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Rhodococcus coprophilus sp. nov.: An Aerobic Nocardioform Actinomycete Belonging to the '*rhodochrous*' Complex

By T. J. ROWBOTHAM AND T. CROSS
Postgraduate School of Studies in Biological Sciences, University of Bradford, Bradford BD7 1DP

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SUMMARY

The aerobic nocardioform actinomycete, found to be common in herbivore dung and aquatic habitats and previously given the trivial name Lspi, was studied along with representative strains of taxa within the '*rhodochrous*' complex. Lspi strains were recovered as a homogeneous cluster which separated from the other reference strains at the 69 % similarity level in a numerical taxonomic analysis. The specific name *Rhodococcus coprophilus* is proposed for this new species. We also consider the generic name *Rhodococcus* Zopf to be appropriate for most of the species currently accommodated within that rather ill-defined taxon known as the '*rhodochrous*' complex and variously classified within the genera *Nocardia*, *Mycobacterium*, *Jensenia*, *Corynebacterium* or *Gordona*.

INTRODUCTION

Willoughby (1969) described three aerobic nocardioform actinomycetes which he isolated from Blelham Tarn, Cumbria, and its environs. One morphological type, termed 'Large spored pink irregular' (Lspi), was the most common nocardioform isolated from the aquatic habitats; its frequency in lake water and mud was confirmed by Johnston & Cross (1976).

Strains now recognized as Lspi were included by Goodfellow (1971) in his numerical taxonomic study of nocardioform bacteria. Two were recovered in his *Nocardia asteroides* cluster, subgroup 1B; the other three strains clustered within the '*rhodochrous*' complex. However, our preliminary studies on the morphology and biochemistry of Lspi strains indicated that they were very similar to each other and belonged to the '*rhodochrous*' complex. In order to further define and classify Lspi strains, we undertook a numerical taxonomic study of our isolates together with related nocardioforms, chosen from the main '*rhodochrous*' clusters recovered by Goodfellow (1971), and species of the genus *Gordona* (Tsukamura, 1971).

METHODS

Strains. These are listed in Table 1. The majority of Lspi strains were isolated from lake water and mud samples on colloidal chitin agar (Cross & Attwell, 1974). Reference strains, from Dr M. Goodfellow, have been fully documented in previous publications (Goodfellow, 1971; Goodfellow & Orchard, 1974; Goodfellow *et al.*, 1974; Mordarski *et al.*, 1976). It was later realized that some strains of *Gordona*, received under different code numbers, were in fact the same strain. They were retained in the study because they had different histories and could indicate the reproducibility of our test methods.

Table 1. *Designations and sources of strains used in the numerical taxonomic study*

(*a*) Lspi strains

CUB118	Lake water, Blelham Tarn, Cumbria. Isolated by Cross & Collins (1966) during a study of *Micromonospora* in lake water
CUB193	
CUB194	
CUB196	
CUB628	Lake mud, Blelham Tarn. L. G. Willoughby, Freshwater Biological Association, Ambleside, Cumbria
CUB671	Lake mud, Blelham Tarn
CUB669	Lake mud, Hawksworth Mere, W. Yorkshire
CUB670	
CUB687	
CUB672	Canal water, Bingley, W. Yorkshire
CUB415	Air, A747. Isolated by J. Lacey from Anderson Sampler plates, exposed when hay bales were opened in the field
CUB686	Spontaneous white mutant of CUB193

(*b*) Other strains

CUB197	*Nocardia* sp. Lake water, Blelham Tarn (Cross & Collins, 1966)
CUB710	*Corynebacterium rubrum* NCIB9433
CUB673	*Nocardia corallina* Batt (Robertson & Batt, 1973)
CUB685	*Nocardia*-like soil bacterium, Lingens A1 (Bachofer, Oltmanns & Lingens, 1973)
CUB711	*N. corallina* A81 (Crawford *et al.*, 1973)
CUB642	*Nocardia erythropolis* N11, NCIB9158
CUB643	*Nocardia pellegrino* N324, ATCC15998
CUB644	*Nocardia opaca* N124, CBS330.61
CUB645	*Rhodococcus rhodochrous* N54, ATCC13808
CUB646	*Mycobacterium rhodochrous* (*N. erythropolis*) N108, ATCC4277
CUB647	*Jensenia canicruria* N53, NCIB8147
CUB674	*Nocardia calcarea* N41, NCIB8863
CUB675	*M. rhodochrous* N66, NCTC8139
CUB712	*N. pellegrino* N325
CUB713	*Nocardia salmonicolor* N5, NCIB9701
CUB677	*Gordona rubra* N655, NCTC10668
CUB679	*G. rubra* N657 (Mordarska T3), NCTC10668
CUB678	*Gordona terrae* N656, NCTC10669
CUB680	*G. terrae* N659 (Mordarska T5), NCTC10669
CUB683	*G. terrae* N662 (Mordarska T6)
CUB676	*Gordona bronchialis* N654, NCTC10667
CUB681	*G. bronchialis* N660 (Mordarska T1), NCTC10667
CUB682	*G. bronchialis* N661 (Mordarska T2)
CUB684	*Gordona aurantiaca* N663, NCTC10741

CUB, Actinomycete Culture Collection, University of Bradford; N, M. Goodfellow, University of Newcastle upon Tyne; NCIB, National Collection of Industrial Bacteria; ATCC, American Type Culture Collection; NCTC, National Collection of Type Cultures; CBS, Centraalbureau voor Schimmelcultures, Baarn, The Netherlands.

All strains were freeze-dried and working cultures were maintained on Bennett's agar.

Growth media and preparation of standard suspensions. Strains were routinely subcultured on Bennett's agar containing $(\mathrm{g\,l^{-1}})$: yeast extract (Difco), 1; beef extract (Lab Lemco powder, Oxoid), 1; Bacto Casitone (Difco), 2; glucose, 10; and agar, 18; final pH 7·3. Bacterial suspensions were grown in shaken flasks of Bennett's broth or of PYG broth, which contained $(\mathrm{g\,l^{-1}})$: Bacto peptone (Difco), 5; yeast extract (Difco), 5; glucose, 10; Casamino acids (Difco), 1; and NaCl, 5; final pH 7·2. All cultures were incubated at 30 °C. Bacteria for chemical analyses were treated with formaldehyde before washing and freeze-drying.

