Letters in Applied Microbiology 1999, **29**, 71–75

Isolation and characterization of an unusual bacterium, allied to the soil bacterium *Bacillus benzoevorans,* from feedlot manure pads in Australia

A.V. Klieve[1], J.J. Plumb[2] and L.L. Blackall[2]

[1]*Queensland Beef Industry Institute, Queensland Department of Primary Industries and* [2]*Department of Microbiology, The University of Queensland, Brisbane, Queensland, Australia*

2096/99: received 15 February 1999 revised 10 May 1999 and accepted 11 May 1999

A.V. KLIEVE, J.J. PLUMB AND L.L. BLACKALL. 1999. Strains of bacteria were obtained by anaerobic enrichment from feedlot manure pads and were phenotypically characterized. Colonies had a cotton wool or hairy appearance. When cultures grown in liquid media were exposed to a drying atmosphere, they produced a pellicle comprised of a cross-meshed array of cells. Colonies on agar media also produced spiral sheets of cells held well above the agar surface. The strains were Gram positive, according to ultrastructural features from transmission electron micrographs and KOH solubility, but Gram negative by Gram stain. The 16S rDNA from strain YEO5 was determined and is 99·3% similar to the type strain of *Bacillus benzoevorans*. The inability of our strains to grow aerobically and lack of endospores differentiated them from previously isolated strains of *B. benzoevorans*. In freshly broken feedlot pad material, a white, hairy coating of the exposed surface appeared within a few hours. We hypothesize that this is due to the insoluble extracellular matrix material produced by this *Bacillus* sp. to avoid desiccation and, additionally, the bacterial covering is responsible for retaining odours within the pad material.

INTRODUCTION

Feedlots for the intensive production of beef cattle comprise pens of beef cattle, at high density, fed grain-based diets and located on open ground. The base of the pen is typically soil, and waste products (faeces and urine) accumulate on this base. With time, the manure is mixed with the top layer of soil and compacted by the animals constant walking to form a surface layer known as the feedlot pad. The manure is periodically removed from the pad, but between times the pads are responsible for the generation of odours regarded as offensive to the public and therefore a nuisance to the industry. Watts and Tucker (1993) demonstrated that the odour concentration from dry feedlots was low, but that 2 d after a significant wetting, the odour concentration peaked 60 times higher than in the dry pad. It was hypothesized that increasing moisture increases microbial activity, initially aerobically until the oxygen present is exhausted and then anaerobically. The anaerobic fermentation of undigested plant material and dead bacteria in the manure produces volatile chemicals, such as the larger chain length fatty acids, that are odoriferous. As the pad dries it aerates, and anaerobic fermentation declines slowly as indicated by the slow decrease in odour emission after 2 d (Watts and Tucker 1993). Although there is considerable data on the pathogenic organisms that occur in animal wastes (L'Hermite *et al*. 1992), little is known of the non-pathogenic bacteria and the dynamics of the ecosystem in feedlot pad material. Knowledge of the microbial processes occurring in the feedlot pad could contribute to future amelioration of odours emanating from feedlots. We report the isolation of an unusual *Bacillus* sp. from feedlot pads that appears closely related to the soil bacterium *Bacillus benzoevorans*. We speculate on the role of this bacterium in extending the period of odour generation in feedlots by slowing the drying and aeration processes following wetting.

Correspondence to: Dr A.V. Klieve Animal Research Institute Locked Mail Bag no. 4 Moorooka Queensland 4105 Australia (e-mail: klievea@dpi.qld.gov.au).

MATERIALS AND METHODS

Isolation

Bacteria were isolated from moist manure pads from cattle feedlots in Toowoomba, Queensland. Samples were taken at

© 1999 The Society for Applied Microbiology

Downloaded from https://academic.oup.com/lambio/article/29/1/71/6703789 by DAF: Dept of Agriculture and Fisheries user on 13 March 2024

a depth of 5 cm below the pad surface. Approximately 2 g manure was mixed with 5 ml feedlot (FL, see below) broth, serially diluted at 10^{-2} increments to 10^{-8}, spread plate inoculated to solidified FL, and incubated at 39 °C, anaerobically (Coy Laboratory anaerobic chamber; 95% CO_2, 5% H_2) overnight. The procedures for handling anaerobic micro-organisms followed those in Hungate (1966) and Holdeman *et al.* (1977). Isolated bacteria were stored frozen at –20 °C, as previously described (Teather 1982).

