Korean J. Microbiol. Biotechnol. (2013), 41(2), 249–252
http://dx.doi.org/10.4014/kjmb.1301.01008
pISSN 1598-642X eISSN 2234-7305

KJMB
Korean Journal of Microbiology and Biotechnology

Immunomodulatory and Antigenotoxic Properties of *Bacillus amyloliquefaciens* KU801

Lee, Na-Kyoung[1], So-Yeon Kim[1], Hyo-Ihl Chang[2], Eunju Park[3], and Hyun-Dong Paik[1]*

[1]*Division of Animal Life Science and Bio/Molecular Informatics Center, Konkuk University, Seoul 143-701, Korea*
[2]*School of Life Sciences and Biotechnology, Korea University, Seoul 136-701, Korea*
[3]*Department of Food Nutrition, Kyungnam University, Masan 631-701, Korea*

Received : January 23, 2013 / Revised : April 2, 2013 / Accepted : April 3, 2013

The *Bacillus* KU801 strain, due to its potential in the field of probiotics for animal use, was isolated from chicken feces. Strain KU801 was identified as *Bacillus amyloliquefaciens* KU801 based on the results of 16S rRNA sequencing. Vegetative and spore cells of *B. amyloliquefaciens* KU801 were resistant to artificial gastric juice and artificial bile acid. *B. amyloliquefaciens* KU801 was found to inhibit the production of nitric oxide (NO) and increase the production of Interleukin-1 alpha (IL-1α). DNA damage induced by N-methyl-Ntion of ninitroso-guanidine (MNNG) was significantly inhibited, in a dose dependent manner, by preincubating MNNG together with *B. amyloliquefaciens* KU801. These results demonstrate the potential use of *B. amyloliquefaciens* KU801 as a feed additive.

Keywords: Identification, probiotic, interleukin-1α, nitritic oxide, DNA damage

Probiotics have been used in animals and human, and their effectiveness has been widely discussed [5]. Bacteria associated with probiotic activity are most commonly lactobacilli and bifidobacteria, but non-pathogenic organisms, such as *Escherichia coli* and yeast, *Saccharomyces boulardii*, have also used. The characteristics of probiotics have been reviewed extensively, including their survival in gastric conditions and colonization of the intestine, reduction of lactose intolerance, prevention of antibiotic-induced diarrhea, prevention of colon cancer, and stimulation of the immune system such as atopic ezema [3, 12, 13].

Most *in vitro* studies of probiotics were confirmed their positive effect in host's digestive tract while their effect could not be guaranteed *in vivo* system. The reduction of probiotic effect *in vivo* system is caused by a complex environment of the lower intestinal pH, various digestive enzymes, and defensive factor in host [2]. The vegetative cell of *Bacillus* sp. does not proliferate in the gastrointestinal tract like other probiotics. However, bacterial spores are inherently robust bioparticles, so they may possess higher survival rates through the stomach as well during transit through the intestine. Therefore, *Bacillus* sp. may possess higher survival rates through the stomach without other expenses during transit through the intestine. *Bacillus* probiotics include commercial strains, such as *Bacillus licheniformis, Bacillus subtilis, Bacillus coagulans,* and *Bacillus polyfermenticus* [6, 10, 16]. Therefore, the aims of this study were to investigate the probiotic properties of isolated *Bacillus* KU801 strain as probiotics for animal use.

Bacillus KU801 strain was isolated in the condition of pH 3 for 1 h and incubation at 80°C for 30 min using tryptic soy broth (TSB, Difco, Sparks, MD, USA) from chicken feces. This strain was cultured in 100 ml of TSB in a 500 ml baffled flask with shaking at 37°C. This strain was stored at -70°C in TSB with 20% (v/v) glycerol.

Bacillus KU801 strain was Gram positive and rod-shaped bacteria. Analysis of 16S rRNA sequences showed that strain KU801 had 97% similarity to *B. amyloliquefaciens* (Fig. 1). Based on these results, strain KU801 was finally identified as *B. amyloliquefaciens* KU801. *B. amyloliquefa-*

***Corresponding author**
Tel: +82-2-2049-6011, Fax: +82-2-455-0381
E-mail: hdpaik@konkuk.ac.kr

ciens was originally reported as an agricultural biomaterial [4], therefore *B. amyloliquefaciens* KU801 was investigated about its potential probiotic properties.

