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原　報

マレーシア発酵食品Temphから単離された乳酸菌*Enterococcus faecalis* TH10の培養液中に産生される抗MRSA活性

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Antimicrobial activity against methicillin-resistant *Staphylococcus aureus* in the culture broth of *Enterococcus faecalis* TH 10, an isolate from Malaysian fermentation food,Temph

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Summary

Bioassay-directed screening for antimicrobial activity against methicillin-resistant *Staphylococcus aureus* (MRSA) led us to the culture broth of *Enterococcus faecalis* TH 10, which was isolated from a Malaysian fermentation food,Temph. The anti-MRSA component was readily extracted with ethyl acetate from the culture broth at pH 3, and the extract retained the activity when incubated overnight with various proteolytic enzymes. In addition,the extract showed poor antimicrobial activity against various lactic acid bacteria,including *Enterococcus faecalis*. These properties strongly suggest that the active compound is distinct from bacteriocin, an extracellularly released peptide or protein which shows potent activity against species closely related to the bacteriocin-producing strain. *E.faecalis* has been reported to produce bacteriocin which has strong hemolytic activity on mammalian erythrocytes, but the active component of the TH 10 strain did not mediate the lysis of rabbit and human erythrocytes.

Key words:*Enterococcus faecalis*, bacteriocin, methicillin-resistant *Staphylococcus aureus*

Lactic acid bacteria, a physiologically related group of gram-positive bacteria, produce a variety of compounds with antimicrobial activity, and they are termed bacteriocin. Bacteriocin is generally defined as extracellularly released peptide or protein that shows a bacteriocidal activity against species closely related to the bacteriocin-producing strain.[1]. It has now become evident, however, that many bacteriocins from lactic acid bacteria show somewhat broader spectrum of activity, affecting also more distantly related species [2]. In fact, bacteriocin-producing lactic acid bacteria appears to interfere with the growth of the food-born pathogen *Listeria monocytogenes* during fermentation process [3-5]. Researchers have long discussed the potential application of bacteriocins in prevention and even in treatment of infectious diseases.

In recent years, there has been a dramatic increase in the incidence of hospital-associated (nosocomial) infections caused by *Staphylococcus aureus* strains that are resistant to multiple antibiotics, and these strains are collectively termed methicillin-resistant *S.aureus* (MRSA). The incidence of MRSA outbreak is particularly high in Japan where the cases are almost four-times that reported in Europe [6]. Although the threat to patient care posed by such organisms has stimulated continuing efforts to search for potent anti-MRSA agents, there has been a growing concern that the pharmaceutical industry may no longer be able to develop novel antibiotics sufficiently quickly.

To exploit the potential of antimicrobial metabolite of lactic acid bacteria for the control and chemotherapy of MRSA, we carried out bioassay directed screening of microorganisms isolated from fermentation foods and

beverages. Several strains were found to produce antimicrobial activity in their culture broth, which inhibited the *in vitro* growth of MRSA on agar medium. *Enterococcus faecalis* TH 10, an isolate from a Malaysian fermentation food, Temph, was especially effective among the lactic acid bacteria isolated. More interestingly, the active component was readily extracted with ethyl acetate, and it retained the activity when treated with proteolytic enzymes. To see whether the anti-MRSA compound falls on the category of bacteriocin, we investigated various properties of the product of *E.faecalis* TH 10 strain : proteolytic treatment using various proteases, and antimicrobial activity against closely related lactic acid bacteria. Hemolytic activity was also assayed for the comparison with previously reported bacteriocin/hemolysin produced by *E.faecalis*.

Materials and methods

1) Materials

Reagents were purchased from the following suppliers; proteinase K,V 8 protease, achromopeptidase were from Wako pure chemical industries, Ltd, and trypsin was from Sigma chemical company. Other chemicals used were analytical grade reagents. Methicillin-resistant *Staphylococcus aureus* type-II strain was used in this study.

2) Culture of *Enterococcus faecalis* TH 10

A stock culture of *E.faecalis* TH 10 was inoculated on 10 ml of the seed medium containing 1.5 % polypepton, 0.5 % yeast extract, 0.25 % NaCl, 1.0 % glucose, 0.05 % sodium thioglycolate, 0.025 % L-cystine, 0.01 % sodium sulfite, 0.01 % sodium carbonate (pH 6.6 before sterilization). After incubation at 37 ℃ for 24 hr, the broth was transferred to one liter of the same medium, and cultivation was carried out at 37 ℃ for 8 days.

3) Extraction of Active Component

The fermentation broth was centrifuged at 4000 xg for 20 min at 4 ℃ to remove cells, and the supernatant solution was extracted with ethyl acetate at pH 3.0. The solvent layer was dried over anhydrous Na_2SO_4, and concentrated under reduced pressure. The residue was dissolved in 2 ml of 2 N NaOH solution, neutralized to pH 7, and the 60 μl portion was loaded on a paper disk. After the paper disk was dried, bioassay was performed by placing the disk on an agar plate and seeded with suspension of test organism. After incubation at 30 ℃ for 24 h, a zone of growth inhibition around the disk was presumed to indicate the presence of active compound.

