Biodegradation **4**: 91–99, 1993.

Metabolism of tannin-protein complex by facultatively anaerobic bacteria isolated from koala feces

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Received 10 June 1992; accepted in revised form 30 November 1992

Key words: tannin-protein complex, gallate, pyrogallol, phloroglucinol, koala

Abstract

The metabolic pathways involved in degradation of tannin-protein complex (T-PC) were investigated in various facultatively anaerobic bacteria, with specific reference to fecal isolates from the koala including T-PC-degrading enterobacteria (T-PCDE), *Streptococcus bovis, Klebsiella pneumoniae,* and *K. oxytoca.* It was demonstrated that T-PCDE and *S. bovis* biotype I were capable of degrading protein complexed with gallotannin (a hydrolyzable tannin), but not that complexed with quebracho (a condensed tannin). Subsequent studies showed that these strains metabolized gallic acid to pyrogallol. Strains of *Klebsiella pneumoniae* and *K. oxytoca,* which did not degrade T-PC, also metabolized gallic acid into pyrogallol. Pyrogallol was not degraded by any strains studied, but it was not detected in fresh feces of the koalas. The majority of strains isolated from feces could degrade phloroglucinol. Based on these findings, we propose that members of the gut microflora of the koala cooperate in the degradation of T-PC.

Introduction

Tannins are phenolic compounds that occur widely in the plant kingdom (Bate-Smith & Lerner 1954; White 1957) and are known to form chemical complexes with proteins in vitro (Hagerman & Butler 1981; McManus et al. 1983). These complexes have been considered to be resistant to degradation within the gut of mammals thus reducing apparent digestibility of protein (Feeny 1976; McLeod 1974; Rhoades & Cates 1976) and increasing fecal nitrogen excretion (Mole & Waterman 1987). On this basis, tannins have been considered as a defense mechanism of plants against herbivory (Rhoades & Cates 1976; Swain 1978).

Tannins are broadly classified into two groups, based on structure: hydrolyzable tannins and condensed tannins (Zucker 1983; Salunkhe et al. 1989). Hydrolyzable tannins contain a central core of carbohydrates (i.e., glucose and polyhydric alcohol) which are esterified by phenolics (i.e. gallic acid). Condensed tannins are structurally more complex, not hydrolyzable, and do not contain carbohydrates.

The koala, *Phascolarctos cinereus,* is an arboreal marsupial inhabiting the forest of eastern Australia. It feeds almost exclusively on the foliage of *Euca-*

lyptus spp. (Eberhard 1978; Eberhard et al. 1975), which are known to have a high content of tannins (Macauley & Fox 1980; Cork & Pahl 1984). This led us to the hypothesis that the koala possesses gut microflora actively metabolizing tannin-protein complex (T-PC). Recently, we have reported that *Streptococcus bovis* biotype I (Osawa 1990; Osawa & Mistuoka 1990; Osawa 1991) and T-PC-degrading enterobacterium (T-PCDE) (Osawa, 1992), both of which occur frequently in the alimentary tract of the animal, had the unique property of degrading protein complexed with a hydrolyzable tannin (tannic acid). However, it is not known how the complex is degraded by these bacteria, and whether those bacteria are also capable of degrading protein complexed with a condensed tannin.

It has been known for some time that gallotannin (a hydrolyzable tannin) was readily hydrolyzed to yield gallic acid by the enzyme called 'tannase (tannin acylhydrolase)' produced by some fungal strains belonging to the genus of *Aspergillus* (Jean et al. 1981; Pourrat et al. 1987) and *Candida* (Aoki et al. 1976). There is also evidence that gallate and relevant trihydroxybenzenoids (i.e. pyrogallol, and phloroglucinol) were metabolized by some rumen bacteria such as *Eubacterium oxidoreducens* (Krumholz & Bryant 1986), *S. bovis* (Tsai & Jones 1975), and *Coprococcus* sp. (Tsai & Jones 1975; Tsai et al. 1976). Krumholz et al. (1987) proposed a pathway for the anaerobic catabolism of gallic acid by *E. oxidoreducens,* in which it was metabolized to pyrogallol, and further to phloroglucinol. Thus, the present study was designed to determine whether such metabolic pathways are involved in the degradation of tannin-protein complex (T-PC) by the koala isolates.

Materials and methods

Bacterial strains

Twelve strains were used in the present study. These included 7 strains isolated from fresh feces of koalas and 5 strains from established cultures for comparison. Those koala isolates were: T-PCDE UQM 3666 and UQM 3667; *Streptococcus bovis* biotype I UQM 3611; *Escherichia coli* LPKS 110 and LPKS 112; *Klebsiella pneumoniae* LPKS 104; *K. oxytoca* LPKS 109. The strains from the established cultures were: *Enterobacter agglomerans* UQM 1615; *S. bovis* biotype II UQM 3539; *S. bovis* biotype I UQM 3546; *E. coli* UQM 845; *K. pneumoniae* UQM 90. All strains were maintained on nutrient agar (Oxoid Ltd., Basingstoke, Hampshire, United Kingdom) with 5% defibrinated horse blood.

