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Occurrence of *Rhodococcus coprophilus* and Associated Actinomycetes in Feces, Sewage, and Freshwater

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Freshwater, sewage, and fecal samples from various sources were examined for *Rhodococcus coprophilus*, associated actinomycetes, *Escherichia coli*, and fecal streptococci. *Rhodococcus coprophilus* was isolated consistently from feces of farm animals, poultry reared in proximity to farm animals, freshwater, and wastewater polluted with animal fecal wastes. It was not isolated from samples of human feces.

The ratio of *R. coprophilus* to total actinomycetes was higher in feces from cattle, sheep, ducks, and geese than in specimens from pigs, horses, and fowl. In samples from two freshwater streams polluted by fecal material from farm animals, the ratios of *R. coprophilus* to total actinomycetes were similar to those found in fecal specimens from cattle and sheep. Ratios of fecal coliform to fecal streptococci could not distinguish between fresh human and animal fecal samples and, furthermore, were not reflected in the stream waters polluted by animal fecal material. *R. coprophilus* has potential in water and dairy bacteriology as a specific indicator organism of fecal pollution due to farm animal wastes.

Rhodococcus coprophilus has been suggested as a specific indicator organism of farm animal fecal contamination (1, 13). In the past, this organism has posed classification problems, and the nomenclature has been somewhat confused (7, 16, 18). However, after an extensive and detailed study of a collection of strains from various sources, the specific name *R. coprophilus* was suggested by Rowbotham and Cross (12) for these aerobic nocardioform actinomycetes commonly found inhabitating herbivore dung and aquatic environments.

Willoughby (18) studied the aerobic nocardioform actinomycetes in water and mud from a small lake, Blelham Tarn, in Cumbria, England, and in several surrounding streams and soils. He described three morphological types of which the commonest was given the trivial name Lspi (large spored pink irregular). Goodfellow (7) included Lspi strains in his numerical taxonomic study of some nocardioform bacteria and recovered some strains in his *Nocardia aesteroides* cluster (group 1), whereas the other strains clustered within the "rhodochrous" complex. In a further numerical taxonomic analysis of Lspi strains chosen from the main "rhodochrous" complex of Goodfellow (7) and species of the genus *Gordona* (16), a homogeneous cluster which separated from the other members of the reference strains at the 69% similarity level led Rowbotham and Cross (12) to propose the specific name *R. coprophilus* for Lspi strains. They suggested that this organism had potential as a specific indicator organism of farm animal pollution, since its natural habitat is herbivore dung. Furthermore, they stated that the ratio of *R. coprophilus* to the other actinomycetes, *Micromonospora* and *Streptomyces*, would provide a useful index to distinguish dairy farm effluents from other nonanimal fecal contamination.

Fecal coliforms, particularly *Escherichia coli*, have traditionally been regarded as the classical indicator organism of fecal contamination. However, this organism is excreted by both humans and animals, and its presence in a water sample cannot distinguish between human and animal fecal contamination. Likewise, fecal streptococci are present in the feces of humans and animals. Ratios of fecal coliforms to fecal streptococci have, however, been used to ascertain whether pollution of water was of human or animal origin (5, 6), although more recent work (10, 17) has shown that ratios of fecal coliforms to fecal streptococci are unreliable. Nevertheless, *Streptococcus bovis* can be used as a specific indicator organism of farm animal pollution (3, 5, 6, 11, 15, 19).

Previous studies on *R. coprophilus* were mainly on its incidence in herbivore dung and aquatic environments (1, 13, 18). Very little information is available on the distribution of this organism in feces from various sources and in

sewage. The purpose of this study was to determine the occurrence of *R. coprophilus* and associated actinomycetes in feces of humans, animals, and birds and in sewage and to evaluate its suitability as a specific indicator organism of animal fecal pollution.

MATERIALS AND METHODS

Samples. Samples were obtained from the Leeds area, West Yorkshire, England, and examined for fecal coliforms, fecal streptococci, *R. coprophilus*, *Micromonospora* spp., and *Streptomyces* spp., using different media. Water and sewage samples were collected in sterile 250- or 500-ml screw-capped bottles. Freshly voided fecal samples were collected, using a sterile spatula, in sterile universal bottles. Precautions were taken so that soil was not included with feces during collection of fecal samples. All samples were transported to the laboratory and examined within 1 h of collection.

