

Detection and analysis of *Lactobacillus paracasei* penicillin-binding proteins revealed the presence of cholate-sensitive penicillin-binding protein 3 and an elongated cell shape in a cholate-sensitive strain

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Penicillin-binding proteins (PBPs) are responsible for peptidoglycan synthesis. By using biotinylated ampicillin, we detected PBPs of *Lactobacillus paracasei* strains. Ten PBPs were identified, 7 of which had apparent molecular sizes similar to those of *Escherichia coli*. In the presence of cholate, strain NRIC 0625 showed an elongated shape, and its putative PBP3 showed cholate-sensitive penicillin-binding activity. Furthermore, this strain was highly sensitive to cefalexin, which is known to inhibit cell division by inactivating PBP3. These results suggest that the septum synthetase PBP3 of lactic acid bacteria can be one of the targets of intestinal bile acid.

Key words: penicillin-binding protein, *Lactobacillus paracasei*, cholate, cell division

Penicillin-binding proteins (PBPs) are cell wall synthetases essential for all bacteria having peptidoglycan. High molecular weight PBPs are bifunctional enzymes consisting of transglycosylases that polymerize glycan chains and transpeptidases that cross-bridge peptide linkages between the sugar chains, as first demonstrated with affinity-purified and gel-purified PBP1Bs of *Escherichia coli* [1–3]. Such activities were also demonstrated by PBPs 2 and 3, but these proteins seemed to require RodA protein and FtzW protein for the side wall and septum peptidoglycan synthesis, respectively [4, 5], as reviewed later [6]. On the other hand, low molecular weight PBPs such as PBPs 5 and 6 only have transpeptidase activity [7]. PBPs are the targets of β -lactam antibiotics, whose chemical structures mimic those of peptide bonds in the peptide bridges of peptidoglycan, and these drugs inactivate PBPs by covalently binding to the active centers of transpeptidase

moieties of PBPs [8].

Up to now, PBPs have been extensively studied for drug development or for understanding bacterial cell proliferation, division, and rod shape formation in pathogenic bacteria or model bacteria such as *E. coli*. However, PBPs of lactic acid bacteria (LAB) have mostly been paid little attention because they are considered to be beneficial to the hosts in most cases and therefore not necessarily to be killed by the drug. On the other hand, the rigid integrity of their cell wall should be important for their survival in the host intestine.

In order to detect PBPs of LAB, we performed penicillin-binding assays using three strains of *Lactobacillus paracasei*: 1. NRIC 0625 obtained from fermented milk (in NODAI Catalogue of Strains, Culture Collection Center, Tokyo), 2. NRIC 1917 isolated from sugarcane wine (Sanchez, P.C.; Studies on sugarcane wine (Basi) in the Philippines, Doctoral Thesis, Tokyo University of Agriculture, Code 270 (1982) pp. 158), and 3. NRIC 1981 isolated as inulin-fermenting LAB from compost by Okada in 1978 (in Nodai Catalogue of Strains, 4th ed., TUA Shuppankai, Tokyo (2009) pp. 34).

In order to prepare biotinylated penicillin, ampicillin sodium salt (Wako Pure Chemical Industries Ltd., Osaka) was biotinylated using succinyl biotin (EZ-Link NHS-LC-Biotin, Thermo Fisher Scientific, Waltham, MA, USA) and then passed through Affi-Gel 102 gel (Bio-Rad

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Laboratories, Hercules, CA, USA) to remove unnecessary materials [9]. Cell extracts of *L. paracasei* strains were prepared by disrupting the cells using a Sonifier (Branson advanced Sonifier 450, Branson, Danbury, CT, USA). Following ultracentrifugation, the membranous pellet was resuspended in 50 mM Tris buffer (pH 7.6) as in previous studies on *E. coli* PBPs [3]. The preparation was then incubated with biotinylated ampicillin for 15 min at 30 °C. A mixture of sarkosyl (Sigma-Aldrich, St. Louis, MO, USA) and non-biotinylated ampicillin (100-fold molar excess relative to the biotinylated ampicillin) was added to the reaction mixture in order to extract membrane proteins and to minimize nonspecific ampicillin bindings, respectively. The samples were then incubated for 5 min at room temperature. Insoluble materials were removed by centrifugation at 12,000 rpm for 5 min. Laemmli buffer was added to the samples followed by boiling for 3 min. The samples were then subjected to SDS polyacrylamide gel electrophoresis (10% or 7.5%). Separated proteins were electro-blotted on nitrocellulose membranes (Amersham Hybond-P, GE Healthcare, Little Chalfont, Buckinghamshire, UK). Western blotting was performed using streptavidin conjugated with peroxidase (Calbiochem, Merck KGaA, Darmstadt, Germany). Following washing with Tris-buffered saline (pH 7.6), PBPs were located on X-ray films using Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare Buckinghamshire, UK).

