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Thermophilic and Mesophilic Actinomycetes in Mouldy Hay

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SUMMARY

Actinomycetes isolated at 40° and/or 60° from mouldy hay included: *Micromonospora vulgaris* Waksman *et al.*, *Thermopolyspora polyspora* Hens., *T. glauca* sp.nov., *Streptomyces thermoviolaceus* Hens., *S. fradiae* (Waksman et Curtis) Waksman et Henrici, *S. griseoflavus* (Krainsky) Waksman et Henrici, *S. olivaceus* (Waksman) Waksman et Curtis, and *S. griseus* (Krainsky) Waksman et Henrici.

INTRODUCTION

Mouldy hay associated with farmer's lung disease contained large quantities of actinomycete spores; over 10^9/g. dry weight hay were reported by Gregory & Lacey (1963*a*). This paper describes the commonest species and gives some information about their occurrence in different grades of hay.

METHODS

Actinomycetes were isolated with the aid of the Andersen sampler (Andersen, 1958) which was loaded with Petri dishes containing medium and suspended horizontally in a small wind tunnel while hay was shaken up-wind in a perforated drum (Gregory & Lacey, 1962, 1963*a*, *b*).

Three media were used for isolation. For incubation at 40° 0·5 mg. actidione/ml. was added to suppress mould growth.

Half-strength nutrient agar contained 14 g. 'Oxoid' nutrient agar granules, 10 g. agar, 1 l. water. *Yeast extract agar* (Pridham *et al.* 1957) contained 4 g. 'Difco' yeast extract, 10 g. malt extract, 4 g. glucose, 20 g. agar, 1 l. water; adjusted to pH 7·3 with KOH. *V8 agar* (Galindo & Gallegly, 1960) contained 200 ml. 'V8' vegetable juice, 4 g. calcium carbonate, 20 g. agar, 800 ml. water; adjusted to pH 7·3 with KOH.

After exposure in the Andersen sampler, Petri dishes were incubated at 40° ($\pm 1°$) and 60° ($\pm 2°$). Colonies were counted after 2, 4 and 8 days. Many isolates were studied on different media and at different temperatures. *Peptone iron agar* (Tresner & Danga, 1958) containing 36 g. peptone iron agar, 1 g. yeast extract, 1 l. water, was used for the melanin test.

RESULTS

Although very many actinomycete colonies developed on the isolation plates, the number of different species was small: seven grew at 40°, of which three also grew at 60°. At both temperatures a few colonies without aerial mycelium developed, but

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most of these showed good growth with aerial mycelium at lower temperatures. These included *Streptomyces griseus* (Krainsky) Waksman et Henrici; others have not yet been identified.

Taxonomy

The taxonomy of thermophilic actinomycetes is now in a state of flux, but like other workers (Tendler, 1959; Erikson, 1952; Henssen, personal communication to P.H.G., 1961) we do not regard thermophily as a good taxonomic character at the generic level. Both thermophilic and mesophilic species are included in the genus *Streptomyces* and we also admit thermophilic species in the genus *Micromonospora*. Tendler (1959) has shown that ability to grow at different temperatures is a function of nutritional requirements rather than a specific character. As morphology seems of primary importance in this group, we follow the classification of Ettlinger *et al.* (1958) for the identification of *Streptomyces*. In the following list those isolates examined in detail are indicated by 'A' and number. Typical cultures have been deposited at the Centraalbureau voor Schimmelcultures (CBS), Baarn, Netherlands, and Eidgenössische Technische Hochschule (ETH), Zürich, Switzerland.

Micromonospora vulgaris Waksman *et al.* Aerial mycelium abundant, cottony, white to light lavender; aerial hyphae mostly simple, emerging from the medium and often dipping back in it after a short arch over the agar (Pl. 1, fig. 4); they seem to empty when the spores are formed. Substrate mycelium light brown, sometimes darker. Spores globose, typically sharp-cornered, 0·5–0·8 μ (Pl. 1, fig. 5*b*), single, sessile (Pl. 1, fig. 5*a*). No reaction on peptone iron agar. Good and rapid growth at 40° and 60°, no growth at 28°. Over 100 isolates were examined which appeared to belong to this species. Their sessile spores disagree with the description of *M. vulgaris*, but with present knowledge of the importance and stability of the sporophore this character seems insufficient for the proposal of a new species. Isolates examined: A64 (CBS 109.62; ETH 31509), A65, etc.

