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Antimicrobial Activity of Enterocins from *Enterococcus faecalis* SL-5 against *Propionibacterium acnes*, the Causative Agent in Acne Vulgaris, and Its Therapeutic Effect

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A lactic acid bacterial strain was isolated from human fecal specimen and identified as *Enterococcus faecalis* SL-5. The isolated strain showed antimicrobial activity against Gram-positive pathogens assayed, especially the highest activity against *Propionibacterium acnes*. The antimicrobial substance was purified and verified as a bacteriocin (named ESL5) of *E. faecalis* SL-5 by activity-staining using *P. acnes* as an indicator. N-terminal sequence of ESL5 was determined (MGAIAKLVAK) and sequence analysis revealed that it is almost identical to the some of enterocins including L50A/B of *E. faecium* L50 and MR10A/B of *E. faecalis* MRR 10-3. From the sequencing data of L50A/B structural genes, the nucleotide sequence showed 100% identity with that of the MR10A/B structural genes, implying that ESL5 is an equivalent of enterocin MR10. Meanwhile, we also tested the therapeutic effect of anti-*P. acnes* activity in patients with mild to moderate acne because of its pathogenic role to acne vulgaris. For this purpose, a concentrated powder of CBT SL-5 was prepared using cell-free culture supernatant (CFCS) of *E. faecalis* SL-5 and included in a lotion for application in the patients. The study showed that CBT SL-5 lotion significantly reduced the inflammatory lesions like pustules compared to the placebo lotion. Therefore our results indicate that the anti-*P. acnes* activity produced by *E. faecalis* SL-5 has potential role to the treatment of acne as an alternative to topical antibiotics.

***Keywords*:** acne vulgaris, antimicrobial activity, cell-free culture supernatant

Probiotic lactic acid bacteria (LAB) are microbes that benefit health of humans and animals, and have long been used in fermented foods. Therefore their safety has been proved empirically and scientifically as well (Sanders, 2003; Floch and Montrose, 2005). Their beneficial roles include modulation of immune system (Schiffrin *et al*., 1995; Kirjavainen *et al*., 1999), anti-mutagenic/anti-carcinogenic activity (Lankaputhra and Shah, 1998; Hirayama and Rafter, 1999), removal of toxins (Kankaanpää *et al.*, 2000), and treatment of constipation (Amenta *et al*., 2006; Liem *et al*., 2007). One of the most important features of LAB that can be exploited in various fields is a wide range of antimicrobial activities against pathogens such as *Escherichia coli* (Sherman *et al*., 2005), *Salmonella* (Fayol-Messaoudi *et al*., 2005), *Listeria* (Touré *et al.*, 2003), *Clostridium* (Kim *et al*., 2007), and *Helicobacter pylori* (Tsai *et al*., 2004). Intensive studies on antagonistic activity of LAB have mainly been performed with *Lactobacilli* and *Bifidobacteria* (Servin, 2004). Although the specific compounds and the mechanisms responsible for the antimicrobial activity remain largely unknown, there are a number of evidences about that activity which is mediated by several factors secreted from LAB. Some well identified factors include bacteriocin, short chain fatty acids, and reuterin (Midolo *et al*., 1995; Jin *et al*., 1996; Aroutcheva *et al*., 2001; Field *et al*., 2007).

The reason that antimicrobial activity of LAB catches the attention is deeply related with increasing concerns about the antibiotics-associated side effects, such as the emergence of antibiotics-resistant strains. To overcome the problem, there has been a strong demand for finding alternatives to antibiotics, especially in food industry and biomedical fields. LAB have been proposed to replace the position of antibiotics because a great number of scientific reports have been accumulated, which indicate powerful and efficient potentials to overcome the antibiotics-associated side effects (Field *et al*., 2007).

Propionibacterium acnes is a Gram-positive, anaerobic, pleomorphic rod-shaped bacteria. Being found as the normal flora on human skin, it is generally known to be a key factor in the pathogenesis of acne vulgaris. The notion is based on the clinical improvement seen following a reduction in the bacterial population density with antibiotic therapy and the finding that a lack of clinical response was associated with the presence of antibiotic-resistant propionibacteria (Eady *et al*., 1989; Thiboutot, 1997). Acne vulgaris is

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a chronic inflammatory dermatosis characterized by non-inflammatory lesions such as open and closed comedones and inflammatory lesions such as papules, pustules, and nodules. Topical therapies such as retinoids, benzoyl peroxide, and antibiotics are generally employed for the treatment of acne. However, treatment with antibiotics led to development of resistant strains of *P. acnes* (Eady *et al*., 1989; Nord and Oprica, 2006). As an alternative to antibiotics for the treatment of acne, cationic antimicrobial peptides have been studied intensively because they have a broad antimicrobial spectrum, and are active against antibiotic resistant pathogens including *P. acnes* (Marta *et al*., 2006).

Although LAB have antimicrobial activity against a wide range of pathogens, the activity against *P. acnes* has been rarely reported. A recent study showed *Lactococcus* sp. HY 449 exerts anti-*P. acnes* activity by the action of bacteriocin (Oh *et al*., 2006). In this study, we isolated a LAB strain, *Enterococcus faecalis* SL-5, from human fecal specimen, and found that it produces bacteriocin(s) having antimicrobial activity against *P. acnes*. To test applicability of the antimicrobial activity against *P. acnes*, we prepared a concentrated powder from cell-free culture supernatant (CFCS) of *E. faecalis* SL-5 and examined its therapeutic effect in the patients with lesions of acne.

