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Construction of a Plasmid Vector for Expression of Bacteriocin N15-encoding Gene and Effect of Engineered Bacteria on *Enterococcus Faecalis*

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ABSTRACT

A 6.09-kb plasmids vector pOri253 was constructed from the plasmid pIL253 (5.2 kb) and a 0.89-kb fragment of *oriColE1* from pBluescript II KS. The bifunctional plasmid pOri253 conferred erythromycin resistance in both *Escherichia coli* and *Enterococcus faecalis*. It has a unique site for each of *Eco*RI, *Bam*HI, *Sal*I and *Pst*I derived from pIL253. The plasmid is quite stable in *E. faecalis* JCM8726 when cultured in Mann Rogosa Sharpe broth without antibiotic. The lactococcal promoter *P23* was inserted at one end of the pOri253 multicloning site. Gene expression was assessed by using an *entAI* gene which coded for bacteriocin N15. The *E. faecalis* harboring constructed plasmid carrying *P23* (pOrient23) had more antibacterial activity than parental *E. faecalis* JCM8726 or its clone harboring non-*P23* containing plasmid (pOrient), as determined by means of an overlay method.

Key words : E*ntAI* - Enterococci - Shuttle vector - pIL253

INTRODUCTION

Bacteriocins and bacteriocin producing strains of lactic acid bacteria (LAB) have been the subject of extensive research due to their potential as biopreservatives (1) and also their effect on the health of humans and animals as probiotics (2,3,4,5). The development of efficient methods for heterologous expression is an important goal in bacteriocin research. It may be used to create strains with improved protective properties that differ only in the ability to produce a certain bacteriocin, thus facilitating the scientific evaluation of the effect of bacteriocins (6,7).

Several studies of heterologous expression of LAB bacteriocins have been described (2,8,9,10,11,12,13). The autoregulatory properties of the *Lactococcus lactis* nisin gene cluster is one of the most widely used (14,15) in which, the level of expression can be controlled by the amount of nisin added for induction (16). However, the systems are still not optimally suited for the above-mentioned aspects of bacteriocin research, *e.g.* the bacteriocin is produced as a chimeric protein and/or the host strains used are not bacteriocin-negative (17). Therefore, in the present experiments we refined the procedure by using the bacteriocin-negative strain (*Enterococcus faecalis* JCM8726; 18) as a recipient microorganism.

The following experiments describe the successful cloning and expression of the bacteriocin N15 (*entAI*) in *E. faecalis* by using a new constructed shuttle vector

between *Escherichia coli* and *E. faecalis*. The functionality of the transferred *entAI* product was assessed by the ability of *entAI*-positive enterococci to show antibacterial spectrum by means of an overlay method.

MATERIALS AND METHODS

Strains and Growth Conditions

Enterococcus faecium N15 and *E. faecalis* JCM8726 (kindly provided by Professor S. Shioya, Osaka University, Japan) (18) was grown at 30°C in MRS broth (Oxoid). *Escherichia coli* strains were grown at 37°C in Luria-Bertani broth (Difco). Whenever appropriate, antibiotics were added to the media as follows: erythromycin (Er) at 10 μg/ml for enterococci, at 200 μg/ml for *E. coli* and ampicillin (Am) at 100 μg/ml for *E. coli*. The solid media were prepared by adition of 1.5% agar (Difco) to the liquid media.

All pure cultures of bacteria **(Table 1)** were kept as stock cultures in 15% glycerol at -70°C.

Table 1. Strains and plasmids.

Strains and plasmids	Relevant characteristics[a]	Source or reference
Strains		
Escherichia coli DH5α	Host strain	Gibco BRL
Enterococcus faecalis JCM8726	Host strain, bac-	(18)
Enterococcus faecium N15	nuka isolate, bacteriocin N15	(18)
Plasmids		
pIL253	5.2 kb, LAB cloning vector; Em^r	(21)
pBluescript II KS	Source of *oriColEI*; Am^r	Stratagene
pKU204	Source of *entAI*	(18)
pOri253	pIL253 with *oriColEI*	This study
pOri23	pOri253 with *P23*	This study
pOrient	pOri253 with *entAI*	This study
pOrient23	pOri23 with *entAI*	This study

[a]Em^r and Am^r, erythromycin and ampicillin resistant, respectively; bac-, bacteriocin non-producing; *P23*, Promoter from *L. lactis.*

Plasmids and Plasmid Constructions

The plasmids and constructed plasmids in this study are listed in **Table 1**. The *entAI* gene (GenBank accession number AB038464) from *E. faecium* N15 (pKU204) (kindly provided by Professor S. Shioya, Osaka University, Japan) (18) was used in this study. The recombinant plasmid pKU204 contained a 0.7-kb insert of the *entAI* gene from *E. faecium* N15. The gene was cloned into *E. coli* or *E. faecium* by using pUC19 or pIL253 as the cloning vector, respectively. The synthesized bacteriocin N15 has a molecular mass of 3-5 kDa (18).