The method of Kobayashi *et al.* [11] was used to analyze the artificial digestive fluid tolerance of *B. amyloliquefaciens* KU801. Vegetative cells of *B. amyloliquefaciens* KU801 was shown low survival rate at gastric juice (pH 2.5 with 1% pepsin), whereas spore cells of *B. amyloliquefaciens* KU801 was shown high survival rate (Fig. 2). Similarly, most described *Bacillus* species cannot proliferate in the gastrointestinal tract, and also have no normal interaction with the gastrointestinal tract [9]. Bacterial spores as probiotics was reported as the strain *B. licheniformis, B. subtilis, B. polyfermenticus* SCD, *B. clausii,* and etc. Spore cells of *B. polyfermenticus* SCD and *B. licheniformis* were reported high survival rate against artificial gastric juice [8, 10]. These spore cells were acid tolerant and reached the intestines without activity loss. In artificial bile acid (0.3% oxgall), vegetative and spore cells of *B. amyloliquefaciens* KU801 was shown high survival rate without activity loss (Fig. 3). In artificial bile acid, *B. polyfermenticus* SCD was reported

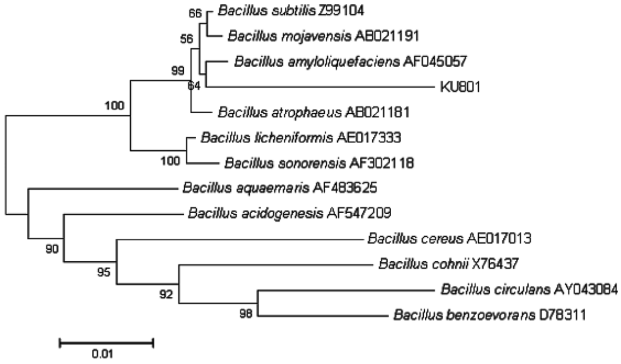

Fig. 1. Phylogenetic tree based on 16S rRNA sequences showing the position of strain KU801 and representatives of some related taxa.
The scale 0.1 substitution per nucleotide position.

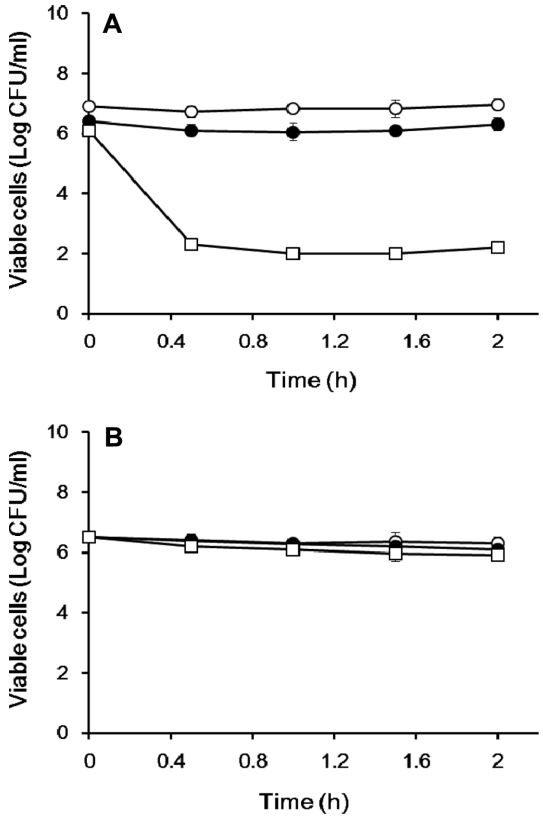

Fig. 2. Survival of (A) vegetative cells and (B) spore cells of *B. amyloliquefaciens* KU801 in artificial gastric juice.
○, Non-treated with artificial gastric juice (control); ●, treated with artificial gastric juice (pH 4.0, 1% pepsin); □, treated with artificial gastric juice (pH 2.5, 1% pepsin).

