APPLIED AND ENVIRONMENTAL MICROBIOLOGY, May 1992, p. 1754–1759 Vol. 58, No. 5
0099-2240/92/051754-06$02.00/0
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Tannin-Protein Complex-Degrading Enterobacteria Isolated from the Alimentary Tracts of Koalas and a Selective Medium for Their Enumeration

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Received 23 October 1991/Accepted 7 March 1992

Tannin-protein complex (T-PC)-degrading enterobacteria (T-PCDE) were isolated from the feces and from a layer of bacteria attached to the cecal wall of koalas. The T-PCDE were facultatively anaerobic, gram-negative, pleomorphic, nonmotile bacilli. The bacteria were also oxidase and catalase negative and resistant to vancomycin, reduced nitrates to nitrites, and grew on MacConkey agar. Growth on tannin-treated agar media showed a distinctive clear zone around the colony. From these observations, a selective agar plate medium (vancomycin- and tannin-treated Wilkins-Chalgren anaerobe agar) was developed to enumerate T-PCDE isolated from the feces of koalas. This medium was highly selective in the enumeration of the fecal T-PCDE and inhibited the growth of concomitant T-PC-degrading *Streptococcus bovis*. The T-PCDE were isolated from 10 of the 12 captive koalas studied; in 8 of these 10 koalas, the facultatively anaerobic bacterial flora was dominated (more than 60%) by T-PCDE. Viable numbers of T-PCDE were, in most of the animals, much larger (more than 100 times) than the numbers of T-PC-degrading *S. bovis*, suggesting that T-PCDE played a more active role in digesting T-PC in the alimentary tracts of koalas.

Tannins occur widely in a variety of plants, including monocotyledons, dicotyledons, and ferns (6). They are known to bind strongly to proteins in vitro and form a compound called tannin-protein complex (T-PC). The complexes are quite resistant to degradation by digestive enzymes, thereby interfering with nitrogen balance in vivo (3, 6, 12).

Microbial degradation of T-PC was first described for *Streptococcus bovis* biotype I isolated from feces of the koala, *Phascolarctos cinereus* (Goldfuss), the food source of which is almost exclusively tannin-rich eucalyptus leaves (7). A tannin-treated selective agar medium was developed (9) in order to investigate the occurrence of T-PC-degrading *S. bovis* in the fecal flora of various animals, and it was found that this bacterium was the dominant streptococcal flora of koalas (9a). The bacterium apparently has an ecological advantage to cleave the protein moiety from the T-PC and thereby satisfy its nitrogen requirement. This, in turn, suggests that the koala has a microbiological mechanism for deriving dietary proteins from the T-PC.

In an attempt to elucidate such a mechanism, I became interested in the possible involvement of bacteria other than *S. bovis* in T-PC degradation in the alimentary tracts of koalas. I report here on the characterization of T-PC-degrading, facultatively anaerobic enterobacteria isolated from the feces and cecal walls of koalas. In addition, I report on the development of selective plate medium for their isolation and enumeration.

MATERIALS AND METHODS

Media. The media used in this study were as follows: (i) Wilkins-Chalgren anaerobe agar (WCAA; Oxoid Ltd., Basingstoke, Hampshire, United Kingdom), the formulation of which included L-arginine as a readily available source of organic nitrogen; (ii) WCAA supplemented with 5% defibrinated horse blood (WCAA+B); (iii) WCAA treated with filter-sterilized 2% tannic acid solution (Kanto Chemical Co., Inc., Nihonbashi, Tokyo, Japan) (T-TWCAA); (iv) T-TWCAA containing vancomycin hydrochloride (Sigma Chemical Co., St. Louis, Mo.) at a final concentration of 2.5 mg/liter (VAT-TWCAA); (v) brain heart infusion agar (Oxoid) containing 0.5% yeast extract (Oxoid) (BHIA); (vi) BHIA supplemented with 5% defibrinated horse blood (BHIA+B); (vii) BHIA treated with filter-sterilized 2% tannic acid solution (T-TBHIA); (viii) T-TBHIA with *Streptococcus* selective supplement (Oxoid) containing colistin sulfate and oxolinic acid (COT-TBHIA).