Thermopolyspora polyspora Hens. Colony yellow. Aerial hyphae whitish, mostly simple, bearing lateral chains of 1 to 10 spores (Pl. 1, fig. 6). Spores globose (Pl. 1, fig. 7) 0·8–1·3 μ; also formed in medium. No reaction on peptone iron agar. No growth at 28°, slow vegetative growth at 40°, and slow vegetative and aerial growth and sporulation at 60°. Isolates examined: A94 (CBS 100.63; ETH 31520), A88, A89, A90, A91, A92, A95.

Thermopolyspora glauca sp.nov. Aerial mycelium abundant, first white, becoming greenish blue and later blue grey; aerial hyphae straight, mostly simple, bearing short lateral spore chains (Pl. 1, figs. 1–3). Vegetative mycelium dark green. Spores oval, 0·8–1·3 × 0·6–0·8 μ, often in pairs, sometimes single, also in chains of 3 or 4; some spores can be found in the medium. No reaction on peptone iron agar. Most isolates were without diffusible pigment but some produced black and some dark brown pigments. Growth very slow but good at 40°, and colonies were first recognizable after 5 or 6 days incubation on nutrient agar. Growth with aerial mycelium also occurs at 28°, but at 60° there is little or no vegetative growth. Type culture: A66 (CBS 110.62; ETH 28797). Other isolates examined: A67, A68, A69, A70.

Henssen (1957) described a bluish thermophilic actinomycete with 4–10 spores in chains under the name of *Thermoactinomyces glaucus* Hens. *T. monosporus* (Schütze) Waksman *et al.* (cf. *Bergey's Manual*, 1948) and *T. viridis* Schuurmans, Olson &

San Clemente (1956) have both single spores and bluish aerial mycelium. Cultivated on various media our isolates never showed more than 4 spores; we therefore consider them as a new species of the genus *Thermopolyspora* Hens. (Micropolyspora Lechevalier, Solotorovsky & McDurmont (1961), described four years later, is considered to be a synonym of Thermopolyspora.)

Streptomyces thermoviolaceus Hens. The description given by Henssen is completed as follows. Aerial mycelium first brown, later ash grey, not abundant, with open loops (Pl. 1, fig. 8) (cf. Henssen, 1957, figs. 7, 8). Spores oval to cylindrical, 0·8–1·2 × 0·6–0·8 μ, surface covered with small hemispherical particles, 0·03 μ (Pl. 1, fig. 9), which are reported for the first time. No reaction on peptone iron agar. Growth rapid and excellent at 40°, good at 60°, and slow and usually without aerial mycelium at 28°. A few colonies (under 1 %) had an intense violet diffusible pigment, but as the aerial mycelium and spore morphology are identical with the other isolates they are classified under the same species. Isolates examined: A71 (CBS 111.62; ETH 28745), A73, A74.

Streptomyces fradiae (Waksman et Curtis) Waksman et Henrici. Aerial mycelium abundant, woolly, pink. Vegetative mycelium yellowish. Spore chains in open spirals (Pl. 2, fig. 10), monopodial branching with a long straight axis. Spores smooth (Pl. 2, fig. 11), 0·7–1·0 × 0·4–0·6 μ. No reaction on peptone iron agar. Good growth at 40°, slower at 28°, and no growth at 60°. Isolates examined: A75 (CBS 112.62; ETH 28746), A76.

Streptomyces griseoflavus (Krainsky) Waksman et Henrici. Aerial mycelium abundant, ash grey. Spore chains in regular spirals (Pl. 2, fig. 12), monopodial branching. Spores 0·8–1·1 × 0·5–0·7 μ, with short (about 0·2 μ) spines (Pl. 2, fig. 13). No reaction on peptone iron agar. Good growth at 40° and 28°, no growth at 60°. Isolates examined: A77 (CBS 113.62; ETH 31510), A78.

