Kasetsart J. (Nat. Sci.) 40 : 13 - 17 (2006)

Acid and Bile Tolerance of *Lactobacillus thermotolerans*, a Novel Species Isolated from Chicken Feces

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ABSTRACT

Five strains of a novel thermotolerant lactic acid bacterium, *Lactobacillus thermotolerans*, isolated from chicken feces were assayed for acid and bile tolerance. Cultures of *L. thermotolerans* were grown anaerobically at 42°C in four different conditions, Man-Rogosa-Sharpe (MRS) broth at pH 6.5, MRS broth at pH 3.0, MRS broth supplemented with 0.3% (w/v) ox gall and MRS broth supplemented with 7 mM sodium taurocholate, respectively. All strains showed an acid tolerance when incubated at pH 3.0 over a period of 4 h. *L. thermotolerans* demonstrated significant changes of absorbance ($p<0.05$) from the control when cultured with 0.3% bile concentration. Strain JCM11427 seemed to show the highest tolerance to bile. However, statistical analyses revealed no significant differences. The conjugated bile salt (sodium taurocholate) at physiological concentration (7 mM) had no effect on growth of the bacterial strains.

Key words: *Lactobacillus thermotolerans*, probiotc, bile, acid tolerance

INTRODUCTION

Probiotics can be defined as live microbial feed supplements which beneficially affect the host animal by improving its intestinal microbial balance (Fuller, 1989). The claims made for probiotics mainly include improvement of growth rate and feed utilization, disease resistance and reduction of gut shedding of enteropathogenic bacteria. Nowadays, research on animal probiotics is mainly focused on calves, pigs and poultry (Ehrmann *et al*., 2002). In the poultry industry, probiotics are use to prevent poultry pathogens and disease so as to improve meat and egg production. Recently, emphasis has been placed on the selection and preparation of *Lactobacillus* strains as probiotics (Chateur *et al*., 1994). Lactobacilli occur among the normal intestinal flora of chicken and other animals from the first few days of their life. The capacity of lactobacilli to colonize the chicken crop by means of adherence has been well documented.

Previously, Niamsup *et al*. (2003) isolated *Lactobacillus thermotolerans*, a novel thermotolerant species, from chicken feces collected in Thailand. The preference of this bacterium for the chicken intestine may be due to the body temperature of chickens, 42°C, which corresponds to the optimum temperature for growth of this bacterium, as determined by measurement of the specific growth rate (Niamsup *et al*., 2003). A highly sensitive and specific real-

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time polymerase chain reaction (PCR) assay for detection of the *L. thermotolerans* presenting in chicken feces was also developed and successfully used to monitor the dynamics of this novel bacterium in chicken feces, thereby providing a powerful tool for studying the distribution and abundance of this bacterium in a complex microbial community. The results also demonstrated that this novel bacterium is a normal member of the chicken intestinal microbiota (Selim *et al*., 2005).

Before using bacterial strains as probiotics, their proper identification is needed. Most bacteria do not survive well at low pH values. The severe acidic conditions of the crop, proventiculus and gizzard could have an adverse effect on the bacteria. Thus, it has been suggested that microbial cultures to be used as probiotics should be screened for their resistance to acidity (Conway *et al*., 1987). Once the bacteria reach the intestinal tract, their ability to survive depends on their resistance to bile (Gilliland *et al*., 1984). Bile entering the duodenal section of the small intestine has been found to reduce survival of bacteria. Hence, the success of a probiotic also depends on the bile-resistant qualities of the selected strains.

The objective of this study was to investigate the acid and bile tolerance of all five strains of *Lactobacillus thermotolerans*, a novel species isolated from chicken feces.

MATERIALS AND METHODS

Bacterial strains and growth condition

Five strains of *Lactobacillus thermotolerans*, JCM11423, JCM11424, JCM11425^T, JCM11426 and JCM11427, previously isolated from chicken feces collected in Thailand (Niamsup *et al*., 2003) were used. They were obtained from the Japan Collection of Microorganisms, Japan. Stock cultures of *L. thermotolerans* strains were stored frozen in Man-Rogosa-Sharpe (MRS) broth (Merck), supplemented with 20% glycerol at -80°C. Working cultures were propagated anaerobically in MRS broth at 42°C for overnight.

