolates and two control strains and the other closely related actinomycetes used as controls.

The serum reactivity test showed that all the environmental isolates possessed antigens recognised by antibodies from the patient with farmer's lung and the asymptomatic farmer, but were not recognised by antibodies from the normal control (Fig. 2).

Discussion

This study shows that simple and rapid procedures can be used to identify *S. rectivirgula*. The most useful tests and procedures are: incubation temperature (52–55°C); growth of beige to orange-brown colonies; chains of spores both on aerial and substrate mycelium; growth on NaCl 10%; confirmation of the type IV cell wall; and production of white aerial mycelium. Presumptive identification of *S. rectivirgula* can be made on these criteria.

The following steps should be taken to isolate *S. rectivirgula* from environments where its presence is being investigated. The first selection step is incubation on TSA at 52–55°C; this eliminates a large number of actinomycetes but, according to Bergey's Manual of Determinative Bacteriology (9th edn) [17], the following species and genera can be cultured under these conditions: *Microbispora* spp., *Microtraspura* spp., *Actinomadura* spp., *Thermomonospora*

Table 3. Acid production from carbohydrates for the 12 environmental isolates and the two control *S. rectivirgula* strains

Substrate	Acid production by strain no.													
	24.1.4	24.1.4A	9.2.1	4.1.2.1.1	A.36.1	A.7.6.1	A.5.3.1	20.1.1	A.35.1	18.2.1	12.2.2	A.21.4.2	T-150	15347 ATCC
Glucose	A	A	A	A	A	A	A	A	A	A	A	A	A	A
Inositol	A	a	A	A	A	A	A	A	A	A	A	A	A	A
Mannitol	A	A	A	A	A	A	A	A	A	A	A	A	A	A
Xylose	A	a	A	A	a	A	A	A	A	a	A	a	a	a
Fructose	A	A	a	a	a	a	A	A	a	a	a	—	—	—
Lactose	A	A	A	A	A	A	A	A	A	A	A	A	a	A
Erythritol	A	A	A	A	A	A	A	A	A	A	A	A	A	A
Galactose	A	A	A	A	A	A	A	A	A	A	A	A	A	A
Glycerol	A	A	A	A	A	A	A	A	A	A	A	A	A	A
Mannose	A	a	—	—	A	A	A	A	—	—	—	—	a	—
Trehalose	A	A	A	A	A	A	A	A	A	A	A	A	A	A

A, high acid production; a, low acid production; —, no acid production.

A, high acid production; a, low acid production; —, no acid production.

Fig. 1. Dendrogram showing the relative distance among the 12 environmental isolates and two control strains of *S. rectivirgula* and control actinomycetes according to their fatty acid composition.

spp., *Thermoactinomyces* spp., *S. hordei* and *S. rectivirgula*.

The second step consists of selection of colonies according to morphology and colour: (beige to orange-brown). Colour selection enables the inclusion of *S. rectivirgula* colonies and eliminates other species, although some thermoactinomycetes could still be included.

Cell-wall analysis should be performed as the third

step. Thermophilic species with type IV cell wall are present only in the family Pseudonocardiaceae: *Actinopolyspora* (not able to grow at 55°C), *Pseudonocardia*, *Saccharomonospora*, *Saccharopolyspora*. All other thermophilic actinomycetes have a type III cell wall [17]. As *Saccharomonospora* spp. typically produce a blue-green aerial mycelium and pigment at 50°C [18], these cannot be confused with *Saccharopolyspora* spp. Finally, cultivation of strains on NaCl 10% eliminates *Micromonospora* (does not produce chains of spores), *S. hirsuta* (will not produce

Fig. 2. OD obtained when IgG response specific to 14 strains (12 environmental and two control strains) was measured in the serum of a normal subject, an asymptomatic seropositive farmer and a patient with farmer's lung. $\diamond$ ATCC 15347; $\oplus$ T-150.

spores on vegetative mycelium) and *S. hordei* (does not produce aerial mycelium). The observation of chains of spores on both aerial and substrate mycelia confirms strains as *S. rectivirgula*.

Fatty acid analysis showed the degree of variation expected for members of the same species. Qualitatively, the averaged data were similar to those found with other members of the genus *Saccharopolyspora* as well as consistent with those described by Korn-Wendisch *et al.* [7]. In the dendrogram (Fig. 1), all strains were grouped with a Euclidian distance of <11 , interpreted to be species level [16]. Two-dimensional analysis of PC1 versus PC2 and PC1 versus PC3 indicated that these strains clustered together at species level and away from other aerobic actinomycetes tested (data not shown). As shown in Fig. 2, all 12 environmental isolates reacted similarly with serum from a patient with farmer's lung and an asymptomatic farmer. These results show that all the environmental isolates possessed the antigens to which the patients were responding. The confirmation of this reaction is important as the purpose of identification and enumeration of environmental *S. rectivirgula* is to quantify the antigenic burden of air sampled and the related risks for a patient.

Physiological properties such as carbohydrate utilisation and acid production pattern have been recommended for identification [6], but in the present study they were found to be time-consuming, difficult to reproduce and incapable of providing a rapid and definitive identification of *S. rectivirgula* strains. Other tests, including macromolecular degradation and antibiotic resistance were of little use to discriminate *S. rectivirgula* strains from other actinomycetes. All isolates were susceptible to the antibiotics. Reactivity

to serum from a patient with farmer's lung and an asymptomatic farmer showed a specific IgG response of the same magnitude for all isolates. It is important to confirm that environmental strains identified as *S. rectivirgula* possess the same antigenic potential as deposited strains. A bacterium could potentially be biochemically and morphologically identical to *S. rectivirgula* but not of medical importance.

This approach to the identification of thermophilic actinomycetes could be generalised to other important environmental and medical species such as *Thermoactinomyces vulgaris*. Such simplified and accurate identification schemes should be established for other frequently isolated species.

This work was supported by the Institut de Recherche en Santé et Sécurité du Travail du Québec (IRSST). C.D. acknowledges the IRSST. We thank Judith Winstansley for her contribution to this work.

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