

Note

**Identification of 3-Phenyllactic Acid As a Possible
Antibacterial Substance Produced
by *Enterococcus faecalis* TH10**

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***Enterococcus faecalis* TH10 is a lactic acid bacterial strain isolated from the Malaysian traditional fermented food, “tempeh”, and has antibacterial activity against various pathogens. To identify the antibacterial substance, the butanol extract of the culture supernatant of *E. faecalis* TH10 was fractionated by HPLC equipped with a reversed-phase partition column, and the elutes were subjected to antibacterial assay. As the activity was observed in a fraction eluted by ca 80% methanol, the fraction was analyzed by gas chromatography/mass spectrometry and 3-phenyllactic acid was identified as the major compound. Fractionation with an optical isomer separation column showed that the preparation contained D- and L-forms of 3-phenyllactic acid at a ratio of 2:1. Authentic 3-phenyllactic acid showed antibacterial activity against various bacteria such as *Staphylococcus aureus* and *Escherichia coli*. These results suggest the possibility that 3-phenyllactic acid is a biopreservative.**

Key words : *Enterococcus faecalis*/Antibacterial substance/Phenyllactic acid.

Various microorganisms coexist with other organisms in the natural environment in antagonistic or symbiotic relationships. Lactic acid bacteria produce many substances which work antagonistically, such as organic acids (e.g., lactic, acetic, and propionic acids), diacetyl, acetaldehyde, 3-hydroxypropion aldehyde, or H₂O₂, and the ability of these substances is thought to be useful for the biopreservation of foods (Earnshaw, 1992) or probiotics (Dunne et al., 1999; Havenaar and Huis In't Veld, 1992; Heller, 2001). Extensive studies have been conducted on bacteriocins, antibacterial substances composed of

peptides produced by lactic acid bacteria (Malik et al., 1994), among which “nisin” has been generally recognized throughout the world to be a safe food preservative (Earnshaw, 1992).

We have isolated *Enterococcus faecalis* TH10, a lactic acid bacterial strain with antibacterial ability, from the Malaysian traditional fermented soybean food, “tempeh”, in which there is a complex bacterial flora (Ohhira et al., 1996). The culture filtrate of *E. faecalis* TH10 had a growth inhibitory effect against various bacteria including methicillin resistant *Staphylococcus aureus* (MRSA) (Ohhira et al., 1996). In this study, the antibacterial substance produced by *E. faecalis* TH10 was surveyed and 3-phenyllactate was identified.

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To obtain the antibacterial substance, *E. faecalis* TH10 was cultivated in MRS broth (Oxoid) at 37°C for 16 hours. Fifty ml of the culture were centrifuged at 4,000 × g and the supernatant was extracted by the same volume of butanol. The water-saturated butanol layer was mixed with the same volume of ether, and the resulting organic solvent layer was dehydrated with Na₂SO₄ and concentrated to 2 ml under vacuum conditions. The concentrated fraction was applied to a reversed-phase partition octyl-bonded polyvinyl alcohol gel column, Shodex Asahipack C8P-50 (250 × 4.6 mm, Showa Denko), at a flow rate of 0.5 ml/minute at room temperature. Twenty-five minutes of a linear gradient elution from 50 to 100% methanol and an additional 5 min elution with 100% methanol were carried out. The absorbance of the column effluent was monitored at 254 nm. The elution was divided into 10 fractions, (a) to (j), each fraction was evaporated in a vacuum centrifuge, and the residue was re-suspended in the original volume of water and subjected to the antibacterial test. Of the 10 fractions, only fraction (e) (a fraction composed of a single peak and eluted with approximately 80% methanol) showed the growth inhibitory effect against MRSA. Namely, when *S. aureus* FSA-1 was cultivated in a mixture of the same volume of each fraction and MRS broth (double strength) at 37°C overnight, adequate growth was observed except with fraction (e) (data not shown).

As mentioned above, the production of bacteriocins composed of peptides by lactic acid bacteria

has been documented by various authors. When fraction (e) was treated with proteinase K, V8 protease, trypsin or achromopeptidase, no reduction of the growth inhibitory effect was observed, even if enough units of the proteases were used to digest the amount of the preparation assumed to be peptide. This suggests the existence of an antibacterial substance other than bacteriocins.

