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Characterization of *Pseudomonas maltophilia* Isolates from Fleece Rot

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A total of 286 isolates of *Pseudomonas maltophilia* was collected from sheep exhibiting brown or yellowish fleece rot and from fly-strike lesions. Enzyme activities for 10 of the isolates were examined by plate tests and with the API ZYM system and compared with the enzymatic profile of a human type strain of *P. maltophilia*. The fact that ovine isolates of *P. maltophilia* are biochemically similar to pathogenic human strains suggests there may be an association between this organism and the brown to yellow type of fleece rot.

As a consequence of the prolonged wetting of sheep fleece, bacteria proliferate in the fleece and at the skin surface, causing superficial dermatitis. The resultant serous exudate causes discoloration and matting of the wool fibers and is termed fleece rot (16). This condition is of economic concern because it not only affects the quality of wool but is a major predisposing factor to blowfly-strike on the body of sheep. When the wool has distinct green coloration, *Pseudomonas aeruginosa* has been isolated as the predominant organism (16), and recent work (4) showed that this organism acts to exacerbate, if not cause, the development of the dermatitis associated with classical, pigmented fleece rot.

It has been claimed that *P. aeruginosa* is often the only organism recovered from fleece rot lesions (12, 13), but this finding has not been confirmed by more recent studies (2, 4). Seddon (16) concluded that in the more common types of fleece rot which showed dingy brown or yellowish discoloration, the bacterial population was very mixed. To understand the etiology of fleece rot and the predisposition to fly-strike, potential causal organisms other than *P. aeruginosa* must be identified.

Little work has been done on the identification of bacteria responsible for fleece rot. Although London et al. (11) recently isolated a number of different *Pseudomonas* species from 11 stained fleece samples, their study was unaccompanied by clinical surveillance of the development of fleece rot and the predisposition to fly-strike.

Pseudomonas maltophilia, a synonym for *Xanthomonas maltophilia* (18), is recognized as the most common pseudomonad after *P. aeruginosa* in human clinical material (8, 9). This study reports the isolation of *P. maltophilia* from fleece rot and fly-strike lesions monitored during three summers in the field. We also examined the enzymatic profiles of a number of our isolates in an attempt to assess the pathogenic potential of the ovine strains.

MATERIALS AND METHODS

Bacterial strains. The 286 strains of *P. maltophilia* were isolated from 721 fleece rot and fly-strike samples obtained from seven locations in three different areas of New South Wales during the summers of 1982, 1983, and 1984. Also collected were 43 unaffected, clean fleece samples. Fleece samples were placed in sterile swab tubes (Johns Products). A suspension of fleece bacteria was obtained by the addition of 2 ml of sterile distilled water to each tube, followed by vigorous agitation with the swab stick. Saturated swabs were streaked onto *Pseudomonas* agar (Oxoid, Australia Pty. Ltd., Heidelberg West, Victoria, Australia) containing 10 μg of cetrimide and fucidin per ml and 50 μg of cephaloridine per ml and incubated at 32°C for 48 h.

Identification. Gram-negative rods which were motile, catalase positive, oxidase negative within 30 s, and which exhibited an alkalinizing reaction in Hugh and Leifson's glucose medium were identified as *P. maltophilia* by the methods of Cowan (6) and by API 20E and 20NE systems (Carter-Wallace [Australia] Pty. Ltd., French's Forest, New South Wales, Australia).

Detection of enzymes. The 10 field isolates and type strain NCTC 10257 used by O'Brien and Davis (15) were grown on horse blood agar for 48 h, and a loopful was inoculated onto a 1% Bacto-Agar plate (Difco Laboratories, Detroit, Mich.) containing the appropriate substrate. Enzyme plate tests were read at 48 h.

The methods previously reported by Burrell and MacDiarmid (3) were used to assay DNase, elastase, phospholipase D, and lecithinase.

Chitinase, mucinase, and collagenase were determined by the method of O'Brien and Davis (15), except that the common medium used was 1% Bacto-Agar and the concentration of collagen was increased tenfold to 0.2%. The method of Smith and Willett (17) was used to assess hyaluronidase and chondroitin sulfatase activities; to eliminate false-positive results, the same procedure was followed by using 1% bovine serum albumin alone.