Media used

Nutrient Broth No. 2 (Oxoid) supplemented with 0.1% yeast extract (Oxoid) was used as basal medium (BM) throughout the present study. Using BM, three kinds of media were prepared as follows.

(1) Plate media: BM was supplemented with 1% agar (Oxoid) and autoclaved. The BM agar (BMA) was allowed to cool to 50°C. The BM was then either mixed with filter-sterilized tannic acid (a hydrolizable tannin; Kanto Chemical Co., Inc., Nihonbashi, Tokyo, Japan) or quebracho tannin (a condensed tannin extracted from wood of *Schinopsis lorentzii* [Anacardiaceae]; Hardie Tradings Ltd., Sydney, Australia) at a final concentration of 0.5% (wt/wt), and poured on to sterile petri dishes to produce two kinds of plate media, hydrolyzable- and condensed-tannin basal medium agar (HTBMA and CTBMA, respectively). The BMA was initially transparent but, with addition of the tannins, it became opaque, indicating that the protein fraction in BMA was bound to the tannins to form T-PC.

(2) Biphasic medium: HTBMA was poured onto multiwell tissue culture plates (24 wells, sterilized flat-bottomed with lid; 0.5 ml per well) and left at 4°C overnight. BM (1.5 ml) was then overlaid on HTBMA in each well (referred to as BM/HTBMA) to prepare a biphasic medium.

(3) Broth media: BM containing either 20 mM gallic acid (GA) or pyrogallol (PY) or phloroglucinol (PH) with or without 20 mM glucose were prepared to produce 6 different broth media (GA, GA+ glucose, PY, PY+ glucose, PH, PH+ glucose, respectively). In addition, BM containing increasing concentrations (0, 20, 40, 60, 80, 100 mM) of gallic acid was prepared. The broth media thus pre-

pared were poured aseptically onto multiwell tissue culture plates (2ml per well).

All media described above were adjusted using NaOH and HCl to an initial pH 6.7 and stored under anaerobic conditions (8–10% CO_2, 10–12% H_2, 78–82% N_2), using Bio-bag (Becton-Dickinson & Co., Cockeysville, Maryland, USA) before use. This anaerobic storage was necessary to avoid undesirable darkening of the media due to oxidation of tannins and phenolics.

Inoculation and incubation

For plate culturing, fresh cultures of the bacterial strains grown on nutrient blood agar were harvested by 1µl loop and inoculated onto HTBMA and CTBMA. The plates were incubated for 7 days.

For bi-phasic and broth culturing, a well established colony of each bacterial strain was suspended in 10ml sterile water. With this bacterial suspension, the media were inoculated (40µl per well) and incubated for 7 days (biphasic media) or 3 days (broth). All incubations were performed anaerobically using Biobag (Beckton-Dickinson & Co.) at 37°C throughout the study.

Analytical methods

Presence of a zone of clearing or decreased cloudiness in the tannin-containing media was used as a qualitative index of degradation of T-PC in culture. The pH of bi-phasic medium before and after the incubation was also measured. Bacterial growth in the broth media was determined by measuring changes in optical density (OD) at 660nm in a spectrophotometer (DMS 200, Varian Pty. Ltd. Mulgrave, Australia).

Reversed-phase high performance liquid chromatography (HPLC) as described by Hoefler & Goggon (1976) was used to quantify end products of the degradation of tannin-protein complex or other phenolic substrates in the spent broth media. A Model 2152 HPLC system (LKB Produkter AB, Bromma, Sweden) equipped with a solvent conditioner was used at ambient temperature (23°C) with flow rate at 1ml/min. Samples were injected onto a Phenomenex µBondpak C18 (10µm) column (300× 3.9mm) with a 30× 3.9mm guard column, through an injector (Model 7125, Rheodyne Inc., Cotati, California, USA) fitted with a 200µl loop. Eluent was monitored using a variable wavelength unit (Model 2151, LKB) set at 254nm, and connected to an integrator (C-R6A Chromatopac, Shimadzu Co., Kyoto, Japan). Solvent for the column, 0.02M citrate-phosphate buffer pH 4.5, and samples were filtered through a 0.45µm millipore filter prior to use. Metabolites were identified by their elution times, which were unaffected by components in the broth.

Detection of gallic acid, pyrogallol, and phloroglucinol in koala feces

Fresh fecal pellets excreted from the anogenital orifice of four adult koalas kept at Lone Pine Koala Sanctuary, Brisbane, Australia, were collected aseptically into a sterile test tube containing 10ml of 0.25 strength Ringer solution (Oxoid). A quantitative and qualitative analysis were made on facultatively anaerobic microflora of these fecal samples. The fecal samples were subsequently analyzed for presence of gallic acid, pyrogallol, and phloroglucinol by HPLC.