Materials and Methods

Bacteria and growth media

E. faecalis SL-5 used in this study was isolated as a lactic acid bacterial strain from fecal specimen of a healthy Korean adult.

E. faecalis SL-5 was grown in MRS broth or in optimized medium (glucose, 3%; soy peptone, 1%; casein peptone, 3%; beef extract, 1%; yeast extract, 1%; L-cysteine-HCl, 0.05%; K_2HPO_4, sodium acetate, 0.05%; diammonium citrate, 0.05%; $MgSO_4$, 0.01%; $MnSO_4$, 0.005%; $CaCl_2$, 0.05%; Tween 80, 0.05%) at 37°C in anaerobic condition. *P. acnes* ATCC 29399 was cultivated in reinforced clostridial medium (RCM) broth (Merck, Germany) at 37°C. *Bacillus cereus* KCTC 3624, *Bacillus subtilis* KFRI 179, *Escherichia coli* O157, *Listeria monocytogenes*, *Salmonella paratyphi* A, *Shigella flexneri*, and *Staphylococcus aureus* KCTC 1972 were cultivated in brain heart infusion (BHI) broth (Becton Dickinson, USA) at 37°C.

Species identification

Fermentation profile of the isolated strain was characterized according to the Bergey's manual of determinative bacteriology using API 20STREP kit (bioMérieux, France). The complete sequence (1,570 bp) of the 16S rDNA gene was amplified by PCR, and determined. The sequence was deposited in the GenBank database and assigned the accession number AY692453.

The 16S rDNA gene sequence was analyzed by a Basic Alignment Search Tool (BLAST) search against the National Center for Biotechnology Information (NCBI) database. The sequences of related species obtained were used to construct the phylogenetic tree by the neighbor-joining method in the MEGA4 package.

Antimicrobial activity test

Antimicrobial activity of *E. faecalis* SL-5 was tested by spot-on-the-lawn method (Kim *et al.*, 2000) using CFCS of the strain. To prepare the CFCS, *E. faecalis* SL-5 was grown overnight in MRS broth at 37°C, cells were removed by centrifugation at 9,940×g for 15 min, and then the resultant supernatant was passed through a cellulose acetate filter (0.45 μm; Advantech MFS, USA). MRS agar plates were overlaid with 0.7% soft agar of RCM or BHI inoculated with 0.5% of the actively growing test strains, allowed to be dried for 30 min, and subsequently spotted with 10 μl of the CFCS. The plates were incubated at 37°C for 24 h in an anaerobic condition. Bacteriocin activity was quantified using two fold serial dilutions of the CFCS in anaerobic solution [42.3 mM Na_2HPO_4, 33.1 mM KH_2PO_4, 0.05% (v/v) Tween 80, 3.2 mM L-cystein·HCl, pH 7.0]. The antimicrobial activity expressed as Arbitrary Units (AU) was determined with the highest twofold dilution that showed a clear inhibitory zone on the MRS agar plate.

Partial purification of bacteriocin-like activity

All the purification steps were carried out at room temperature. The antimicrobial activity was purified from a 2-L culture of *E. faecalis* SL-5 grown in the optimized medium described above. After the culture was cultivated at 37°C for 8 h, cells were removed by centrifugation (9,940×g/15 min/4°C) and subsequent passage through a cellulose acetate filter (0.45 μm). Ammonium sulfate was added to the supernatant to achieve 70% of saturation. Following equilibration the precipitate was collected by centrifugation (20,000×g/30 min/4°C). The obtained pellet was dissolved in Tris-HCl buffer (50 mM, pH 7.0), resulting in a 70 ml of solution which was subsequently dialyzed (cut-off of 500 Da) against the same buffer with buffer exchange for a total of three changes over 48 h period. The desalted sample was five-fold concentrated by ultrafiltration (cut-off of 3 kDa). Eight milliliters of the concentrate (7 mg/ml) was applied to a 64 ml (3 ml×9 ml) CM Sepharose™ Fast Flow column (GE Healthcare, Sweden) pre-equilibrated with Tris-HCl buffer (50 mM, pH 7.0), and eluted with a NaCl gradient (0 M∼1 M) at flow rate of 1 ml/min. Three-milliliter fractions were collected in a fraction collector (BioLogic LP, BIO-RAD, USA) and the bacteriocin-like activity was eluted with 0.5 M NaCl in the same buffer.

Protein concentration was determined according to the method of Bradford with bovine serum albumin (BSA) as a standard (Bradford, 1976). The antimicrobial activity in each fraction was assayed by spot-on-the-lawn method, as described above.

Activity staining

Tricine-SDS-PAGE was performed according to the method of Schägger (2006) using 16% gels. Following CM Sepharose™ Fast Flow column, each 800 ng of protein from the fraction showing the highest activity, along with 10 μl of SeeBlue Plus2 (Invitrogen, USA) as protein standard, was loaded onto two consecutive lanes and analyzed on the duplicated gels. Each of the gels was either stained with Coomassie blue R-250 or placed on RCM agar plate overlaid with RCM soft agar containing 0.5% *P. acnes*, which then incu-

bated at 37℃ for 24 h.

Determination of N-terminal sequence

Two micrograms of protein from the fraction described above were electrophoresed on 16% Tricine-SDS-PAGE gels. The gels were either stained with Coomassie blue R-250 or electroblotted onto PVDF membranes (0.22 μm). The portions of the membrane carrying the bands which showed bacteriocin-like activity were cut out and subjected to N-terminal sequencing.