Media

Feedlot pad material (250 g) was homogenized with 500 ml distilled water in a Waring Blender (Waring Products Division Dynamics Corporation of America, New Hartford, Connecticut, USA) for 60 s. This was diluted to 1:1 with distilled water and allowed to stand for 1 h at room temperature prior to centrifugation at 10 000 × g for 30 min. The supernatant replaced rumen fluid in a habitat simulating medium (Klieve *et al.* 1989) and was called feedlot (FL) broth. Peptone, yeast extract, glucose (PYG) medium, with and without additives, was also employed (Holdeman *et al.* 1977).

Phenotypic characterization

Characters such as colony and cellular morphology, ability to produce spores, motility, and Gram reaction were determined for micro-organisms growing anaerobically on FL agar or broth at 39 °C overnight. The Gram reaction was determined by staining (Holdeman *et al.* 1977) and by KOH solubility (Fahy and Haywood 1983). The Microbact AN24 anaerobe system (Disposable Products, Adelaide, Australia) was used to determine biochemical characteristics.

Electron microscopy

Negative staining was used to visualize flagellation and thin sectioning to visualize cell wall structure. Both were standard procedures. Negative staining was on butvar coated grids with 2% phosphotungstic acid at pH 6·5. Samples for thin sectioning were fixed with gluteraldehyde and osmium tetroxide, dehydrated with acetone, embedded in spurs epoxy resin, and stained with uranyl acetate and lead citrate. Samples were examined using a Philips CM10 transmission electron microscope.

16S rDNA sequencing and phylogenetic analysis

The direct lysis and polymerase chain reaction (PCR) amplification of the 16S rDNA from a selected pure culture (YE05) were as previously described (Blackall 1994; Blackall *et al.* 1994). The purified gene was sequenced by an automated DNA sequencer and the data phylogenetically analysed using methods from Blackall *et al.* 1994. The sequence determined was deposited in EMBL under accession number Y14693.

RESULTS

Phenotypic characterization

Several pure cultures of micro-organisms growing anaerobically on FL agar had a characteristic ‘cotton wool’ or ‘hairy’ appearance. Aerial filaments extending from the colonies could be seen at × 40 magnification under the microscope (Fig. 1a). Individual cells of the organisms growing anaerobically in FL broth appeared as long Gram negative rods (using the Gram stain technique), either singly or in pairs, with slightly rounded or clubbed ends (Fig. 1b). The rods were either straight or slightly curved, exhibited a twitching type of motility and spores were never observed. Cells of these organisms on FL agar appeared to grow in cross-meshed (Fig. 1c), spiral sheets held well above the agar. The sheets comprised alternating parallel chains of cells surrounded by a sheath of amorphous, highly refractile material (Fig. 1d). The growth of the organism in sheets was not observed in sealed Hungate tubes of anaerobically prepared liquid FL medium. However, if the caps of the tubes were removed in a drying atmosphere (incubator at 39 °C), the matrix comprising the sheets was produced in a period of ≈ 2 h and a pellicle could be macroscopically observed at the liquid surface. A similar response was elicited when tubes were left open in the anaerobic chamber. The two growth habits were interchangeable. When matrix enclosed cells from either liquid or solid media were inoculated into sealed tubes of FL liquid media, they grew as individual motile cells. When these latter cells were inoculated to solid media, the sheets were microscopically observed and the colonies appeared hairy.