As shown in Fig. 1, the PBPs of *L. paracasei* (LpPBPs) were compared with those of *E. coli*. There were 10 major PBPs, with apparent molecular sizes ranging between 30 kDa and 100 kDa (39, 40, 47, 53, 58, 66, 75, 85, 90, and 96 kDa). Although the bands were less sharp as compared with those observed in the PBP assays using radioactive [¹⁴C] penicillin G from previous studies [3, 10], they were reasonably reproducible. In terms of size, 96, 66, 58, 47, and 40/39 kDa bands resemble PBPs 1, 2, 3, 4, and 5/6 of *E. coli*, respectively, which were designated as LpPBPs as indicated in Fig. 1. The 75 and 53 kDa bands, indicated by one and two asterisks, respectively, are relatively prominent in *L. paracasei*, but they did not show any resemblance to the major *E. coli* PBPs in terms of size. In line with this, others reported that an additional 55 kDa PBP was observed in *E. coli* only when using biotinylated ampicillin for detection but not in reactions using [¹²⁵I]-labeled penicillin V, and the band may be nonspecific because it could not be competed out with non-labelled ampicillin or penicillin V [9]. When the genome of *L. paracasei* BL23 (standard strain) was sought for PBPs, three PBP2 proteins with molecular weights ranging from 75 to 77

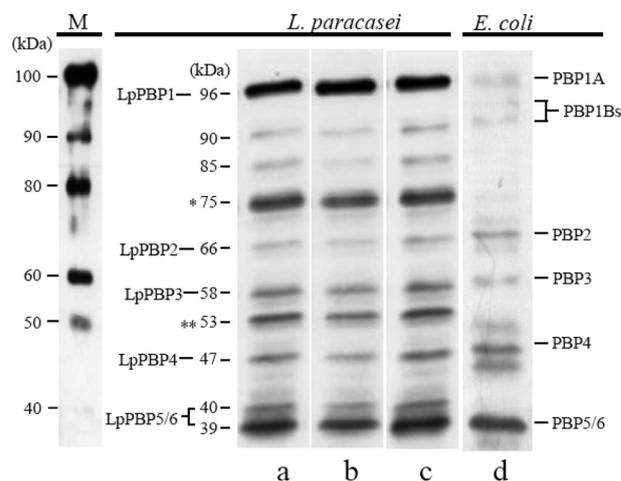


Fig. 1. Penicillin-binding proteins of *L. paracasei* and *E. coli*.

The penicillin-binding reactions and detection were performed with biotinylated ampicillin followed by western blotting using streptavidin-coupled peroxidase.

The strains used were as follows: a, *L. paracasei* NRIC 0625; b, *L. paracasei* NRIC 1981; c, *L. paracasei* NRIC 1917; d, *E. coli* JM 109. The indicated numbers are estimated molecular weights in kDa. LpPBPs assigned in this study are shown to the left of the estimated molecular size numbers of the bands. The far left lane (M) shows molecular weight markers and their sizes.

kDa were determined. The 75 kDa protein in the PBP pattern might represent the tentatively annotated PBP2 species in *L. paracasei*, although we propose that the 66 kDa protein is LpPBP2 based on its size resemblance to *E. coli* PBP2 (Fig. 1). Functional information is not available for these LpPBPs. Moreover, the draft genome data of the genes annotated as putative penicillin-binding protein homologs in *L. paracasei* strains used in this study [11] exhibited very low homology (with calculated sizes ranging between 75–83 kDa) when compared with those of genes for *E. coli* PBPs (data not shown). Instead, the LpPBPs demonstrated in this study gave a tangible image of “penicillin-binding” proteins in *L. paracasei* (with apparent molecular weights ranging between 39–96 kDa), appeared after SDS gel electrophoresis and subsequent localization by immunoblotting (Fig. 1).

