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Formation of a Clear Zone on Tannin-Treated Brain Heart Infusion Agar by a *Streptococcus* sp. Isolated from Feces of Koalas

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Gram-positive cocci, isolated from the feces of koalas and identified as *Streptococcus bovis* biotype I, formed a distinct clear zone on tannin-treated brain heart infusion agar, suggesting that this isolate has the unique characteristic of degrading the tannin-protein complex.

The koala, *Phascolarctos cinereus* (Goldfuss), is an arboreal marsupial inhabiting the forests of eastern Australia. It is entirely folivorous, feeding almost exclusively on the foliage of *Eucalyptus* spp. (5, 6), which are known to have high concentration of tannins (3, 11). Tannins are a diverse group of soluble phenolic compounds that form chemical complexes with proteins. These complexes are considered to be resistant to degradation (i.e., by protease) within the guts of mammals, thereby interfering with digestion and the utilization of dietary protein (8, 13, 16, 18). Cork et al. (2) found a low tannin level in koala feces relative to that in the diet and suggested that the tannin fraction of the total phenolic compounds was degraded to a considerable extent in the alimentary tracts of koalas. Investigation of the functions of the ceca of the koala (12) and the ring tail possum, *Pseudocherius peregrinus*, another marsupial feeding on eucalyptus diets (A. Lomdahl, Hons. thesis, Monash University, Clayton, 1983), led to the claim that bacterial degradation of tannin-protein complexes in the cecum may make protein available to these animals.

Fresh fecal pellets were collected directly from the anogenital orifice of 12 captive koalas (nine adult males and three adult females) kept at the Lone Pine Koala Sanctuary. The animals were provided daily with fresh eucalyptus leaves ad libitum. The major species of eucalyptus leaves fed to the animals included forest red gum (*Eucalyptus tereticornis*), tallow wood (*E. microcorys*), and grey gum (*E. punctata*). Fecal pellets collected from each animal were weighed aseptically, and approximately 1 to 2 g (wet weight) was taken into a tube containing 20 ml of sterile 0.25-strength Ringer solution (Oxoid Ltd., Basingstoke, Hampshire, United Kingdom) and thoroughly mixed with a homogenizer and a Vortex test-tube mixer.

Approximately 5 ml of filter-sterilized 2% tannic acid solution (Kanto Chemical Co., Inc., Nihonbashi, Tokyo, Japan) was overlaid on a plate of brain heart infusion agar medium (Oxoid) containing 0.5% yeast extract (Oxoid) for 20 min. After this treatment, the surface of the medium was highly opaque, indicating that the overlaid tannic acids were bound to a protein fraction available on the surface of the medium, forming insoluble complexes. The overlaid tannic acid solution was then removed with an aspirator, and the surface of the medium was rinsed with sterile 0.25-strength Ringer solution three times to ensure removal of residual tannic acids from the surface of the medium. The tannin-treated brain heart infusion agar medium thus prepared (T-TBHIA) was stored anaerobically in a Bio-bag (Marion Laboratories, Inc., Kansas City, Mo.), since we found that the surface of the T-TBHIA became dark after prolonged exposure to the air (possibly due to oxidization of tannin-protein complexes). This anaerobic storage method successfully prevented the darkening, and the surface of the medium remained opaque. About 0.1 ml of the fecal suspension was spread onto the T-TBHIA and incubated anaerobically at 37°C for 72 h.

After the incubation, smooth-surfaced white colonies 2 to 3 mm in diameter grew on the T-TBHIA plates for 8 of 12 koalas. These colonies had distinct, clear zones extending just beyond their edges (Fig. 1). The purified isolates from the colonies showed gram-positive cocci. Subsequent biochemical tests by the API 20 STREP system (API System, Montalieu, Vercieu, France) revealed that the isolates (from eight plates) were *Streptococcus bovis* biotype I.

Table 1 summarizes the growth, formation of clear zones on T-TBHIA, and other characteristics of these isolates and the other species (*Streptococcus faecium*, *S. faecalis*) and strains (*S. bovis* biotypes I and II/1) used for comparison. *S. bovis* biotype I (UQ I) grew on T-TBHIA with a clear zone, whereas *S. bovis* biotype II/1 (UQ II) grew on this medium but did not form a clear zone. Strains of *S. faecalis* (QUT I) and *S. faecium* (QUT II) hardly grew on the T-TBHIA. This suggests that *S. bovis* biotype I is capable of degrading the tannin-protein complex. Such microbial degradation might help koalas obtain dietary protein from tannin-rich eucalyptus, but further work in enumerating this isolate in the

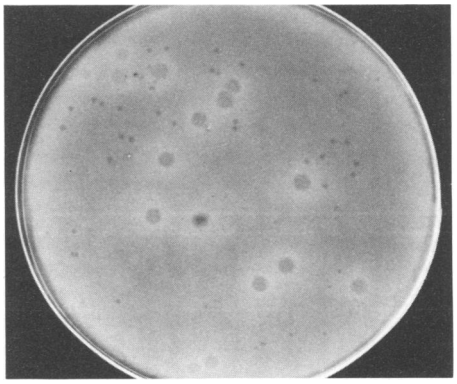

FIG. 1. Growth of bacteria forming clear zones around their colonies on T-TBHIA (after 72 h of incubation at 37°C).

