FULL PAPER *Bacteriology*

Screening of the Enterocin Genes and Antimicrobial Activity against Pathogenic Bacteria in *Enterococcus* Strains Obtained from Different Origins

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ABSTRACT. Antimicrobial activities of 139 *Enterococcus* isolates (48 *E. faecium* and 91 *E. faecalis*) obtained from canine feces, boiler meat samples, swine feces, wild waterfowl feces, and human feces were examined against respective bacteria, including *Streptococcus pyogenes*, *Staphylococcus aureus, Bacillus subtilis, Listeria monocytogenes, Salmonella* Enteritidis, and *Escherichia coli*. Bacteriocin (BAC) production assay revealed that the antimicrobial activity against at least one of 6 indicator strains (BAC+ phenotype) was found in 51 (37%) isolates (29 *E. faecium* and 22 *E. faecalis*). Twenty-four of 46 isolates positive for at least one of the enterocin structural genes (*entA, entB, entL50AB*, and *cylL*) showed a BAC+ phenotype. The existence of other enterocins or nonenterocin factors was implied because the BAC+ phenotype was detected in a total of 27 *Enterococcus* isolates that had none of the enterocin genes tested. The antimicrobial activity against Gram-negative strains (*Salmonella* Enteritidis and *E. coli*) was detected in the 6 *Enterococcus* isolates that had either the *entA, entB, entL50AB* or *cylL* genes. Moreover, the proportion of the antimicrobial activity against *L. monocytogenes* among the *cylL*-positive *E. faecalis* isolates showing beta-hemolysis (10/16) was significantly ($p<0.01$) higher than among those lacking beta-hemolysis (2/15). The results suggested that certain characteristics are likely to be associated with the antimicrobial activity against specific organisms.

KEY WORDS: antimicrobial activity, cytolysin, enterocin, *Enterococcus*.

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Enterococci are commensal bacteria of the gastrointestinal tract of mammals and other warm-blooded animals [14, 29, 30]. *Enterococcus faecium* and *E. faecalis* are occasionally used in foods for the purpose of the ripening cheeses [15, 24] and black olives [13], and also used in probiotic products [17, 33]. However, organisms of the genus *Enterococcus*, in particular *E. faecalis* and *E. faecium*, are of medical relevance because of their increased incidence as a cause of disease, and have also become a significant cause of nosocomial infections [22, 27]. One factor expressed by many *E. faecalis* isolates from clinical specimens is cytolysin [4, 18, 19] which has been associated with virulence of this organism in animal infection models [21]. Although cytolysin confers beta-hemolytic activity, it exhibits a broad target cell range, including both eukaryotic and prokaryotic cells [29], especially Gram-positive organisms [2, 31].

A number of enterocins, bacteriocins produced by enterococci, have been characterized especially in *E. faecalis* (cytolysin [32], bacteriocin 31 [34], and bacteriocin AS-48 [16, 25]) and *E. faecium* (enterocin A [1], enterocin B [3], enterocin L50A/L50B [6], enterocin P [5], and enterocin Q [7]). Bacteriocins inhibit the growth of other bacteria that are usually related to bacteriocin-producing organism [20]. The ability to produce bacteriocins may play an important physiological role in providing an ecological advantage over others that inhabit the same ecosystem but do not produce these peptides [28]. Thus, it is of interest to determine whether certain genotypic and phenotypic profiles of enterocins are associated with enterococcal isolates from specific origins, such as foods, food animals, and wild animals. In this study, we screened *E. faecalis* and *E. faecium* isolates for the enterocin genes described above and antimicrobial activity against representative pathogenic bacteria to determine a possible association between combinations of antimicrobial activity against different bacteria, the enterocin genotypes, species of *Enterococcus*, and origins of the isolates.

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MATERIALS AND METHODS

Bacterial strains: This study included 139 *Enterococcus* isolates (48 *E. faecium* and 91 *E. faecalis*) from different origins (46 boiler meat samples, 44 canine fecal samples, 11 swine fecal samples, 19 wild waterfowl feces, and 19 human fecal samples). The broiler meat samples were from carcasses originally processed in Japan, China, or Brazil from 2000 to 2005. Canine fecal samples were obtained from animals admitted to a private veterinary hospital and the veterinary teaching hospital of Tottori University in Japan between 2001 and 2004. Swine fecal samples were obtained in October 2005 from different-aged animals on a commercial farm in Japan. Wild waterfowl feces were collected in