Results

Degradation of Tannin-protein complex

Table 1 shows the results of bacterial degradation of tannin-protein complex (T-PC) in nutrient broth medium. Although all strains grew on hydrolyzable-tannin basal medium agar (HTBMA) and condensed-tannin basal medium agar (CTBMA), formation of a clear zone around colonies was observed only with tannin-protein complex-degrading enterobacteria (T-PCDE) and *Streptococcus bovis* biotype I strains on HTBMA (Fig. 1) and the size of the colonies was larger than that of any other strains. A similar sign of T-PC degradation was also observed in the solid layer of the bi-phasic medium

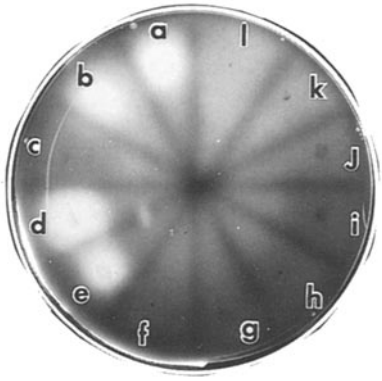

Fig. 1. Formation of a clear zone by T-PCDE and *S. bovis* biotype I on nutrient agar containing 0.5% tannic acid (hydrolyzable tannin).

Legends for strains: a, T-PCDE UQM 3666; b, T-PCDE UQM 3667; c, *Enterobacter agglomerans* UQM 1615; d, *Streptococcus bovis* biotype I UQM 3546; e, *S. bovis* biotype I UQM 3611; f, *S. bovis* biotype II UQM 3539; g, *Escherichia coli* UQM 845; h, *E coli* LPKS 110; i, *E. coli* LPKS 112; j, *Klebsiella pneumoniae* UQM 90; k, *K. pneumoniae* LPKS 104; l, *K. oxytoca* LPKS 104.

(BM/HTBMA) inoculated with T-PCDE and *S. bovis* biotype I strains, with opacity of the layer slightly decreased.

Subsequent analysis of the aqueous layer of BM/HTBMA revealed that T-PCDE and *S. bovis* biotype I strains produced pyrogallol from T-PC. Pyrogallol was also produced by *Klebsiella pneumoniae* and *Klebsiella oxytoca* strains but the amount was much smaller than that produced by T-PCDE and *S. bovis* biotype I strains. Gallic acid was detected in all samples including the control sample (not inoculated with any bacterial strain) except for those inoculated with T-PCDE and *S. bovis* biotype I strains. HPLC analysis indicated that tannic acid used in the present study did not consist solely of gallotannin but contained 8–9% (wt/wt) free gallic acid. Thus, it is most likely that the gallic acid detected in these samples is the free gallic acid eluted from the solid layer containing tannic acid. Phloroglucinol was never observed in any samples. Final pH of bi-phasic media that were inoculated with the bacterial strains was in the range of 7.06–7.47, slightly higher than that of the un-inoculated medium (pH 6.91). The pH of the media inoculated with these T-PC-degrading strains (Lk-1, Lk-2, Sb-1, and Sb-2; see Table 1 for abbrevation of strain identification) was, in most cases, slightly lower than pH of those inoculated with the enterobacteria.

Table 1. Bacterial degradation of tannin-protein complex (T-PC).

Medium/test	Strain												
	Lk-1	Lk-2	Ea	Sb-1	Sb-2	Sbt	Ec-1	Ec-2	Ec-3	Kp-1	Kp-2	Ko	Cont
CTBMA													
growth	+	+	+	+	+	+	+	+	+	+	+	+	
clear zone	–	–	–	–	–	–	–	–	–	–	–	–	
HTBMA													
growth	+	+	+	+	+	+	+	+	+	+	+	+	
clear zone	+	+	–	+	+	–	–	–	–	–	–	–	
BM/HTBMA													
Product conc. (mM)													
gallic acid	0	0	1.8	0	0	1.9	1.6	1.1	1.1	0.3	0.2	0.5	1.2
pyrogallol	4.4	3.7	0	3.6	3.4	0	0	0	0	0.8	0.8	1.1	0
phloroglucinol	0	0	0	0	0	0	0	0	0	0	0	0	0
Final pH	7.11	7.06	7.07	7.08	7.11	7.10	7.09	7.36	7.47	7.22	7.28	7.37	6.91

Strains used: Lk-1, T-PCDE UQM 3666; Lk-2, T-PCDE UQM 3667; Ea, *Enterobacter agglomerans* UQM 1615; Sb-1, *Streptococcus bovis* biotype I UQM 3546; Sb-2, *S. bovis* biotype I UQM 3611; Sbt, *S. bovis* biotype II UQM 3539; Ec-1, *Escherichia coli* UQM 845; Ec-2, *E coli* LPKS 110; Ec-3, *E. coli* LPKS 112; Kp-1, *Klebsiella pneumoniae* UQM 90; Kp-2, *K. pneumoniae* LPKS 104; Ko, *K. oxytoca* LPKS 104. Cont = control.