N-terminal sequencing was conducted by Edman degradation on a liquid-phase automatic protein sequence analyzer (Procise 491HT, Applied Biosystems, USA) at the Korea Basic Science Institute in Seoul.

Mode of action

P. acnes was inoculated into RCM broth at the final concentration of 3.5×10^6 CFU/ml, followed by the addition of partially purified bacteriocin to the culture at various concentrations ranging from 0 to 800 AU/ml. Subsequently, the cultures were maintained at 37℃ for 3 h. An equal volume of aliquots from the cultures was taken and plated on RCM agar to determine viable cell count.

Preparation of a concentrated powder from the CFCS

The CFCS was prepared from 2-L culture, as described above, and concentrated by ultrafiltration using a membrane with molecular weight cut-off of 3 kDa. The retentate after filtration was mixed with maltodextrin to a final concentration of 10% (w/v). The mixture was tyndalized at 105℃ for 10 min followed by 90℃ for 10 min and subsequently subjected to lyophilization to make a concentrated powder named CBT SL-5.

Effect of CBT SL-5 on cytotoxicity

Cytotoxicity of CBT SL-5 was evaluated by assaying mitochondrial metabolic activity according to the method of Carmichael *et al*. (1987) with minor modifications. Briefly, HaCat cells (human keratinocyte cell line) were cultured in Dulbecco's Modified Eagle's Medium (DMEM; WelGene Inc., Korea) supplemented with 10% fetal bovine serum (HyClone, USA) under the controlled atmosphere (5% CO_2, 100% humidity) at 37℃. The cells (5×10^4 CFU/ml) were inoculated onto a 24-well micro-plate. The micro-plate was then incubated at 37℃ for 24 h. CBT SL-5 was dissolved in 50 mM Tris-HCl buffer (pH 6.8) at various concentrations and filter-sterilized. The bacteriocin solutions were applied to the wells, which were then incubated at 37℃ for 24 h. MTT reagent [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide; Sigma, USA] (0.5 mg/ml) was added to each well, which was incubated at 37℃ for 4 h. The absorbance was measured at 540 nm by using Biotrack II microplate reader (Biochrom Ltd., UK). Control was treated with buffer only.

Patients and the study design

A 8-week, double-blind, randomized, placebo-controlled, phase III study was conducted under identical protocols to evaluate the therapeutic effect and safety of *E. faecalis* SL-5 for the treatment of mild to moderate acne. The study was performed from September 2006 to January 2007. A total of 70 patients were recruited to the department of dermatology, Kangnam St. Mary's hospital, college of medicine, the Catholic University of Korea. Those patients 12 years of age or older with a clinical diagnosis of acne vulgaris involving the face were enrolled in this study. The patients should have 5 to 50 inflammatory lesions (defined to include papules or pustules) and 5 to 100 noninflamatory lesions (comedones) above the mandibular line at the study's baseline. Individuals with severe cystic acne, acne conglobata or 10 or more active or developing nodules were excluded from participation.

Table 1. Composition of the CBT SL-5 lotion

Ingredients	Composition (%)
Water	86.62
Butylene glycol	4.00
Pentylene glycol	3.00
PEG-60 hydrogenated castor oil	0.50
Xanthan gum	0.20
Melaleuca alternifolia (tea tree) leaf oil	0.10
Methylparaben	0.25
Phenoxyethanol	0.20
Allantoin	0.10
Disodium EDTA	0.03
CBT SL-5	6,400 AU
Total	100

The eligible patients were randomly assigned to either the CBT SL-5 group or the placebo group. The composition of CBT SL-5 lotion is listed in Table 1. The composition of the two treatment lotions was identical except that water was added to the placebo lotion instead of CBT SL-5. CBT SL-5 has been registered as LACTOPAD on the International Nomenclature Cosmetic Ingredient (INCI) list and registered as a raw material for cosmetics by the US CTFA (Cosmetics, Toiletry, and Fragrance Association) (CTFA file No. 9082). The patients were instructed to apply a thin layer of CBT SL-5 lotion or placebo lotion twice a day to the acne-involved areas of the face. After washing with a cleanser, each study lotion was applied once in the morning and again at least 1 h before bedtime to the entire affected area and it was rubbed in until it completely disappeared. The patients were examined at baseline and at the end of week 2, 4, and 8 in the treatment. At each visit, two special dermatologists independently counted the number of noninflammatory and inflammatory acne lesions present. The therapeutic effect of the treatment was evaluated by the percentage of reduction of the acne lesions at baseline and at each visit in the CBT SL-5 group, compared with the placebo group.

Adverse events, local signs and symptoms (adverse reactions of facial oiliness, peeling, dryness, and erythema), and the physical examination findings were monitored throughout the study. The patients were specifically queried at each visit.

The data are presented as means with 95% confidence intervals (CIs) or medians with an interquartile range. The λ^2-test was used to evaluate the final efficacy of the treatment between the CBT SL-5 group and the placebo group.