Acid tolerance test

The acid tolerance test was performed as described previously (Ehrmann *et al*., 2002). Bacteria were grown anaerobically in MRS broth at 42°C for overnight, then subcultured into fresh MRS broth and incubated for another 24 h. The cultures were centrifuged at 5,000 g for 10 min at 4°C. The pellets were washed twice, and resuspended in sterile phosphate buffer saline (PBS), pH 7.2. Each strain culture was diluted 1/100 in PBS at pH 3.0. After the incubation period of 4 h, the bacteria were transferred to MRS broth and incubated anaerobically at 42°C. The optical densities (OD_{600}) were monitored at 2h-interval for 24 h. Each strain cultured in MRS broth at optimal pH (6.5) was run as controls. All tests were carried out in triplicate.

Bile tolerance test

The method used for testing bile tolerance was similar to that described by Gilliland *et al*. (1984). The bacteria were grown overnight in MRS broth and then 2% freshly-prepared overnight cultures were inoculated into tubes containing MRS broth with 0.3% ox gall (Sigma), MRS broth containing 7 mM sodium taurocholate (Sigma), and MRS broth without bile (as controls). The inoculated tubes were incubated at 42°C. Growth was monitored at 2h-interval for 24 h (OD_{600}). All tests were carried out in triplicate.

Statistical analyses

Statistical analysis of acid and bile tolerance test was performed by using SPSS software (SPSS Inc., Chicago, IL). A Chi-square test was used for comparative studies; $p<0.05$ was considered significant.

RESULTS AND DISCUSSION

It is important that the probiotic microorganisms are able to reach the gastrointestinal tract (GIT) and remain viable there for 4 h or more (Ouwehand *et al*., 1999). Figure 1 showed the results responding to acid tolerance assay at pH 3 for the strains. All strains of *L. thermotolerans* survived during an incubation period of 4 h with delay in growth comparing to the controls. However each strain maintained an acceptable final cell concentration level at about 10^6 cfu ml^{-1} (data not shown). These results were comparable to those reported by Jin *et al*. (1998) that most of *Lactobacillus* strains isolated from GIT of chickens were tolerant to acid. Comparison to humans and domestic animals such as pigs and cattle, the alimentary tract of chicken is shorter. The time required for feed to pass through the entire alimentary canal is as short as 2.5 h (Duke, 1977). Therefore, acid tolerance for bacterial strains in chickens is not as crucial as for those in other animals where the feed passage rate is much slower.

Bile tolerance of the five strains was also investigated (Figure 1). All strains of *L. thermotolerans* demonstrated significant changes of absorbance ($p<0.05$) from controls when cultured with 0.3% bile concentration. *L. thermotolerans* JCM 11427 showed the highest tolerance among the five strains. The presence of 7 mM sodium taurocholate had no significant effect on survival and growth of *L. thermotolerans*. The concentration of 7 mM sodium taurocholate was chosen because the small bowel of chicken contains about 21 mM conjugated bile acids, of which approximately one-third are conjugated to taurine (Tannock *et al*., 1994). Resistance to bile salts is of great importance to survival and growth of bacteria in the intestinal tract, and thus, is a prerequisite for bacteria to be used as probiotics (Havenarr, 1992). The average bile concentration is around 0.3%, and may range up to the extreme of 2.0% during the first hour of digestion (Gotcheva *et al*., 2002). The effect of bile on the survival of lactobacilli has been investigated by several authors and is thought to be linked to the ability to de-conjugate bile acids (Tannock *et al*., 1989). Bile resistance of some strains is related to specific enzyme activity-bile salt hydrolase (BSH) which helps hydrolyze conjugated bile, thus reduce its toxic effect (Du Toit *et al*., 1998). BSH activity has most often been found in organisms isolated from the intestines or feces of animals (Tanaka *et al*., 1999). The reason for bile salt hydrolase production by lactobacilli remains enigmatic. Neither of the molecules resulting from the hydrolysis of taurocholate can be utilized by the lactobacilli (Tannock *et al*., 1997).

CONCLUSIONS

The results obtained in the present study showed the tolerance of the novel species, *L. thermotolerans*, in the conditions of high ox gall concentration and low pH value. From this study, it could be concluded that all the five strains of *L. thermotolerans* were (i) resistant or tolerant to pH 3, and (ii) tolerant to 0.3% bile, while *L. thermotolerans* JCM11427 seemed to be more tolerant than the other strains.

ACKNOWLEDGEMENTS

This work was done by the collaboration in a Core University Programme between Yamaguchi University and Kasetsart University, Supported by the Scientific Cooperation Programme agreed by Japan Society for the Promotion of Science (JSPS) and National Research Council of Thailand (NRCT).