Metabolism of gallic acid, pyrogallol, and phloroglucinol

Table 2 shows the results of bacterial degradation of gallic acid, pyrogallol, and phloroglucinol. In the BM containing gallic acid, T-PCDE, *K. pneumoniae,* and *K. oxytoca* strains metabolized gallic acid to pyrogallol. The conversion appeared to be complete with T-PCDE strains since the amount of pyrogallol produced by these strains equalled that of gallic acid consumed. This was, however, not the case with *K. pneumoniae* and *K. oxytoca* strains: these strains consumed a large amount of gallic acid but produced pyrogallol at much lower concentration than what was expected.

By contrast, *K. pneumoniae* and *K. oxytoca* strains metabolized gallic acid to pyrogallol completely when the medium was supplemented with glucose. With this glucose supplementation, *S. bovis* biotype I strains also showed a capacity to metabolize gallic acid to pyrogallol.

Pyrogallol could not be further metabolized by any strains used in the present study. The strains of *S. bovis* biotype I metabolized phloroglucinol in the medium without glucose. This metabolic property of *S. bovis* was not evident when the medium was supplemented with glucose. In the glucose-supplemented medium, T-PCDE UQM 3666, *Escherichia coli* UQM 845, and *K. pneumoniae* LPKS 104 metabolized phloroglucinol. *S. bovis* biotype II strain UQM 3539 metabolized phloroglucinol regardless of presence of glucose in the medium.

Metabolism and growth of the strains at increasing concentrations of gallic acid

Table 3 shows the bacterial metabolism of gallic acid at varying concentrations. In agreement with the observations described above, T-PCDE strains metabolized gallic acid to pyrogallol much more actively and completely than did *K. pneumoniae* and

Table 2. Bacterial degradation of gallic acid (GA), pyrogallol (PY), and phloroglucinol (PH).

Substrate	Benzonoids (mM) detected after 3 days	Bacterial strain											
		Lk-1	Lk-2	Ea	Sb-1	Sb-2	Sbt	Ec-1	Ec-2	Ec-3	Kp-1	Kp-2	Ko	Cont
Ga														
	GA	0*	0*	18.0	17.7	19.3	20.4	19.3	17.1	17.5	0*	0*	11.9*	19.6
	PY	19.8*	21.4*	0	0	0	0	0	0	0	13.8*	12.6*	3.0*	0
	PH	0	0	0	0	0	0	0	0	0	0	0	0	0
GA+ glucose														
	GA	0*	0*	21.0	0*	0*	20.7	18.6	18.7	19.8	0*	0*	0*	20.2
	PY	18.5*	18.8*	0	19.5*	20.5*	0	0	0	0	19.0*	20.8*	21.0*	0
	PH	0	0	0	0	0	0	0	0	0	0	0	0	0
PY														
	PY	19.3	19.2	20.5	17.8	20.4	18.9	19.9	20.4	19.2	20.5	18.4	20.7	19.9
	PH	0	0	0	0	0	0	0	0	0	0	0	0	0
PY+ glucose														
	PY	18.9	18.9	20.2	20.4	19.8	19.7	20.6	19.4	19.9	19.6	20.6	19.3	20.4
	PH	0	0	0	0	0	0	0	0	0	0	0	0	0
PH														
	PH	21.0	20.8	21.7	14.6*	14.5*	14.4*	20.8	19.6	20.7	21.3	21.5	20.8	20.8
PH+ glucose														
	PH	14.3*	20.4	16.1	20.5	20.2	15.2*	16.4*	19.0	20.2	20.3	11.6*	19.1	20.5

The data are means of triplicate tests.

*Significantly different (Student *t*-test, $P < 0.01$) from concentration in the controls.

See Table 1 for abbreviations of bacterial names.

Cont= control.

Growth conditions: in basal medium, incubated anaerobically at 37°C for 3 days.

K. oxytoca. For example, the amounts of pyrogallol produced by T-PCDE and *K. pneumoniae* in the medium initially containing 100mM gallic acid were, on average, 100mM and 55mM, respectively, despite the fact that nearly all of the gallic acid appeared to have been consumed by both groups of bacteria. Pyrogallol was also detected in the spent broth of *S. bovis* biotype I but the amount (less than 0.1mM) was negligible when compared to that produced by the above strains. Phloroglucinol was never detected in any samples.

Figures 2a, b, c, and d show maximum growth yields (OD) of the bacterial strains with different concentration of gallic acid added to BM. Growth yields of T-PCDE strains were linear with gallic acid concentration up to 100mM while those of *E. coli, K. pneumoniae,* and *K. oxytoca* strains did not seem to be affected by either the presence or concentration of gallic acid. BM used in the present study did not seem to support the growth of either *E. agglomerans* or *S. bovis* strains of both biotypes regardless of presence of gallic acid.

Gallic acid, pyrogallol, and phloroglucinol in feces

The facultatively anaerobic microflora of koala feces consisted mainly of T-PCDE, *S. bovis* biotype I, *E. coli, K. pneumoniae* (or *K. oxytoca*) as enumerated by cell-froming units. No gallic acid, pyrogallol, or phloroglucinol was detected in these fecal samples.