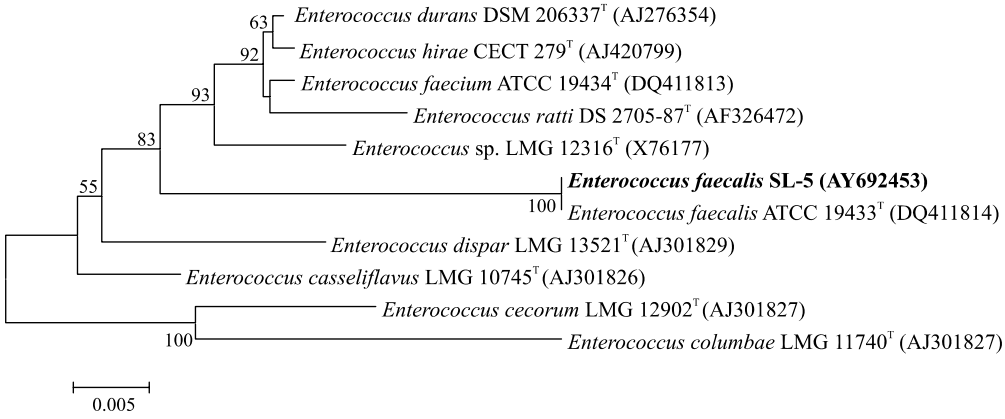

Fig. 1. Phylogenetic relationship between *E. faecalis* SL-5 and its related species based on 16S rRNA gene sequence analysis. The phylogenetic tree was generated by the neighbor-joining method in the MEGA4 package. The numbers at the nodes are the confidence levels expressed as percentages of occurrence in 1,000 bootstrap replicates of the original sequence data. The GenBank accession no. are shown in parentheses. Bootstrap values greater than 50% are shown at the branch points. Bar, 0.005 accumulated changes per nucleotide.

P-values less than 0.05 were considered significant.

Results and Discussion

Identification of the isolate

The isolated strain was taxonomically identified as *E. faecalis* based on Gram-staining, API kits, and the relevant phylogenetic relationship via the Neighbor-joining method (Fig. 1).

On the other hand, due to concern about vancomycin-resistant enterococci (VRE), we examined whether *E. faecalis* SL-5 has antibiotic resistance to vancomycin. In the experiments, we observed that no colonies were formed on plates containing vancomycin at concentration of 2.5 μg/ml and PCR amplification with primers specific to *van*A or *van*B gene using total DNA as template failed to detect the genes (data not shown). From these results, we concluded that *E. faecalis* SL-5 is sensitive to vancomycin.

Antimicrobial spectrum of the isolate

E. faecalis SL-5 was found to have antimicrobial activity against some positive pathogens including *P. acnes* (Table 2). *B. cereus* KCTC 3624, *B. subtilis* KFRI 179, *L. monocytogenes*, *P. acnes* ATCC 29399, and *S. aureus* KCTC 1927 were inhibited by the antimicrobial activity but *E. coli* O157 and *S. flexneri* were not susceptible to it. *P. acnes* ATCC 29399 showed the highest sensitivity to the activity among the four susceptible strains (Fig. 2). Gram-negative strains were not inhibited by the antimicrobial activity from *E. faecalis* SL-5. This indicates that bacteriocin is likely to be the substance responsible for inhibition of target strains. The reason is because LAB bacteriocins display activity towards closely related Gram-positive bacteria in general. In addi-

Table 2. Antimicrobial spectrum of *E. faecalis* SL-5

Bacteria	Inhibition
Bacillus cereus KCTC 3624	+
Bacillus subtilis KFRI 179	+
Escherichia coli O157	-
Listeria monocytogenes	+
Propionibacterium acnes ATCC 29399	+
Salmonella paratyphi A	-
Shigella flexneri	-
Staphylococcus aureus KCTC 1927	+

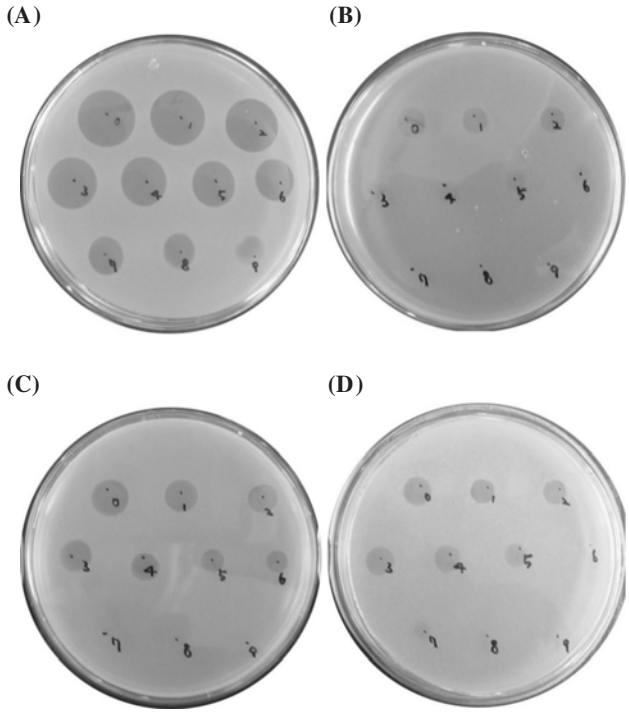

Fig. 2. Antimicrobial activity of *E. faecalis* SL-5 against pathogens. The antimicrobial activity was assayed using CFCS of *E. faecalis* SL-5 by spot-on-the-lawn method, as described in 'Materials and Methods'. *P. acnes* ATCC 29399 (A), *B. subtilis* KFRI 179 (B), *B. cereus* KCTC 3624 (C), and *S. aureus* KCTC 1927 (D) were used as the test strain.