PCR amplification of DNA fragments was carried out using a Perkin-Elmer apparatus (GeneAmp PCR System 9700; Perkin-Elmer, Norwalk, Conn.). The reactions consisted of 100 ng of template DNA, 0.5 μM specific primers (Invitrogen, USA), 0.2 mM dNTPs, 10×PCR buffer, 2 mM $MgCl_2$ and 2 U *Taq* DNA polymerase (Life Technologies). The following conditions were applied for 30 cycles using specific primer pairs listed in **Table 2** (19). The *oriColEI* from pBluescript II KS (Stratagene) or *entAI* from pKU204 were amplified for 30 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 1 min. The lactococcal promoter *P23* was amplified from *L. lactis* chromosome at 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s. PCR fragments were purified by the QiaQuick purification Kits (Qiagen). Digested inserts corresponding to the various PCR fragments were then isolated on agarose gel and ligated to the corresponding recipient vectors **(Table 1)** after digestion with appropriate restriction enzymes **(Table 2)** (Invitrogen). Ligation was carried out for 1 h at 30°C using T4 DNA ligase (Life Technologies). All constructions and plasmids were established using *E. coli* as an intermediate host. The plasmids from *E. coli* were prepared by the Qiaprep Spin Miniprep Kit (Qiagen) according to the manufacturers directions. Methods for molecular cloning techniques and transformation of *E. coli* were conducted as described previously (20). The same procedure was applied to DNA extraction from enterococci, except that the enterococcal walls were digested with 10 mg/ml of lysozyme. Enterococci electroporations were performed by Gene pulser II (BioRad), using 50 μF, 600 Ω and 2.5 kV in ice-cold electroporation cuvette (2-mm electrode gap). The transformants were selected for determining the plasmid stability.

Table 2. List of the primers used for PCR amplification of various DNA fragments.

Gene	Primers[a]	Site	Size (bp)
OriColEI	GCTCTAGACGCTCTTCCGCTTCCTCG	*Xba*I	891
	GCTCTAGATTTAAATTTAAAAGGATCTAGGTGA	*Xba*I	
P23	CGGAATTCGAAAAGCCCTGACAACCC	*Eco*RI	180
	CGGGATCCAACATCATTGTCATTCATATTTTT	*Bam*HI	
entAI	CGGGATCCTTATGTACGAAGTGCATTCTC	*Bam*HI	700
	TTCTGCAGTGAGACATATTTAATATTATA	*Pst*I	

[a]Primer sequences are written 5′→ 3′.
Nucleotides underlined corresponding for restriction enzyme(s); bp, base pair(s).

Determination of Plasmid Stability

The segregrational stability of plasmids was examined by growing the transformants in MRS broth with daily transfer to fresh MRS broth without antibiotic. On certain days, 0.1 ml of each culture was plated on both MRS agar and MRS agar containing Em to determine the number of Em-resistant colonies. Em-resistant colonies were recorded as a percentage of total colony forming units (cfu) on MRS without Em [*i.e.*, % Em-resistant colonies = (Em-resistant cfu/total cfu) ×100].

Preparation of Concentrated Culture Filtrate

E. faecium N15 or transformants were inoculated to a concentration of 10^8 cells/ml in a 100 ml MRS broth in a 500 ml Duran bottle, and incubated as a still culture at 30°C for 48 h. The culture was centrifuged (12,000 rpm, 20 min, 4°C; Hettich Zentrifugens), and then filter sterilized (0.45 μm poresize; Millipore). The sterile cell-free supernatant was concentrated by using a concentrator tube (1 kDa molecular weight cutoff; PALL Life Sciences) to reduce the volume to 1 ml. Culture filtrate from type strain *E. faecium* N15 was used as a control.

Bacterial Inocula

Bacterial cell inocula was prepared from washed cultures grown in MRS broth (Oxoid) as still cultures at 30°C for 2 days. Bacterial concentrations were determined using Mcfarland No.0.5, and adjusted to 10^6 per ml of sterile peptone water (2%).

Antibacterial Activity Assay

The overlay method was performed duplicately using MRS agar plates on which transformants were inoculated as 2 cm long lines and incubated at 30°C for 2 to 15 days in 5% CO_2 atmosphere. The plates were then overlaid with 5 ml of MRS soft agar (MRS broth, 0.7% agar; Oxoid) containing 10^6 bacterial cell per ml. The plates were then incubated aerobically at 30°C for 24-48 h. The plates were examined for inhibition zones around the tranformants of enterococci streaks. The inhibition zone was scored as follows: -, no suppression; +, no bacterial growth on 0.1 to 3% of the plate area per bacterial streak; ++, no bacterial growth on 3 to 8% of plate area per bacterial streak; or +++, no bacterial growth on >8% of plate area per bacterial streak.

