Biochemical and Antigenical Characterization of Tannin-Protein Complex Degrading Enterobacteria Isolated from Koalas, *Phascolarctos cinereus*

Yutaka NAKAI, Masashi NIIMI, Tomonori KANAI, Ro OSAWA[1)], Tamio INAMOTO, Tasuke ANDO, and Keiji OGIMOTO
Department of Animal Microbiology and Parasitology, Tohoku University, 1–1 Tsutsumidori-Amamiyacho, Aoba-ku, Sendai 981 and [1)]Department of Nutritional Hygiene, Tokushima University, 3–18–15 Kuramotocho, Tokushima 770, Japan
(Received 31 August 1994/Accepted 3 February 1995)

ABSTRACT. Biochemical and antigenical characteristics of tannin-protein complex degrading enterobacteria (T-PCDE) isolated from Koalas, *Phascolarctos cinereus*, were investigated. T-PCDE had a specific profile of characteristics, and T-PCDE was distinguished from those of 12 type strains of Enterobacteriaceae used.—KEY WORDS: Enterobacteriaceae, koala, tannin-protein complex degrading enterobacteria.

J. Vet. Med. Sci. 57(3): 559–561, 1995

The koala, *Phascolarctos cinereus*, inhabits the forests of eastern Australia, and a number of koalas have been imported and reared in zoos in Japan. It feeds almost exclusively on *Eucalyptus* spp. leaves [2, 3] which have high concentrations of tannins [1, 6]. Tannins readily form chemical complexes with proteins and the resulting complexes are resistant to degradation within the gut of mammals. Recently, Osawa [7] isolated an enterobacterium that can degrade tannin-protein complexes (tannin-protein complex degrading enterobacteria, T-PCDE) from feces of koalas. T-PCDE is similar to Enterobacteriaceae in some characteristics, e.g. facultatively anaerobic and gram-negative. We, therefore, compared the biochemical and antigenical characteristics of T-PCDE with those of species of Enterobacteriaceae.

Three strains (LX1, LX2, and LX3; strain names in Australian Collection of Microorganisms were UQM3666, UQM3667, and UQM3682, respectively) of T-PCDE isolated from koalas were used [7]. As the reference strains of Enterobacteriaceae, type strains of 12 species (*Proteus vulgaris* ATCC13315, *P. mirabiris* ATCC29906, *P. myxofaciens* ATCC19692, *P. penneri* ATCC33519, *Providencia alcalifaciens* ATCC9886, *P. heimbachae* ATCC35613, *P. rettgeri* ATCC29944, *P. rustigianii* ATCC33673, *P. stuartii* ATCC29914, *Pantoea agglomerans JCM1236, Klebsiella pneumoniae* JCM1662 and *Escherichia coli* JCM1649) were used. Biochemical characteristics were determined by routine methods [4, 5]. Gas production from glucose and H_2S production were determined with triple sugar iron agar. Motility, gelatin hydrolysis and deoxyribonuclease activity were investigated at 37°C for 2 days, 22°C and 37°C, respectively. Oxidase activity was determined by the methods of Kovacs. Tannase activity was determined by Osawa's methods [7, 8]. Anti-T-PCDE serum was prepared from rabbits immunized with formaldehyde treated cells of LX-1 strain of T-PCDE. Enzyme linked immunosorbent assay (ELISA) was performed by modified methods of Uchida *et al.* [9] with the antiserum. As antigens, bacteria which were cultured for 48 hr at 37°C and washed three times with phosphate-buffered saline (PBS), were suspended to be 1.5×10^8 CFU/m*l* were used. Each well of a microtiter plastic plate (Costar, #3590) was coated with 100 μl of the antigen suspension, then coated with 200 μl of 3.0% bovine serum albumin (BSA: Wako Pure Chemical Industries) in PBS, and washed 5 times with PBS containing 0.1% Tween 20 (PBST). The serum which was diluted 10,000 times in PBST containing 0.1% BSA were added to the wells (100 μl/well), and incubated for 1 hr at room temperature. After washed 5 times, 100 μl of horseradish peroxidase conjugated goat anti-rabbit IgG (H+L chain specific, Cappel Laboratories) diluted to 1:1,000 was added to be incubated for 1 hr at room temperature. After washing, the reaction was developed with 2,2'-azino-di[3-ethyl-benzthiazoline sulfonate (6)] (ABTS) peroxidase substrate (Kirkegaard & Perry Laboratories) for 30 min at room temperature. Absorbances at 405 nm were measured by means of a microplate reader (BIO-RAD Laboratories).