Standard bacterial suspensions for all growth and utilization tests were stored in semi-solid agar at 4 °C (Kützner, 1972). Strains were grown in Bennett's broth in shake flasks for

5 days, washed twice with distilled water, and resuspended to give an E_{660} value of 0·4. Samples (5 ml) of this suspension were added to screw-capped bottles each containing 5 ml warm (40 °C) water agar [Oxoid agar no. 3 (2·5 g l^{-1}) containing thiamin.HCl (8 mg l^{-1})]. Fresh inocula of most strains were prepared monthly; however, the viability of three Lspi strains (CUB669, CUB193 and CUB686) and one strain of *Gordona bronchialis* (CUB682) declined rapidly and, for these, fresh inocula were prepared as required.

The basal medium for utilization tests was that of Stevenson (1967), but with a higher concentration of K_2HPO_4 (3 g l^{-1}) to raise the pH. All test media had a final pH of 7·0. Most nocardioform bacteria tested required thiamin for growth and accordingly membrane-filtered thiamin.HCl solution was added at 4 mg l^{-1} to all media deficient in this vitamin.

Morphological characters. Plates of Bennett's agar were inoculated for single colonies and examined microscopically after 24 h with a × 40 (N.A. 0·57) long working distance objective (Vickers Instruments, York). The presence of aerial hyphae was noted and the microcolonies were divided into two categories: either predominantly composed of rods and branching fragments, or remaining essentially mycelial. After 3 days, smears were stained by Hucker's modification of the Gram stain (Conn, Bartholomew & Jennison, 1957). The diameter of isolated colonies was measured after 7 days and the presence of substrate mycelium, and rhizoid or asteroid colonies with a central papilla was noted. Tubes of Bennett's broth, incubated for 3 days, were examined for the presence of a pellicle. Strains with growth extending across the broth surface and up the sides of the tube were scored as positive, no surface growth or few isolated colonies as negative.

Chemotaxonomic characters. Mycolic acids were detected using a thin-layer chromatographic method which distinguishes between two of the lipid LCN-A (lipid characteristic of *Nocardia*) analogues, LCN-A type a (asteroides) and LCN-A type c (calcarea) (Mordarska, Mordarski & Goodfellow, 1972; Minnikin & Goodfellow, 1976). The isomers of diaminopimelic acid in whole-cell hydrolysates were detected by paper chromatography (Becker, Lechevalier & Lechevalier, 1965) using the solvent system methanol/water/pyridine/98 % (v/v) formic acid (80:19:10:1, by vol.) (Perkins, 1965). Sugars were detected by paper chromatography using the solvent system butan-1-ol/pyridine/water/toluene (5:3:3:4, by vol.) (Lechevalier, 1968). Freeze-dried preparations of *Nocardia asteroides* NCTC8595, *N. brasiliensis* ATCC19296, *Rhodococcus rhodochrous* CUB645 and *Streptomyces griseus* CUB575 were used as reference standards.

Utilization of organic compounds as sole sources of carbon and energy. Test compounds (listed in Tables 2 and 3) in the solidified basal medium in divided polystyrene Replidishes (Sterilin, Richmond, Surrey) were inoculated with standard suspensions by means of a multipoint inoculator (Denley Instruments, Bolney, Sussex). Plates containing basal medium only and basal medium plus 0·1 % (w/v) glucose were also inoculated as controls. Results were recorded after 1, 2 and 3 weeks incubation. Tests were performed once but were repeated if the results were inconclusive. A positive result was registered if growth on the test medium was greater than on the control medium without a carbon source, and negative where growth was less than that on the basal medium plus glucose.

Thiamin requirement and growth on wax. Bacteria grown in Bennett's broth were washed and resuspended for 40 h in a glucose/nitrate mineral salts starvation medium lacking thiamin. The resultant suspension was washed repeatedly, resuspended in phosphate buffer pH 7·0 to give $E_{660} = 0{\cdot}2$, and 0·05 ml samples were then added to 10 ml McClung's (1960) carbon-free mineral salts medium in Universal bottles containing wax-coated glass slides (12 × 75 mm). Duplicate bottles containing added thiamin (4 mg l^{-1}) were also inoculated. After 3 weeks incubation in the dark, slides were compared. Growth on the paraffin wax in the thiamin supplemented medium, which usually occurred at the air/liquid/wax interface, and little or no growth on the control slide was taken to indicate a requirement for thiamin. Slide cultures were incubated for a further week, one set in the dark and another in continuous bright light in order to detect any increase in pigmentation indicating partial photochromogenicity.

This method was also used to compare the ability of strains to grow on paraffin wax, cetyl alcohol and stearic acid as sole carbon sources.

Resistance to inhibitory agents. The effects of NaCl, sodium azide and *p*-nitrophenol in Bennett's agar were recorded after 3 weeks incubation.

Hydrolysis of insoluble organic compounds. Agar media, containing a suspension of the test compound, were inoculated with 0·05 ml standard suspension and examined for clear zones around the inoculum after incubation. The hydrolysis of adenine, elastin, guanine, xanthine, tyrosine or casein was detected in the basal media and by methods used by Gordon (1968). Tyrosinase activity was determined in plates incubated at 20 °C and 30 °C because some strains showed evidence of enzyme activity only at the lower incubation temperature. Chitin hydrolysis was tested in a colloidal chitin agar (Cross & Attwell, 1974), and cellulose hydrolysis in the basal medium of Eggins & Pugh (1962) containing 1·0 % (w/v) cellulose powder (MN-300; Macherey, Nagel & Co., Germany).

Antibiotic sensitivity tests. Filter paper discs containing antibiotics (Goodfellow & Orchard, 1974) were placed on Diagnostic Sensitivity Test agar (Oxoid), with added thiamin (4 mg l^{-1}), spread with 0·1 ml standard suspension of the test organism per 90 mm diam. plate. Plates were incubated for 5 days. Strains showing inhibition zones >0·5 mm were recorded as sensitive.

Additional tests. Hydrolysis of Tween 80 was detected by the method of Sierra (1957) and of DNA by the method of Jeffries, Holtman & Guse (1957). Urease was detected in the medium of Gordon & Mihm (1959) solidified with agar. Bacterial growth on Bennett's agar was used for catalase and oxidase tests (Steel, 1961). Ability to grow on M3 isolation medium containing nitrate, propionate and thiamin (Rowbotham & Cross, 1977) was also recorded.

Collection and coding of data. For each strain, 110 unit characters were studied. The results of 32 tests of no discriminatory value (Table 2) were not included for computation. All tests were designed to have only two states, positive or negative; a result that was still inconclusive after retesting was coded as negative.