Sterile quarter-strength Ringer solution, pH 7.0 (Oxoid Ltd.), was used to prepare serial dilutions of samples of water or sewage. Feces (1 g) were emulsified in 9 ml of Ringer solution; serial dilutions were then made, and appropriate volumes were filtered or spread on the surface of media. All tests were done in triplicate, and the geometric mean results were calculated.

Heat treatment. The methods of Rowbotham and Cross (13) were used. Water and sewage samples (2- to 10-ml portions) were first immersed in a water bath at 55°C for 6 min to reduce contaminating bacteria other than actinomycetes. Further dilutions were then made, and 0.2-ml volumes were spread on the surface of the appropriate medium. Fecal samples (1 g) were homogenized in 9 ml of quarter-strength Ringer solution (Oxoid) containing gelatin (0.01% wt/vol), and this was then heat treated. Samples (0.2 ml) of appropriate dilutions were spread on the surface of media by means of sterile, L-shaped glass rods.

Media and incubation. Fecal coliforms were enumerated by membrane filtration as described in *Standard Methods for Examination of Water and Wastewater* (2). Membrane filters (type HAWG 047; Millipore Corp.) were incubated on pads saturated with 0.1% (wt/vol) sodium lauryl sulfate broth (8, 14). The medium was similar to that recommended in *Report 71* (4) and was prepared from dehydrated base-membrane-enriched Teepol broth (Oxoid). It was rehydrated with water containing 0.1% sodium lauryl sulfate instead of 0.4% Teepol. Incubation was at 30°C for 4 h, followed by 44°C for a further 18 to 20 h (4).

Fecal streptococci were assayed by membrane filtration on KF *Streptococcus* agar of Kenner et al. (9). Incubation was at 37°C for 4 h, followed by incubation at 44°C for 44 h (4).

R. coprophilus and associated actinomycetes were enumerated on MM3 agar (Table 1), a modification of M3 agar suggested by Rowbotham and Cross (13). Preliminary studies on M3 agar indicated that the medium was insufficiently selective for *R. coprophilus* and *Micromonospora*, being frequently overgrown by other bacteria; this problem was overcome by supplementing M3 agar with 5 mg of nalidixic acid and 3.5 mg of sodium azide per liter (higher concentrations of these supplements were found to inhibit the growth of *Micromonospora*). Plates of MM3 agar were incubated at 30°C for 12 to 14 days and then exposed to sunlight (intensity range, 500 to 1,500 lx) on the bench for a further 4 to 7 days, as *R. coprophilus* is partially photochromogenic (13); color production was found to be intensified after this exposure to sunlight and greatly aided enumeration. The long incubation period was found to be necessary for identifiable colony formation by *R. coprophilus*; colonies were formed by the actinomycetes within 5 to 7 days, but these were small and generally lacked their characteristic color and form so that identification was difficult. Moreover, the actinomycetes count was commonly much lower than that obtained after incubation for 12 to 14 days.

TABLE 1. *Modified M3 agar*[a]

Substance	Amt/liter
KH_2PO_4	0.466 g
Na_2HPO_4	0.732 g
KNO_3	0.1 g
NaCl	0.29 g
$MgSO_4 \cdot 7H_2O$	0.1 g
$CaCO_3$	0.02 g
Sodium propionate	0.2 g
$FeSO_4 \cdot 7H_2O$	200 μg
$ZnSO_4 \cdot 7H_2O$	180 μg
$MnSO_4 \cdot 4H_2O$	20 μg
Sodium azide	3.5 mg
Nalidixic acid	5 mg
Agar (Difco)	18 g
Distilled water	1,000 ml

[a] The agar was prepared by autoclaving at 120° for 15 min and cooling to ~50°C, after which 10 ml of 0.5% (wt/vol) cycloheximide and 1 ml of 0.4% (wt/vol) thiamine (previously sterilized by membrane filtration) were added. This mixture was then poured into 90-mm-diameter petri dishes. The pH of the agar was 7.0 ± 0.1.