To identify the antibacterial substance(s), gas chromatography/mass spectrometry (GC/MS) was carried out. GC was conducted using an HP6890 series gas chromatograph (Hewlett-Packard, Inc.) with a computer-controlled M-Station mass spectrometer (JEOL Co., Ltd.). An SPB-Octyl column (60 m × 0.25 mm i.d, SUPELCO, Inc.) was used for the separation, with pentafluoroalkane as a mass standard. The chromatography was performed by raising temperatures from 60 to 250°C at 30°C/min, then from 250 to 280°C at 3°C/min. Temperatures for the injector, separator, and ion source were 230, 270, and 280°C, respectively. Ionizing energy was 70 eV. The sample was methylated by heating it at 120°C for 1 h with HCl-methanol (5:95) in a sealed tube and applied to GC. When the non-methylated fraction (e) was applied to GC, no peak was observed (Fig.1A), whereas an obvious peak with some minor peaks was observed in the chromatogram of the methylated sample (Fig.1B). Referring to the chromatogram of the authentic standards (Sigma-Aldrich Chem: Fig. 1C-E), the major peak of Fig.1B was thought to be that of 3-phenyllactate. As shown in Fig.2, MS spec-

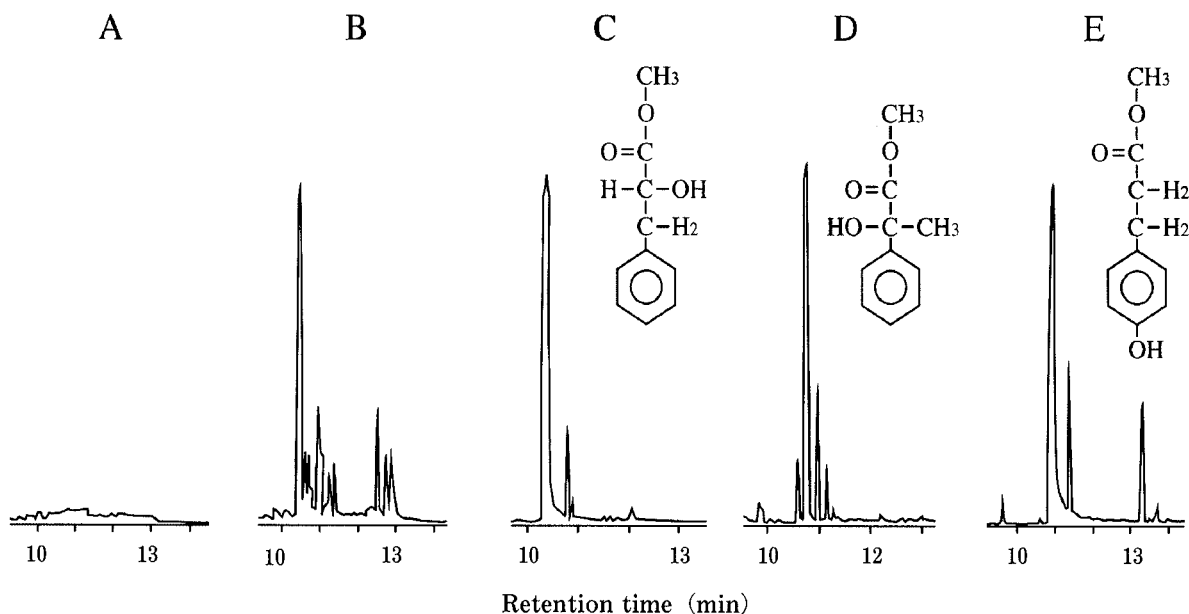


FIG. 1. Total ion chromatograms of the fraction (e) having antibacterial activity and reference compounds on GC/MS. A, non-methylated fraction (e); B, methylated fraction (e); C, methylated phenyllactic acid; D, methylated atrolactic acid; E, methylated 3-(4-hydroxyphenyl) propionic acid.

trums of the methylated sample and 3-phenyllactate methyl-ester well agreed, confirming the above result. The antibacterial activity of authentic 3-phenyllactate was then tested by the disc method.