Data analysis and presentation. The simple matching coefficient (S_{SM}) (Sokal & Michener, 1958) was used and the unsorted similarity matrix produced was converted to a sorted similarity matrix and phenogram (Fig. 6) by a highest single linkage procedure (Lessel & Holt, 1970).

RESULTS AND DISCUSSION

Morphology and growth requirements

Lspi strains did not produce a pellicle on Bennett's broth after 3 days incubation, although isolated floating colonies sometimes occurred. This is in contrast to most of the rhodochrous strains studied (Table 3) which formed a definite pellicle. On Bennett's agar plates inoculated for single colonies, three colony forms of Lspi were often observed (Fig. 1): effuse rhizoidal colonies with a central orange papilla (A); colonies with a large central papilla and a narrow mycelial fringe (B); and colonies with a much larger, irregular, wrinkled papilla and no mycelial fringe (C). Well-isolated colonies on rich media, such as Bennett's agar, tended to be of form C; colonies on poor media or where nutrients were limited because of overcrowding were of form A. Intermediate forms between A and C occurred and reflected

Table 2. *Tests of no discriminatory value* (*total* 32) *when comparing Lspi with 'rhodochrous' strains*

(*a*) Tests for which all 36 strains were positive

1	Gram stain (positive)	
2	Catalase	
3	Growth on M3 isolation medium	
Sole carbon source (%, w/v):		
4	Sodium acetate	(0·1)
5	Sodium propionate	(0·1)
6	Sodium isobutyrate	(0·1)
7	Sodium valerate	(0·1)
8	D-Glucose	(1·0)
9	D-Fructose	(1·0)
10	D-Mannose	(1·0)

(*b*) Tests for which all 36 strains were negative

1	Oxidase	
2	Growth on Bennett's agar + *p*-nitrophenol	(0·05 %, w/v)
Hydrolysis of:		
3	Casein	
4	Cellulose	(1·0 %, w/v)
5	Chitin	
6	Elastin	(0·3 %, w/v)
7	Guanine	(0·2 %, w/v)
8	Xanthine	(0·2 %, w/v)
Sole carbon source (%, w/v):		
9	Sodium glycollate	(0·1)
10	Sodium mandelate	(0·1)
11	Sodium tartrate	(0·1)
12	Sodium 2,2-dichloropropionate ('Dalapon' herbicide)	(0·1)
13	*o*-Hydroxybenzaldehyde	(0·1)
14	Biphenyl	(0·05)
15	Nicotinamide	(0·1)
16	Pyrazinamide	(0·1)
17	Ethane-1,2-diol	(1·0)
18	D-Cellobiose	(1·0)
19	Dulcitol	(1·0)
20	Inulin	(1·0)
21	Cholesteryl acetate	(0·1)
22	β-Carotene	(0·05)

nutrient availability. To facilitate recognition and enumeration of Lspi, the concentration of the sole carbon source in the M3 isolation medium (Rowbotham & Cross, 1977) was adjusted so that the characteristic form A colonies were produced. Unlike most other rhodochrous strains (Table 3), growth of Lspi strains extended into the agar (Fig. 1).

Colony pigmentation was enhanced by exposure to light indicating partial photochromogenicity. Lspi strains required thiamin to utilize paraffin wax as a sole carbon source. Growth and pigmentation were reduced on media deficient in thiamin.

Impression smears, prepared from the central papilla, contained branched chains and complex aggregates of Gram-positive, non acid-fast coccoid elements (1·0 to 1·5 μm diam.) (Fig. 2). In old cow-dung and other materials containing high numbers of Lspi, similar coccoids were seen. Consequently, a colony-forming unit of Lspi from dung or other substrates may be one or a short chain of coccoids still joined together. The diameter of the vegetative mycelium rarely exceeded 1·0 μm although short lengths of swollen, irregular hyphae sometimes occurred. Large spherical bodies or cystites were occasionally seen in the

Table 3. *Frequency of positive characters (numbers of strains positive) in clusters defined at the* 80 % *similarity level*

		Lspi†	Lspi. Hypothetical Mean Organism	*Nocardia erythropolis* *Jensenia canicruria*	*Nocardia calcarea*	*Nocardia pellegrino*	*Gordona rubra*	*Gordona terrae*	*Gordona bronchialis*	*Rhodococcus rhodochrous*
Cluster		1		2	7	3	4	5	6	
No. of strains tested		11		4	3	3	2	3	3	1
Characters										
*1 Bennett's broth (3 days, 30 °C), presence of pellicle		0	–	4	2	3	2	3	0	1
Bennett's agar (30 °C):										
*2 Microcolonies, mycelial (24 h)		11	+	0	0	3	0	0	0	0
*3 Microcolonies, rods and branching fragments (24 h)		0	–	4	3	0	2	3	3	1
*4 Rudimentary aerial mycelium (24 h)		9	+	0	0	2	0	0	0	1
*5 Sub-surface mycelium (7 days)		11	+	0	0	3	0	0	0	1
*6 Asteroid colonies with central papilla (7 days)		11	+	0	1	0	0	0	0	0
*7 Separate colonies < 2·5 mm diam. (7 days)		11	+	0	0	0	0	0	2	0
Growth on Bennett's agar in the presence of:										
8 Sodium chloride (5 %, w/v)		7	+	4	3	3	2	3	3	1
*9 Sodium chloride (7 %, w/v)		0	–	0	0	2	2	3	3	1
10 Sodium azide (0·01 %, w/v)		11	+	4	3	3	2	3	2	1
Hydrolysis of:										
*11 L-Tyrosine (0·2 %, w/v)		0	–	3	3	3	2	3	3	0
*12 Adenine (0·5 %, w/v)		0	–	4	3	0	0	0	0	0
13 [Inhibition by adenine]		11	+	0	0	0	0	0	0	0
14 DNA		7	+	0	0	0	0	0	0	0
15 Urea		6	–	4	3	0	2	3	3	0
*16 Tween 80		11	+	3	3	3	0	0	1	1
Sole carbon source (%, w/v):										
17 Stearic acid		6	–	4	3	3	2	3	3	1
18 Cetyl alcohol		10	+	4	3	3	2	3	3	1
19 Paraffin wax (m.p. 60 °C)		11	+	4	3	3	2	3	3	1
*20 Thiamin required for Paraffin wax to be used as sole carbon source		11	+	1	1	3	0	3	0	1
*21 [Partially photochromogenic]		11	+	0	0	3	0	3	0	1
22 Sodium butyrate	(0·1)	11	+	3	2	2	2	3	3	1
*23 tri-Sodium citrate	(0·1)	0	–	4	3	2	2	3	1	1
24 Sodium lactate	(0·1)	5	–	4	3	3	2	3	3	1
*25 Sodium L-malate	(0·1)	0	–	4	3	3	2	3	3	1
*26 Sodium succinate	(0·1)	0	–	4	3	3	2	3	3	1
27 Sodium benzoate	(0·1)	5	–	1	1	3	2	3	3	1
*28 Sodium *m*-hydroxybenzoate	(0·1)	11	+	2	2	0	0	0	0	1
*29 Sodium *p*-hydroxybenzoate	(0·1)	0	–	4	3	3	0	0	0	1
*30 Acetamide	(0·1)	0	–	4	3	3	0	0	3	0
31 Benzamide	(0·1)	0	–	0	1	0	0	0	0	0
32 L-Alanine	(0·1)	0	–	4	1	0	0	0	1	1
33 D-Alanine	(0·1)	0	–	4	3	0	0	0	2	1
*34 DL-Norleucine	(0·1)	0	–	4	3	3	0	0	0	1
35 L-Proline	(0·1)	0	–	4	2	0	2	3	1	0
36 L-Serine	(0·1)	0	–	3	3	0	2	1	3	0
37 L-Tyrosine	(0·1)	0	–	3	2	3	0	0	0	1
38 Propane-1,2-diol	(1·0)	7	+	3	3	3	0	2	3	1
*39 Butane-1,3-diol	(1·0)	0	–	4	3	2	2	3	2	1
40 Butane-1,4-diol	(1·0)	0	–	0	0	0	2	0	1	0
*41 Glycerol	(1·0)	0	–	4	3	3	2	3	3	1