marshes and paddy fields in the western Japan between December 2004 and February 2005, and between November and December 2005 where swans and wild ducks migrate every winter. Enrichment cultures of swab samples from broiler meat and animal fecal samples using heart-infusion broth were plated onto EF agar (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) and bile esculin azide agar (Difco, Becton Dickinson, Sparks, Md., U.S.A.) and *E. faecium* or *E. faecalis* isolates were retained. Identification was carried out by PCR using specific primers for these species [11]. Human isolates were randomly selected from fecal isolates that were recovered from in-patients in Tottori university hospital in 2005. A total of 6 strains of six different bacterial genera, as follows, were used as enterocin production indicators. *Streptococcus pyogenes* strain 940055 was isolated from a throat swab sample from a human patient. *Staphylococcus* aureus strain ATCC29213 was purchased from the American Type Culture Collection (ATCC, Manassas, VA, U.S.A.). *Listeria monocytogenes* strain L98–76P2 was isolated from a pork meat sample. *Bacillus subtilis* used in this study was isolated from an environmental sample. *Salmonella* Enteritidis IFO3313 and *Escherichia coli* IFO3301 were purchased from the Institute of Fermentation, Osaka. *S. pyogenes, S. aureus, L. monocytogenes*, and *Salmonella* Enteritidis strains above were used as representative pathogenic organisms.

Detection of enterocin structural genes by PCR: Presence of the *entA, entB, entAS-48, bac31*, and *cylL* genes were detected by a PCR assay as reported previously [33], using the specific primer pairs described by De Vuyst *et al.* [10]. Because activation of cytolysin is regulated by the *cylM, cylA*, and *cylB* gene products [12, 23], the *cylM* [12], *cylA* [23], and *cylB* [23] genes were also screened by PCR. To amplify the *cylA*, and *cyl B*, a total of 30 cycles of amplification were carried out and each of cycle consisted of 97°C denaturation for 1 min, 52°C annealing for 30 sec, and 72°C extension for 1 min. PCR amplifications of the *cylM* was performed by an initial cycle of denaturation at 94°C for 2 min, annealing at 56°C for 2 min, and elongation at 72°C for 2 min, followed by 29 cycles of denaturation at 92°C for 15 sec, annealing at 56°C for 15 sec and extension for 72°C for 15 sec. PCR analyses were performed to determine the presence of structural genes of enterocins 50A and 50B (*entL50A/B*), enterocin P (*entP*), and enterocin Q (*entQ*) using the specific primer pairs described by [7]. Amplification condition was the same that used for the *cylA* and *cylB* genes, except that annealing was carried out at 60°C. PCR products were resolved by electrophoresis on a 1.5% agarose gel (1X Tris-acetate-EDTA buffer). *E. faecium* strain GIFU8355 was used as positive control for detection the enterocin A (*entA*), and enterocin B (*entB*) genes.

Pulsed-field gel electrophoresis (PFGE): Subtyping of *E. faecium* isolates from swine feces were carried out using PFGE with *Sma*I-digested chromosomal DNA [26]. PFGE was performed with a 1.0% agarose gel by using a CHEF-DRII apparatus (Bio-Rad Laboratories, Hercules, Calif.) in 0.5× Tris-borate-EDTA buffer at 14°C at 200 V. A linearly ramped switching from 0.5 to 20 sec was applied for 20 hr.

Enterocin and beta-hemolysin assay: Enterocin detection was carried out by the method previously described by Del Campo *et al.* [9]. The antimicrobial activity due to enterocins in enterococcal isolates from different origins was visually detected by observing clear inhibition zones around the producer strain on the agar plate seeded with each of the indicator strains. Those strains in which antimicrobial activity were observed, were designated Bac+. This assay does not discriminate between single and multiple enterocin production. Beta-hemolytic activity was examined using Columbia blood agar base (Becton, Dickinson and Co., Sparks, MD) plates supplemented with 5% (v/v) of either sheep blood or horse blood. The presence of a clear zone of hemolysis around the colonies in both the blood plates was referred as a positive.

Statistical analysis: Statistical analysis of the proportion of antilisterial activity among the *cylLMBA* harboring *E. faecalis* isolates positive or negative for beta-hemolysis was done by the Fisher's exact test, and statistical significance was determined at $p<0.05$.

RESULTS

The enterocin assay revealed that antimicrobial activity against at least 1 of 6 indicator strains (BAC+ phenotype) was shown in 51 of the 139 *Enterococcus* isolates tested (Table 1). Of 48 *E. faecium*, 29 (60%) were BAC+, whereas this phenotype was detected in only 22 of 91 (24%) *E. faecalis* isolates. Eight of 9 *E. faecium* isolates obtained from pig feces were BAC+ phenotype. Because these isolates were obtained from different-aged pigs in the same farm, the possible relationships among the BAC+ *E. faecium* were investigated using PFGE. *Sma*I-digested PFGE patterns of the 8 *E. faecium* isolates showed multiple band differences (data not shown), indicating that these isolates were genetically unrelated.