Enumeration and confirmation of colonies. For the enumeration of fecal coliforms, all yellow colonies were counted. Confirmation was done by random isolation of colonies which were first purified and then inoculated into tubes of tryptone water (for indole production at 44°C) and lactose peptone water containing Durham's tube (for gas production).

All red or maroon colonies growing on membranes incubated on KF agar were counted as fecal streptococci. Confirmation was done by direct microscopic examination for typical short-chained streptococci and characteristic colonies after subculture onto MacConkey agar (4).

Colonies of *R. coprophilus* appeared as stellate colonies with bright orange central papillae. Confirmation of presumptive positive colonies was done by observation on a stereoscopic-zoom Olympus microscope for characteristic aesteroidal appearance.

RESULTS

A total of 97 fecal specimens were obtained from different individuals, farm, laboratory, and domestic animals, and also from various avian

species reared under farm or laboratory conditions. These were tested for fecal coliforms, fecal streptococci, *R. coprophilus*, and associated actinomycetes. Four fecal specimens collected from droppings inside four cages containing five to nine mice per cage were also processed for these bacteria. Additional specimens from humans, hens, and 14-day-old chicks were assayed for *R. coprophilus* and associated actinomycetes only. The results in Table 2 show the ranges and

TABLE 2. *Ranges and geometric means of fecal coliforms, fecal streptococci, and actinomycetes per gram of sample from human, animal, and avian feces*

Source of sample	No.	No. (geometric mean) per gram of sample				
		Fecal coliform	Fecal streptococci	*R. coprophilus*	*Micromonospora*	*Streptomyces*
Human	18	1.3×10^5–9.0×10^9 (6.3×1^7)	4.0×10^3–4.7×10^7 (1.1×10^5)	0	0	0
	16	ND[a]	ND	0	0	0
Cattle	8	1.5×10^5–6.5×10^6 (1.0×10^6)	3.2×10^3–4.2×10^6 (5.0×10^4)	6.9×10^4–6.9×10^5 (2.0×10^5)	0–2.4×10^5 (4.0×10^3)	3.4×10^3–2.2×10^5 (1.3×10^4)
Sheep	7	1.8×10^5–5.6×10^7 (2.7×10^6)	4.7×10^3–7.3×10^6 (4.4×10^6)	1.7×10^4–2.5×10^6 (1.1×10^5)	2.3×10^3–5.1×10^5 (4.0×10^4)	0–3.0×10^4 (5.4×10^2)
Pig	8	4.9×10^6–6.0×10^8 (3.9×10^7)	1.1×10^4–2.9×10^6 (1.9×10^6)	30–9.3×10^4 (7.8×10^3)	0	1.0×10^3–2.1×10^5 (1.4×10^4)
Horse	5	6.3×10^2–3.4×10^3 (1.0×10^3)	2.7×10^4–3.7×10^7 (1.8×10^6)	1.8×10^4–2.2×10^5 (7.6×10^4)	2.4×10^4–2.0×10^5 (5.2×10^4)	8.0×10^3–2.7×10^5 (3.3×10^4)
Cat	5	8.9×10^4–2.6×10^9 (6.3×10^7)	8.8×10^5–8.9×10^{10} (9.5×10^8)	0	0	0
Dog	5	4.1×10^6–4.3×10^9 (5.0×10^8)	3.5×10^7–7.9×10^9 (4.0×10^8)	0–150 (2.5)	0–65 (4)	0–135 (5)
Rabbit	4	2.8×10^3–4.9×10^4 (1.0×10^4)	2.6×10^3–1.1×10^5 (1.6×10^4)	0	0	0
Rat	4	5.6×10^4–6.3×10^5 (1.6×10^5)	1.1×10^5–1.1×10^6 (3.2×10^5)	0	0	0
Mouse	4 (28)[b]	4.7×10^6–1.0×10^7 (6.3×10^6)	2.8×10^7–7.1×10^7 (4.0×10^7)	0	0	2.0×10^2–6.7×10^2 (3.2×10^2)
Hen[c]	4	1.9×10^3–3.2×10^4 (7.9×10^3)	2.6×10^3–5.6×10^4 (1.0×10^4)	3.9×10^3–1.7×10^4 (6.3×10^3)	1.4×10^4–1.7×10^4 (1.3×10^4)	8.0×10^2–1.5×10^3 (1.3×10^3)
Hen/cock[d]	9	3.7×10^6–1.5×10^7 (6.3×10^6)	1.0×10^5–2.3×10^5 (1.6×10^5)	0	0–5.0×10^2 (6)	1.2×10^3–2.9×10^4 (5.8×10^3)
Chicken[e] (14-day-old)	4	ND	ND	0	0	1.5×10^2–8.7×10^2 (4.5×10^2)
Duck[c]	5	8.8×10^6–4.9×10^7 (2.0×10^7)	6.8×10^5–3.0×10^6 (1.3×10^6)	1.1×10^4–2.9×10^5 (7.9×10^4)	1.3×10^4–1.2×10^5 (4.0×10^4)	1.6×10^3–3.3×10^4 (7.9×10^4)
Goose[c]	3	9.7×10^2–6.6×10^4 (6.3×10^3)	1.4×10^5–5.6×10^6 (1.3×10^6)	1.2×10^5–2.6×10^5 (1.6×10^5)	5.2×10^3–1.2×10^5 (2.5×10^4)	1.2×10^4–1.9×10^4 (1.6×10^4)
Turkey[e] (21-day-old)	6	2.6×10^8–2.0×10^9 (7.9×10^8)	5.6×10^7–2.8×10^8 (1.6×10^8)	0	0–2.4×10^3 (7)	0–5.9×10^3 (4.8×10^2)
Seagull	6	1.7×10^2–2.7×10^5 (6.3×10^3)	2.0×10^2–4.2×10^4 (5.0×10^3)	0–6.5×10^4 (32)	6.5×10^4–3.5×10^6 (5.0×10^4)	8.7×10^5–2.3×10^7 (2.0×10^6)