Table 3. (*contd.*)

		Lspi†	Lspi. Hypothetical Mean Organism	*Nocardia erythropolis Jensenia canicruria*	*Nocardia calcarea*	*Nocardia pellegrino*	*Gordona rubra*	*Gordona terrae*	*Gordona bronchialis*	*Rhodococcus rhodochrous*
*42 D-Mannitol	(1·0)	0	−	4	3	3	2	3	1	1
*43 D-Sorbitol	(1·0)	0	−	4	3	3	2	3	1	1
*44 *meso*-Inositol	(1·0)	0	−	4	3	0	0	0	3	0
45 D-Glucosamine.HCl	(1·0)	1	−	4	2	0	0	0	0	1
46 L-Arabinose	(1·0)	0	−	0	0	0	0	0	0	0
47 D-Galactose	(1·0)	0	−	0	0	2	0	0	0	0
48 Lactose	(1·0)	0	−	0	0	0	0	0	0	0
49 D-Maltose	(1·0)	11	+	3	2	3	2	3	3	0
50 D(+)-Melezitose	(1·0)	8	+	0	0	0	2	3	0	1
51 D-Raffinose	(1·0)	8	+	2	2	2	1	1	3	0
52 L(+)-Rhamnose	(1·0)	0	−	0	0	0	0	3	0	0
53 D-Salicin	(1·0)	0	−	4	3	3	1	0	1	0
54 D(+)-Sucrose	(1·0)	7	+	4	3	3	2	3	3	0
55 D(+)-Trehalose	(1·0)	7	+	4	3	3	2	3	3	1
56 D-Xylose	(1·0)	0	−	0	2	1	0	0	0	0
57 Dextrin	(1·0)	8	+	0	0	0	0	1	0	0
58 Testosterone	(0·1)	11	+	4	3	3	2	3	1	1
Antibiotic sensitivity‡:										
59 Gentamycin	(10)	11	+	4	3	3	2	0	3	1
60 Kanamycin	(10)	9	+	0	0	3	2	3	2	1
61 Neomycin	(50)	10	+	3	2	3	2	3	3	1
62 Streptomycin	(100)	11	+	0	3	3	2	3	1	1
63 Tobramycin	(50)	11	+	1	0	3	2	1	3	1
64 Rifampicin	(50)	11	+	4	3	3	2	0	3	1
65 Cephaloridine	(100)	10	+	1	0	3	2	3	2	0
66 Lincomycin	(100)	11	+	4	3	2	2	3	2	1
67 Erythromycin	(50)	11	+	4	3	3	2	3	3	1
68 Oleandomycin	(50)	9	+	2	2	3	2	0	0	1
69 Novobiocin	(50)	6	−	0	0	2	0	0	0	0
*70 Anoxicillin	(500)	11	+	0	1	3	0	0	3	0
71 Capreomycin	(100)	10	+	3	2	3	2	3	1	1
72 Polymyxin B	(100)	9	+	0	0	0	0	0	0	0
73 Fusidic acid	(100)	11	+	3	3	3	2	3	3	1
74 Doxycycline	(100)	11	+	4	3	3	2	3	2	1
75 Minocycline	(50)	11	+	4	3	3	2	3	2	1
76 Vancomycin	(50)	11	+	3	1	3	2	1	0	1
77 Dapsone	(500)	11	+	2	3	3	2	3	3	1
78 Septrin	(500)	11	+	0	2	3	2	2	3	0
Additional sole carbon source tests§ (0·1 %, w/v):										
Sodium adipate		3	−	4	3	3	0	2	0	1
* Sodium gluconate		0	−	4	3	0	2	2	0	0
* Sodium γ-aminobutyrate		0	−	4	3	3	0	0	0	0
* Sodium phenylacetate		0	−	4	3	0	2	0	0	0
* L-Phenylalanine		0	−	4	3	0	0	0	0	0
* L-Asparagine		0	−	4	3	0	0	1	0	0
Glycine		0	−	0	0	0	0	1	0	0

* Selected distinguishing characters for Lspi.
† CUB686, a white mutant of CUB193, was not included.
‡ Numbers in parentheses indicate the concentration of antibiotic (μg ml^{-1}) in which the disc was dipped.
§ Also found useful for separating species.