The pH of the medium was adjusted to 7.1 ± 0.1. The methods of treatment with tannic acid and storage of the tannin-treated media were detailed by Osawa (7). WCAA+B was used for the maintenance of most strains throughout the study.

Isolation of T-PC-degrading strains. A fresh fecal pellet (1 g, wet weight) was collected from the anogenital orifice of an apparently healthy koala kept at Lone Pine Koala Sanctuary. A portion (approximately 3 by 3 cm) of fresh cecal wall was also obtained from another adult koala, which had been euthanized because of severe traumatic injuries. The cecal wall was rinsed gently with sterile diluent several times. After being rinsed, the surface of the cecal wall retained a dark matted appearance, due to a thin layer of cecal content which covered the cecal epithelium. A subsequent microscopic examination of the surface of the cecal wall revealed that the layer consisted mainly of very long bacilli, one end of which adhered to the cecal epithelium. This bacterial layer was scraped off the wall with a sterile scalpel blade. The fecal and cecal samples that were collected were placed separately in individual tubes containing 20 ml of sterile 0.25-strength Ringer solution (Oxoid; used extensively as "diluent" for the resuspension of cells throughout the present investigation) and emulsified thoroughly with a homogenizer and a vortex mixer. Approximately 0.1 ml of the suspension was spread onto WCAA+B, and the plates were

incubated for 3 days at 37°C. Colonies differing in morphology were isolated from both samples, and these isolates were then subcultured onto T-TBHIA. The plates were incubated in an atmosphere enriched with 8 to 10% CO_2, 8 to 10% H_2, and 80 to 85% N_2 by using Bio-bags Type A (Becton Dickinson and Co., Cockeysville, Md.; referred to as anaerobic incubation hereafter) at 37°C for 3 days.

After incubation, many isolates developed very fine colonies on the plates, with clear zones extending just beyond their edges. These isolates from fecal and cecal samples were designated strains UQM 3666 and UQM 3667, respectively.

Bacterial strains. *Enterobacter agglomerans* UQM 1615 (from Department of Microbiology, University of Queensland, St. Lucia, Queensland, Australia) and the isolated strains UQM 3666 and UQM 3667 (collectively referred to as T-PC-degrading enterobacteria [T-PCDE] hereafter) were used throughout this study. Two T-PC-degrading strains of *S. bovis* I, UQM 3546 (strain NCDO 2019 from the National Collection of Dairy Organisms, Reading, United Kingdom) and UQM 3611 (isolated from koala feces), were also used to compare clear zone formations on various tannin-treated media.

Determination of strain properties. The microscopic and colonial morphologies of the strains were determined by using Gram stain and growth on WCAA+B, respectively. A well-established colony of each strain on WCAA was inoculated onto T-TBHIA, COT-TBHIA, T-TWCAA, and VAT-TWCAA and incubated anaerobically at 37°C; 5 days later, the colonies were examined for growth and clear zone formation. Biochemical properties, including fermentation of carbohydrates and enzymatic activities, were determined with commercial identification kits (Api 24 E and Api 50 CHE; API System, Montalieu, Vercieu, France). Motility and fermentation of glucose were tested with the commercial kits M Medium (API System) and OF Medium (API System), respectively. Growth on MacConkey agar (Oxoid) and Simmons citrate agar (Oxoid) supplemented with 0.2% glucose was also determined.

Growth requirements for blood and oxygen were tested by spreading a series of 10-fold dilutions (10^{-1} to 10^{-4}) prepared from a single colony of each anaerobically cultured strain (2 days old) onto WCAA, WCAA+B, BHIA, and BHIA+B with the aid of sterile L-shaped glass rods. These inoculated plates were incubated aerobically, anaerobically, and in an atmosphere of reduced oxygen (5 to 15% O_2) by using Bio-bags Cfj (Becton Dickinson and Co.; referred to as microaerophilic incubation hereafter) at 37°C for 3 days for surface plate counts. All counts were done in triplicate.