The biochemical characteristics are summarized in Table 1. The characteristics of three strains of T-PCDE coincide with each other. Three strains of T-PCDE had some characteristics in common with strains of Enterobacteriaceae. They were negative in oxidase production and positive in D-glucose utilization and nitrate reduction. However, three strains of T-PCDE did not produce the catalase which was commonly produced by reference strains of Enterobacteriaceae.

Reactivity of anti-T-PCDE-LX1 serum with bacterial strains was investigated (Fig. 1). The serum reacted strongly with T-PCDE of each strain (LX1, LX2 and LX3). The serum showed relatively strong cross reactivity with *P. rustigianii* ATCC33673, *P. myxofaciens*

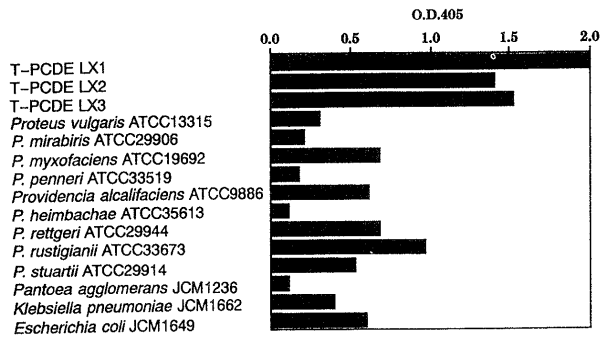

Fig. 1. Optical densities (O.D.) in ELISA of anti-T-PCDE-LX1 serum for bacterial strains.

Table 1. Biochemical characteristics of strains of T-PCDE and Enterobacteriaceae[a)]

	LX1	LX2	LX3	A	B	C	D	E	F	G	H	I	J	K	L
Biochemical characteristics															
Citrate (Simmons)	−	−	−	−	+	+	−	+	−	+	−	+	+	+	−
D-Glucose, oxidative	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Glucose, gas production	−	−	−	−	+	−	−	−	−	−	−	−	−	+	+
H_2S production	−	−	−	+	+	+	−	−	−	−	−	−	−	−	−
Indole production	−	−	−	−	−	−	−	−	−	+	+	+	−	−	+
KCN, growth	−	−	−	+	+	+	+	+	+	+	+	+	+	+	−
Malonate utilization	−	−	−	+	±	±	±	±	±	+	+	±	+	+	±
Methyl red	−	−	−	+	+	+	+	+	+	+	+	+	−	−	+
Motility	−	−	−	+	+	+	+	+	+	+	+	+	+	−	+
Mucic acid utilization	−	±	−	−	−	−	−	−	−	−	−	−	+	+	+
Tartaric acid utilization	±	+	±	−	−	−	−	−	−	−	−	−	−	−	−
Urea hydrolysis	−	−	−	+	+	+	+	−	−	+	+	−	−	+	−
Voges-Proskauer	+	+	+	−	−	−	−	−	−	−	−	−	+	+	−
Production of:															
Arginine dihydrolase	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+
Catalase	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+
Deoxyribonuclease	−	−	−	+	+	+	+	+	+	+	+	+	−	−	−
Esculin hydrolysis	+	+	+	+	−	−	−	−	−	+	−	−	+	+	+
β-Galactosidase	+	+	+	+	−	+	−	−	−	−	−	−	+	+	+
Gelatin hydrolysis	−	−	−	+	−	+	+	−	−	−	−	−	−	−	−
Lipase	−	−	−	−	+	+	−	−	−	−	−	−	−	−	−
Lysine decarboxylase	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+
Nitrate reduction	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Ornithine decarboxylase	−	−	−	−	+	−	−	−	−	−	−	−	−	−	+
Oxidase	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Phenylalanine deaminase	−	−	−	+	+	+	+	+	+	+	+	+	−	−	−
Tannase	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
Acid production from:															
Adonitol	−	−	−	−	−	−	−	+	+	+	−	−	−	+	−
L-Arabinose	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+
D-Arabitol	−	−	−	−	−	+	−	−	+	+	−	−	+	+	−
Cellobiose	−	−	−	−	+	+	+	−	+	−	−	−	−	+	+
Dulcitol	−	−	−	−	−	+	−	−	−	−	−	−	−	+	−
Erythritol	−	−	−	−	−	−	+	−	−	+	−	−	−	−	−
Fructose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Galactose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Glycerol	+	+	+	+	+	+	+	−	±	+	−	+	−	+	+
Glycogen	±	+	±	−	−	±	−	−	−	−	−	−	−	−	−
myo-Inositol	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−
Inulin	±	±	±	−	−	−	−	−	−	−	−	−	−	±	−
Lactose	+	+	+	−	−	−	−	−	−	−	−	−	+	+	+
Maltose	+	+	+	+	−	−	−	−	−	−	−	−	+	+	+
D-Mannitol	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+
D-Mannose	−	−	−	−	−	−	+	+	+	+	+	+	+	+	+
Melezitose	−	−	−	+	−	+	+	−	−	−	−	−	−	−	−
Melibiose	+	+	+	−	−	−	−	−	−	−	−	−	−	+	+
α-Methyl-D-glucoside	+	+	+	+	−	+	+	−	−	−	−	−	−	+	−
Raffinose	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−
L-Rhamnose	+	+	+	−	−	−	−	−	−	−	−	−	+	+	+
Salicin	+	+	+	+	−	−	−	−	−	−	−	−	+	+	+
D-Sorbitol	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+
L-Sorbose	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+
Starch	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Sucrose	+	+	+	+	−	+	+	−	−	−	+	−	+	−	+
Tagatose	+	+	+	−	−	−	−	−	−	−	−	−	−	+	+
Trehalose	−	−	−	+	+	+	+	−	−	−	−	−	+	+	+
Turanose	−	−	−	+	−	+	+	−	−	−	−	−	−	+	−
D-xylose	+	+	+	+	+	−	+	−	−	−	−	−	+	+	−