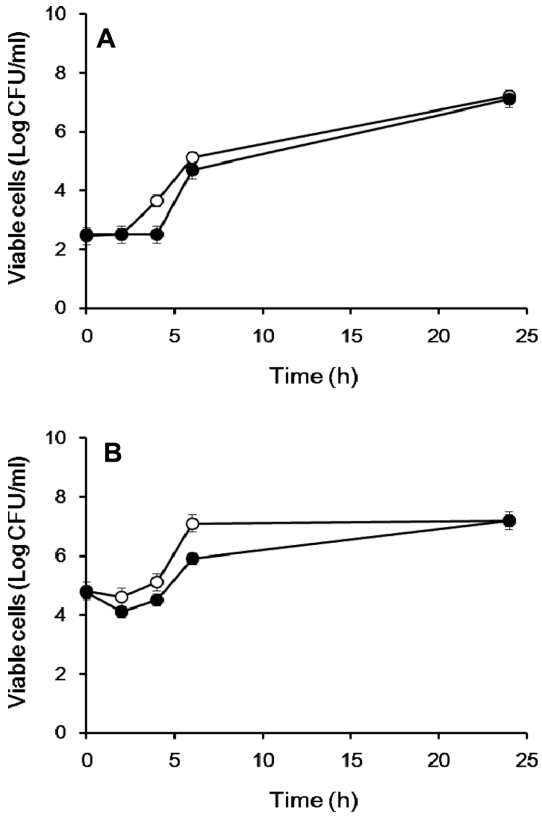

Fig. 3. Survival of (A) vegetative cells and (B) spore cells of *B. amyloliquefaciens* KU801 in artificial bile acid after treated with artificial gastric juice (pH 2.5, 1% pepsin) at 37°C for 2 h.
○, non-added bile acid after treated with artificial gastric juice (control); ●, treated with artificial gastric juice and bile acid.

http://dx.doi.org/10.4014/kjmb.1301.01008

high survival rate against artificial gastric juice [8], but *B. licheniformis* was shown low viability below 25% [9].

The enzyme production of *B. amyloliquefaciens* KU801 was investigated by using API ZYM kit (BioMerieux, Lyon, France). *B. amyloliquefaciens* KU801 did not produce the carcinogenic enzyme, β-glucuronidase. Alkaline phosphatase, esterase, and esterase lipase were produced.

Murine macrophage cell line (RAW 264.7, KCLB 40071) was used for NO and IL-1α production, cultured in Dulbecco's Modified Eagle Medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Gibco) and penicillin G (100 U/ml, Sigma, St. Louis, MO, USA)/ streptomycin (100 μg/ml, Sigma) at 37°C in a humidified 5% CO_2 incubator. These supernatant or concentrates of *B. amyloliquefaciens* KU801 were treated in RAW 264.7 cell line. The production of NO was measured by using Griess reagent (Sigma, St. Louis, MO, USA). IL-1α production was measured by the quantitative ELISA with commercially available kits (Koma Biotech Inc., Seoul, Korea). NO production of polysaccharides, flavonoids, and phenolic compounds in Lipopolysaccharides (LPS)-activated RAW 264.7 macrophages was reported [7, 13]. The supernatants of *B. amyloliquefaciens* KU801 suppress NO production in LPS-activated macrophages. LPS treatment of macrophage cells elevated the oxidative product of NO (34.53 ± 2.20 μmol/l) was compared to LPS-untreated negative control (3.81 ± 0.09 μmol/l). Also, treatment using the supernatant of *B. amyloliquefaciens* KU801 in LPS-activated macrophages decreased oxidative stress by suppressing NO production (12.22 ± 0.22 and 8.50 ± 0.18 μmol/l in case of 50 and 100 μl of *B. amyloliquefaciens* KU801 supernatant, respectively). IL-1α stimulates the growth and action of immune system cells that fight disease. The treatment of *B. amyloliquefaciens* KU801 produced 444 pg/ml of IL-1α. Chungkukjang fermented with *Bacillus* spp. strains produced about 100 pg/ml of IL-1α [1]. The non-lipopolysaccharide component of *Lactobacillus acidophilus* (La 1) stimulates the production of IL-1α and TNF-α by mouse macrophages in vitro as our results [14].