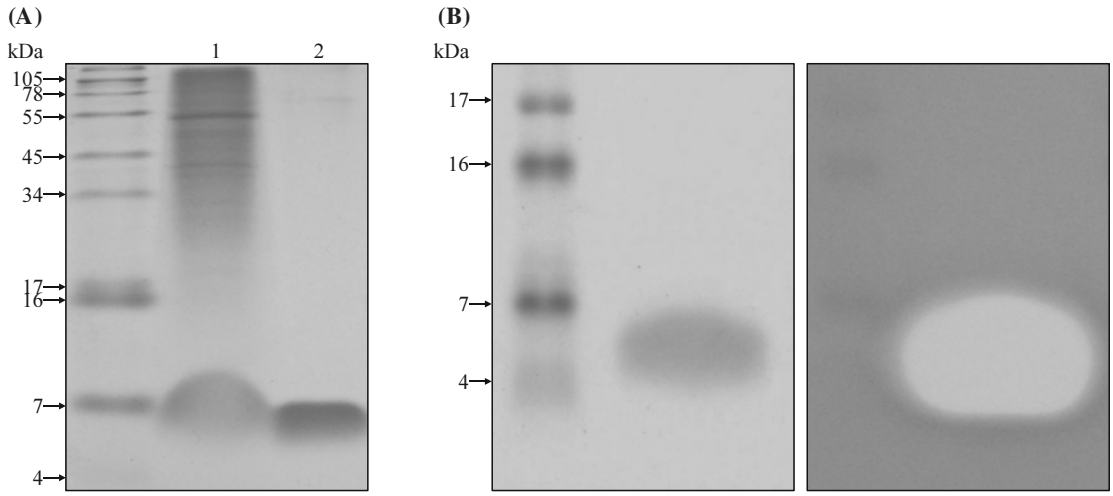

Fig. 3. Tricine-SDS-PAGE of partially purified enterocin from the culture supernatant and detection of antimicrobial activity. (A) The concentrate (lane 1) loaded on the CM Sepharose column and partially purified bacteriocin (lane 2) from the column were analyzed on 16% Tricine-SDS-PAGE gel which was then stained with Coomassie blue R-250. Ten and 0.8 micrograms of protein were loaded on the gel, respectively. (B) Partially purified bacteriocin (right lane) was analyzed on 16% Tricine-SDS-PAGE gels, along with protein standards (left lane, sizes indicated on the left). The gel was stained with Coomassie blue R-250 (left). One of the duplicate gels was placed on RCM agar plate and overlaid with RCM soft agar containing *P. acnes* (right).

tion, a narrow spectrum of activity may be due to interaction with a specific receptor on the cell membrane of target strain (Van Belkum *et al*., 1991). However, we do not rule out the possibility that bacteriocin of *E. faecalis* SL-5 might have activity against Gram-negative bacteria other than the strains used in this study since only three pathogenic Gram-negative bacteria were employed for the assay.

Identification of the antimicrobial substance

To identify the substance responsible for antimicrobial activity *E. faecalis* SL-5, the culture supernatant was fractionated by ultrafiltration, ammonium sulfate precipitation, and CM Sepharose column. The highest anti-*P. acnes* activity was eluted with 0.5 M NaCl in CM Sepharose column (data not shown). When an aliquot of the fraction with the highest activity was analyzed on 16% Tricine-SDS-PAGE, a strong band having molecular weight of about 5 kDa was observed (Fig. 3A). The purified protein was verified to be the bacteriocin of *E. faecalis* SL-5 by activity-staining of one of the duplicate gels using *P. acnes* as an indicator (Fig. 3B). Thus, we named the bacteriocin ESL5.

The bands showing anti-*P. acnes* activity were electroblotted onto PVDF membrane, cut out, and subjected to protein sequence analyzer, as described in 'Materials and Methods'. The first 10 amino acids at the N-terminus were determined and the sequence is as follows: Met-Gly-Ala-Ile-Ala-Lys-Leu-Val-Ala-Lys. The obtained sequence was compared with protein sequences held in the NCBI protein database by a BLAST. The result showed that the obtained N-terminal sequence has 100% identity with that of some enterocins such as enterocins MR10A/B (Martín-Platero *et al*., 2006) and enterocin L50A (Cintas *et al*., 1998) and 90% identity with that of L50B (data not shown).

Mode of action

To investigate the action mode of ESL5 against *P. acnes*, partially purified bacteriocin was added to the culture of test strain at various concentrations. The viable cell count of the test strain was determined at specific times after addition of the bacteriocin. As shown in Fig. 4, addition of ESL5 resulted in large decrease in the number of viable *P. acnes* cells compared to that of control. Over 99% cells in the culture were killed in a concentration-dependent manner within 1 h after the addition of bacteriocin. The result indicates that the mode of action of ESL5 against *P. acnes* is bacteriocidal.