One or more enterocin structural genes (*cylL, entA, entB,* and *entL50A/B*) were detected in 46 (33%) *Enterococcus* isolates (12 *E. faecium* and 34 *E. faecalis*) (Table 1). All the *cylL*-positive isolates had the *cylM, cylB*, and *cylA* genes. The *cyl* genes were mainly detected in *E. faecalis* strains, while the *entA* and *entB* genes were detected in strains of both *E. faecium* and *E. faecalis*. Of 33 *Enterococcus* isolates (31 *E. faecalis* and 2 *E. faecium*) that had the *cyl* genes, 19 *E. faecalis* strains did not show BAC+. All the 9 *E. faecium* strains positive for the *entA* or *entB* genes were BAC+, whereas all the 3 *E. faecalis* strains with these genes had no antimicrobial activities. None of *Enterococcus* isolates studied had the *entP, entQ, bac31*, and *entAS-48* genes. Of 93 *Enterococcus* strains negative for the enterocin genes investigated, 27 strains (10 *E. faecalis* and 17 *E. faecium*) were found to be BAC+.

The results of antimicrobial activities against different indicator strains for 51 BAC+ *Enterococcus* isolates are shown in Table 2. Various combinations of antimicrobial activities against 6 indicator strains were found in *Entero-*

Table 1. Antimicrobial activity and enterocin genes detected in *Enterococcus* isolates from different origins

Species	BAC+ phenotype	Enterocin gene	Isolates from: Broiler meat	Canine feces	Swine feces	Wild waterfowl feces	Human
E. faecium	BAC+	*cylLMBA*		2			
		entA+entB		1		1	
		entA		4	2		1
		entL50AB			1		
		None	7	4	5	1	
	Non-BAC+	None	10	7	1	1	
E. faecalis	BAC+	*cylLMBA*		7		1	4
		None	6	2		2	
	Non-BAC+	*cylLMBA*	3	9		3	4
		entA+entB	1	1			
		entA		1			
		None	19	6	2	10	10

Table 2. Antimicrobial activity against a series of 6 indicator strains detected in BAC+ isolates

Indicator strain	*E. faecium* (left) and *E. faecalis* (right) from: Broiler meat	Canine feces	Swine feces	Wild waterfowl feces	Human
S. pyogenes	7/7[a)] 0/6	5/11 1/9	5/8 0/0	1/2 0/3	0/1 2/4
S. aureus	7/7 0/6	8/11 1/9	8/8 0/0	1/2 0/3	0/1 0/4
L. monocytogenes	0/7 0/6	4/11 8/9	1/8 0/0	1/2 3/3	1/1 4/4
B. subtilis	0/7 5/6	0/11 4/9	1/8 0/0	0/2 1/3	0/1 3/4
S. Enteritidis	0/7 0/6	0/11 1/9	3/8 0/0	1/2 0/3	0/1 1/4
E. coli	0/7 0/6	0/11 1/9	1/8 0/0	1/2 0/3	0/1 0/4

a) Number of isolates with antimicrobial activity against each of the indicator strains/ number of BAC+ isolates.

Table 3. The presence of beta-hemolytic activity and antilisterial activity in the *cylLMBA*-positive *E. faecalis* isolates from different origins

Beta-hemolysis	Antilisterial activity	*cylLMBA*-positive isolates from: Broiler meat	Canine feces	Wild waterfowl feces	Human
Yes	Yes		7	1	2
Yes	No		2	3	1
No	Yes				2
No	No	3	7		3

coccus isolates studied. One *E. faecalis* isolate with the *cyl* genes obtained from a dog and one *E. faecium* with the *entL50A/B* genes obtained from a pig showed antimicrobial activities against all 6 indicator strains. Six isolates that showed antimicrobial activities against Gram-negative bacteria (*E. coli* and/or *Salmonella* Enteritidis) had one of the e*ntA, entB, cylLMBA*, and *entL50A/B* genes. *Enterococcus* isolates obtained from broiler meat showed antimicrobial activities only against the Gram-positive indicator strains used. The antimicrobial activity against *L. monocytogenes* was found in a high proportion of *E. faecalis* isolates obtained from humans (4/4), dogs (8/9), and wild waterfowl feces (3/3). Of the 18 *E. faecalis* isolates positive for the antilisterial activity, 12 isolates had the *cyl* genes (Table 3). Remaining 6 isolates (2 from wild waterfowl feces, 3 from boiler meat and 1 from dog) were negative for all enterocin genes tested. Beta-hemolysis was found in 16 *E. faecalis* isolates and 2 *E. faecium* isolates with the *cyl* genes, although 15 of the 31 *E. faecalis* isolates positive for these genes did not show beta-hemolysis (Table 3). The incidence of antimicrobial activity against *L. monocytogenes* found in the *cyl* genes-positive *E. faecalis* isolates showing beta-hemolytic activity (10/16) was significantly ($P<0.01$) higher than that found in the isolates without beta-hemolytic activity (2/15). Of 7 *E. faecium* isolates positive for antilisterial activity (Table 2), 1 isolate from dog feces was positive for the *cyl* genes, 1 and 3 isolates from a human and dog feces, respectively, had the *entA*, and 1 isolate from swine feces had the *entL50AB* gene.