4) Proteolysis Treatment

The ethyl acetate extract (10 mg in dry weight) was treated overnight at 37 ℃ with 0.1 mg of proteinase K, V8 protease, trypsin, and achromopeptidase, respectively in 100 μL of 20 mM potassium phosphate buffer at pH 7.5, loaded on paper disks and dried for the bioassay. The amount of the proteolytic enzymes used corresponds to 2.2 units for proteinase K, 1,100 units for trypsin, 2.2 units for V 8 protease, and 100 units for achromopeptidase.

5) Hemolysis Assay

Hemolysis assay was performed with rabbit and human erythrocytes as described for hemolysin/bacteriocin of *E. faecalis* [7].

Results and Discussion

1) Properties of anti-MRSA component in the culture broth of *E. faecalis* TH 10.

Enterococcus faecalis TH 10, an isolate from a Malaysian fermentation food Temph, was especially effective among the lactic acid bacteria tested. The active component was readily extracted with ethyl acetate from the culture broth of the TH 10 strain at pH 3. The extract showed potent growth inhibition against MRSA, and a moderate activity against methicillin-sensitive *S. aureus* (Table). Since the extract contains various compounds in it, the results does not prove that an identical substance inhibited MRSA and methicillin-sensitive *S. aureus*. It is rather more important to note that the extract failed to show inhibitory activity against the lactic acid bacteria tested except for two species, *Streptococcus salivarius* and *Pediococcus acidilactici*. *Enterococcus* species were not inhibited by the extract, either. These antimicrobial spectrum indicates that the anti-MRSA component appears to be distinct from bacteriocin molecules which are usually inhibitory against closely related lactic acid bacteria.

Proteolytic enzymes are frequently used for

Table Inhibitory spectrum of ethyl acetate extract of the culture of *E. faecalis* TH10.

Test Organism	Dosage (mg) a		
	20	10	5
MRSA	+	+	—
Methicillin-sensitive Stapylococcus aureus	+	—	
Enterococcus faecalis RIMD 3116001	—	—	
Enterococcus faecium	—	—	
Streptococcus salivarius	+	—	
Pediococcus acidilactici	+	—	
Pediococcus pentosaceus	—	—	
Lactococcus lactis subsp. lactis	—	—	
Leuconostoc mesenteroides subsp. mesentroides OR-1	—	—	
Leuconostoc mesenteroides subsp. Dextranicum 7-1-9	—	—	
Lactobacillus plantarum	—	—	
Lactobacillus delbrueckii	—	—	

a Dry weight of the ethyl acetate extract loaded on paper disk. +, Growth inhibition of test organism around paper disk; —, no inhibition.

conventional diagnosis for bacteriocin production ; proteolytic enzymes catalyze hydrolysis of protein or peptide-like components, and cause dramatic decrease in the bacteriocidal activity. We found that the ethyl acetate extract retained the anti-MRSA activity after the treatment with V 8 protease, proteinase K, trypsin, and achromopeptidase. These proteolytic enzymes alone do not affect the growth of MRSA. The results suggest that the active component is not a peptide or a protein, but it rather appears to be an acidic low molecular weight substance, which is extracted with ethyl acetate at pH 3 .

Thus, the active component produced in the culture broth of *E.faecalis* TH 10 strain appears to differ from bacteriocins, a protein or peptide-like antimicrobial metabolite with potent activity against species closely related to the bacteriocin-producing strain.

2) Hemolysis Assay.

In previous papers, *E.faecalis* has been isolated from natural food samples such as traditional French cheese [8], mozzarella cheese whey[9], and foods in a retail supermarket [10]. The species has also been recognized as human pathogens with multiple antibiotic-resistance and high-level aminoglycoside resistance [11-13]. These clinical isolates of *E.faecalis* are reported to produce bacteriocin which mediates the lysis of a broad range of gram-positive bacteria [14-16], and the molecule also acts as a hemolysin which effectively lyses human, rabbit, and horse erythrocytes [7]. The anti-MRSA component of the TH10 strain, however, failed to mediate the lysis of human and rabbit erythrocytes, indicating that the anti-MRSA compound is distinct from ever reported bacteriosins produced by *E.faecalis*.

Our present study shows that *E.faecalis* produces a potent anti-microbial product against MRSA, whose identity appears to be a low molecular weight substance. Racemic lactate did not show antimicrobial activity against MRSA even when 30 mg of the sodium salt was loaded on the paper disk, so the commonly distributed metabolite of lactic acid bacteria can be ruled out as the candidate for the active substance. Further study is in progress to purify and characterize the active substance using silica gel column, activated charcoal, and anion-exchange resin, and the results will be reported soon. In the current situation where the discovery of novel antimicrobial agents is becoming increasingly difficult, the present study suggests that food-born lactic acid bacteria may offer some potential applicability in chemotherapy and control of MRSA.

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