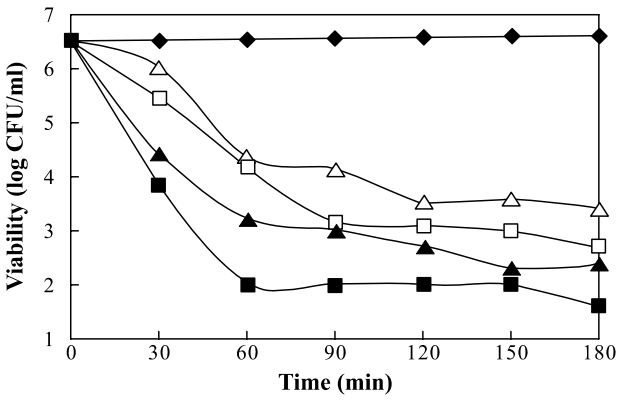

Fig. 4. Inhibitory mode of bacteriocin produced by *E. faecalis* SL-5. The survival rate of *P. acnes* was determined by adding bacteriocin at the final concentration of 100 AU/ml (△), 200 AU/ml (□), 400 AU/ml (▲), or 800 AU/ml (■) to growing cultures. The viable cell counts were determined every 30 min. For control, no bacteriocin was added (◆).

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  1 ATGGGAGCAATCGCAAAATTAGTAGCAAAGTTTGGATGGCCAATTGTTAAAAAGTATTAC   60
    M  G  A  I  A  K  L  V  A  K  F  G  W  P  I  V  K  K  Y  Y

 61 AAACAAATTATGCAATTTATTGGAGAAGGATGGGCAATTAACAAAATTATTGATTGGATC  120
    K  Q  I  M  Q  F  I  G  E  G  W  A  I  N  K  I  I  D  W  I

121 AAAAAACATATTTAAAAATAAGGATGTGTTAATGTATGGGAGCAATCGCAAAATTAGTAG  180
    K  K  H  I  *                    M  G  A  I  A  K  L  V  A

181 CAAAGTTTGGATGGCCATTTATTAAAAAATTCTACAAACAAATTATGCAGTTTATCGGAC  240
      K  F  G  W  P  F  I  K  K  F  Y  K  Q  I  M  Q  F  I  G  Q

241 AAGGATGGACAATAGATCAAATTGAAAAATGGCTAAAAAGACATTAA               287
      G  W  T  I  D  Q  I  E  K  W  L  K  R  H  *
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Fig. 5. Nucleotide sequence of the amplified 287 bp PCR product and deduced protein sequences. Primer sequences are underlined.

Characterization of the structural gene

To identify the structural gene for ESL5, PCR amplification was performed using primers based on the structural genes for L50A and L50B (Cintas *et al.*, 1998) with total genomic DNA as template. From the PCR reaction, 287-bp PCR product was amplified and sequenced. The nucleotide sequence of the PCR product showed 100% identity with that of the structural genes for enterocin MR10A and MR10B. Thus, it contains two structural genes encoding equivalents of MR10A and MR10B, respectively (Fig. 5). The molecular weight of the enterocins, ESL5A and ESL5B, of *E. faecalis* SL-5 based on the deduced amino acid sequences were calculated to be 5176.36 Da and 5182.29 Da, respectively. Because enterocin MR10A and MR10B were co-purified with each other (Martín-Platero *et al.*, 2006) and the first 14 residues of the deduced amino acid sequences of the ESL5 structural genes are identical to each other, it is very likely that the purified bacteriocin, described above, was a mixture of ESL5A and ESL5B.

The optimal condition for production of the antimicrobial activity

To find the optimal condition for production of ESL5, we optimized the medium for *E. faecalis* SL-5, as described in 'Materials and Methods', where the highest cell growth occurred (data not shown). Next, we determined the optimal pH and cultivation time where the highest antimicrobial activity against *P. acnes* was achieved (Fig. 6). The optimal condition for production of the highest antimicrobial activity was observed when *E. faecalis* SL-5 was cultivated for 8 h at pH 6 in the optimized medium.

Table 3. Concentration of antimicrobial activity of *E. faecalis* SL-5

Concentration step	Activity (AU/ml)	Concentration fold
Culture	25,600	1
Ultrafiltration	819,200	32
Mixture with maltodextrin	409,600	16
Tyndalization	409,600	16
Lyophilization	3,276,800 (AU/g)	128

Production of CBT SL-5 and its physicochemical properties

It is well known that bioactive peptides generated by the action of peptidases of LAB can contribute to antimicrobial activity or/and immunomodulatory activity (Meisel and Bockelmann, 1999). Thus culture supernatant of LAB contains these additional traits, which implies that CFCS of *E. faecalis* SL-5 might be more effective in treating acne to the patients. Based on the idea, we decided to concentrate various activities including anti-*P. acnes* activity in culture supernatant of *E. faecalis* SL-5 for wide applications.

A concentrated powder was prepared using CFCS of *E. faecalis* SL-5. As shown in Table 3, CFCS of *E. faecalis* SL-5 was processed through ultrafiltration, tyndalization, and lyophilization, producing highly concentrated powder named CBT SL-5. It should be noted that any live cells of *E. faecalis* SL-5 were completely removed by centrifugation and filtration, as described in 'Materials and Methods', to make CBT SL-5 free of live bacteria.

Physicochemical properties of CBT SL-5 were examined under various conditions. The anti-*P. acnes* activity in CBT SL-5 remained stable with no loss in the activity over a wide range of temperature tested (Table 4). We also examined stability of the powder in the pH range 2 to 12 and the anti-*P. acnes* activity in CBT SL-5 remained quite stable over the pH range for as long as 90 days (data not shown).