API ZYM tests. The 19 enzymes were semiquantitatively assayed with the API ZYM kit (Analytab Products, Plainview, N.Y.) according to the instructions of the manufacturer.

Production of antiserum. Field isolate MCPM001 was grown on horse blood agar for 48 h at 32°C. The cells were scraped off the plate into sterile 0.85% saline and killed by adding 40% formaldehyde to a final concentration of 1%. For immunization, a suspension of cells at a concentration of 1.4×10^9/ml in 0.85% saline containing 0.5% Formalin was mixed with an equal volume of alhydrogel adjuvant. Two subcutaneous doses, each of 2 ml, were administered to a sheep at a 1-month interval. Serum was collected before the first vaccination and 2 weeks after the second vaccination and tested for slide agglutination with 30 *P. maltophilia* strains.

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TABLE 1. Enzyme plate test results for 10 ovine *P. maltophilia* strains after 48 h of incubation at 32°C

Enzyme	Test result (no. of strains)		
	Negative	Weakly positive	Positive
Chitinase	2[a]	8	0
Collagenase	0	1	9[b]
DNase	0	1	9[b]
Elastase	1	0	9[b]
Hyaluronidase	1	0	9[b]
Chondroitin sulfatase	1	1	8[b]
Mucinase	0	1	9[b]
Phospholipase	0	0	10[b]
Albuminase	8	2	0

[a] Weakly positive after 5 days.
[b] Enzyme activity obtained with human type strain NCTC 10257.

RESULTS

Clinical appearance. Lesions in the fleece from which *P. maltophilia* was recovered ranged from yellow banding of the staple to a severe serous exudate which caused matting of the wool fibers. Fly-strikes were often subsequently observed in the latter type of lesion.

Clean fleece. Numbers of bacteria obtained from clean fleeces were very low. *P. maltophilia* was present in 7 of 43 samples.

Plate tests. The results of the enzyme plate tests are summarized in Table 1. All 10 strains produced collagenase, DNase, mucinase, and phospholipase. Chitinase was weakly positive for eight strains. Two strains showed no chitinolytic activity at 48 h, but slight clearing was present within 5 days. Only one strain failed to produce elastase, hyaluronidase, and chondroitin sulfatase. Type strain NCTC 10257 (UQM 497) produced collagenase, elastase, DNase, phospholipase, hyaluronidase, and chondroitin sulfatase, but no chitinolytic activity was observed.

API ZYM. The results with the API ZYM system are shown in Table 2. All strains produced acid and alkaline phosphatase, esterase lipase C8, leucine arylamidase, and naphthol-AS-BI-phosphohydrolase. The human type strain produced these same enzymes and in addition was weakly positive for trypsin.

Agglutination tests. All strains but one were strongly agglutinated by the anti-*P. maltophilia* serum produced in sheep but not by the prevaccination serum used as a control.

DISCUSSION

The results of the present study suggest that *P. maltophilia* is a potential causal organism of the nonpigmented variety of fleece rot previously described by Seddon (16). The organism was recovered from 40% of fleece lesions ranging from slight yellow banding of the fleece to severe exudation, whereas only 16% of unstained fleece samples yielded *P. maltophilia*. This is to be compared with the recovery of *P. aeruginosa* from 26.5% of these lesions and from 23% of clean fleece samples.

Of the *Pseudomonas* species, *P. maltophilia* is regarded as being second in importance to *P. aeruginosa* in human infections (8). Comparisons of the enzymatic profiles of our ovine isolates of *P. maltophilia* with a human type strain suggested that ovine isolates may have similar potential as pathogens.

Ovine strains of *P. maltophilia* very closely resembled human isolates in their biochemical profiles (15), and the API 20NE system was found to be a reliable means of identification. Esculin and gelatin hydrolysis were important diagnostic reactions, and all isolates tested were able to assimilate mannose, malate, and citrate. Of the ovine isolates, 76% reduced nitrates, compared with 37% quoted for human strains by API and 59% observed by Holmes et al. (8).