When fraction (e) was applied to an optical isomer separation column, Nucleosil Chiral-1 (Macherey-Nagel, Germany), D- and L-phenyllactic acids were obtained at a ratio of 2:1. Therefore, a mixture of D- and L-phenyllactic acids at this ratio was subjected to the test. D- and L-phenyllactic acids were purchased from Sigma-Aldrich Chem. Co. and Tokyo Kasei Kogyo Co., Ltd., respectively. The antimicrobial disc susceptibility test (National Committee for Clinical Laboratory Standards 1993) was used for the assay.

Briefly, a paper disc (8 mm diameter) loaded with 60 μ l of the sample solution was placed on a nutrient agar plate or Luria Bertani agar plate on which test organisms were inoculated. After overnight cultivation at 37°C, the diameter of the clear zone around the disc formed by the growth inhibitory effect was measured. Samples were adjusted to pH 6.8, 6.0 and 5.5. In addition to *S. aureus* FSA-1, *Bacillus cereus* JCM 2152, *Escherichia coli* FEC-83 (O157:H7), *Yersinia enterocolitica* JCM 1677, *Salmonella enterica* serovar Choleraesuis JCM 6977 and *Clostridium perfringens* JCM 1290 were used as test organisms. As shown in Table 1, the growth inhibitory effect of phenyllactic acid was observed against all test organisms except

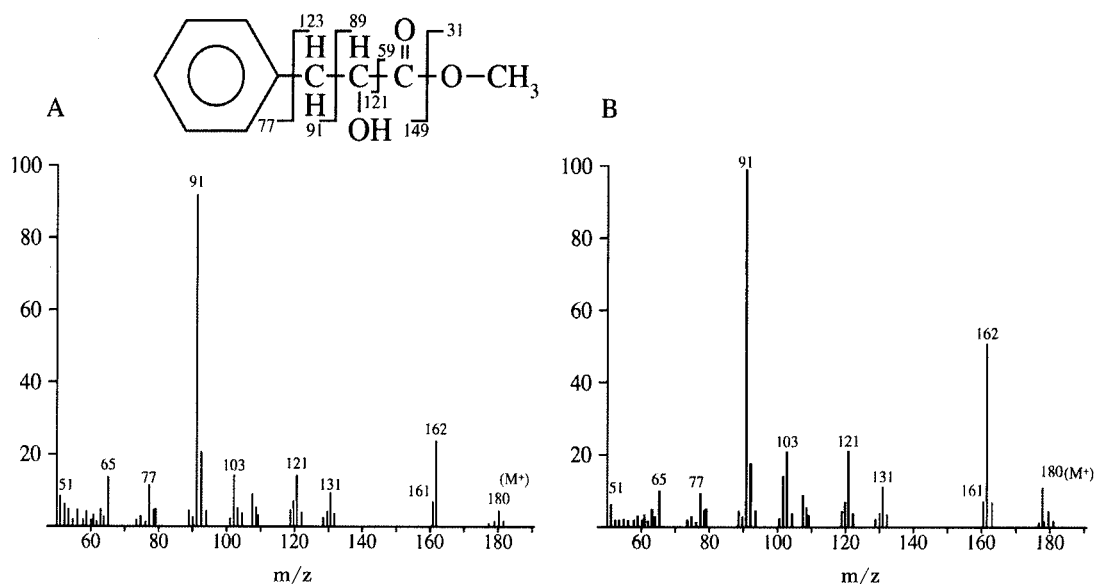


FIG. 2. Mass-fragment spectrums of phenyllactic acid methyl ester (A) and methylated fraction (e) (B) having antibacterial activity.

TABLE 1. Antibacterial activity of 3-phenyllactic Acid^a.