Fig. 4 shows the antigenotoxic effect of *B. amyloliquefaciens* KU801 against MNNG (Fluka Co., Buchs SG, Switzerland) in phagocyte cells using the comet assay [15, 16]. Firstly, lyophilized *B. amyloliquefaciens* KU801 (vegetative or spore cells) was suspended in Hank's balanced salt solution (HBSS) at a concentration of 0, 10, 25, and 50 mg/ml. Each suspension was then preincubated with MNNG at

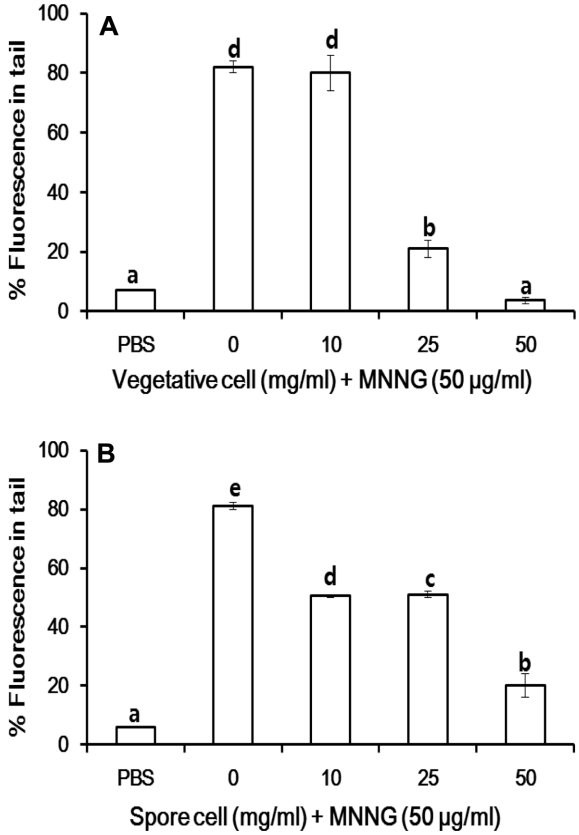

Fig. 4. Protective effect of (A) vegetative cells and (B) spore cells of *B. amyloliquefaciens* KU801 against DNA damage in lymphocyte.

a final concentration of 50 μg/ml for 30 min in a shaking incubator (37°C, 150 rpm). The increased DNA damage induced by MNNG was significantly inhibited by preincubating MNNG together with the vegetative cells of *B. amyloliquefaciens* KU801 in a dose dependent manner. The tail intensity of vegetative and spore cells of *B. amyloliquefaciens* KU801 was significantly decreased by 21% and 51% versus the positive control at 25 mg/ml, respectively. Notably, the extent of comet formation in cells treated with 50 mg/ml of *B. amyloliquefaciens* KU801 was lower than that seen in the PBS-treated negative control. These results provide new insights into the mechanism of the anticancer properties of *B. amyloliquefaciens* KU801. As a result, *B. amyloliquefaciens* KU801 could be used as feed additives having immunomodulatory effects.

Acknowledgements

This study was funded by a research grant (Code #

20070401034025) from Biogreen 21 program, Rural Development Administration, Korea and supported by Priority Research Centers Program through the National Research Foundation of Korea funded by the Ministry of Education, Science and Technology (2012-0006686).