Streptomyces olivaceus (Waksman) Waksman et Curtis. Aerial mycelium abundant, ash grey. Spore chains in straight hyphae (Pl. 2, fig. 14), monopodial branching. Spores smooth, 0·4–0·6 × 0·7–1·0 μ (Pl. 2, fig. 15). No reaction on peptone iron agar. Good growth at 40° and 28°, no growth at 60°. Isolate examined: A79 (CBS 114.62; ETH 28748).

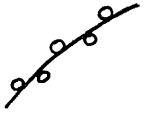
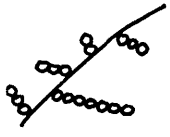
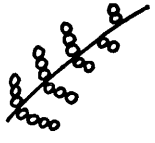
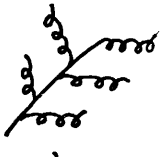
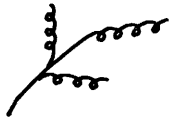
Table 1 gives in diagrammatic and tabular form the diagnostic features as an aid to identification.

Numbers of colonies on different media

Tables 2 and 3 show the importance of the medium for quantitative studies of the different species of actinomycetes. Except for sample W at 60°, many more colonies of *Micromonospora vulgaris* occurred on nutrient agar than on yeast agar or 'V8' agar; the opposite is true for *Streptomyces thermoviolaceus* and *S. griseoflavus*. More colonies of *Thermopolyspora glauca* developed on nutrient agar than on either yeast or 'V8' agars. More colonies of *S. fradiae* developed on yeast agar than on nutrient agar, but 'V8' agar gave fewer colonies than nutrient agar. Although the occurrence of *T. polyspora* was not recorded in the tests on which Tables 2 and 3 are based, its abundance in self-heated mouldy hay was similar to *M. vulgaris*. Both have been isolated thousands of times and are particularly characteristic of hay associated with farmer's lung disease.

Good hay contains few actinomycetes. In mouldy hays, which have obviously heated, actinomycetes are very abundant, particularly the thermophilic species

Table 1. *Diagnostic features of seven thermophilic actinomycetes*

Species	Spores		Colonies		Temperature and growth rate
Micromonospora vulgaris A64	Single	 Globose		Flat, large; aerial myc. light pink, veg. myc. brown	 **40–60°,*** fast
Thermopolyspora polyspora A94	Short chains (3–10 spores)	 Globose		Very small; aerial myc. whitish, veg. myc. yellow	 *40–60°*, slow
T. glauca A66	Short chains (2–4 spores)	 Oval, smooth		Small; aerial myc. blue-green, veg. myc. dark green	 (28)–**40°**, slow
Streptomyces thermoviolaceus A71	In long loops	 Oval with hemispherical particles		Large; aerial myc. ash grey, veg. myc. dark	 (28)–**40**–*60°*, fast
S. fradiae A75	Spirals on long straight hyphae	 Oval, smooth		Middle never flat; aerial myc. woollish, pink, veg. myc. yellow	 **28–40°**, medium
S. griseoflavus A77	Spirals	 Oval, spiny		Flat; aerial myc. ash grey, veg. myc. ± grey	 **28–40**–*55°*, medium
S. olivaceus A79	Straight hyphae	 Oval, smooth		Flat; aerial myc. ash grey, veg. myc. yellowish	**28–40°**, medium

* (28°), only slight growth; 28°, poor growth; *55°*, fairly good growth; **40°**, optimum growth.

Table 2. *Number of colonies on six Andersen sampler plates exposed to different hay samples shaken in the wind tunnel*

Incubated at 60°.

Hay sample reference number	Exposure time (sec.)	Sample weight (g.)	Medium*	*Micromonospora vulgaris* No. colonies	*Streptomyces thermoviolaceus* No. colonies
H 65. Farmer's lung hay	5	41	N	46	4
			Y	31	47
H 65. Farmer's lung hay	5	—	N	161	2
			V 8	22	39
F. Mouldy hay	5	39	N	236	15
			Y	181	545
F. Mouldy hay	5	—	N	192	1
			V 8	22	17
W. Mouldy hay	5	52	N	101	6
			Y	134	218
G. Good hay	15	41	N	61	0
			Y	4	6
SB. Good hay	15	38	N	22	2
			Y	6	9

* N = Nutrient agar, Y = yeast extract agar, V 8 = V 8 agar.