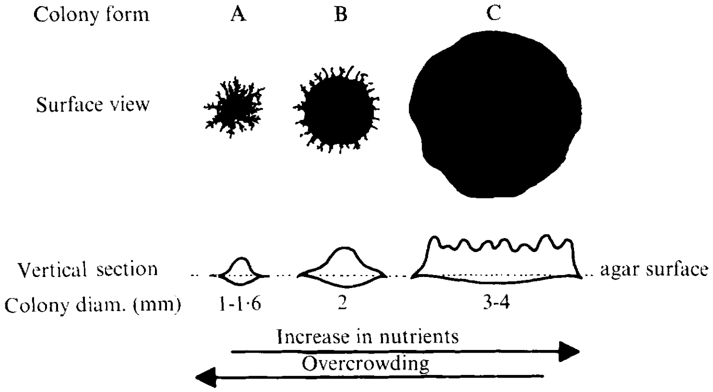
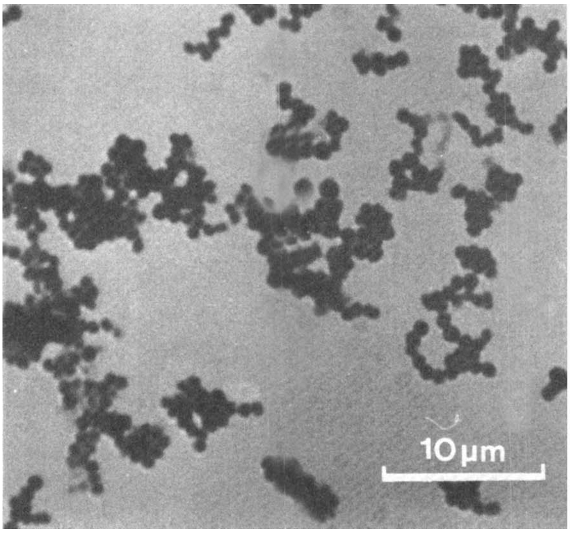


Fig. 1. Colony forms of an Lspi strain observed on Bennett's agar incubated for 2 weeks at 30 °C: A, effuse rhizoidal colonies with a central orange papilla; B, colonies with a large central papilla and a narrow mycelial fringe; C, colonies with a larger, irregular, wrinkled papilla and no mycelial fringe.



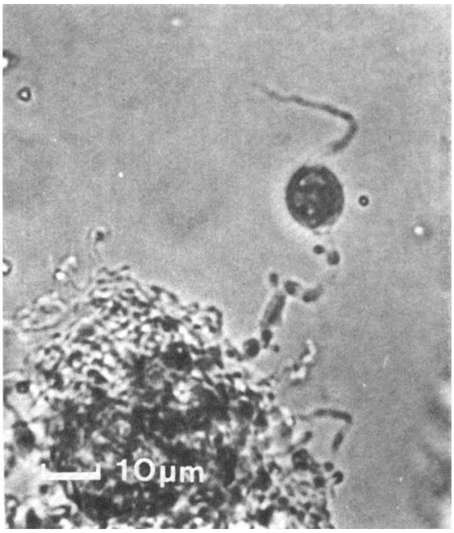


Fig. 2. Fig. 3.

Fig. 2. Gram-stained impression smear from the central papilla of an Lspi colony: strain CUB687 grown on Bennett's agar at 30 °C for 1 week.

Fig. 3. A cystite at the edge of an Lspi colony: strain CUB670 grown on Bennett's agar at 25 °C for 2 weeks.

mycelial fringe around colonies; they were usually subterminal (Fig. 3) and occurred more frequently in certain strains (e.g. CUB670).

Colonial development. A uniform and just visibly turbid suspension of CUB687 in Bennett's broth was prepared from the central papilla of a 1-week-old colony on Bennett's agar. One drop of this suspension, spread on a 90 mm plate of the same agar, was incubated on a microscope stage at 30 °C. The growth of one coccoid was photographed at 0 h and at hourly intervals from 12 h to 24 h (Fig. 4). By 12 h most of the coccoids had produced several germ tubes which developed into a branched primary mycelium. Growth into the agar occurred after 15 h and production of the central papilla commenced after 18 h. One aerial

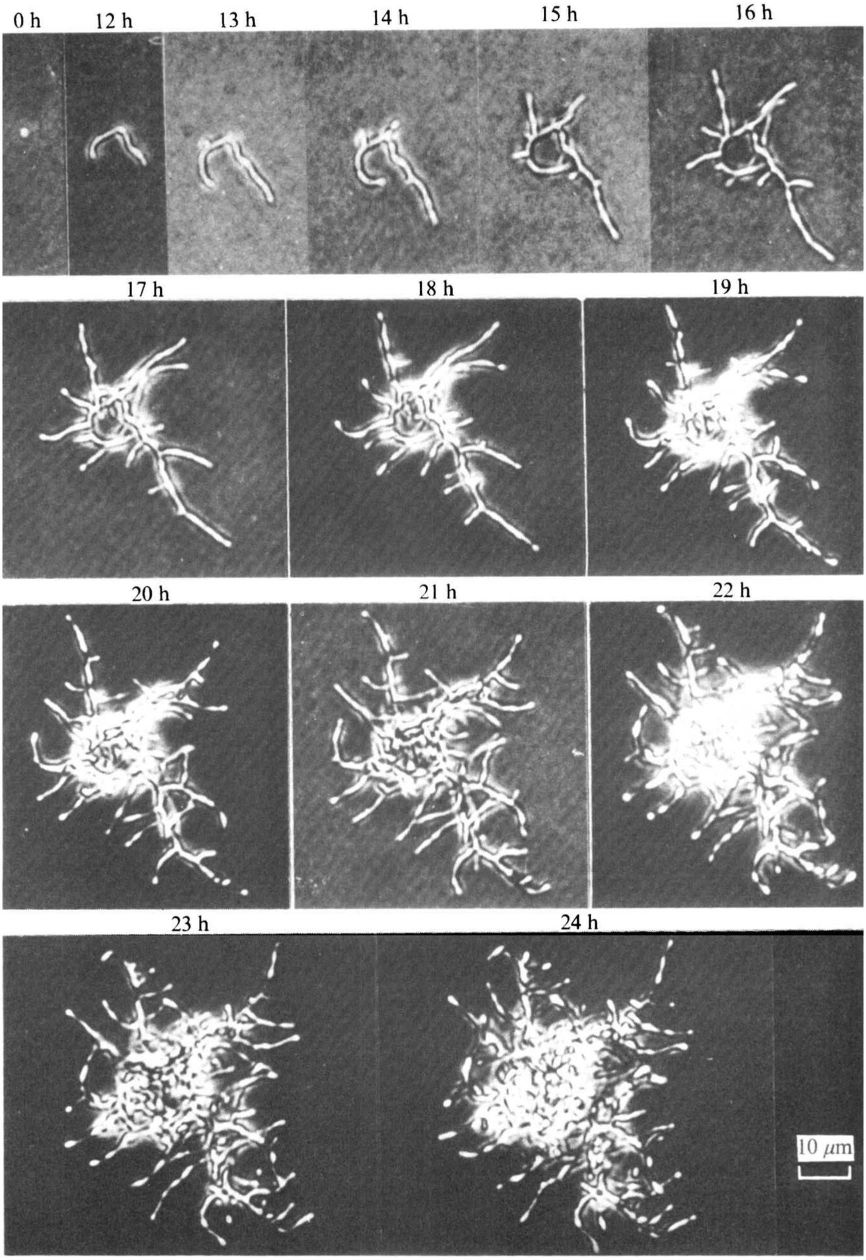


Fig. 4. Mycelial growth of an Lspi strain (CUB687) on Bennett's agar during 24 h incubation at 30 °C.