Like human strains of *P. maltophilia* (15), ovine strains produced a range of extracellular enzymes. Of 10 ovine isolates, 9 produced elastase. This is substantially more than the 7 of 31 elastase-positive human strains reported by Nord et al. (14), although the type strain was a strong elastase producer.

O'Brien and Davis (15) reported that human isolates were unable to digest collagen. However, when the concentration of collagen was increased from 0.02% to 0.2%, collagenolytic activity was detected in all ovine strains.

Hyaluronidase and chondroitin sulfatase have been proposed as virulence factors for other gram-negative organisms such as *Pasteurella multocida* (5) and *Moraxella bovis* (7). We found that 9 of 10 ovine strains of *P. maltophilia* also produced these enzymes. This compares well with the findings of O'Brien and Davis (15), although their human strains were devoid of chondroitin sulphatase activity. We were able to demonstrate chondroitin sulfatase activity after 4 days of incubation with the human type strain.

O'Brien and Davis (15) found the method used by Smith and Willett (17) for estimating hyaluronidase and chondroitin sulfatase activities could yield false-positive reactions, because some strains hydrolyzed the albumin conjugate. However, we found this method useful when plates were compared with an albumin substrate plate. Two ovine strains exhibited weak albuminase activity but were potent hyaluronidase and chondroitin sulfatase producers, and it is unlikely that the large zones of clearing could be attributed to albumin hydrolysis alone.

The isolate which failed to produce hyaluronidase and

TABLE 2. Enzyme activities of 10 *P. maltophilia* strains from sheep after testing with the API ZYM system

Enzyme	Test result (no. of strains)		
	Negative	Weakly positive[a]	Positive
Alkaline phosphatase	0	0	10[b]
Esterase C4	1	6	3[b]
Esterase lipase C8	0	1	9[b]
Esterase lipase C14	10	0	0
Leucine arylamidase	0	6	4
Valine arylamidase	6	4	0
Cysteine arylamidase	10	0	0
Trypsin	10	0	0
Chymotrypsin	10	0	0
Acid phosphatase	0	0	10[b]
Naphthol-AS-BI-phosphohydrolase	0	4	6[b]
α-galactosidase	10	0	0
β-galactosidase	10	0	0
β-glucuronidase	10	0	0
α-glucosidase	10	0	0
β-glucosidase	8	2	0
β-*N*-acetyl-D-glucosaminidase	10	0	0
α-mannosidase	10	0	0
α-fructosidase	10	0	0

[a] Weakly positive corresponds to color reaction 1 on the API scheme. Reactions of 2 to 5 are positive.
[b] Enzyme activity obtained with human type strain NCTC 10257.

chondroitin sulfatase also showed no elastase activity, suggesting that it may have been less virulent than the other isolates we tested.

The API ZYM test is a simple method for identifying the ability of bacteria to produce 19 different enzymes. All ovine isolates exhibited strong phosphatase activity. These enzymes have been isolated from other bacteria (6) and are thought to contribute to pathogenicity by eliciting an allergic response (1).

Ovine isolates of *P. aeruginosa* are serologically related to human isolates; up to 14 serotypes have been recognized (2, 3). Sheep antisera produced with formalinized *P. aeruginosa* cells also reacted with the Difco *P. aeruginosa* antisera scheme (unpublished data). However, 29 of 30 ovine isolates of *P. maltophilia* were agglutinated by antiserum similarly prepared against a single isolate, suggesting that ovine strains are less serologically diverse than the human isolates examined by Hugh and Ryschenkow (10).

In this study we demonstrated an association between *P. maltophilia* and the dingy brown to yellow type of fleece rot lesion and showed that ovine strains are biochemically similar to a pathogenic human strain. The possibility that *P. maltophilia* is a later and opportunistic agent remains unresolved. A causal role for *P. aeruginosa* in the development of exudative dermatitis associated with classical, pigmented fleece rot has been demonstrated previously (4), and similar studies on the pathogenicity of *P. maltophilia* may further define its role in fleece rot.

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