Discussion

Hydrolyzable tannins such as tannic acid and gallotannin have an optimum pH (pH 4–6) for their protein-binding property. Acidic and alkaline shifts away from this optimum pH-cause dissociation of the complex (Van Buren & Robinson 1969; Van Sumere et al. 1975). Condensed tannins such as sorghum tannin and quebracho bind to protein almost independently of pH in the range 3.5–7.0 (Barry & Manley 1984; Van Sumere et al. 1975), thus forming relatively stable (undegradable) complexes with protein in the alimentary tract of mammals (Zucker 1983). In the present study, the degradation of tan-

Table 3. Bacterial degradation of gallic acid at varying concentration.

Benzenoid (mM) detected after 3 days	Initial concentration (mM) of gallic acid	Bacterial strain											
		Lk-1	Lk-2	Ea	Sb-1	Sb-2	Sbt	Ec-1	Ec-2	Ec-3	Kp-1	Kp-2	Ko	Cont
Gallic acid														
	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	20	0*	0*	18.0	17.7	19.3	20.4	19.3	17.1	17.5	0*	0*	11.9*	19.6
	40	tr*	0*	37.3	36.0	39.2	39.6	40.3	36.8	35.8	0*	0*	38.1	38.8
	60	tr*	tr*	57.3	52.4	56.8	57.4	60.9	58.8	51.5	4.0*	0*	53.9*	54.3
	80	tr*	tr*	80.3	79.7	81.1	77.6	80.1	79.0	74.8	3.8*	1.5*	50.3*	78.3
	100	tr*	tr*	91.2	101.0	101.8	100.2	94.0	95.4	93.3	0.4*	1.0*	69.2*	98.6
Pyrogallol														
	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	20	19.8	21.4	0	0	0	0	0	0	0	13.8	12.6	3.0	0
	40	36.8	43.1	0	tr	tr	0	0	0	0	18.1	16.4	2.4	0
	60	58.0	63.9	0	tr	tr	0	0	0	0	50.0	30.2	2.2	0
	80	83.6	83.6	0	tr	tr	0	0	0	0	53.1	42.3	4.8	0
	100	99.2	103.1	0	tr	tr	0	0	0	0	62.8	49.8	7.6	0

tr, trace (<0.1mM).

*Significantly different (Student *t*-test, $P < 0.01$) from concentration in the controls.

See Table 1 for abbreviations of bacterial names.

Growth conditions: in basal medium gallic acid, incubated anaerobically at 37° C for 3 days.

Cont = control.