Therapeutic effect of CBT SL-5

After the Institutional Review Board approval, we evaluated the therapeutic effect of CBT SL-5 in the patients suffering from mild to moderate acne using a lotion including CBT SL-5. A total of seventy patients were enrolled in this study and they were randomly assigned to either the CBT SL-5 group (n=37) or the placebo group (n=33). A total of 9

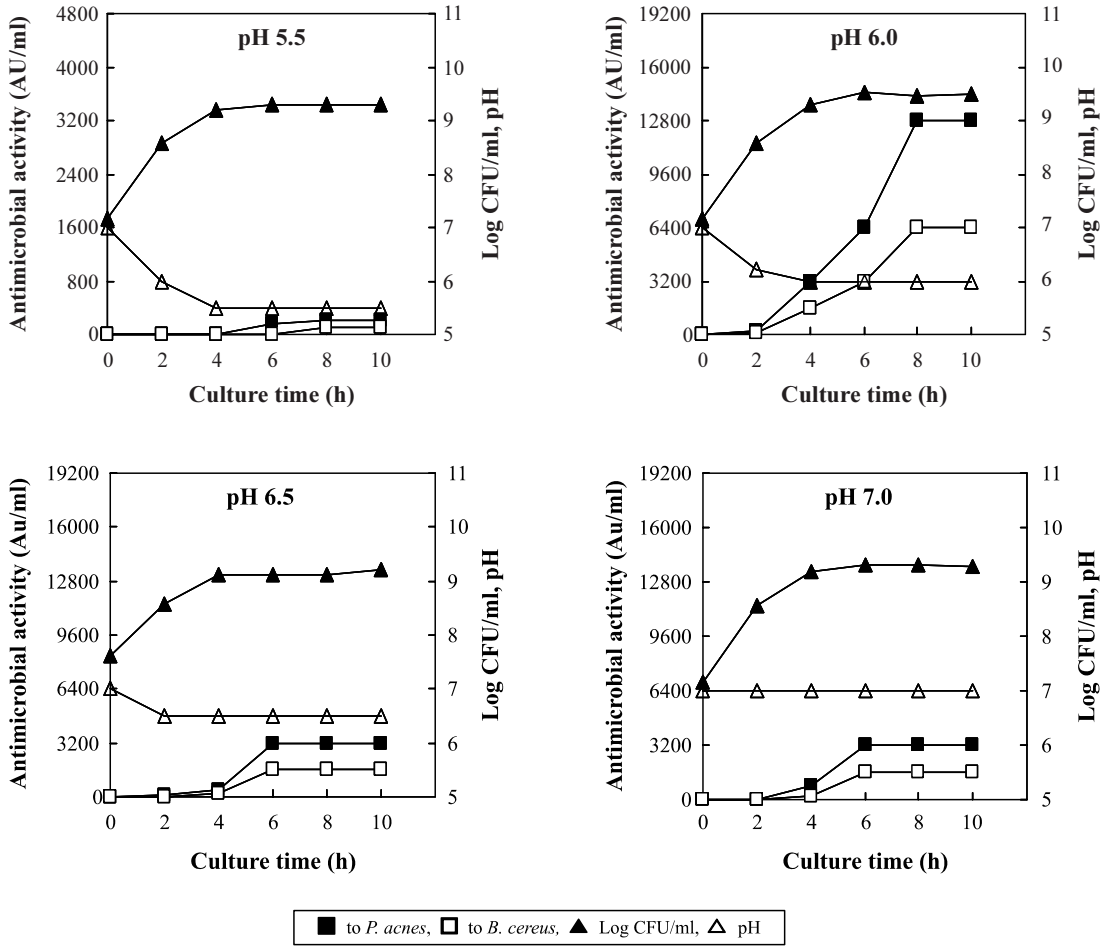

Fig. 6. Optimization of production of the antimicrobial activity. *E. faecalis* SL-5 was anaerobically cultivated in the optimized media, as in Materials and Methods, at 37°C with gentle agitation (150 rpm) using a pH-controlled fermenter. In the course of cultivation, the antimicrobial activity against *P. acnes* (■) and *B. cereus* (□), viable cell counts (▲), and pH (△) were determined every 2 h.

Table 4. Stability of anti-*P. acnes* activity in CBT SL-5 at different temperatures

Day	Storage temp. (°C)	Antimicrobial activity (AU/ml)
0	4	12,800
	25	12,800
	40	12,800
7	4	12,800
	25	12,800
	40	12,800
14	4	12,800
	25	12,800
	40	12,800
21	4	12,800
	25	12,800
	40	12,800
28	4	12,800
	25	12,800
	40	12,800

patients from this study discontinued treatment prematurely: 8.1% (3/37) of the CBT SL-5 group and 18.2% (6/33) of the placebo group. The vast majority of premature study discontinuations (88.9%, 8/9) were for administrative reasons, including loss to follow up and treatment noncompliance. Only one (11.1%, 1/9) patient treated with placebo lotion discontinued because of a lack of efficacy, aggravation of acne and an adverse event (skin irritation).

In the study, the number of lesions in the patients was counted periodically in the course of treatment. For non-inflammatory lesions such as comedones, the percentage of remaining lesions decreased in the CBT SL-5 group, whereas there were few changes in the placebo group. However, the difference between the CBT SL-5 and placebo groups was not statistically significant (Fig. 7A).