Although staining suggested the cells were Gram negative, they were not solubilized by KOH solution, indicating they were Gram positive. They grew anaerobically in sealed pre-reduced, anaerobically sterilized FL liquid medium and on FL agar in anaerobic jars. No growth was evident when cells were inoculated to FL agar allowed to equilibrate to aerobic conditions for 24 h and incubated aerobically. However, limited growth was observed when cells were inoculated to anaerobically prepared plates, inoculated in the anaerobic chamber and subsequently incubated in sealed plastic bags aerobically, indicating that although the bacterium could not grow under strictly aerobic conditions it could grow in the presence of limited oxygen.

Biochemical tests using the Microbact 24AN system showed strain YE05 did not ferment carbohydrates, hydrolyse esculin or starch, produce indole, or possess catalase, but did show weak gelatinase activity and reduced nitrate to nitrite. Based on these results and by comparison to known anaerobic

Downloaded from https://academic.oup.com/lambio/article/29/1/71/6703789 by DAF: Dept of Agriculture and Fisheries user on 13 March 2024

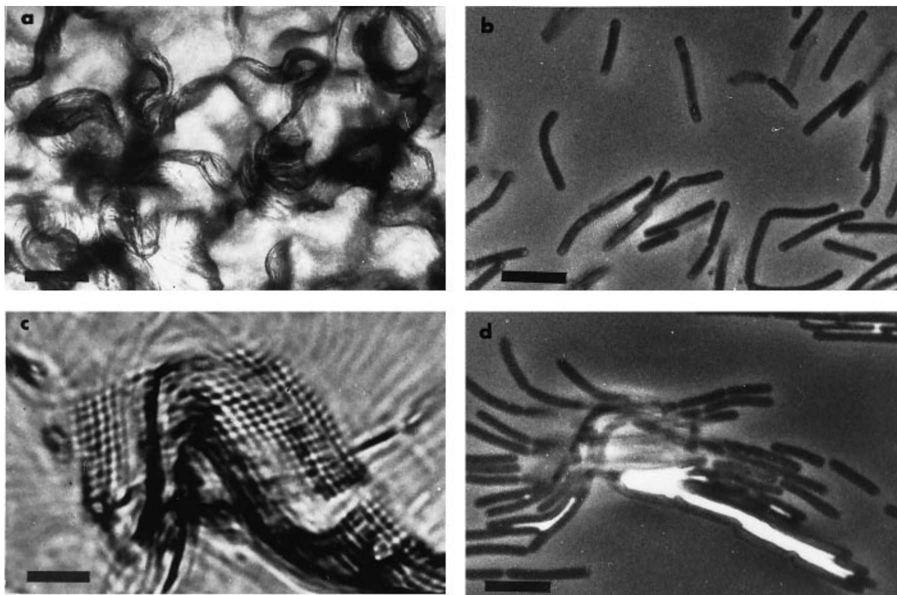

Fig. 1 Growth characteristics of *Bacillus* sp YE05: (a) growth on agar plates showing aerial filaments (dark material) extending from the colony, Bar = 50 μm; (b) motile cells in broth culture, Bar = 5 μm; (c) an aerial filament showing cross-meshed sheets of cells, Bar = 20 μm; and (d) parallel chains of bacteria with the highly refractile matrix material produced between chains, Bar = 5 μm

species, (Holdeman *et al.* 1977) the organism's identity was closest to *Vibrio succinogenes*.

Electron microscopy

Negative staining of FL broth-grown motile cells shows that cells are motile by means of peritrichous flagella. From thin sectioned cells, the cell wall structure appears to be characteristic of Gram positive cells (Fig. 2).

16S rDNA sequencing and phylogenetic analysis

The near complete 16S rDNA sequence (1525 nucleotides) from YE05 was used in phylogenetic analyses. Figure 3 shows the evolutionary distance tree and the phylogenetic placement of strain YE05.

DISCUSSION

The 16S rDNA similarity between strain YE05 and the type strain of *B. benzoevorans* (NCIMB12555) is 99·3%. According to the 16S rDNA sequence data and phylogenetic analysis, strain YE05 is most likely a representative of *B. benzoevorans.* The phylogenetic tree presented in Fig. 3 is a part of a larger tree prepared by comparison of 33 *Bacillus* species 16S rDNA sequences. Our tree from the extended dataset was identical in topology and arrangement to that presented by Ash *et al.* (1991).