Table 3. *Number of colonies on six Andersen sampler plates exposed to different hay samples shaken in the wind tunnel*

Incubated at 40°. Pairs of results were from the same experiment.

Hay sample reference number	Exposure time (sec.)	Sample weight (g.)	Medium†	*Micromonospora vulgaris*	*Thermopolyspora glauca*	*Streptomyces fradiae*	*Streptomyces** with greyish aerial mycelium
H 65. Farmer's lung hay	5	41	N	27	10	2	13
			Y	3	5	4	61
H 65. Farmer's lung hay	5	—	N	30	20	21	2
			V 8	1	1	4	94
H 44. Farmer's lung hay	5	30	N	*c.* 700	69	4	89
			Y	30	0	22	*c.* 900
H 44. Farmer's lung hay	5	—	N	431	52	12	27
			V 8	0	1	26	337
F. Mouldy hay	5	39	N	*c.* 495	41	8	28
			Y	50	2	14	*c.* 430
F. Mouldy hay	5	—	N	18	2	13	5
			V 8	1	0	8	108
W. Mouldy hay	5	52	N	105	20	5	9
			Y	39	2	21	*c.* 400
G. Good hay	15	41	N	12	0	1	1
			Y	7	0	3	7
SB. Good hay	15	38	N	19	3	1	4
			Y	3	0	2	7

* Mainly *Streptomyces thermoviolaceus* and *S. griseoflavus*.
† N = Nutrient agar, Y = yeast agar, V 8 = V 8 agar.

(Gregory & Lacey, 1963*a*, Table 3), where grey colonies were not counted at 60° because only nutrient agar was used.

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EXPLANATION OF PLATES

Electron micrographs of spores of Actinomycetes from mouldy hay; and photomicrographs showing aerial growth at edge of colony (photographed in air).

PLATE 1

Fig. 1. *Thermopolyspora glauca*, edge of colony. ×375.

Fig. 2. *T. glauca*, spore formation. ×13,500.

Fig. 3. *T. glauca*, spores. ×13,500.

Fig. 4. *Micromonospora vulgaris*, edge of colony. ×375.

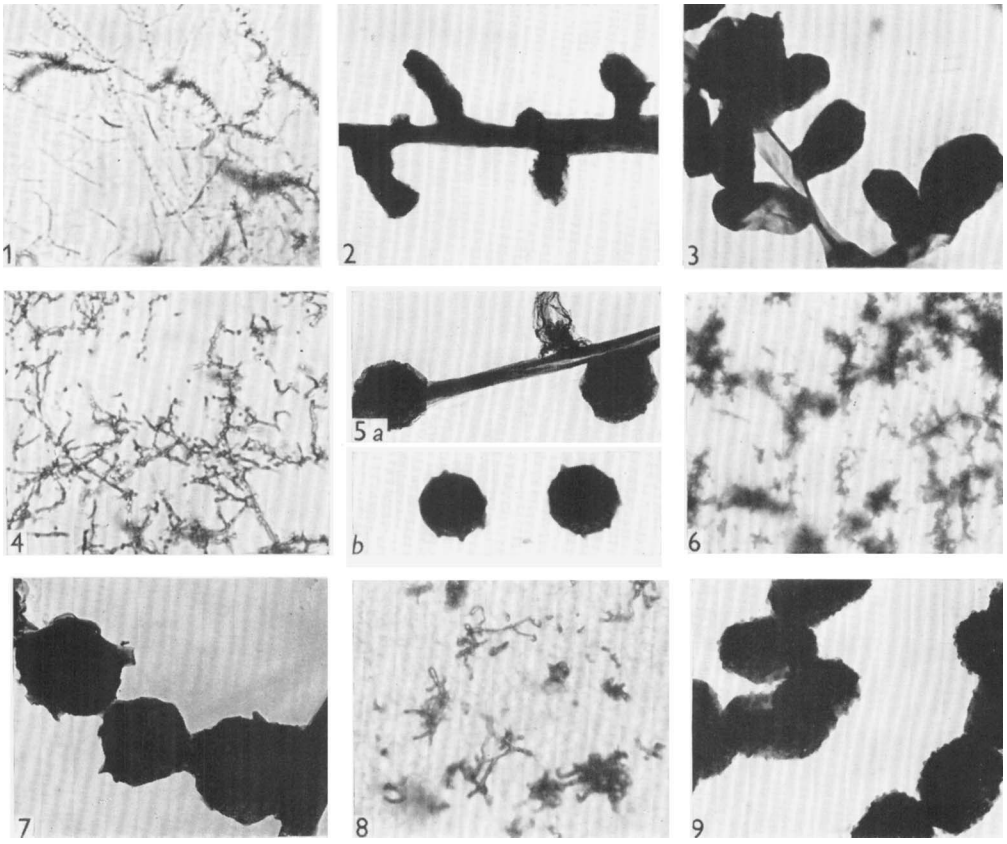
Fig. 5. *M. vulgaris*, spores. ×13,500.

