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

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**ABSTRACT.** Biochemical and antigenical characteristics of tannin-protein complex degrading enterobacteria (T-PCDE) isolated from Koalas, *Phascolarctos cinererus*, 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.


The koala, *Phascolarctos cinererus*, 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, Osaka [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 (P. *mirabilis* ATCC13315, P. *mirabilis* ATCC29906, P. *myxofaciens* ATCC19692, P. *penneri* ATCC33519, *Providencia alcalifaciens* ATCC8866, P. *heimbuchae* ATCC35613, P. *retgeri* ATCC29544, P. *rugimani* ATCC33673, P. *suaari* 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 H2S 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 Osaka’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 antisera. 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 × 108 CFU/ml 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 was 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. rugimani* ATCC33673, *P. myxofaciens*
Table 1. Biochemical characteristics of strains of T-PCDE and Enterobacteriaceae\(^a\)

<table>
<thead>
<tr>
<th>Biochemical characteristics</th>
<th>LX1</th>
<th>LX2</th>
<th>LX3</th>
<th>A</th>
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<td>D-Glucose, oxidative</td>
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<td>D-Glucose, gas production</td>
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<td>H(_2)S production</td>
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<td>Indole production</td>
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<td>Methyl red</td>
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<td>Mucic acid utilization</td>
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<td>Tartaric acid utilization</td>
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Production of:

- Arginine dihydrodase
- 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-Arabinol
  - Cellobiose
  - Dulcitol
  - Erythritol
  - Fructose
  - D-Galactose
  - Glycerol
  - Glycogen
  - myo-Inositol
  - Inulin
  - Lactose
  - Maltoolose
  - D-Mannitol
  - D-Mannose
  - Maltose
  - Melibiose
  - α-Methyl-D-glucoside
  - Raffinose
  - L-Rhamnose
  - Salicin
  - D-Sorbitol
  - L-Sorbosse
  - Starch
  - Sucrose
  - Tagatose
  - Trehalose
  - Turanose
  - D-xyllose

\(^a\) A = Proteus vulgaris ATCC 13315; B = P. mirabilis ATCC 29906; C = P. myxofaciens ATCC 19692; D = P. penneri ATCC 33519; E = Providencia alcalifaciens ATCC 9886; F = P. heimboeae ATCC 35613; G = P. retgeri ATCC 29944; H = P. ruginosum ATCC 33673; I = P. stuartii ATCC 29914; J = Pantoea agglomerans JCM 1236; 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 taxa should be performed.

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REFERENCES