Bacillus benzoevorans was discovered by Pichinoty (1983) and then more fully described by Pichinoty and Asselineau (1984) and Pichinoty *et al.* (1984). Eleven strains of the organism were isolated from pasteurized soil by aerobic enrichment at 32 °C. Cells of *B. benzoevorans* grew as unbranched flexible filaments, 1·8 μm in diameter and sometimes greater than 1000 μm in length, with cell septa along the filament being discernible. Endospores that did not deform the cell mor-

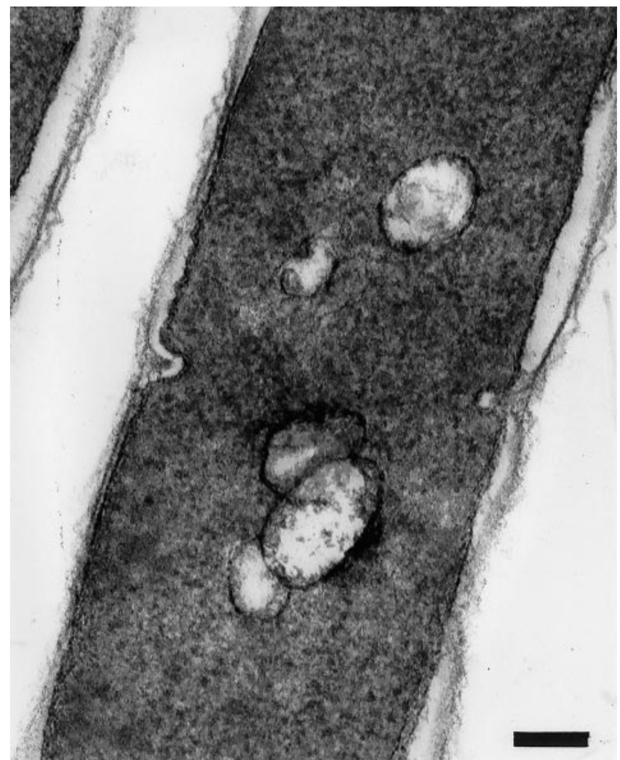

Fig. 2 Thin sectioned cells of *Bacillus* sp YE05, Bar = 0·1 μm

Downloaded from https://academic.oup.com/lambio/article/29/1/71/6703789 by DAF: Dept of Agriculture and Fisheries user on 13 March 2024

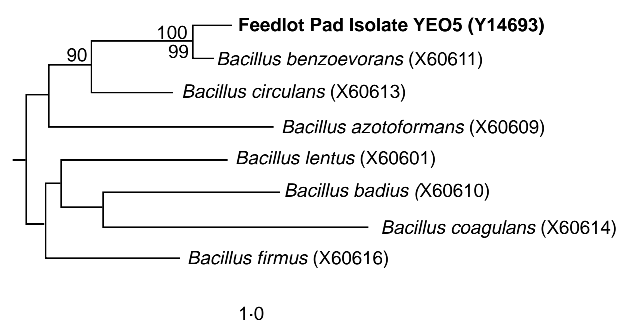

Fig. 3 Evolutionary distance tree of feedlot pad isolate YE05, and its closest phylogenetic relatives in the genus *Bacillus* based on the comparative analysis of 1370 nucleotide positions. Database accession numbers are in parentheses after species or strain names. Bootstrap values greater than 74% (100 bootstrap resampling) from distance (upper) and parsimony (lower) analyses are presented at nodes

phology and a thin, translucid sheath were visible (Pichinoty 1983). The later publications reported cells of *B. benzoevorans* were motile by peritrichous flagella and exhibited a variable Gram reaction but were Gram type positive. In vigorously shaken liquid culture, separate rods with rounded ends predominated, but in stationary liquid medium, the culture had a mycelial aspect and a thick pellicle was formed at the surface. The sheath was found to comprise 73% proteins, 12·9% reducing sugars, and 8% lipids. Other data are shown in Table 1. Where comparative phenotypic data exists, strain YE05 (and three additional isolates) appear similar to *B. benzoevorans*, with the notable exceptions that we did not observe endospores, the strains did not grow aerobically, were unable to use benzoic acid as a carbon and energy source, and pellicle formation was dependent on a gaseous interface where the gaseous phase is apparently not saturated. The lack of sporulation could well be due to culture conditions selecting against the organisms producing endospores. While *B. benzoevorans* is typically aerobic, strain YE05 was unable to grow in fully aerobic conditions.