Test bacteria	3-Phenyllactate ^b concentration									
	50mM			35mM			10mM			
	pH ^c	6.8	6.0	5.5	pH	6.8	5.5	pH	6.8	5.5
<i>Staphylococcus aureus</i> (MRSA) FSA-1		2.7 ^d	3.7	4.2		1.8	4.0		1.7	3.8
<i>Bacillus cereus</i> JCM 2152		2.0	3.5	4.0		1.8	4.5		1.4	3.6
<i>Escherichia coli</i> O157:H7 (95-07)		3.0	3.0	3.5		1.7	3.0		1.8	2.7
<i>Yersinia enterocolitica</i> JCM 1677		4.0	4.3	5.1		3.8	4.5		2.3	3.9
<i>Salmonella enterica</i> serovar Choleraesuis JCM 6977		2.0	2.5	3.7		2.1	3.2		1.0	2.3
<i>Clostridium perfringens</i> JCM 1290		0.0	0.0	0.0		0.0	0.0		0.0	0.0

^aA paper disc (8 mm) loaded 60 μ l of 3-phenyllactate solution was placed on an agar plate on which test bacteria were inoculated, and the plate was incubated at 37°C overnight.

^bA mixture of D- and L-forms at a ratio of 2:1 was used.

^cpH of medium.

^dNumbers indicated are the diameter of the clear zone formed by growth inhibitory effect of 3-phenyllactate.

a spore-forming bacterium *C. perfringens*. Although the effect was higher in acidic pH (5.5), it was observed also in neutral pH (6.8), suggesting that phenyllactic acid has its own growth inhibitory effect in addition to the effect due to the reduction of pH. Phenyllactic acid may act as a complex form in neutral pH, but the actual form is unknown at the present.

When cells of *B. cereus* were treated with 5 mM phenyllactic acid at pH 6.8, washed with saline and inoculated in nutrient broth, no growth was observed even with only a few minutes of treatment. This suggests that the effect is bactericidal.

Although it has been published that phenyllactic acid is produced by some bacteria including *Brevibacterium* or *Corynebacterium* (Kamata et al., 1986; Marayan and Rao, 1974), information on its antibacterial activity is still inadequate. Dieuleveux et al. (1998a,b) reported that *Geotrichum candidum*, a yeast-like organism in natural milk flora, produced two anti-*Listeria* compounds, D-3-phenyllactic acid and D-3-indolactic acid. They also suggested that phenyllactic acid plays a role as a biopreservative in cheese by inhibiting the growth of coexisting bacterial flora. Lavermicocca et al (2000) reported an antifungal effect of phenyllactic acid produced by a lactic acid bacterium, *Lactobacillus plantarum*. Dieuleveux et al. (1998a) showed that L-3-phenyllactic acid also had antibacterial activity, although slightly weaker than that of D-form. They showed that *G. candidum* produced only the D-form of 3-phenyllactic acid. It is of interest that our preparation from *S. faecalis* TH10 contained D- and L-forms of 3-phenyllactic acid at a ratio of 2:1. The effect of the ratio of the optical isomers on antibacterial activity remains to be investigated.

Lactic acid bacteria are effective organisms for biopreservation because of their production of various antimicrobial substances including organic acids, such as lactic, acetic and propionic acids. Phenyllactic acid produced by *E. faecalis* TH10 is also thought to be one of the compounds. We monitored the antimicrobial activity of the various fractions of *E. faecalis* TH10 culture using *S. aureus* as the test organism and identified 3-phenyllactic acid. However, the possibility still remains that antimicrobial substance(s) other than 3-phenyllactic acid exist. Further study is underway in our laboratory.

E. faecalis TH10 was isolated from the Malaysian traditional fermented food, "tempeh" and identified to produce effective antimicrobial substance(s) (Ohhira et al. 1996). *E. faecalis* TH10 is an organism in the normal flora of tempeh, indicating it is harmless for humans. Although *E. faecalis* is known as not only a bioreservatic and probiotic organism but also an

opportunistic pathogen, the strain TH10 does not produce hemolysin, a virulent factor (Ohira et al., 1996), and has no toxic activity against some cultured mammalian cells (unpublished observation). Thus *E. faecalis* TH10 or its products such as 3-phenyllactic acid are useful for biopreservation. In this study, antimicrobial activity was assayed with food-borne pathogens, such as *S. aureus*, *B. cereus*, *E. coli*, *Salmonella* and *Y. enterocolitica*. Especially in *S. aureus* and *E. coli*, their hyper pathogenic phenotype or serotype, MRSA and O157:H7, respectively, were used. The effectiveness of *E. faecalis* TH10 or its products against these pathogens suggests its usefulness.

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