In addition, LpPBP3 (58 kDa) was detected in *L. paracasei* (Fig. 1). In *E. coli* and other tested bacteria, it was previously reported that this protein was identified as the primary target of specific β -lactam antibiotics such as cephalexin, which induces formation of filamentous cells, indicating the role of PBP3 in septum formation upon bacterial cell division [12]. Cephalexin was reported to have high affinity to PBP3, causing formation of filamentous multinuclear cells, but not killing the bacteria

[10].

We examined the sensitivity of 3 *L. paracasei* strains isolated from different sources and *E. coli* strain JM109 (as a control) to antibiotics that target cell division (cefalexin) and rod cell formation (mecillinam) [10] by monitoring their growth curves in the presence of each drug. As shown in Fig. 2A, NRIC 0625 had the highest sensitivity to 50 µg/ml cefalexin followed by NRIC 1917 and 1981. No growth was observed with *L. paracasei* strains at 200 µg/ml cefalexin, while *E. coli* strain JM109 grew at the same concentration to some extent but was not able to propagate at the concentration of 400 µg/ml.

As for mecillinam sensitivity, *L. paracasei* NRIC 0625 had the highest sensitivity at the concentration of 200 µg/ml, followed by NRIC 1917 and 1981 (Fig. 2B). *E. coli* JM109 was highly sensitive to the drug, and severe growth inhibition was observed even at 0.2 µg/ml. This finding is in accordance with a previously reported study indicating that *E. coli* is highly sensitive to mecillinam [10].

As expected, treatment of *E. coli* cells with cefalexin induced filamentous cell formation, and mecillinam (0.4 µg/ml) caused generation of short or round cells (Fig. 3A).

For *L. paracasei* strains, 100 µg/ml cefalexin caused filamentous cell formation in strain NRIC 0625, but this was not so evident in NRIC 1917 and 1981 (Fig. 3B). For the cell division of *E. coli*, it was proposed that more than 35 proteins function altogether, including PBP3, by forming the putative divisome [15]. Information on the cell division of LAB is still limited, but at least cefalexin led to filamentous cell formation as shown in this study, indicating that PBP3 has an important role in cell division (Fig. 3B).

In contrast to the effects on *E. coli*, mecillinam did not prominently alter the rod shape of LABs. However, there was a slight tendency for partially round cell formation in NRIC 0625 at 200 µg/ml and in all *L. paracasei* strains at 400 µg/ml (Fig. 3C).

In *E. coli*, the involvement of RodA, PBP2, and MreB in rod cell formation has been elucidated [10, 12]. Mecillinam targets PBP2, whereas A22 (S-(3,4-dichlorobenzyl)isothiourea) targets the cytoskeletal protein MreB, and administration of either drug to *E. coli* causes round cell formation [13, 14]. Our data implied that putative mecillinam-sensitive protein did not function in rod cell formation of LAB, but a certain by-pass pathway seemed to exist for the maintenance of rod shape even at a high concentration of mecillinam. As it has been reported that penicillin-binding activities of *E. coli* have different sensitivities when exposed to

various detergents [16], we examined the sensitivity of LpPBPs following treatment with cholate, the major fat dissolving agent contained in the bile acid secreted in the host intestine with amphipathic properties that debilitate intestinal Gram-positive microbiota. Following scanning and quantitation of the PBP bands on film with the GelAnalyser 2010 software (gelanalyser.com, <http://en.bio-soft.net>), it was shown that treatment of the membrane fractions of *L. paracasei* with 1% w/v cholate resulted in partial inactivation of their penicillin-binding activity (Fig. 4). Among all the LpPBPs in all the strains, marked inactivation (up to 40%) of the binding capacity of LpPBP3 of NRIC0625 was observed (Fig. 4, far left panel, indicated by asterisk). This indicated that NRIC 0625 had a cholate-sensitive putative counterpart of PBP3 (LpPBP3) in terms of penicillin-binding activity. The second most sensitive LpPBP3 was that of NRIC 1981, with about 20% inactivation. Interestingly, the PBP3 of *E. coli* was the most sensitive one to the treatment with 1% w/v deoxycholate in similar experiments reported previously [16], suggesting the vulnerability of PBP3 to bile-originated detergent.