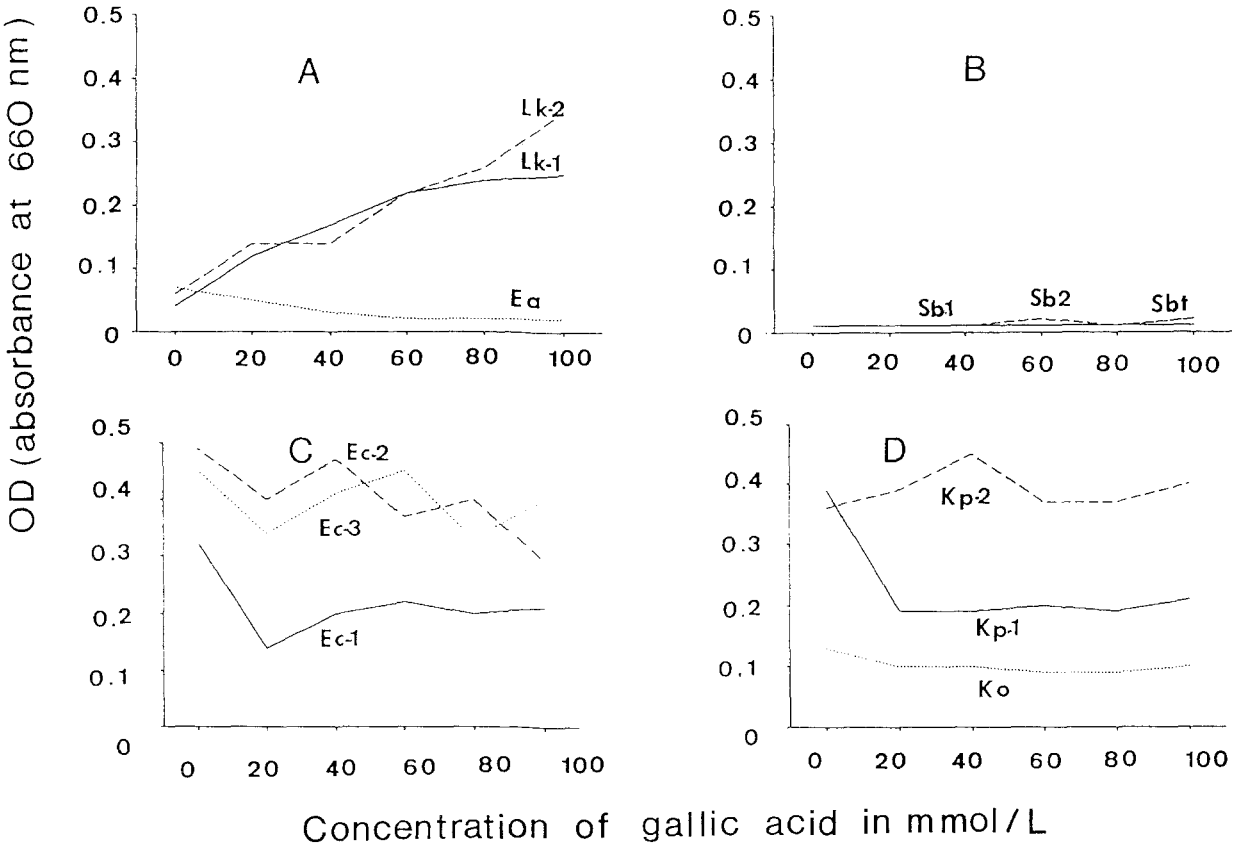

Fig. 2. Maximum growth of bacteria with different levels of gallic acid as a substrate in basal medium.

A: Lk-1, T-PCDE UQM 3666; Lk-2, T-PCDE UQM 3667; Ea, *Enterobacter agglomerans* UQM 1615.
B: Sb-1, *Streptococcus bovis* biotype I UQM 3546; Sb-2, *S. bovis* biotype I UQM 3611; Sbt, *S. bovis* biotype II UQM 3539.
C: Ec-1, *Escherichia coli* UQM 845; Ec-2, *E coli* LPKS 110; Ec-3, *E. coli* LPKS 112.
D: Kp-1, *Klebsiella pneumoniae* UQM 90; Kp-2, *K. pneumoniae* LPKS 104; Ko, *K. oxytoca* LPKS 104.

nin-protein complex (T-PC) was observed for the protein complexed with gallotannin but not with quebracho. From this evidence, it was initially considered that the alkaline change in pH of the medium due to bacterial metabolism might have simply weakened the binding, and resulted in a dissociation of the T-PC. However, this was considered unlikely because there was no significant difference in pH between the spent medium with apparent T-PC degradation and the one without (Table 1).

As described earlier, hydrolyzable tannins are readily hydrolyzed by tannase (tannin acylhydrolase) produced by some fungal strains (Aoki et al. 1976; Jean et al. 1981; Pourrat et al. 1987). Its presence or activity can be determined simply by measuring gallic acid liberated from the gallotannin or methylgallate (Jean et al. 1981; Pourrat et al. 1987). Recently, we observed Osawa, unpublished hydrolysis of methylgallate to gallic acid by tannin-protein complex-degrading enterobacteria (T-PCDE) and *Streptococcus bovis* biotype I strains, indicating positive tannase activity in these bacterial strains.

Bacterial metabolism of gallic acid was reported with *Klebsiella aerogenes* (Grant and Patel 1969), *Citrobacter* sp. (Yoshida et al. 1982), *Eubacterium oxidoreducens* (Krumholz et al. 1987), and *Pleobacter acidigallici* (Samain et al. 1986). The metabolism involves decarboxylation of gallic acid to produce pyrogallol (Booth et al. 1959; Grant and Patel 1969; Scheline 1966; Krumholz et al. 1987). In the present

study, the decarboxylation was observed with *Klebsiella* strains as well as T-PCDE and *S. bovis* biotype I. Furthermore, a higher growth yield was observed with increasing concentration of gallate in T-PCDE strains. Such gallate-dependent growth was reported with *Citrobacter* sp. (Yoshida et al. 1982), *Pelobacter acidigallici* (Samain et al. 1986), and *Eubacterium oxidoreducens* (Krumholz & Bryant 1986). This was, however, not observed with the pyrogallol producing *S. bovis* biotype I and *Klebsiella* strains in the present study: conversion of gallic acid to pyrogallol by these strains was negligible or incomplete, respectively, when the culturing medium did not contain glucose.

Transformation of pyrogallol to phloroglucinol has been demonstrated in *E. oxidoreducens* (Krumholz et al. 1987) and *P. acidigallici* (Samain et al. 1986). However, none of the strains used in the present study showed such a capability, although pyrogallol was never detected in the fresh feces of koalas. Rayudu et al. (1970) reported that pyrogallol had a severe depressing effect on growth of chicks and that about 100% mortality was observed when the dietary level of the compound reached 2%. Since the koala thrives on a diet likely to yield high levels of this toxin, the suggestion can be made that the pyrogallol may be degraded by other members of microflora (i.e. obligate anaerobes) in the alimentary tract of the koala. Alternatively, the detoxification may take place in the liver of the animals. Further investigation is necessary to distinguish between these possibilities.