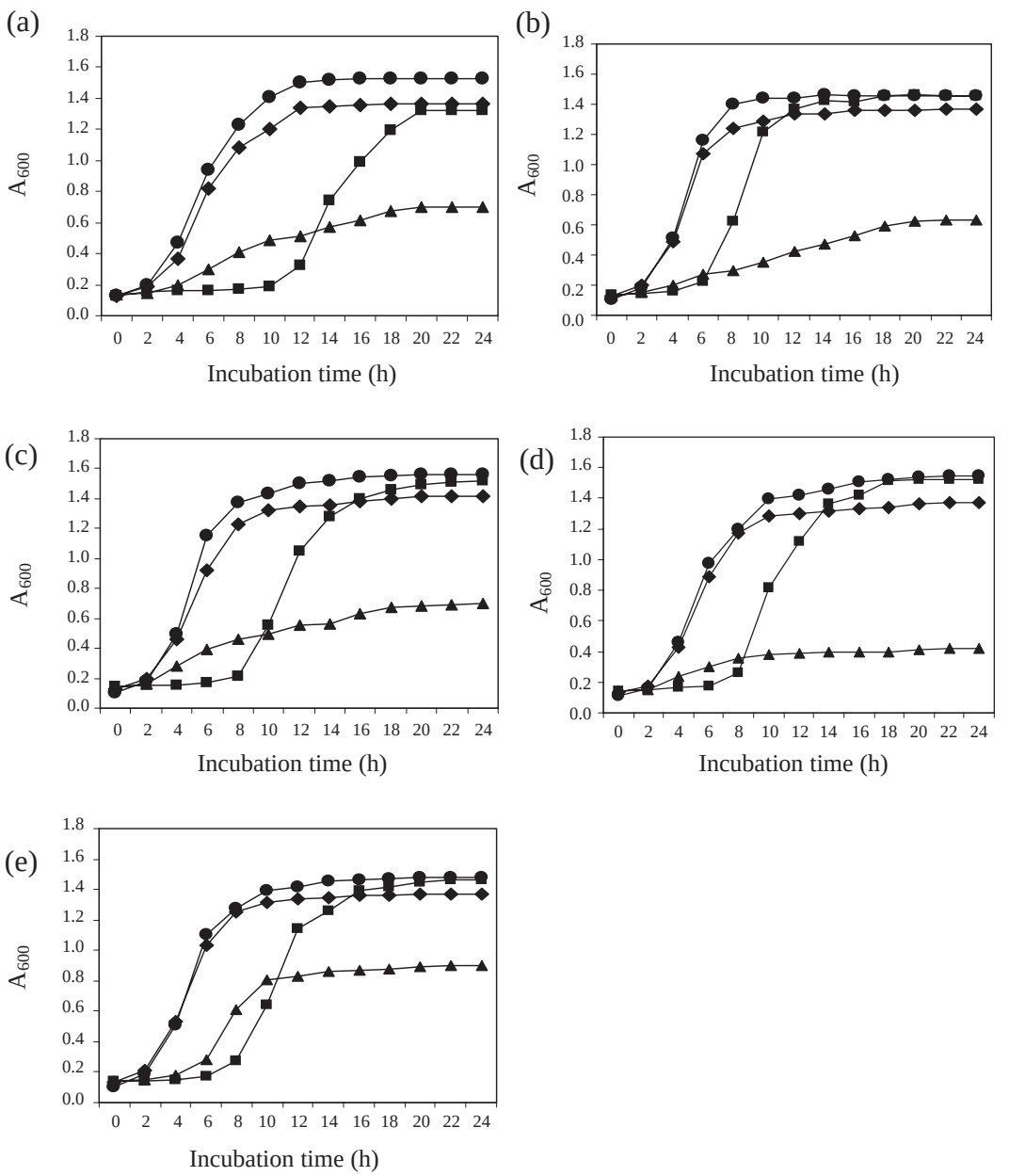

Figure 1 Growth of *Lactobacillus thermotolerans* cultured in MRS medium pH 6.5, and containing no bile (●), MRS pH 3.0 (■), MRS containing 0.3% ox gall (▲) and MRS containing 7 mM sodium taurocholate (◆). (a) JCM 11423; (b) JCM11424; (c) JCM 11425^{T}; (d) JCM11426; (e) JCM11427

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