Cholate has been implicated in damage to cell membrane primarily via its surface-active properties, but it was reported that treatment of LAB with cholate could cause disruption of the cell wall that results in generation of a bubble-like structure on cell surface, suggesting its toxic effect on cell wall maintenance [17]. In order to examine the relation between cholate sensitivity and cell wall synthesis by PBPs as mentioned above, we treated *L. paracasei* cells in culture with cholate. LABs were exposed to 1 mM, 2 mM, 3 mM, 4 mM, and 5 mM cholate in MRS medium, and the OD₆₆₀ was measured during a 24 hr period (Fig. 5). Among the 3 tested strains, NRIC 1917 turned out to be the most resistant strain to 2 mM cholate, while strains NRIC 0625 and 1981 seemed to be nearly equally sensitive when looking at their sudden growth declines at 2 mM cholate. Growth monitoring by OD measurement provided images reflecting the real-time effect of the stress, but at the same time, this may not precisely reflect the survival state, especially for cephalixin, which causes cell elongation. Therefore, we also measured cell viability with the time course by counting the surviving cell numbers at different time points following serial dilution and plating according to a classical method [18] and compared the results with those for cholate stress (Fig. 6). In terms of cefalexin, the order of sensitivity appeared to be NRIC 0625>1917>1981, as shown in the right panel of Fig. 6. On the other hand, in terms of cholate sensitivity, the sensitivities of strains NRIC 0625 and 1981 turned out to be almost the same.