In the present study, phloroglucinol was degraded by some, though not all, strains belonging to T-PCDE, *S. bovis, Enterobacter agglomerans, Escherichia coli,* and *Klebsiella pneumoniae* strains. Degradation of phloroglucinol was reported for many bacterial strains including *Rhodopseudomonas gelatinosa* (Whittle et al. 1976), *Streptococcus bovis* (Tsai & Jones 1975; Tsai et al. 1976), *Coprococcus sp.* (Tsai & Jones 1975; Tsai et al. 1976), *Pelobacter acidigallici* (Schink & Pfennig 1982; Samain et al. 1986), and *Eubacterium oxidoreducens* (Krumholz et al. 1987), most of which produced acetate as an end product of the substrate. Consistent with the finding presented by Tsai & Jones (1975), all *S. bovis* strains used in the present study degraded phloroglucinol. It was, however, not determined why those *S. bovis* biotype I strains failed to do so with the presence of glucose in the media.

Cork et al. (1983) measured high digestibility of phenolics in koalas fed on a species of eucalypt known to contain hydrolyzable tannin. They interpreted this to mean that much of the tannin was degraded in the stomach. Various studies and reviews have suggested that hydrolyzable tannins can be degraded in the stomach and small intestine of mammals (Glick and Joslyn 1970; McLeod 1974; Robbins et al. 1987). However, Cork et al. (1983) found significant concentration of phenolics in the contents of the caecum/proximal colon, indicating that not all degradation occurred in the upper gut.

Recently (Osawa and Walsh, unpublished), we observed that tannic acid is apparently not hydrolyzed by highly acidic (pH 1–3) treatments. The evidence supports that at least some hydrolyzable tannin contained in the eucalypt foliage escapes hydrolysis in the acid of the stomach and reaches the caecum/proximal colon in the koala. Thus, it is most likely that the predominant members of the microflora, at least the facultatively anaerobic bacteria in the alimentary tract of this species cooperate in the degradation of T-PC and in the metabolism of the phenolics produced during the course of this activity.

References

Aoki K, Shinke R & Nishira H (1976) Purification and some properties of yeast tannase. Agric. Biol. Chem. 40: 79–85

Barry TN & Manley TR (1984) The role of condensed tannins in the nutritional value of *Lotus pedunculatus* for sheep. Bri. J. Nutr. 51: 493–504

Bate-Smith EC & Lerner NH (1954) Leucoanthocyanidins. 2. Systematic distribution of leucoanthocyanins in leaves. Biochem. J. 58: 126–132

Booth AN, Masri MS, Robbins DJ, Emerson OH, Jones FT & De Eds F (1959) The metabolic fate of gallic acid and related compounds. J. Biol. Chem. 234: 3014–3016

Brune A & Schink B (1990) Pyrogallol to phloroglucinol conversion and other hydroxyl transfer reactions catalyzed by cell extracts of *Pelobacter acidigallici.* J. Bacteriol. 172: 1070–1076

Cork SJ, Pahl L (1984) The possible influence of nutritional factors on diet and habitat selection by the ringtail possum (*Pseudocheirus peregrinus*). In: Smith AP & Hume ID (eds) Pos-

sums and Gliders, pp 269–276. Chipping Norton Inc., Sydney, Australia

Cork SJ, Hume ID & Dawson TJ (1983) Digestion and metabolism of a mature folilar diet (*Eucalyptus punctata*) by an arboreal marsupial, the koala (*Phascolarctos cinereus*). J. Comp. Physiol. 153: 181–190

Eberhard IH (1978) Ecology of the koala, *Phascolarctos cinereus* (Goldfuss) Marsupialia: Phascolarctidae, in Australia. In: Montgomery GG (ed) The Ecology of Arboreal Folivore, pp. 315–327. Smithsonian Institution Press, Washington, DC

Ebarhard IH, McNamara J, Pearse RJ, Southwell IA (1975) Ingestion and excretion of *Eucalyptus punctata* D.C. and its essential oil by the koala, *Phascolarctos cinereus* (Goldfuss). Aust. J. Zool. 23: 169–179

Feeny P (1976) Plant apparency and chemical defense. Recent Adv Phtochem. 10: 1–40

Grant DJW, Patel JC (1969) The non-oxidative decarboxylation of p-hydroxybenzoic acid, gentisic acid, protocatechuic acid and gallic acid by *Klebsiella aerogenes*. Antonie van Leeuwenhoek 35: 325–343

Glick Z & Joslyn MA (1970) Food intake depression and other metabolic effects of tannic acid in the rat. J. Nutr. 100: 509–515

Hagerman AE & Butler LG (1981) The specificity of proanthocyani din-protein interactions. J. Biol. Chem. 256: 4494–4497

Hoefler AC & Coggon P (1976) Reversed-phase high-performance liquid chromatography of tea constituents. J. Chromatogr. 129: 460–463

Jean D, Pourrat H, Pourrat A & Carnat A (1981) Assay of tannase (tannin acylhydrolase EC 3.1.1.20) by gas chromatography. Analyt. Biochem. 110: 369–372

Krumholz LR & Bryant MP (1986) *Eubacterium oxidoreducens* sp. nov. requiring H_2 or formate to degrade gallate, pyrogallol, phloroglucinol and quercetin. Arch. Microbiol. 144: 8–14

Krumholz LR, Crawford RL, Hemling ME & Bryant MP (1987). Metabolism of gallate and phloroglucinol in *Eubacterium oxidoreducens* via 3-hydroxy-5-oxohexanoate. J. Bacteriol. 169: 1886–1890

Macauley BJ, Fox LR (1980) Variation in total phenols and condensed tannins in eucalyptus: leaf phenology and insect grazing. Aust. J. Ecol. 5: 31–35

McLeod MN (1974). Plant tannins – their role in forage quality. Nutr. Abst. Rev. 44: 803–815.