hypha was visible after 21 h and three after 24 h. Cross-walls were seen after 12 to 14 h incubation (Fig. 5). Hyphae in the region of the central papilla became subdivided at regular intervals, and the units enlarged to form the spherical coccoids. These development stages indicate that Lspi strains could be included in McClung's (1954) Group II morphological subdivision of the nocardiae.

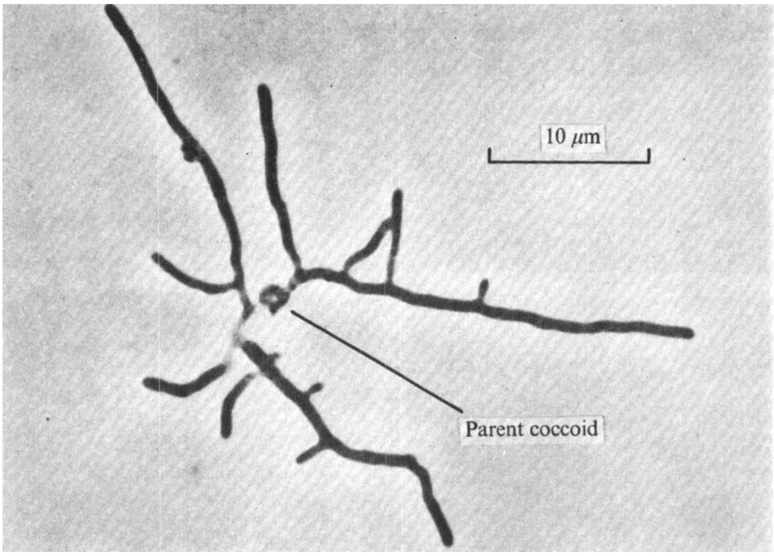


Fig. 5. Microcolony of an Lspi strain (CUB687) on Bennett's agar after 14 h incubation at 30 °C showing the parent coccoid and mycelium with few cross-walls.

The hyphae of CUB118 divided into irregular fragments instead of coccoids. When transferred to fresh media these fragments or portions of them enlarged to form club- or lemon-shaped bodies which later germinated. Growth of CUB687 on the EGYA medium of Cure & Keddie (1973) was slower with a more extended mycelial phase than on Bennett's agar; no cross-walls were seen at 24 h.

The microcolonies of Lspi strains were similar to those of *Nocardia pellegrino* strains but quite distinct from those of other rhodochrous strains examined (Table 3).

Chemotaxonomy

Whole-organism material from the 12 Lspi strains contained *meso*-diaminopimelic acid and large amounts of arabinose and galactose, consistent with a type IV wall composition (Becker *et al.*, 1965). This wall composition is found in the genera *Corynebacterium*, *Mycobacterium*, *Nocardia* and members of the '*rhodochrous*' complex; however, the mycolic acids of these taxa vary in chain length and structure. Lspi strains contained lipid LCN-A with a lower R_F than that of *Nocardia asteroides* and *Nocardia brasiliensis* but with a mobility similar to that of LCN-A from *Rhodococcus rhodochrous*. The DNA base composition of Lspi strains was 60 to 64 mol % GC (Mordarski *et al.*, 1977).

Numerical taxonomy

At the 90 % similarity level (%S) there were five main clusters: Lspi, *Nocardia erythropolis/Jensenia canicruria*, *N. pellegrino*, *Gordona rubra* and *G. terrae* (Fig. 6). At 83 % S the three *G. bronchialis* strains united with themselves (cluster 6) and with clusters 4 and 5. At 87 to 88 % S, three strains including *N. calcarea*, called cluster 7, joined cluster 2 containing *N. erythropolis* and *J. canicruria* (Fig. 6). Table 3 shows the frequency of positive characters in clusters defined at the 80 % similarity level.

Cluster 1: *Lspi*. The Lspi strains formed a homogeneous cluster which was clearly