TABLE 1. Growth and clear zone formation on T-TBHIA and other biochemical characteristics of gram-positive cocci isolated from koala feces[a]

Strain	Isolated (or obtained) from:	Final identification by API 20 STREP	Biotype	Gram stain	Growth	Clear zone	Catalase	β-Hemolysis[b]	VP	HIP	ESC	PYRA	α-GAL	β-GUR	β-GAL	PAL	LAP	ADH	RIB	ARA	MAN	SOR	LAC	TRE	INU	RAF	AMD	GLYG
K-1	Koala	*S. bovis*	I	+	+	+	−	−	+	−	+	−	+	−	−	−	+	−	−	−	+	−	+	+	−	+	+	+
K-4	Koala	*S. bovis*	I	+	+	+	−	−	+	−	+	−	+	−	−	−	+	−	−	−	+	−	+	+	−	+	+	+
K-7	Koala	*S. bovis*	I	+	+	+	−	−	+	−	+	−	+	−	−	−	+	−	−	−	+	−	+	+	−	+	+	+
K-10	Koala	*S. bovis*	I	+	+	+	−	−	+	−	+	−	+	−	−	−	+	−	−	−	+	−	+	+	−	+	+	+
K-13	Koala	*S. bovis*	I	+	+	+	−	−	+	−	+	−	+	−	−	−	+	−	−	−	+	−	+	+	−	+	+	+
K-16	Koala	*S. bovis*	I	+	+	+	−	−	+	−	+	−	+	−	−	−	+	−	−	−	+	−	+	+	−	+	+	+
K-31	Koala	*S. bovis*	I	+	+	+	−	−	+	−	+	−	+	−	−	−	+	−	−	−	+	−	+	+	−	+	+	+
K-35	Koala	*S. bovis*	I	+	+	+	−	−	+	−	+	−	+	−	−	−	+	−	−	−	+	−	+	+	−	+	+	+
UQ 1	UQ	*S. bovis*	I	+	+	+	−	−	+	−	+	−	+	−	−	−	+	−	−	−	+	−	+	+	+	+	+	+
UQ II	UQ	*S. bovis*	II/1	+	+	−	−	−	+	+	+	+	+	−	−	−	+	+	−	−	−	−	+	+	+	+	+	+
QUT I	QUT	*S. faecalis*	1	+	−	−	−	−	+	+	+	+	−	−	−	−	+	+	+	−	+	+	−	+	−	−	−	−
QUT II	QUT	*S. facium*	1	+	−	−	−	−	+	+	+	+	+	−	+	−	+	+	+	+	+	+	−	+	−	−	−	−

[a] Abbreviations: UQ, University of Queensland; QUT, Queensland University of Technology; VP, acetoin production; HIP, hippurate hydrolysis; ESC, esculin hydrolysis; PYPRA, pyrrolidonylarylamidase; α-GAL, α-galactosidase; β-GUR, β-glucuronidase; β-GAL, β-galactosidase; PAL, alkaline phosphatase; LAP, leucine arylamidase; ADH, arginine dihydrolase; RIB, ribose; ARA, L-arabinose; MAN, mannitol; SOR, sorbitol; LAC, lactose; TRE, trehalose; INU, inulin; RAF, raffinose; AMD, starch; GLYG, glycogen.

[b] On Columbia blood agar medium.

koala's alimentary tract and determining the pathway for the degradation is necessary to substantiate this view.

The results also suggest that the formation of a clear zone is a phenotypic character specific to *S. bovis* biotype I. A number of strains of *S. bovis* have been isolated from the alimentary tracts of ruminants, including cattle (9), sheep (9), water buffaloes (17), goats (4), and mule deer (14), and nonruminants such as rhesus monkeys (1) and pigs (15). However, recent taxonomic studies have indicated that the current classification of *S. bovis* is problematic. For example, many bovine strains of *S. bovis* differed in physiologic characteristics from strains derived from humans (10). Farrow et al. (7) demonstrated that some atypical *S. bovis* strains from straw and cattle formed a distinct group on the basis of DNA-DNA homologies. Further work with a wider range of type strains of *S. bovis* and other relevant species with known DNA status is in progress to determine whether the formation of a clear zone is specific to *S. bovis* biotype I.

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