When the effect of CBT SL-5 on inflammatory lesions such as pustules was examined, a statistically significant reduction was observed in the CBT SL-5 group compared with the placebo group (Fig. 7B, $P<0.05$). The result indicates that topically administered CBT SL-5 may function in the treatment of acne by killing the *P. acnes* and in turn reducing

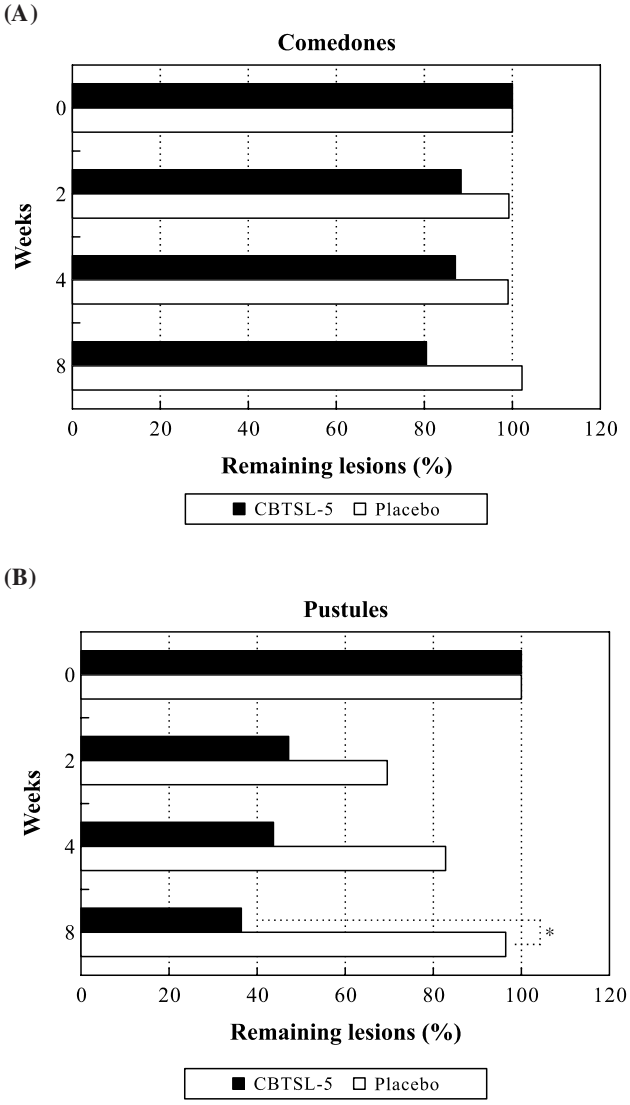

Fig. 7. The therapeutic effect of CBT SL-5 on (A) the non-inflammatory lesions (comedones) and (B) the inflammatory lesions (pustules). The number of lesions in the patients was counted over the period. $^{*}P<0.05$

the production of inflammatory mediators that are synthesized and released form the pathogen. Another possible mechanism might be direct suppression of inflammation.

Safety

We evaluated cytotoxicity of CBT SL-5 using HaCat cells, human keratinocyte cell line. When HaCat cells were treated with increasing amount of the CBT SL-5 up to 800 AU/ml, only 10% higher inhibition was observed in the cell treated with 800 AU/ml of the CBT SL-5 compared to control (treated with buffer) (Fig. 8). This result indicates that the CBT SL-5 may not have adverse effects when used for human application.

Safety of CBT SL-5 lotion was also evaluated in the course of the clinical test. Adverse events were monitored

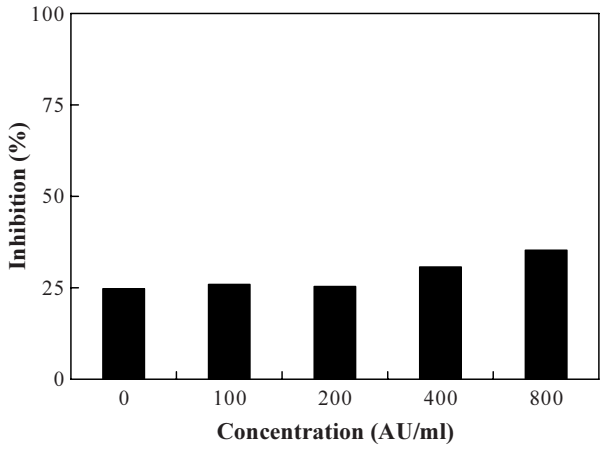

Fig. 8. Cytotoxicity of CBT SL-5 on HaCat cells (human keratinocyte cell line). CBT SL-5 was dissolved in 50 mM Tris-HCl buffer (pH 6.8) to make solutions containing 100, 200, 400, or 800 AU/ml. Proliferation of HaCat cells was monitored by MTT assay described in 'Materials and Methods'. For control, 50 mM Tris-HCl buffer (pH 6.8) without CBT SL-5 was treated. All the experiments were performed at three replicates and average values were shown without error bars.

by the investigators at each visit. Patients were asked at each visit about local signs and symptoms, including unpleasant odor, skin dryness, erythema, and other irritation such as stinging, sensitivity to touch, and peeling on the application-site. There was no serious or significant adverse events noted during this study. Only one patient of the placebo group discontinued because of lack of efficacy, aggravation of acne and skin irritation. Two patients of the CBT SL-5 group experienced mild and temporary irritation such as stinging and erythema on the application site. Mild unpleasant odor and skin dryness were reported by several patients in both treatment groups. All of the events were of mild intensity and they were resolved during treatment, and they did not result in treatment discontinuation. The CBT SL-5 lotion was well tolerated by most patients.

Taken together, our results indicate that anti-*P. acnes* activity of *E. faecalis* SL-5 has potential role to the treatment of acne as an alternative to topical antibiotics.

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