[a] ND, Not determined.
[b] Each cage contained from 5 to 9 mice.
[c] Fecal specimens obtained from Brookland farm, West Yorkshire, England.
[d] Fecal specimens obtained from the animal house of the Department of Animal Nutrition and Physiology, Leeds University, West Yorkshire.
[e] Fecal specimens obtained from the animal house of the Department of Agricultural Sciences, Leeds University, West Yorkshire.

geometric means of bacterial counts from fecal specimens from man, animals, and birds. Significantly, *R. coprophilus* was not recovered from fecal specimens from humans, cats, rabbits, rats, mice, or turkeys. In fecal specimens from cattle, sheep, horses, pigs, hens, ducks, and geese (farm specimens), the numbers ranged from 3.9×10^3 to 2.5×10^6 per g of feces. Lower numbers were associated with pigs and seagulls. The frequency of isolation of *R. coprophilus* was found to be 100% in feces from cattle, sheep, pigs, and horses. Differences in numbers and frequency of isolation emerged in results from fowls. In droppings obtained from birds reared under laboratory conditions, *R. coprophilus* was not detected, whereas in samples from farm-raised specimens, the organism was consistently isolated in high numbers. The numbers of *R. coprophilus* in stream water from Adel and Meanwood becks, near Leeds, which are both polluted with farm wastes, were usually above 10^3/100 ml (Table 3). Similar results were obtained in samples of raw sewage from Barwick-in-Elmet Sewage Treatment Works and samples of final effluent from Knostrop Sewage Treatment Works in Leeds. The frequency of positive samples was higher when effluent from the slaughterhouse was being received at the works, and the negative samples were collected at other times.

The ratios of fecal coliform to fecal streptococci were generally above 4 in fecal specimens from humans, cattle, sheep, pigs, dogs, ducks, turkeys, and fowl reared in the laboratory, whereas fecal coliform/fecal streptococci ratios of less than 1 were obtained in fecal samples from horses, cats, rats, mice, and geese (Table 4). The ratios obtained in samples of stream water from Adel and Meanwood becks (Table 5) ranged from 0.5 to 42.5, with a median ratio of 8.0, whereas in samples of raw sewage and final effluent they ranged from 0.8 to 7.1.