Given the considerable differences in phenotype between YE05 and typical *B. benzoevorans* strains it is not possible at this stage to assign YE05 and similar isolates to a recognized taxon. Further characterization is required to clarify the taxonomic positions of this bacterium within the genus *Bacillus*.

Pellicle formation and the production of an insoluble matrix between chains and sheets of cells by YE05, appears to be associated with desiccation and occurred both on solid media and liquid media, but only when anaerobic tubes were uncapped. This response could not be attributed to the presence of oxygen as the pellicle formed as readily under strict anaerobic conditions as when exposed to air. As the only difference between capped and uncapped tubes was the lack of a saturated atmosphere in the uncapped tubes, it is suggestive that desiccation is the trigger. Pichinoty and Asselineau (1984) found that pellicle formation was always present except in vigorously shaken cultures. However, as their cultures were aerobically grown it was not necessary for culture vessels to be sealed in a manner that would maintain a saturated atmosphere. The individual motile cellular growth, reported previously only in vigorously shaken cultures (Pichinoty and Asselineau 1984), was a constant feature with the feedlot manure isolates when grown in capped anaerobic tubes.

Table 1 Characteristics of *Bacillus benzoevorans* (11 strains) and of strain YE05 isolated in this study

Characteristic	*Bacillus benzoevorans*	Strain YE05
Spore formation	+	–
Motile by means of peritrichous flagella	+	+
Aerobic growth	+	–
Production of extracellular sheath/matrix	+	+
Ability to grow in media containing peptone, yeast extract and either acetate or benzoate as C and E sources	+	–
Ability to use carbohydrates as C and E sources	–	–
NO_3^- as electron acceptor	+	+
NO_2^- as electron acceptor	–	–
mol% G+C in the DNA	41·3 ± 1·1	ND
Urease	+	ND
Catalase	–	–

ND = Not determined.

The use of extracellular polysaccharide to resist desiccation is well known amongst soil bacteria (Hepper 1975; Dudman 1977; Roberson and Firestone 1992), and we speculate that the *B. benzoevorans*–like strains that inhabit the feedlot manure pad produce an insoluble extracellular matrix as an aid to resist desiccation. It has been observed in the field (S. Lott, personal communication) that the pad stratifies into layers, with air between, upon drying, and the surfaces of these layers become coated with a white hairy material similar to the appearance of *Bacillus* sp. YE05 colonies on agar plates. It was also observed that once the pad was damaged the broken surface rapidly developed (within a matter of hours) the white hairy coating. Microscopic examination of white material on small pieces of pad, the large stratified layers from which the field observations were made was not available, showed that true fungi as well as cells similar to the *Bacillus* sp. contributed to the white hairy material. Extrapolating from the results and observations presented it is possible that

Downloaded from https://academic.oup.com/lambio/article/29/1/71/6703789 by DAF: Dept of Agriculture and Fisheries user on 13 March 2024

when the feedlot pad is wet *Bacillus* sp.YE05 is motile and colonizes much of the pad. As the pad dries the organism produces the extracellular matrix at surfaces to protect itself from desiccation. If this hypothesis is correct the pad may become partially sealed, slowing the process of drying and aeration, which in turn would prolong odour generation from the anaerobic fermentative bacteria in the manure ecosystem.

The lack of a noticeably strong odour from cultures of the *Bacillus* grown on laboratory media, and the apparent lack of a fermentative metabolism, suggests that the bacterium itself contributes little to the odour problem.