DISCUSSION

Almost 37% of *Enterococcus* isolates used in this study showed BAC+ activity. The incidence of BAC+ isolates was higher among *E. faecium* (60%) than among *E. faecalis* (24%) isolates. According to Poeta *et al.* [28], 49% of *E. faecium* and 37% of *E. faecalis* isolates of different wild animal origin showed antimicrobial activity against at least one of the tested indicator strains. On the contrary, Del Campo *et al.* [9] found a higher proportion of BAC+ isolates among *E. faecalis* (80.6%) than among *E. faecium* (21.6%) isolates obtained from human clinical and fecal samples, sewage, and chicken samples. Similarly, De Vuyst *et al.* [10] reported that bacteriocin production was found among 58.7% and 68.3% of *E. faecium* and *E. faecalis* strains, respectively, of different origins, including food, feed, animals, and clinical and nonclinical human isolates. Inconsistency of the BAC+ phenotype incidence among these species may be partly due to difference in indicator strains used. Moreover, environmental factors, such as feeds, conditions found in the gastrointestinal tract, and the balance of organisms in the intestinal flora are likely to affect the presence and persistence of enterococci because BAC+ phenotype was found in 8 of 9 genetically unrelated *E. faecium* isolates in our study that were obtained from pigs kept in a single farm.

The results of the antimicrobial activity and the PCR results imply the existence of other enterocin genes that were not tested in this study. None of enterocin genes tested were found in all of the 7 *E. faecium* and 6 *E. faecalis* with BAC+ phenotype that were obtained from broiler meat. Five of the 8 isolates with BAC+ phenotype (exclusively against Gram-positive indicator strains) from pig feces had no detectable enterocin genes. Nucleotide substitutions in the enterocin genes may prevented PCR amplification with the selected primers used in this study because possible sequence divergence of the *cyl* genes were suggested [29]. Except for the above observation, similar tendencies in distribution of enterocin genes tested were not found among enterococcal isolates of different origins.

Marked variations of inhibitory spectra were shown among *Enterococcus* isolates with identical enterocin genotypes. Nevertheless, antimicrobial activities against Gram-negative bacteria (*E. coli* and *Salmonella* Enteritidis) were found in 6 *Enterococcus* isolates that had one of the *cylLMBA, entA*, and *entL50AB* genes. We have previously demonstrated [33] that the inhibitory activity against *Salmonella* Enteritidis was found in one *E. gallinarum* strain that was negative for all of the above genes [33]. Therefore, association of other enterocins or nonenterocin factors with the inhibitory effect against *Salmonella* Enteritidis in this study can not be excluded. Enterocin A has been reported to show antimicrobial activity against *L. monocytogenes* [1], although in the current study, no antilisterial activity was detected in more than half (5/9) of *E. faecium* and all of the 3 *E. faecalis* isolates possessing the *entA* gene. Other researchers have noted that phenotypic testing revealed the existence of apparently silent genes [10, 12].

Possible correlation between beta-hemolytic strains of *E. faecalis* positive for the *cylLMBA* genes and antimicrobial activities against *L. monocytogenes* was demonstrated in the present study. The incidence of antilisterial activity among the *cyl* genes-positive isolates with beta-hemolytic activity was significantly higher than among beta-hemolysis negative isolates. In this study, beta-hemolytic activity was exclusively shown in the strains with the *cylL* gene. Interestingly, cytolysin is not considered to be active against *L. monocytogenes* [8]. Thus, it remains to be determined whether beta-hemolysis and antilisterial activity are actually mediated by the same agent, although the beta-hemolytic characteristic may be intimately associated with the production of unknown agents possessing antilisterial activity, even new enterocins.

In summary, observations in our study suggest that the antimicrobial activity may be associated with other enterocin genes that was not studied or nonenterocin factors. Investigating the correlation between a certain characteristic and antimicrobial activity against specific organism is likely to offer a first step in identifying novel agents exhibiting such activity.

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