Susceptibility to various antibiotics, including kanamycin, colistin, vancomycin, chloramphenicol, erythromycin, oxytetracycline, penicillin, gentamicin, rifampin, and ampicillin, was determined by the presence of an inhibition zone around antibiotic-impregnated discs (Oxoid) on WCAA+B (see Table 1 for amount of each antibiotic in the discs).

Evaluation of a selective medium. A series of 10-fold dilutions (10^{-1} to 10^{-4}) of a single colony of each anaerobically cultured T-PCDE strain was made in diluent. From each dilution, 0.1-ml samples were spread onto WCAA and VAT-TWCAA in the manner described above. The plates were then incubated microaerophilically at 37°C for 3 days for surface plate counts and evaluation of clear zones.

Fresh fecal pellets were collected from 12 male koalas kept at Lone Pine Koala Sanctuary in August 1991. A series of 10-fold dilutions (10^{-1} to 10^{-5}) of the emulsified feces (1 g) was spread onto WCAA, VAT-TWCAA, and COT-TBHIA in the manner described above. The plates of WCAA and VAT-TWCAA and the plates of COT-TBHIA were incubated microaerophilically and anaerobically, respectively, at 37°C for 3 days to enumerate total facultatively anaerobic bacteria, T-PC-degrading gram-negative bacilli, and T-PC-degrading streptococci. Phenotypic characteristics of T-PC-degrading gram-negative bacilli isolated from the koalas were characterized in the same manner as with the T-PCDE.

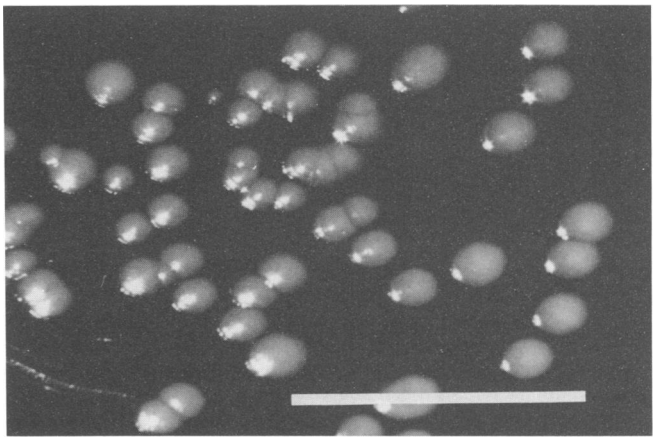

FIG. 1. Surface colonies on WCAA+B incubated aerobically at 37°C for 72 h. Bar, 10 mm.

RESULTS

Colony and cellular morphology. The T-PCDE strains formed smooth grey to white colonies 1 to 3 mm in diameter without hemolysis on WCAA+B and BHIA+B after 3 days of aerobic incubation (Fig. 1). The strains were gram-negative bacilli of various sizes (Fig. 2) and included coccoidal rods (0.5 by 2.0 μm), short rods (0.5 by 3 to 5 μm), and long filamentous rods (0.5 by 50 to 100 μm).

Growth and clear zone formation on tannin-treated media. The presence of oxygen also supported the growth of T-PCDE but caused severe darkening of the surface of tannin-treated medium (possibly because of the oxidization of T-PC), which made it technically difficult to observe the formation of clear zones on the plate. This problem was,