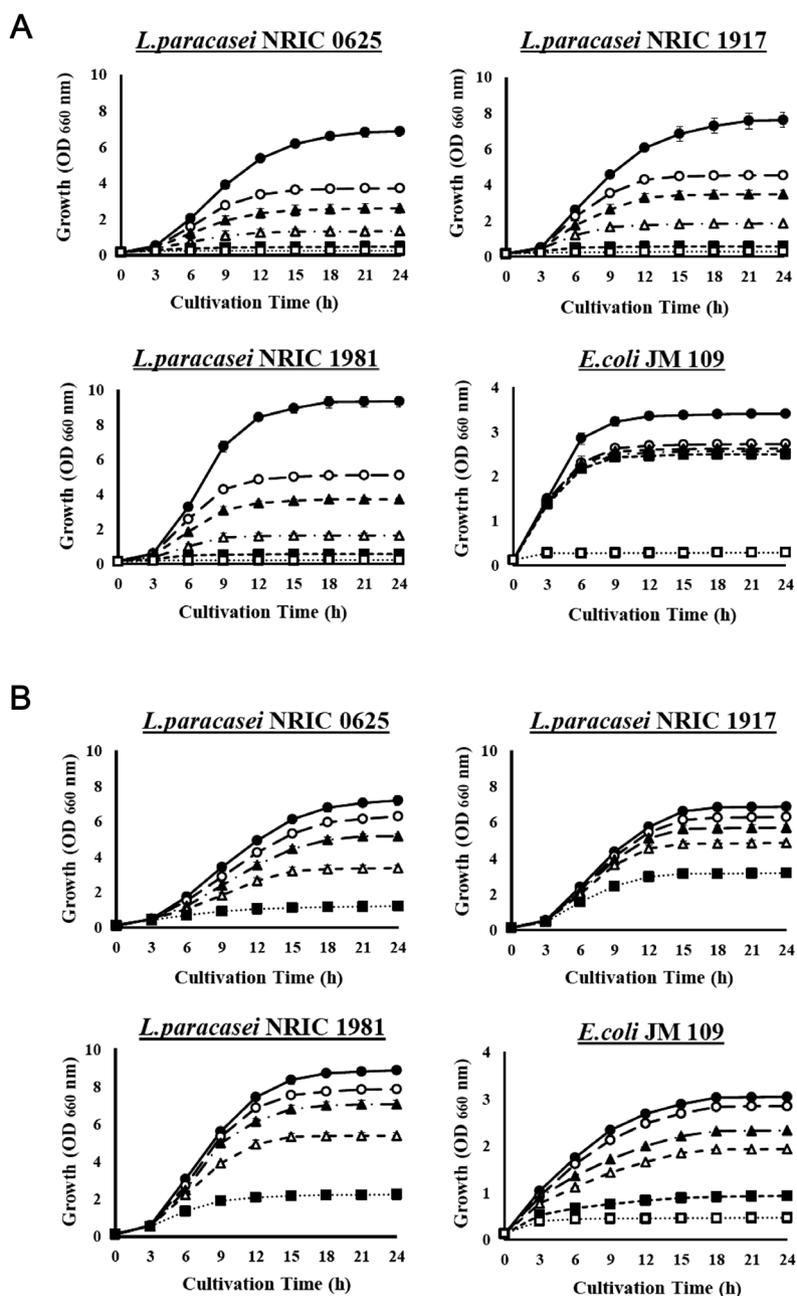


Fig. 2. Sensitivity of *E. coli* JM109 and *L. paracasei* strains used in this study to cefalexin (cell division inhibitor) and mecillinam (rod formation inhibitor).

A: Growth curves in the presence of different concentrations of cefalexin.

Concentrations of cefalexin: ●, 0 µg/ml; ○, 25 µg/ml; ▲, 50 µg/ml; △, 100 µg/ml; ■, 200 µg/ml; □, 400 µg/ml

B: Growth curves in the presence of different concentrations of mecillinam.

Concentrations of mecillinam: For *L. paracasei*, ●, 0 µg/ml; ○, 50 µg/ml; ▲, 100 µg/ml; △, 200 µg/ml; □, 400 µg/ml; for *E. coli*, ●, 0 µg/ml; ○, 0.04 µg/ml; ▲, 0.08 µg/ml; △, 0.1 µg/ml; ■, 0.2 µg/ml; □, 0.4 µg/ml

Interestingly however, when the morphological state was examined, strain NRIC 0625 showed an elongated cell shape already at the concentration of 2 mM, while NRIC

1981 did not show elongated cells, but rather showed slightly swollen cells (Fig. 7). At 4 mM cholate, strain NRIC 0625 cells were elongated, but both NRIC

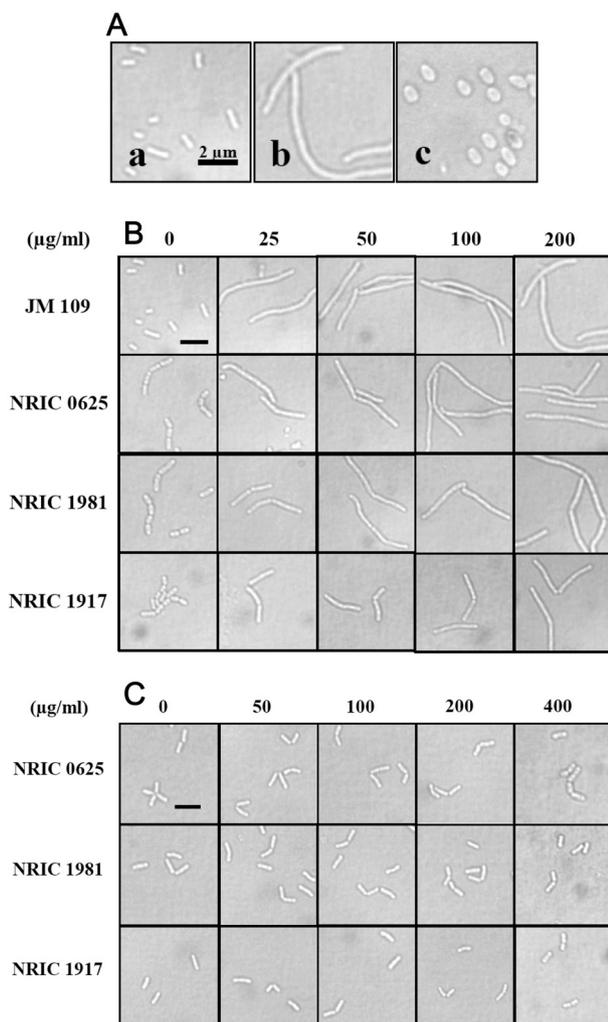


Fig. 3. Cellular morphology of *E. coli* JM109 and *L. paracasei* strains used in this study in the presence of cefalexin and mecillinam.