References

1. Chang, J. H., Y. Y. Shim, S. H. Kim, K. M. Chee, and S. K. Cha. 2005. Fibrinolytic and immunostimulating activities of *Bacillus* spp. strains isolated from c*hungkuk-jang*. *Korean J. Food Sci. Technol.* **37**: 255-260.
2. Chen, S., Q. Zhao, L. R. Ferquson, Q. Shu, I. Weir, and S. Garg. 2012. Development of a novel probiotic delivery system based on microencapsulation with protectants. *Appl. Microbiol. Biotechnol.* **93**: 1447-1457.
3. Cho, I. J., N. K. Lee, and Y. T. Hahm. 2009. Characterization of *Lactobacillus* spp. isolated from the feces of breast-feeding piglets. *J. Biosci. Bioeng.* **108**: 194-198.
4. Chung, S. and S. D. Kim. 2005. Biological control of phytopathogenic fungi by *Bacillus amyloliquefaciens* 7079: suppression rates are better than popular chemical fungicides. *J. Microbiol. Biotechnol.* **15**: 1011-1021.
5. Fernández, M. F., S. Boris, and C. Barbés. 2003. Probiotic properties of human lactobacilli strains to be used in the gastrointestinal tract. *J. Appl. Microbiol.* **94**: 449-455.
6. Hong, H. A., L. H. Duc, and S. M. Cutting. 2005. The use of bacterial spore formers as probiotics. *FEMS Microbiol. Rev.* **29**: 813-835.
7. Ippoushi, K., H. Itou, K. Azuma, and H. Higashio. 2002. Effect of naturally occurring organosulfur compounds on nitric oxide production in lipopolysaccharide-activated macrophages. *Life Sci.* **71**: 411-419.
8. Jun, K. D., H. J. Kim, K. H. Lee, H. D. Paik, and J. S. Kang. 2002. Characterization of *Bacillus polyfermenticus* SCD as a probiotic. *Korean J. Microbiol. Biotechnol.* **30**: 359-366.
9. Kim, J. W., K. D. Jun, J. S. Kang, J. S. Jang, B. J. Ha, and J. H. Lee. 2005. Characterization of *Bacillus licheniformis* as a probiotic. *Korean J. Biotechnol. Bioeng.* **20**: 359-362.
10. Kim, S. S., J. H. Lee, Y. S. Ahn, J. H. Kim, and D. K. Kang. 2003. A fibrinolytic enzyme from *Bacillus amyloliquefaciens* D4-7 isolated from *chungkook-jang*; its characterization and influence of additives on thermostability. *Korean J. Microbiol. Biotechol.* **31**: 271-276.
11. Kobayashi, Y., K. Toyama, and T. Terashima. 1974. Tolerance of the multiple antibiotic resistant strains, *L. casei* PSR 3002, to artificial digestive fluids. *Jpn. J. Microbiol.* **29**: 691-697.
12. Lim, S. D., K. S. Kim, S. A. Cho, and J. R. Do. 2010. Physiological characteristics and immunomodulating activity by *Lactobacillus paracasei* subsp. *paracasei* BF146 isolated from new-born infant feces. *Korean J. Food Sci. Ani. Resour.* **30**: 223-231.
13. Lin, C. C., M. J. Lu, S. J. Chen, and S. C. Ho. 2006. Heavy fermentation impacts NO-suppressing activity of tea in LPS-activated RAW 264.7 macrophages. *Food Chem.* **98**: 483-489.
14. Rangavajhyala, N., M. Shahani, G. Sridevi, and S. Srikumaran. 1997. Nonlipopolysaccharide components of *Lactobacillus acidophilus* stimulate the production of interleukin-1a and tumor necrosis factor-a by murine macrophages. *Nutr. Cancer* **28**: 130-134.
15. Singh, N. P., M. T. McCoy, R. R. Tice, and E. L. Schneider. 1988. A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp. Cell Res.* **175**: 184-191.
16. Sreekumar, G. and K. Soundarajan. 2010. Isolation and characterization of probiotic *Bacillus subtilis* SK09 from dairy effluent. *Ind. J. Sci. Technol.* **3**: 863-866.

국문초록

면역조절능과 유전독성 억제능을 가지는 *Bacillus amyloliquefaciens* KU801. 이나경[1], 김소연[1], 장효일[2], 박은주[3], 백현동[1]*. *[1]건국대학교 동물생명과학대학 생명분자정보학센터, [2]고려대학교 생명환경과학대학, [3]경남대학교 식품영양학과*

닭분변으로부터 면역활성능이 뛰어난 KU801 균주를 분리하고 16S rRNA 서열분석을 통해 *Bacillus amyloliquefaciens* KU801로 동정하였다. *B. amyloliquefaciens* KU801의 영양세포와 아포세포는 인공위액 (pH 2.5, 1% pepsin)과 인공담즙 (0.3% oxgall)에 대한 저항성을 나타내었다. *B. amyloliquefaciens* KU801은 산화질소 (NO)의 생산을 감소시켰으나 인터루킨-1α (IL-1α)의 생산은 증가시키는 것을 확인하였다. Commet assay를 통한 유전독성능에 미치는 영향을 확인한 결과, *B. amyloliquefaciens* KU801을 첨가하였을 때 DNA 손상을 처리 농도에 비례하여 감소시키는 것을 확인하였다. 이들 결과를 토대로, *B. amyloliquefaciens* KU801은 사료용 정장제로서의 이용 가능성을 확인할 수 있었다.

http://dx.doi.org/10.4014/kjmb.1301.01008