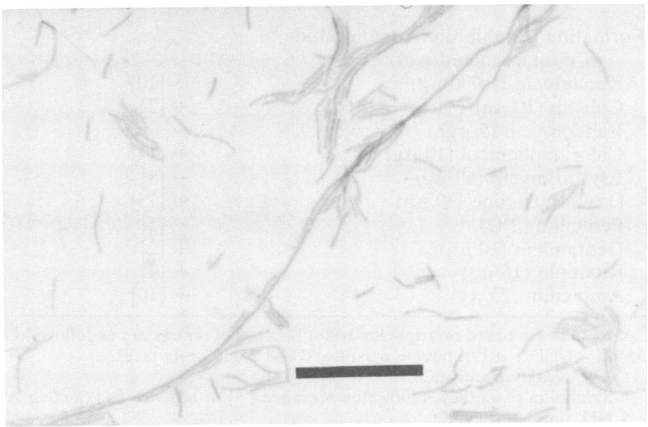

FIG. 2. Gram-stained cells, including an extremely long cell, from WCAA+B incubated aerobically at 37°C for 48 h. Bar, 20 μm.

TABLE 1. Comparison of phenotypic characteristics of T-PCDE strains isolated from alimentary tracts of koalas and of other T-PC-degrading strains

Characteristic	Results with[a]:				
	T-PCDE strains		*E. agglomerans* UQM 1615	*S. bovis* biotype I	
	UQM 3666	UQM 3667		UQM 3546	UQM 3611
Growth and clear zone formation on:					
T-TBHIA	+w[b] (+)[c]	+w (+)	+ (−)	+ (+)	+ (+)
COT-TBHIA	−	−	−	+ (+)	+ (+)
T-TWCAA	+ (+)	+ (+)	+ (−)	+ (+)	+ (+)
VAT-TWCAA	+ (+)	+ (+)	+ (−)	−	−
Growth on MacConkey agar	+w	+w	+	−	−
Ammonium as sole nitrogen source	−	−	+	+	+
Motility	−	−	+	−	−
Enzyme reactions					
β-Galactosidase	+	+	+	ND[d]	ND
Arginine dihydrolase	−	−	−	ND	ND
Lysine decarboxylase	−	−	−	ND	ND
Ornithine decarboxylase	−	−	−	ND	ND
Citrate utilization	−	−	+	ND	ND
H_2S production	−	−	−	ND	ND
Urease	−	−	−	ND	ND
Tryptophan desaminase	−	−	−	ND	ND
Indole production	−	−	−	ND	ND
Voges-Proskauer reaction	+w	+w	+w	ND	ND
Gelatin hydrolysis	−	−	−	ND	ND
Reduction of nitrates	+	+	+	ND	ND
Oxidase	−	−	−	−	−
Catalase	−	−	−	−	−
Acid production from:					
D-Glucose (oxidative)	+	+	+	ND	ND
D-Glucose (fermentative)	+	+	+	ND	ND
Mannitol	−	−	+	ND	ND
Inocitol	−	−	+	ND	ND
Sorbitol	−	−	+	ND	ND
Rhamnose	+	+	+	ND	ND
Sucrose	+	+	+	ND	ND
Melibiose	+	+	+	ND	ND
Amygdaline	+	+	+	ND	ND
L-Arabinose	+	+	+	ND	ND
Glycerol[e]	−	+	+	ND	ND
D-Arabinose[e]	−	+	−	ND	ND
D-Xylose[e]	−	+	+	ND	ND
Adonitol[e]	+	−	−	ND	ND
Amidon[e]	+	−	−	ND	ND
D-Arabitol[e]	+	−	+	ND	ND
Gluconate[e]	+	−	+	ND	ND
Formation of inhibition zone around disc impregnated with:					
Kanamycin (1,000 μg)	+ [10][f]	+ [11]	+ [7]	ND	ND
Colistin (10 μg)	+ [2]	+ [4]	+ [2]	ND	ND
Vancomycin (5 μg)	−	−	−	ND	ND
Chloramphenicol (10 μg)	+ [11]	+ [12]	+ [8]	ND	ND
Erythromycin (60 μg)	+ [5]	+ [6]	+ [4]	ND	ND
Oxytetracycline (30 μg)	+ [15]	+ [11]	+ [7]	ND	ND
Penicillin (2 U)	+ [5]	+ [2]	−	ND	ND
Gentamicin (10 μg)	+ [2]	+ [2]	+ [3]	ND	ND
Rifampin (15 μg)	+ [4]	+ [4]	+ [4]	ND	ND
Ampicillin (25 μg)	+ [10]	+ [9]	−	ND	ND

[a] Results are based on triplicate tests. Sources of strains are as follows: UQM 3666, koala feces; UQM 3667, koala cecal wall; UQM 1615, not known; UQM 3546 (= NCDO 2019), bovine mastitis; UQM 3611, koala feces.