Cells were observed after 12 hours of exposure to antibiotics.

A: Morphological examination of *E. coli* under light microscope ($\times 1,000$).

a, control; b, cefalexin 400 $\mu\text{g/ml}$; c, mecillinam 0.4 $\mu\text{g/ml}$.

B: Morphological examination of *L. paracasei* in the presence of cefalexin ($\times 1,000$).

C: Morphological examination of *L. paracasei* in the presence of mecillinam ($\times 1,000$).

Bars indicate 2 μm .

and 1917 cells were swollen. These data implied that although cholate sensitivity did not directly correlate with cefalexin sensitivity, the elongated shape formation of strain NRIC 0625 may directly reflect the sensitivity of its LpPBP3 to cholate.

Others observed that when a library of random insertion mutants was constructed using *Enterococcus faecalis* and analyzed for bile sensitivity, the mutations

fell into proteins related to cell wall integrity among other functions such as DNA repair and transcriptional regulation [19]. In *Nocardia lactamdurans*, it was reported that one of the mutations affected in a gene coding 36.7 kDa protein similar to PBP4 resulted in septation anomalies [20]. Although the 58 kDa putative LpPBP3 reported here may not be the paralog of the PBP4 of *N. lactamdurans* or *E. faecalis*, its cholate-sensitive penicillin-binding activity and filamentous cell formation of strain NRIC 0625 in the presence of cholate indicate a causative relation to the function of septum forming PBP and survival in the host intestine.

Our results implied that LpPBP3 might have a role in the maintenance of peptidoglycan structure under cholate stress conditions, in addition to the known role in septum formation. The damage on peptidoglycan may be caused by the function of lytic enzymes, which would be triggered to work under stress conditions. In line with this, it should be noted that PBP4 of *Bacillus subtilis* was reported to function as a peptidoglycan hydratase under high salt stress [21]. Furthermore, others reported that *L. plantarum* cells showed a shrunken, empty, or bubbling appearance on the cell surface upon treatment with bile, suggesting that local cell wall breakdown occurred on the peptidoglycan [17].

Aside from peptidoglycan synthesis, one of the plausible molecules playing a role in bile resistance is the efflux transporter that pumps out bile from cells, and such significance was documented by using deletion mutants of a major facilitator superfamily transporter protein, multidrug resistance transporter, and ABC transporter in *L. acidophilus* [22]. In addition, by investigating bile tolerance in six different *L. casei* strains, which have different bile sensitivities isolated from different sources, major bile tolerance biomarkers were shown to fall into proteins with assigned functions in membrane modification, detoxification, and central metabolism [23]. These previous studies indicate complexity and mutual cross functions of bile tolerance mechanisms employed in lactic acid bacteria against bile stress. To our knowledge, cholate sensitivity of LpPBPs has not been demonstrated in previous studies, and we assume the PBPs may function in bile tolerance in concert with other mechanisms reported therein.

In short, our results indicated that LpPBP3 (58 kDa) may be one of the key factors involved in cholate resistance functioning in the maintenance of rigid peptidoglycan under bile stress conditions. On the other hand, LpPBP2 (which appeared as a 66 kDa PBP in Fig. 1 but was differently annotated as 75–77 kDa proteins in the data bank), at least in *L. paracasei*, may not be