Fig. 6. *Thermopolyspora polyspora*, edge of colony. ×375.

Fig. 7. *T. polyspora*, spores. ×13,500.

Fig. 8. *Streptomyces thermoviolaceus*, edge of colony. ×375.

Fig. 9. *S. thermoviolaceus*, spores. ×13,500.



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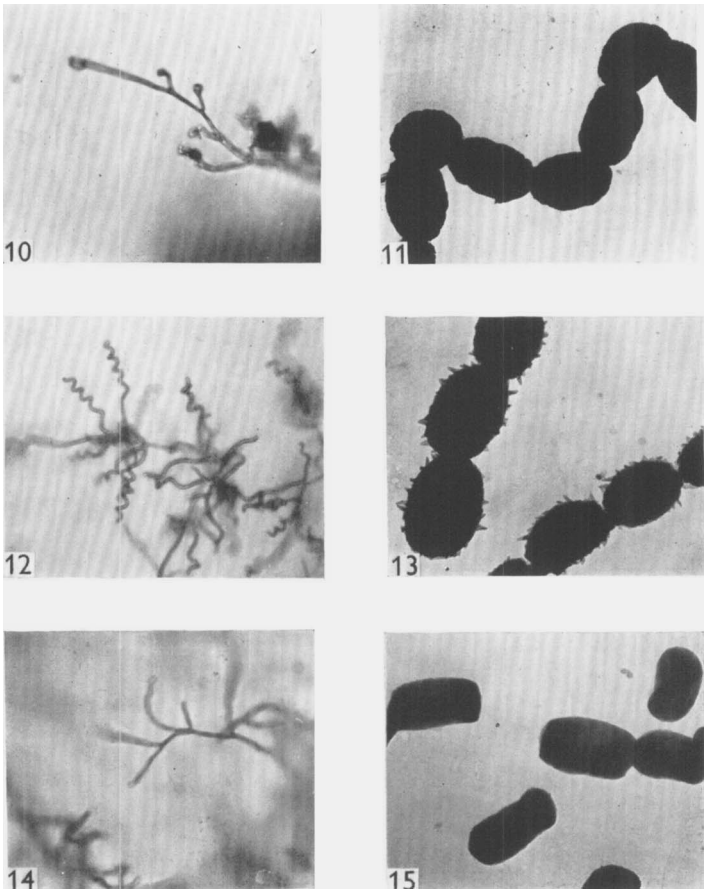
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PLATE 2

Fig. 10. *S. fradiae*, edge of colony. ×375.

Fig. 11. *S. fradiae*, spores. ×13,500.

Fig. 12. *S. griseoflavus*, edge of colony. ×375.

Fig. 13. *S. griseoflavus*, spores. ×13,500.

Fig. 14. *S. olivaceus*, edge of colony. ×750.

Fig. 15. *S. olivaceus*, spores. ×13,500.