[b] +w, weakly positive.

[c] Symbol in parentheses indicates presence (+) or absence (−) of clear zone.

[d] ND, not determined.

[e] Determined by Api 50 CHE (API System).

[f] Number in brackets is average size of inhibition zone (expressed in millimeters).

TABLE 2. Recovery of pure cultures on WCAA, WCAA+B, BHIA, and BHIA+B under aerobic (O+), microaerophilic (O+w), and anaerobic (O−) conditions

Strain	Dilution of primary suspension[a]	Atmospheric condition	No. of colonies on[b]:			
			WCAA	WCAA+B	BHIA	BHIA+B
UQM 3666	10^{-5}	O+	69	67	57	73
		O+w	76	69	56	64
		O−	0*[c]	0*	0*	0*
UQM 3667	10^{-5}	O+	118	130	76	111
		O+w	118	117	0	120
		O−	0*	0*	0*	0*
UQM 1615	10^{-5}	O+	52	55	56	56
		O+w	48	55	62	53
		O−	49	49	56	57

[a] Primary suspension was prepared by suspending a well-established colony in a 10-ml diluent.
[b] Each result is the mean of triplicate counts.
[c] 0*, weak growth was observed with the lower dilutions ($10^{-1} \sim 10^{-3}$).

however, circumvented by incubating the inoculated plate microaerophilically to keep the darkening to a minimum. Growth and clear zone formation by the strains on various tannin-treated media are summarized in Table 1. The T-PCDE strains grew on T-TBHIA, T-TWCAA, and VAT-TWCAA with the formation of a clear zone but failed to grow on COT-TBHIA. It should be noted that the growth and clear zone formation shown by both strains were much smaller on T-TBHIA than on other media. The strain *E. agglomerans* UQM 1615 grew on all tannin-treated media except COT-TBHIA but did not form a clear zone. Strains of T-PC-degrading *S. bovis* grew with the formation of a clear zone on all tannin-treated media except VAT-TWCAA.

Physiological and biochemical properties. Physiological and biochemical properties of the T-PCDE strains and of *E. agglomerans* UQM 1615 are summarized in Table 1. The T-PCDE were characterized by positive tests for β-galactosidase, Voges-Proskauer reaction, and reduction of nitrates and by negative tests for motility, oxidase, gelatin hydrolysis, citrate utilization, and indole production. They grew poorly on MacConkey agar medium and failed to grow on Simmons citrate agar supplemented with 0.2% glucose, indicating that they are not capable of utilizing inorganic nitrogen (ammonium) as a sole source of nitrogen. Both strains shared the same characteristic pattern by Api 24 E (API System) but were differentiated by additional tests for hydrolysis of carbohydrates by using Api 50 CHE (API System). The APILAB software (API System) indicated that the pattern of the reactions of both strains identified them as *E. agglomerans*. The strains were susceptible to all antibiotics tested except vancomycin. *E. agglomerans* UQM 1615 demonstrated the same susceptibility pattern except that it was resistant to ampicillin.

Requirements of blood and oxygen for growth. The results for the T-PCDE strains and for *E. agglomerans* UQM 1615 after 3 days of growth on BHIA, BHIA+B, WCAA, and WCAA+B under three different atmospheric conditions are shown in Table 2. The recovery of T-PCDE was very poor on any medium incubated anaerobically. For aerobic and microaerophilic incubations, recoveries of both strains on the different media were comparable, except that recovery of UQM 3667 was significantly ($P < 0.01$ by Student's t test) lower on BHIA than on other media. Recoveries of *E. agglomerans* 1615 were comparable regardless of the type of media or atmospheric conditions.