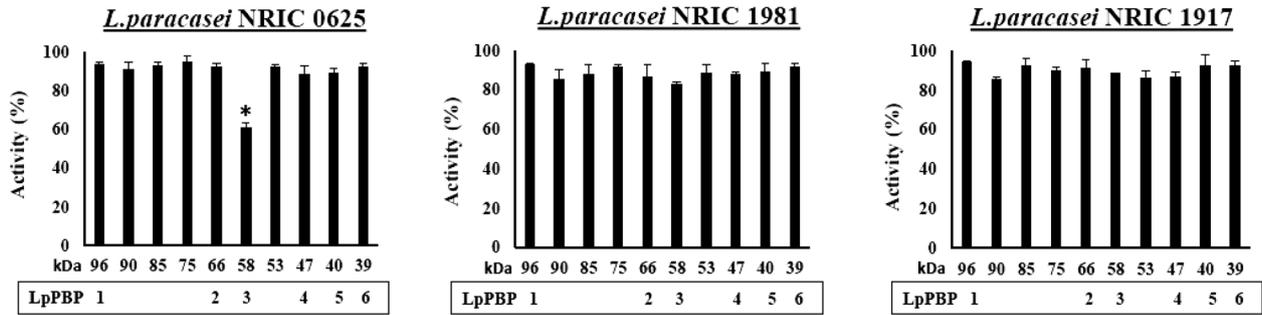


Fig. 4. Inactivation of *L. paracasei* PBPs by cholate.

Penicillin-binding reactions were performed in the presence of 1% cholate. The band intensities on the film were quantified with the GelAnalyser 2010 software.

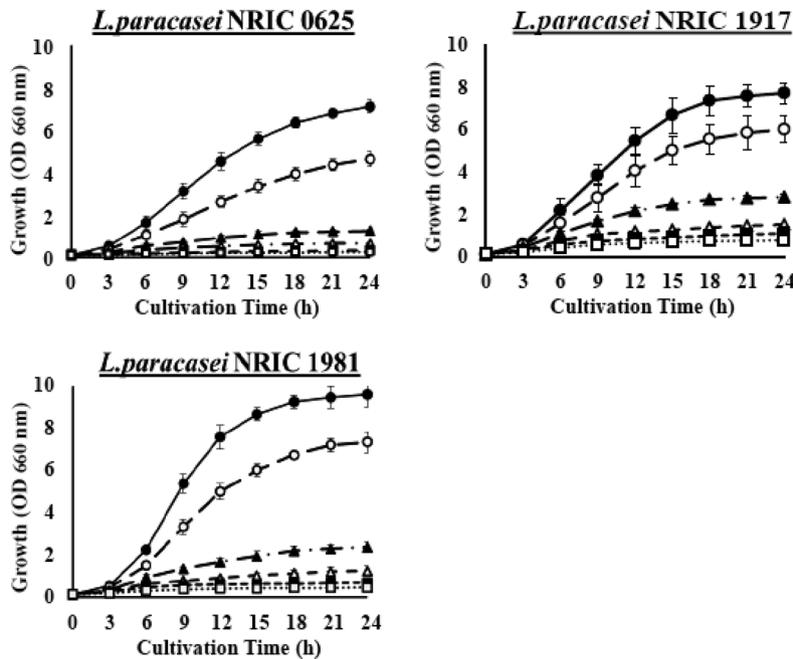


Fig. 5. Sensitivity of *L. paracasei* strains used in this study to different concentrations of cholate.

Concentrations of cholate: ●, 0 mM; ○, 1 mM; ▲, 2 mM; △, 3 mM; □, 4 mM; ■, 5 mM

directly involved in the rod cell shape formation.

The information given in this report may serve as criteria for the selection of *L. paracasei* strains as probiotic agents.

Conflict of interest

We declare that we have no conflicts of interest concerning the study described in this report.