ACKNOWLEDGEMENTS

The authors wish to thank Robin White for technical assistance, Howard Prior for electron microscopy and assistance with photography, Jenny Cassady and Emma Puttick of the DNA Sequence Analysis Facility and Rick Webb of the Centre for Microscopy and Microanalysis. They also wish to thank Peter Watts, Robyn Tucker and Simon Lott (Feedlot Services, QDPI, Toowoomba, Qld), for assistance with field work and for useful discussions on feedlot waste management. This work was funded by the Meat Research Corporation of Australia.

REFERENCES

Ash, C., Farrow, J.A.E., Wallbanks, S. *et al.* (1991) Phylogenetic heterogeneity of the genus *Bacillus* revealed by comparative analysis of small-subunit ribosomal RNA sequences. *Letters in Applied Microbiology* **13**, 202–206.

Blackall, L.L. (1994) Molecular identification of activated sludge foaming bacteria. *Water Science and Technology* **29**, 35–42.

Blackall, L.L., Seviour, E.M., Cunningham, M.A. *et al.* (1994) '*Microthrix parvicella*' is a novel, deep branching member of the *actinomycetes subphylum*. *Systematic and Applied Microbiology* **17**, 513–518.

Dudman, W.F. (1977) The role of surface polysaccharides in natural environments. In: Surface carbohydrates of the prokaryotic cell, ed. *Sutherland I*, pp. 357–414. London: Academic Press Inc Ltd.

Fahy, P.C. and Hayward, A.C. (1983) KOH solubility test. In: Plant bacterial diseases-a diagnostic guide, ed. Fahy P C And Persley G J, pp. 342–343. Sydney: Academic Press Inc.

Hepper, C.M. (1975) Extracellular polysaccharides of soil bacteria. In: Soil Microbiology, ed. Walker N., pp. 93–110. London: Butterworth and Co Ltd.

Holdeman, L.V., Cato, E.P. and Moore, W.E.C. (1977) Anaerobe Laboratory Manual, 4th edn., Blacksburg: Virginia Polytechnic Institute and State University.

Hungate, R.E. (1966) *The Rumen and its Microbes*. London: Academic Press Inc.

Klieve, A.V., Hudman, J.F. and Bauchop, T. (1989) Inducible bacteriophages from ruminal bacteria. *Applied and Environmental Microbiology* **55**, 1630–1634.

L'Hermite, P., Sequi, P. and Voorburg, J.H. (1992) *Scientific basis for an environmentally safe and efficient management of livestock farming*. Report of the Scientific Committee of the European Conference Environment, Agriculture and Stock Farming in Europe. Brussels, Belgium.

Pichinoty, F. (1983) Isolement d'une bacterie de grandes dimensions, aerobie, sporulee, engainee, par culture elective a partir du sol. *Annales de Microbiologie (Paris)* **134B**, 443–446.

Pichinoty, F. and Asselineau, J. (1984) Morphologie et cytologie de Bacillus benzoevorans, une nouvelle espece filamenteuse, engainee et mesophile, degradant divers acides aromatiques et phenols. *Annales de Microbiologie (Paris)* **135B**, 199–207.

Pichinoty, F., Asselineau, J. and Mandal, M. (1984) Caracterisation biochimique de *Bacillus benzoevorans* sp. nov., une nouvelle espece filamenteuse, engainee et mesophile, degradant divers acides aromatiques et phenols. *Annales de Microbiologie (Paris)* **135B**, 209–217.

Roberson, E.B. and Firestone, M.K. (1992) Relationship between dessication and exopolysaccharide production in a soil *Pseudomonas* sp. *Applied and Environmental Microbiology* **58**, 1284–1291.

Teather, R.M. (1982) Maintenance of laboratory strains of obligately anaerobic rumen bacteria. *Applied and Environmental Microbiology* **44**, 499–501.

Watts, P.J. and Tucker, R.W. (1993) The effect of ration on waste management and odour control in feedlots. *Recent Advances in Animal Nutrition in Australia* **1993**, 117–129.

Downloaded from https://academic.oup.com/lambio/article/29/1/71/6703789 by DAF: Dept of Agriculture and Fisheries user on 13 March 2024