VAT-TWCAA as a selective medium for enumeration. The results of microaerophilic incubation of the T-PCDE strains indicated that the counts of both strains on VAT-TWCAA did not differ significantly ($P > 0.9$ by Student's t test) from the corresponding counts obtained with WCAA+B. Colonies formed on VAT-TWCAA had distinctive clear zones extending beyond their edges, but their mean size was slightly smaller (1.0 mm in diameter) than that on WCAA+B (1.5 mm).

T-PC-degrading gram-negative bacilli were isolated from 10 of 12 animals studied (Table 3). They formed distinctive clear zones on VAT-TWCAA (Fig. 3) and had the same morphological and biochemical characteristics as the T-PCDE strains, as tested by API 24 E (API System), except that two of the isolates gave a negative test for hydrolysis of rhamnose. The viable counts obtained from VAT-TWCAA were in good agreement with those obtained from WCAA+B ($r = 0.99$; $P < 0.001$). The viable counts (log CFU/g of wet feces) of T-PCDE from the 10 koalas ranged from 4.7 to 7.9, and these bacteria predominated (more than 60% in 8 koalas) the facultatively anaerobic fecal flora (Table 3).

The remaining flora consisted mainly of enterobacteria, including *Escherichia coli* and *Klebsiella pneumoniae*, which also grew on VAT-TWCAA, but their colonies did not form clear zones.

TABLE 3. Viable counts of total facultative anaerobic bacteria (TFAB), T-PCDE, and T-PC-degrading *S. bovis* (T-PCDSB)

Animal no.	Viable counts (log CFU/g [wet wt] of feces) of:				T-PCDE/TFAB ratio (%)	T-PCDE/T-PCDSB ratio (fold)
	TFAB on WCAA+B	T-PCDE on WCAA+B	T-PCDE on VAT-TWCAA	T-PCDSB on COT-TBHIA		
K-1	7.1	7.1	7.1	4.3	114	6.8×10^2
K-2	6.6	6.6	6.6	3.9	117	6.0×10^2
K-3	7.3	7.3	7.3	4.0	93	1.5×10^3
K-4	5.8	5.6	5.6	3.6	65	1.0×10^2
K-5	5.7	5.6	5.4	2.7	80	5.0×10^2
K-6	5.2	4.9	4.9	4.8	38	1.3
K-7	7.3	7.3	7.3	4.0	102	2.0×10^3
K-8	4.4	Absent	Absent	4.1		
K-9	7.9	7.9	7.9	2.6	97	1.8×10^5
K-10	4.3	Absent	Absent	3.7		
K-11	5.8	5.7	5.4	4.0	83	4.4×10^1
K-12	5.6	4.7	4.7	4.7	13	1.5

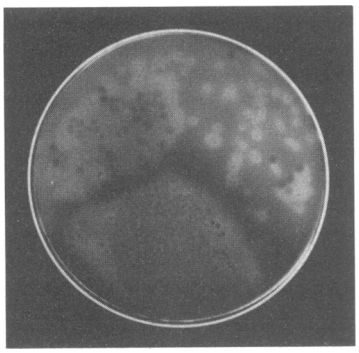

FIG. 3. Growth of T-PCDE forming clear zones around their colonies on VAT-TWCAA (after 72 h of incubation at 37°C). Note that three serial 10-fold dilutions (10^{-1}, 10^{-3}, and 10^{-4}) of koala feces were spread on equally divided areas of the plate.

T-PC-degrading *S. bovis* was isolated in all 12 animals; the viable counts ranged from 2.6 to 4.8, but they were less than 1/100 of the corresponding counts of T-PCDE in the 7 of 10 animals that also had both types of T-PC-degrading bacteria in their feces (Table 3).