a) A=*Proteus vulgaris* ATCC13315; B=*P. mirabiris* ATCC29906; C=*P. myxofaciens* ATCC19692; D=*P. penneri* ATCC33519; E=*Providencia alcalifaciens* ATCC9886; F=*P. heimbachae* ATCC35613; G=*P. rettgeri* ATCC29944; H=*P. rustigianii* ATCC33673; I=*P. stuartii* ATCC29914; J=*Pantoea agglomerans* JCM1236; K=*Klebsiella pneumoniae* JCM 1662; L=*Escherichia coli* JCM 1649.
All strains oxidatively utilized D-glucose and produced acid from fructose, D-galactose and starch.

ATCC19692, and *P. rettgeri* ATCC29944, but the level of optical density was obviously lower than that of T-PCDE. In a preliminary experiment, western blotting of T-PCDE showed 60, 37, 36.8, 36 and 31 kDa antigens to LX1 serum, and the antigen profile of T-PCDE in western blotting was different from those of tested Enterobacteriaceae. These results of ELISA and western blotting indicate that T-PCDE is antigenically different from species of Enterobacteriaceae tested. On the other hand, 60 kDa antigen was detected in all strains of T-PCDE and Enterobacteriaceae in western blotting. This common antigen may be responsible for the cross reactivity between T-PCDE and Enterobacteriaceae.

In conclusion, although T-PCDE has some common characteristics of species of Enterobacteriaceae, T-PCDE has specific characteristics by which it can be biochemically or antigenically differentiated from species of Enterobacteriaceae. For taxonomic designation of T-PCDE, genetical comparison, i.e. analysis of guanine plus cytosine contents and DNA-DNA hybridization, between T-PCDE and a wider range of taxons should be performed.

ACKNOWLEDGEMENTS. We thank Lone Pine Koala Sanctuary, Brisbane, Australia, for financial support. We also thank Dr. L. I. Sly of the Department of Microbiology, the University of Queensland, for providing T-PCDE strains for the study.

REFERENCES

1. Cork, S. J. and Pahl, L. 1984. pp. 269–276. *In*: Possums and Gliders (Smith, A. P. and Hume, I. D. eds.), Chipping Norton Inc., Sydney.
2. Eberhard, I. H. 1978. pp. 315–327. *In*: The Ecology of Arboreal Folivores (Montogomery, G. G. ed.), Smithsonian Institution Press, Washington, D. C.
3. Eberhard, I. H., McNamara, J., Pearse, R. J., and Southwell, I. A. 1975. *Aust. J. Zool.* 23: 169–179.
4. Ewing, W. H. 1986. *In*: Edwards and Ewing's Identification of Enterobacteriaceae, 4th ed. (Edwards, P. R. and Ewing, W. H. eds.), Elsevier Science Publishing Co., Inc., New York.
5. Kelly, M. T., Brenner, D. J., and Farmer III, J. J. 1985. *In*: Manual of Clinical Microbiology, 4th ed. (Lennette, E. H., Balows, A., Hausler W. J. Jr., and Shadomy, H. J. eds.), ASM, Washington, D. C.
6. Macauley, B. J. and Fox, L. R. 1980. *Aust. J. Ecol.* 5: 31–35.
7. Osawa, R. 1992. *Appl. Environ. Microbiol.* 58: 1754–1759.
8. Osawa, R. 1993. *Appl. Environ. Microbiol.* 59: 1251–1252.
9. Uchida, T., Hasbullah, Nakamura, T., Nakai, Y., and Ogimoto, K. 1994. *J. Vet. Med. Sci.* 56: 1021–1023.