The proportion of *R. coprophilus* to the total actinomycetes was generally higher in specimens from cattle (median, 0.62) and sheep (median, 0.59) than in specimens from pigs and horses (median, 0.4 and 0.43, respectively). It is particularly interesting to note that the ratios obtained from samples of water and sewage closely resembled those obtained from farm animals (Tables 4 and 5). Ratios of fecal coliforms to *R. coprophilus* and fecal streptococci to *R. coprophilus* were found to be too variable and inconsistent to be of any use (Tables 4 and 5).

DISCUSSION

Although Rowbotham and Cross (13) recommended the use of M3 agar for enumerating *R. coprophilus* and associated actinomycetes, initial trials carried out in our laboratory indicated that this medium was not sufficiently selective for work involving sewage. The modified M3 agar (MM3; Table 1) was found to be adequate for enumerating *R. coprophilus* and associated actinomycetes, although complete inhibition of contaminating bacteria was not achieved. The concentration of azide was found to be critical. Nalidixic acid could be added up to a concentration of 9 μg/ml without interfering with the numbers of *R. coprophilus*. The numbers of *Micromonospora* were, however, significantly reduced at this concentration.

It has been suggested (13) that the natural habitat of *R. coprophilus* is herbivore dung. While this may be true, the high counts obtained (Table 2) indicate that this nocardioform actinomycete can also occur in the gut of some avian

TABLE 3. *Ranges and geometric means of fecal coliforms, fecal streptococci, and associated actinomycetes in 100-ml samples of stream water and sewage*

Source of sample	No.	No. (geometric mean) in 100 ml of sample				
		Fecal coliform	Fecal streptococci	*R. coprophilus*	*Micromonospora*	*Streptomyces*
Streamwater						
Adel Beck	8	3.6×10^2–2.7×10^4 (4.0×10^3)	1.3×10^2–6.3×10^3 (7.9×10^2)	6.7×10^3–1.5×10^5 (1.6×10^4)	0–2.3×10^4 (2.0×10^3)	0–4.5×10^3 (2.0×10^2)
Meanwood Beck	6	7.4×10^2–7.5×10^3 (2.0×10^3)	1.7×10^2–7.5×10^3 (5.0×10^2)	3.9×10^3–2.5×10^4 (1.3×10^4)	1.1×10^3–3.0×10^4 (7.9×10^3)	0–4.3×10^3 (4.0×10^2)
Sewage						
Barwick-in-Elmet sewage treatment works (raw sewage)	5	8.7×10^6–3.8×10^7 (1.6×10^7) 2.5×10^6–2.7×10^7 (5.0×10^6)	7.0×10^3–9.0×10^6 (7.9×10^4)	0–2.7×10^6 (1.4×10^2)	0–2.7×10^5 (1.0×10^2)	
Knostrop sewage treatment works (raw sewage)	7	2.1×10^6–3.4×10^7 (1.1×10^7)	1.1×10^6–9.4×10^6 (5.4×10^6)	0–6.4×10^4 (3.7×10^3)	0–2.0×10^4 (45)	0–1.7×10^4 (34)
Final effluent	3	1.8×10^5–3.0×10^5 (2.5×10^5)	4.4×10^4–1.2×10^5 (7.9×10^4)	1.2×10^3–8.4×10^3 (3.2×10^3)	2.4×10^3–4.5×10^3 (3.2×10^3)	6.5×10^2–1.2×10^3 (1.0×10^3)

TABLE 4. *Ranges and arithmetic means of ratios of fecal coliform, fecal streptococci, R. coprophilus, and total actinomycetes in samples of feces from various sources*