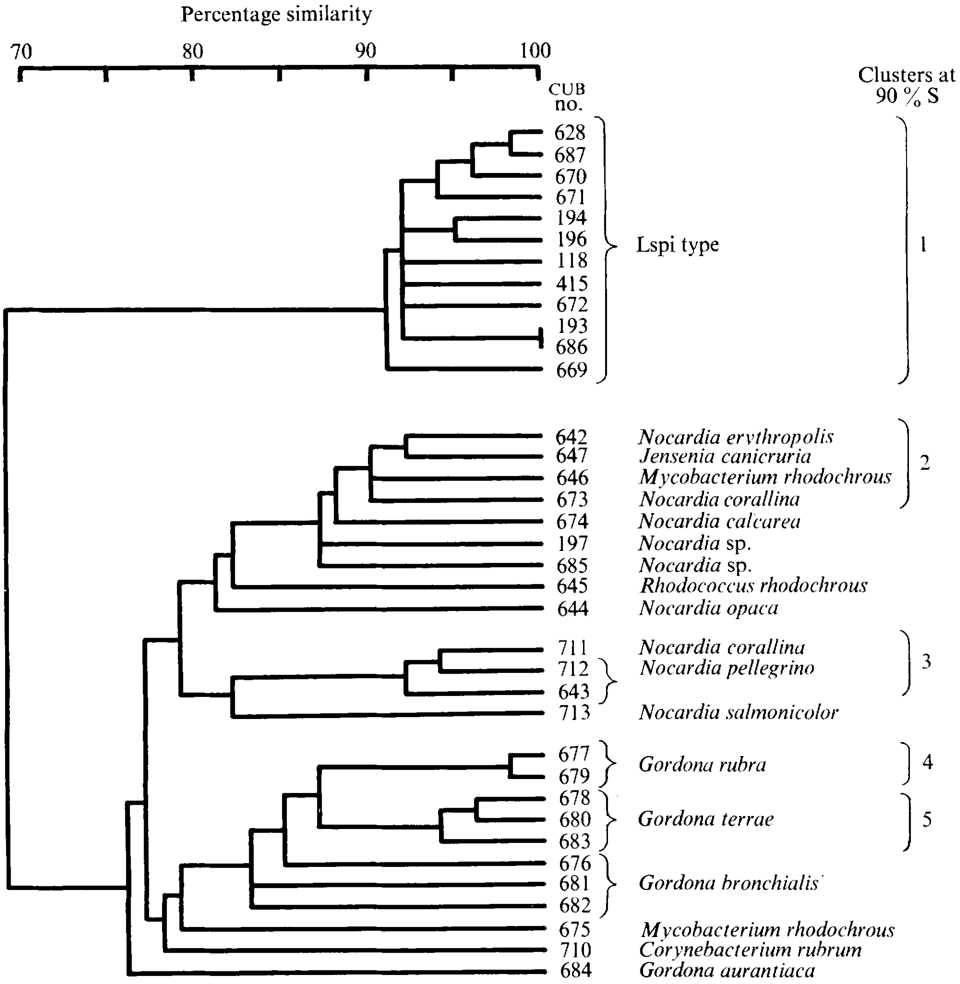


Fig. 6. Cluster analysis by the highest single linkage method.

separated from the other nocardioforms tested, and did not include any of the reference strains studied (Fig. 6).

Goodfellow (1971) included five Lspi strains in his numerical taxonomic studies on nocardioform bacteria. These early strains had been inadequately characterized; the colony morphology and presence of a visible mycelium had suggested that some strains might belong to the then ill-defined species *Nocardia asteroides*. Consequently, he received CUB118 (N249) as '*N. asteroides* subgroup', CUB415 (N265) as a '*Nocardia* sp. *asteroides* type', and CUB193, 194 and 196 as '*Nocardia* sp.'. Because of the large number of strains, Goodfellow carried out his analysis in two parts, one containing the potentially pathogenic nocardiae, the other the so-called soft-growing nocardioform organisms. Thus CUB118 and CUB415 were included in the first analysis, where they grouped with *N. asteroides* strains in cluster subgroup 1B. The other Lspi strains included in the second analysis were allocated to the '*rhodochrous*' cluster, subgroup 14G, a heterogeneous subgroup containing mainly unnamed strains.

In a further numerical study (Goodfellow, Fleming & Sackin, 1972), the Lspi strains CUB193 and CUB194 were again recovered outside the main clusters of rhodochrous strains, but did not fall within the mycobacteria or coryneforms. Strains CUB193 and CUB194 are very similar to the other Lspi strains and their position at the edge of the '*rhodochrous*' complex was confirmed by our studies.

Lspi strains differ from *Nocardia asteroides* in their morphology. None of those examined in this study, or subsequently isolated from a variety of habitats (Rowbotham & Cross, 1977), produced a visible aerial mycelium on agar media. The very short and infrequent aerial hyphae that were observed could only be seen with the aid of a microscope. The orange–red colonies did not excrete melanin or other water-soluble pigments and elements of the fragmenting mycelium enlarged to give the characteristic cocci observed by Willoughby (1969). Other differences noted included the lower mobility of the lipid LCN-A on thin-layer chromatograms and the inability of Lspi strains to utilize succinate, glycerol or mannitol as the sole carbon source. Lspi strains were also more sensitive to the antibiotics benzylpenicillin and cephaloridine.

Lspi strains require thiamin for growth and the lack of this vitamin in some of the test media used by Goodfellow (1971) possibly influenced the disparate clustering of Lspi by introducing a high number of negative results. When Goodfellow excluded the negative matches, a greater weighting was placed on the fewer positive matches, and the morphological characters used contributed to the clustering of the two Lspi strains with *Nocardia asteroides*.

Clusters 4, 5 and 6 were recognized by Tsukamura (1973, 1974) as different species of the genus *Gordona*. It is therefore reasonable to equate cluster 1 to a species. Lspi strains can be assigned to the '*rhodochrous*' complex on morphological, chemotaxonomic and numerical taxonomic grounds. None of the other nocardioforms clusters with Lspi, nor has a literature search or communications with other workers revealed any named strains identifiable as Lspi. Lspi strains thus appear to be a new species belonging to the '*rhodochrous*' complex.

A good type strain should be typical of its species and have a high similarity to other strains of that species. Type strains can be selected by taking the strain which has the highest average similarity to all the other strains, the centrotype, or by constructing a hypothetical mean organism (HMO; Tsukamura & Mizuno, 1968) and taking the strain which has the highest similarity to it as the type strain. The typical Lspi strain CUB687 was selected by both methods, it had an average similarity to the other Lspi strains of 91 % and was 96 % similar to the HMO. We therefore propose that CUB687 become the type strain of the taxon Lspi. Selected distinguishing tests for Lspi are indicated by an asterisk in Table 3.

Cluster 2: *Nocardia erythropolis/Jensenia canicruria*. The similarity between *Nocardia erythropolis* (CUB646 and CUB642) and *Jensenia canicruria* (CUB647), reported by several workers, was again confirmed (Fig. 6). These three strains were also recovered, closely clustered together, by Goodfellow (1971) in his subgroup 14D, and Bradley (1971) allocated CUB646 and CUB647 to his *N. erythropolis* group. *Nocardia corallina* (CUB673) of Batt & Woods (1951) also belongs to cluster 2. Three strains (cluster 7) join cluster 2 at a slightly lower similarity level: CUB197, an isolate from Blelham Tarn mud, placed by Goodfellow (1971) on the edge of his subgroup 14D; CUB685, and a fungicide degrader isolated from soil by Bachofer *et al.* (1973); and CUB674, *N. calcarea*.

Cluster 3: *Nocardia pellegrino*. This clearly defined cluster is equivalent to cluster 14 subgroup A of Goodfellow (1971) and Goodfellow *et al.* (1972); it was also recovered by

Tacquet *et al.* (1971). Strain CUB711, provisionally named *Nocardia corallina*, belongs to this group.

Cluster 4: *Gordona rubra.* The strains CUB677 and CUB679 are both strains of *G. rubra* NCTC1C668 (ATCC25593).