McManus J, Lilley TH & Haslam E (1983). Plant polyphenols and their association with protein. In: Hedin PA (ed) Plant Resistance to Insects, pp 123–137. ACS symposium Series 208, American Chemical Society, Washington

Mole S & Waterman PG (1987) Tannins as antifeedants to mammalian herbivores – Still an open question? In: Waller GR (ed) Allelochemicals: Role in Agriculture and Forestry, pp 572–587. American Chemical Society, Washington DC

Osawa R (1990) Formation of a clear zone on tannin-treated brain heart infusion agar by a *Streptococcus* sp. isolated from feces of koalas. Appl. Environ. Microbiol. 56: 829–831

Osawa R & Mitsuoka T (1990) Selective medium for enumeration of tannin-protein complex degrading *Streptococcus* spp. in feces of koalas. Appl. Environ. Microbiol. 56: 3609–3611

Osawa R (1991) An investigation of streptococal flora in feces of koalas. J. Wildl. Manage. 55: 623–627

Osawa R (1992) Tannin-protein complex degrading enterobacteria isolated from the alimentary tracts of koalas, and a selective medium for their enumeration. Appl. Environ. Microbiol. 58: 1754–1759

Pourrat H, Regerat F, Morvan P & Pourrat A (1987) Microbiological production of gallic acid from *Rhus corriaria* L. Biotheh. Let. 9: 731–734

Rayudu GVN, Kadirvel R, Vohra P & Kratzer FH (1970) Toxicity of tannic acid and its metabolites for chickens. Poult. Sci. 49: 957–960

Rhoades DF & Cates RG (1976) A general theory of plant antiherbivore chemistry. Recent Adv. Phytochem. 10: 168–213

Robbins CT, Mole S, Hagerman AE & Hanley TA (1987) Role of tannins in defending plants against ruminants: reduction in dry matter digestion? Ecol. 68: 1606–1615

Salunkhe DK, Chavan JK & Kadam SS (1989) Dietary Tannins: Consequences and Remedies. CRC Press, Inc, Boca Raton, Florida

Samain E, Albagnac G & Dubourguier H-C (1986) Initial stepts of catabolism of trihydroxybenzenes in *Pelobacter acidigallici*. Arch. Microbiol. 144: 242–244

Scheline RR (1966) Decarboxylation and demethylation of some phenolic benzoic acid derivatives by rat caecal contents. J. Pharm. Pharmac. 18: 664–669

Schink B, Pfenning N (1982) Fermenation of trihydroxybenzenes by *Pelobacter acidigallici* gen. nov. sp. nov., a new strictly anaerobic, non-sporeforming bacterium. Arch. Microbiol. 133: 195–201

Swain T (1978) Plant-animal coevolution: a synoptic view of palezoic and mesozoic. In: Harborne JB (ed) Biochemical Aspects of Plant and Animal Co-evolution, pp 1–19. Academic Press, Inc., New York

Tsai CG & Jones GA (1975) Isolation and identification of rumen bacteria capable of anaerobic phloroglucinol degradation. Can. J. Microbiol. 21: 749–801

Tsai CG, Gates DM, Ingledew WM, Jones GA (1976). Products of anaerobic phloroglucinol degradation by *Coprococcus* sp. Pe_15. Can. J. Microbiol. 22: 159–164

Van Buren JP, Robinson WB (1969) Formation of complexes between protein and tannic acid. J. Agr. Food. Chem. 17: 772–777

Van Sumere CF, Albrecht J, Dedonder A, DePooter H & Pé I (1975) Plant protein and phenolics. In: Harborne JB & Van Sumere CF (eds) Annu. Proc. Phytochem (pp 211–261) Academic Press. San Diego

White T (1957) Tannins-their occurrence and significance. J. Sci. Food. Agric. 8: 377–385

Whittle PJ, Lunt DO & Evans WC (1976) Anaerobic photo metabolism of aromatic compounds by *Rhodopseudomonas* sp. Biochem. Soc. Trans. 4: 490–491

Yoshida H, Tani Y & Yamada H (1982) Isolation and identification of a pyrogallol producing bacterium from soil. Agric. Biol. Chem. 46: 2539–2546

Zucker WV (1983) Tannin: Does structure determine function? An ecological perspective. Am. Nat. 121: 335–365