Source of sample	No.	Range (arithmetic mean) of ratio of:[a]			
		FC/FS	FC/RC	FS/RC	RC/TA
Human	17	16.6–12,353 (1,778)			
Cattle	8	1.2–688 (102)	1.3–63.8 (13.7)	0.04–0.69 (0.33)	0.2–0.9 (0.62)
Sheep	7	0.02–3,404 (524)	0.2–295 (101)	0.05–30 (12.3)	0.11–0.99 (0.59)
Pig	8	14.8–16,364 (2,562)	114–86,947 (53,202)	1.4–1,000 (521)	0.02–0.98 (0.40)
Horse	5	0.00002–0.04 (0.01)	0.008–0.04 (0.02)	0.4–649 (209)	0.15–0.58 (0.43)
Cat	5	0.0001–0.77 (0.36)			
Dog	5	0.008–91.4 (25.9)			
Rabbit	4	0.08–18.8 (4.9)			
Rat	4	0.43–0.57 (0.50)			
Mouse	28	0.14–0.17 (0.16)			
Fowl (Brookland farm)	4	0.3–3 (1.3)	0.45–6.8 (2.6)	0.45–11.9 (4.51)	0.05–0.2 (0.13)
Fowl (laboratory reared)	9	17–75 (45)			
Duck	5	14–59 (31)	30–3,909 (1,036)	2.6–273 (78)	0.39–0.78 (0.59)
Goose	3	0.002–0.01 (0.006)	0.008–0.3 (0.09)	1.2–21.5 (12.6)	0.65–0.86 (0.76)
Seagull	5	0.04–6.4 (2.7)			
Turkey	5	3.7–7.7 (4.9)			

[a] FC, Fecal coliform; FS, fecal streptococci; RC, *R. coprophilus*; TA, total actinomycetes.

TABLE 5. *Ranges and arithmetic means of ratios of fecal coliform, fecal streptococci, R. coprophilus, and total actinomycetes in 100-ml samples of stream water and sewage*

Source of sample	No.	Range (arithmetic mean) of ratios of:[a]			
		FC/FS	FC/RC	FS/RC	RC/TA
Stream water (Adel and Meanwood becks)	14	0.5–42.5 (8.0)	0.05–186.7 (14.1)	0.004–0.53 (0.12)	0.27–1.0 (0.62)
Raw sewage					
Barwick-in-Elmet sewage treatment works	5	0.8–7.1 (4.5)	2.2–3,000 (1076)	0.3–3,857 (858)	0.1–1.0 (0.75)
Knostrop sewage treatment works	7	0.8–3.9 (2.3)	127–2,429 (1187)	66–1,119 (517)	0–1.0 (0.62)
Final effluent					
Knostrop sewage treatment works	3	1.8–4.1 (2.8)	62–168 (88)	14–92 (40)	0.17–0.70 (0.43)

[a] FC, Fecal coliform; FS, fecal streptococci; RC, *R. coprophilus*; TA, total actinomycetes.

species. However, it should be pointed out that the avian species were reared in close proximity with farm animals, whereas birds reared under laboratory conditions did not harbor *R. coprophilus*. It seems likely that the birds reared in farms may have ingested *R. coprophilus* from the environment and that eventually these organisms multiplied in the gut to reach the high proportions encountered in fecal droppings. There is a need to examine a wider range of avian species to establish the incidence of this organism in droppings and in various parts of the alimentary tract.

Table 4 indicates that fecal coliform/fecal streptococci ratios of more than 4 are common findings in fecal specimens from humans, cattle, sheep, pigs, ducks, horses, and turkeys. Similar ratios were obtained in samples of stream water from Adel and Meanwood becks. These streams run through agricultural land and receive mainly agricultural effluents and storm water runoff from fields grazed by sheep and cattle. Therefore, the present studies confirm our earlier finding that this ratio cannot be used to distinguish between human and animal fecal pollution (17).

At present, very scanty information is available on the characteristics of *R. coprophilus* with reference to survival and resistance to disinfectants, and it is therefore now impossible to determine whether it fulfills the requirements expected of an ideal indicator organism. In view of the fact that this organism occurs in the feces of farm animals in high numbers and is absent in human feces, it seems reasonable to suggest that *R. coprophilus* is a potential indicator organism of farm animal pollution. One limitation of the use of this organism as a specific indicator organism is the long incubation period it takes to

grow into visible colonies. Nevertheless, the potential public health significance of *R. coprophilus* in aquatic, food, and especially dairy bacteriology cannot be ignored.

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