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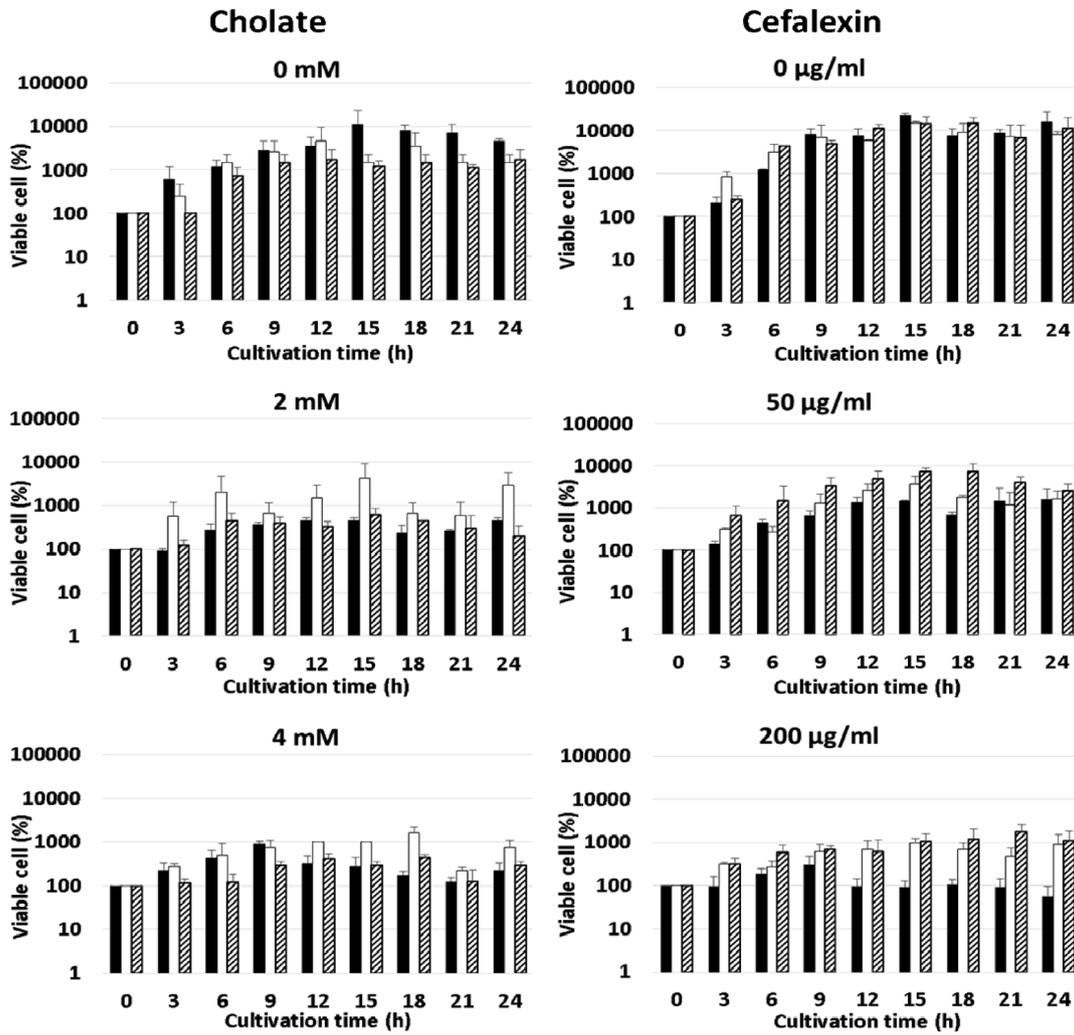


Fig. 6. Viability test of *L. paracasei* strains against cholate and cefalexin.

Viable cells were counted at each indicated time point and at different concentrations of the stress substance following serial dilution and plating. Solid bar, NRIC 0625; empty bar, NRIC 1917; diagonal line bar, NRIC 1981.

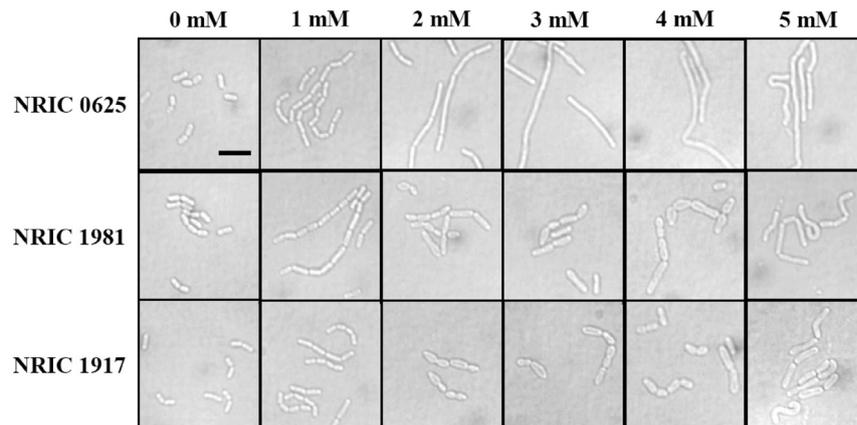


Fig. 7. Cellular morphology of *L. paracasei* strains used in this study in the presence of cholate.

Cells were observed after 24 hours of exposure to the indicated concentration of cholate in the medium. The bar indicates 2 µm.

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