DISCUSSION

Taxonomic considerations. The T-PCDE strains are likely to fall within the family *Enterobacteriaceae* because they are facultatively anaerobic, oxidase-negative, gram-negative bacilli. They reduce nitrates to nitrites and grow on MacConkey agar. These observations indicate that the strains resemble *E. agglomerans*. *E. agglomerans* is a well-established human pathogen (10) and is also isolated from many plants and animals (4, 11), but its taxonomic status is poorly defined because of its heterogeneity, and the species was subclassified into a number of biogroups (2). The present isolates, however, do not belong to any of these biogroups, and their nonmotility is inconsistent with any species belonging to the genus *Enterobacter* as described by Brenner (1). The frequent occurrence of extremely long filamentous cells (more than 0.1 mm) is also a distinct morphological characteristic of these koala isolates. Thus, the T-PCDE may belong to a new genus, but a formal designation should await further genetical and phenotypic examinations on the strains.

Ecological considerations. It has recently been demonstrated (9a) that koalas have a significantly high proportion of T-PC-degrading *S. bovis*. It has been also reported that this bacterium dominated the fecal streptococcal flora of koalas which apparently fed on eucalyptus leaves with higher tannin content (8). On the basis of this, the possibility was considered that there is an active adaptive strategy based on a microbial degradation of T-PC in the koala's alimentary tract. In the present study, it was found that T-PCDE were predominant in the facultatively anaerobic microflora and that their viable counts were significantly higher than the counts for T-PC-degrading *S. bovis* in most koalas. The evidence suggests that the T-PCDE play a much more active role in the digestion of dietary T-PC than does T-PC-degrading *S. bovis*.

McKenzie (5) presented scanning electron micrographs showing that a variety of microbes were attached to the surface of the cecal epithelia of koalas. In the present study, the T-PCDE strain UQM 3667 was, in fact, isolated from a "bacterial layer" on the cecal wall. Thus, it is likely that the T-PCDE colonize the cecum and that dead or unattached microbes are washed into the colon. This may explain why the T-PCDE were absent in the fecal flora of some of the koalas studied. An immunohistological study using rabbit antiserum against the T-PCDE is in progress to determine the specific colonization.

Evaluation of VAT-TWCAA as a selective medium for enumeration. The newly developed VAT-TWCAA is based on the ability of the T-PCDE strains to form well-established colonies with distinctive clear zones on T-TWCAA but not on T-TBHIA and on the T-PCDE strains' resistance to vancomycin.

In the course of developing a selective medium for T-PC-degrading *S. bovis*, Osawa and Mitsuoka (9) considered the possibility that T-TBHIA would allow the growth of only the least nutritionally demanding bacteria such as *S. bovis*, *E. coli*, and *Proteus mirabilis*, since the available source of nitrogen would be in the form of amino acids and inorganic nitrogen, which were not bound to tannins. As described earlier, the T-PCDE strains are not capable of utilizing inorganic nitrogen as a sole source of nitrogen, and therefore an amino-acid-supplemented medium such as T-TWCAA would be necessary to ensure sufficient growth of T-PCDE strains.

The VAT-TWCAA is a useful medium for selective enumeration of T-PCDE strains in koala feces. The inclusion of vancomycin prevented the growth of T-PC-degrading *S. bovis*, thus facilitating the identification of T-PCDE. The use of this medium together with COT-TBHIA could be applied to the evaluation of T-PC-degrading activity of the alimentary microflora in other animals.

ACKNOWLEDGMENTS

This study was conducted under permits from the Queensland National Parks and Wildlife Service.

I thank P. Bird of the Department of Dentistry, Oral Biology and Pathology, University of Queensland, and W. Blanshard, Veterinary Service and Research, Lone Pine Koala Sanctuary for their valuable comments and criticism on an earlier draft of this paper. I also thank L. I. Sly and D. Grassick of the Department of Microbiology, University of Queensland for providing and maintaining strains of bacteria for this study.

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