Cluster 5: *Gordona terrae.* Strains CUB678 and CUB680 are duplicates of NCTC10669 (ATCC25594). The 100 % similarity between strain CUB193 and its white mutant CUB686, and the high similarity between duplicates of *G. rubra* (98 %) and *G. terrae* (96 %) indicate that the majority of tests were reproducible. The tests in which the duplicates differed were utilization of sole carbon sources, e.g. raffinose.

Cluster 6: *Gordona bronchialis.* This cluster was more heterogeneous. Strains CUB681 and CUB676, supposed to be duplicates of NCTC10667 (ATCC25592), were found to be only 83 % similar. Strain CUB682 grew poorly on many media, and tended to die quickly on storage.

Other strains studied remained distinct and their affinities require further studies in a programme involving many more strains of the '*rhodochrous*' complex.

Identification of the Lspi cluster

Taxonomic studies indicate that members of the '*rhodochrous*' complex form a taxon quite distinct from the genera *Mycobacterium*, *Nocardia* and *Corynebacterium* (Bradley, 1971; Goodfellow, 1971; Tacquet *et al.*, 1971; Goodfellow *et al.*, 1972; Cross & Goodfellow, 1973; Ridell & Norlin, 1973; Tsukamura, 1973, 1974; Goodfellow *et al.*, 1974; Jones, 1975; Alshamaony *et al.*, 1976; Mordarski *et al.*, 1976). The discovery of a common and hitherto unnamed nocardioform actinomycete, which undoubtedly falls within this complex, demands the choice of a suitable generic name before we can propose a new specific epithet. We have considered the possible names for this genus, namely *Rhodococcus* Zopf 1891, *Jensenia* Bisset & Moore 1950, *Proactinomyces* Jensen 1931 and *Gordona* Tsukamura 1971.

Proactinomyces, advocated by Bradley & Bond (1974), would have priority over *Jensenia* but its use would create considerable confusion. A recent classification of the actinomycetes (Krassilnikov, 1970) uses *Proactinomyces* to include aerobic and anaerobic nocardioforms and species currently classified in several alternative genera (Buchanan & Gibbons, 1974). This dilemma can be overcome by observing rules of priority and choosing *Rhodococcus*. An authentic type species is available, *Rhodococcus rhodochrous* ATCC13808 (see Gordon, 1966), which was recovered in the '*rhodochrous*' clusters 14C (Goodfellow, 1971) and 1*a* (Goodfellow *et al.*, 1974) during the course of extensive numerical studies. This opinion is supported by Tsukamura (1974).

The status of the genus *Gordona* (Tsukamura, 1971) remains a problem. We feel that the later suggestion (Tsukamura, 1973) that rhodochrous strains, including *Rhodococcus rhodochrous*, should be accommodated in *Gordona* was premature. The alternative should now be considered: can all the Gordona species be included within the genus *Rhodococcus*? Gordona species have been separated from rhodochrous strains in antibiotic sensitivity studies (Goodfellow & Orchard, 1974), in lipid analyses (Alshamaony *et al.*, 1976) and in DNA reassociation experiments (Mordarski *et al.*, 1976). However, in a numerical taxonomic study using 109 characters, Tsukamura (1974) showed that strains of the '*rhodochrous*' complex, including *Rhodococcus rhodochrous* ATCC13808, and *Gordona* spp. formed a cluster at the 87 % similarity level. He concluded that the appropriate generic name now appeared to be *Rhodococcus* and accordingly reclassified his *Gordona* spp. as species of *Rhodococcus*. Our results would not support the retention of a separate genus *Gordona*.

The early descriptions of Gordona species (Tsukamura, Inagaki & Kondo, 1970; Tsukamura, 1971) emphasized that the organisms were weakly or slightly acid-fast, particularly when grown on Lowenstein–Jensen or Ogawa egg media. Acid-fastness had been noted previously in species belonging to the '*rhodochrous*' complex when grown in milk or on agar media containing glycerol (Gordon & Mihm, 1957, 1959). The earlier studies of Erikson (1949) had emphasized the importance of the growth medium; strains then classified in the genus *Proactinomyces* produced acid-fast elements more often in media containing complex protein and fat and in chemically defined media containing paraffin or large quantities of glycerol.

There appear to be other recognizable species within the genus *Rhodococcus*. Our clusters including *Nocardia erythropolis* and *N. pellegrino* are obvious candidates together with *R. rhodochrous*, but the very limited studies reported here could not be used as a basis for such proposals. We do propose that cluster 1, the Lspi cluster, should be considered a new species within the genus *Rhodococcus* and that it be named *Rhodococcus coprophilus* sp. nov. The specific name *coprophilus* (Gr.n. *copros* dung; Gr.adj. *philus* loving; M.L.adj. *coprophilus* dung loving) refers to the key role herbivore dung plays in the ecology of this organism (Rowbotham & Cross, 1977).

Rhodococcus coprophilus sp. nov.

Type strain CUB687, NCIB11211, NCTC10994, ATCC29080.

An aerobic, catalase positive, oxidase negative, mesophilic, thiamin-requiring nocardioform actinomycete. *Rhodococcus coprophilus* has a type IV wall composition, a DNA base composition of 60 to 64 mol % GC, and contains lipid LCN-A type c. On Bennett's agar, after 2 weeks incubation at 30 °C, it forms small 2 mm diam. rhizoid colonies with a central orange papilla; growth into the agar also occurs. Pigmentation is enhanced by light. The young microcolonies on Bennett's agar are mycelial and after 24 h sparse, non-sporulating aerial hyphae are usually present. No macroscopic aerial mycelium, extracellular pigments or characteristic odours are produced. The central papilla of mature colonies is composed of complex aggregations of Gram-positive, non acid-fast, non-motile coccoids (1 to 1·5 μm diam.). Cystites may occur in the mycelial fringe of colonies on Bennett's agar. No pellicle is produced on the surface of Bennett's broth although isolated floating colonies may occur.

In contrast to many similar organisms, *R. coprophilus* does not utilize citrate, L-malate, succinate, butane-1,3-diol, glycerol, D-mannitol, sorbitol or any of the amino acids tested as the sole source of carbon and energy. It does not hydrolyse tyrosine and is more sensitive to polymyxin than related species.

Rhodococcus coprophilus grows on herbivore dung; it has been isolated from the dung of cows, sheep, goats, horses and donkeys. It is common on grass and in the soil beneath grazed pastures, and is washed into streams and lakes where it can accumulate in the sediment.

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