The agar well diffusion assay was performed using MRS agar plates containing 10^6 /ml of *E. faecalis* JCM8726. The agar was cut into holes using a steriled cork-borer and a droplet of warm agar was added to each well in order to avoid leakage. The 100 μl of each sample in duplicate manner were added to each well and allowed to diffuse for 5 h at room temperature, followed by aerobic incubation at 30°C for 24-48 h. The antibacterial effect was graded as follows: -, no suppression; +, weak suppression around the wells; ++, strong suppression, with detectable clear zone around the well; or +++, very strong suppression, with large clear zone around the well.

RESULTS AND DISCUSSION

Construction of the *E. coli*-enterococci Shuttle Vector

The shuttle vector pOri253 (6.09 kb) as shown in **Table 1** and **Figure 1** was designed to be able to replicate both in *E. coli* and enterococci. It was constructed from pIL253 (21) carrying an erythromycin resistant gene (Em^r) which can confer erythromycin resistance to both enterococci and *E. coli*, including lactococci (22). However, pIL253 **(Figure 1A)** does not replicate autonomously in *E. coli*. To overcome the problem, pIL253 was equipped with the high copy-number *oriColEI*

replicon of pBluescript II KS. The *OriColEI* was amplified by PCR using the primers presented in **Table 2**, and inserted at the *Xba*I restriction site of pIL253. The resulting shuttle vector pOri253 **(Table 1, Figure 1B)** can successfully replicate in both *E. coli* and *E. faecalis*. The plasmid contains four cloning sites of *Eco*RI, *Bam*HI, *Sal*I, and *Pst*I.

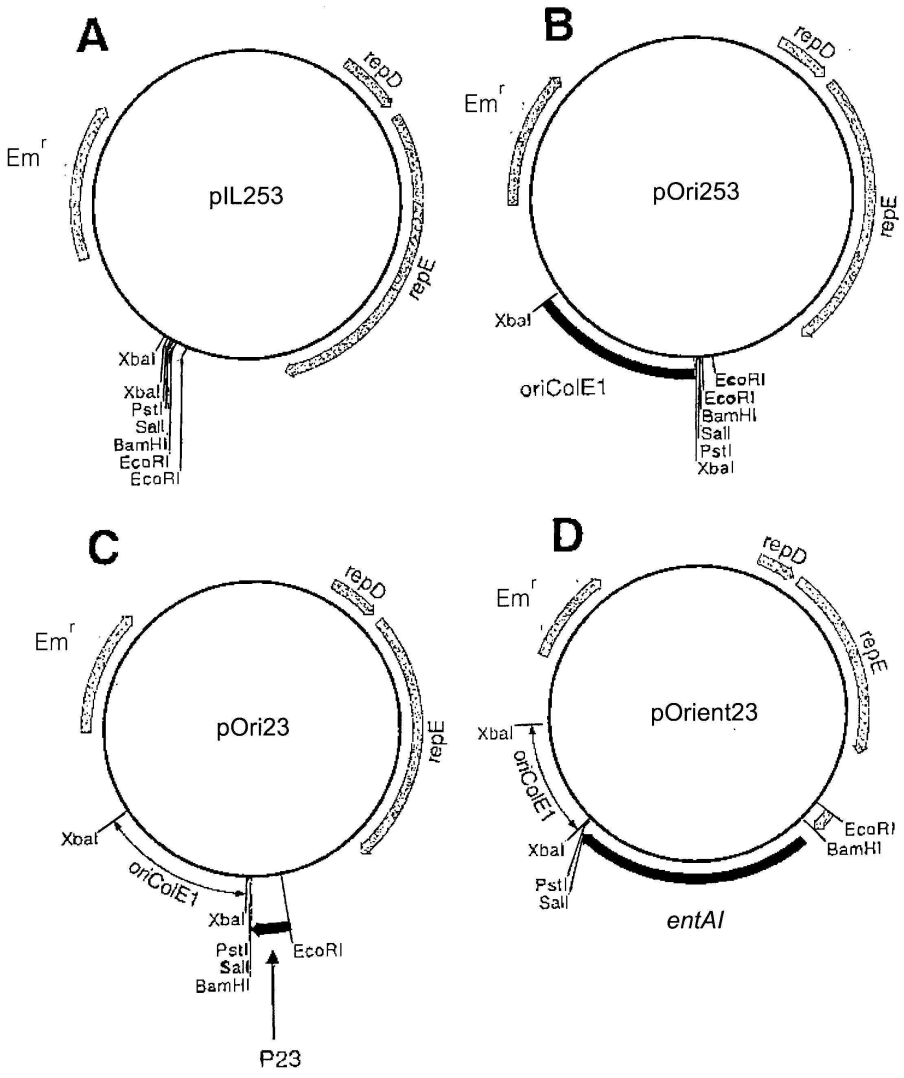

Figure 1. Restriction map of the shuttle plasmid vectors generated and utilized in this study **(Table 1)**. The original lactococcal plasmid pIL253 (A) was used to construct the *E. coli*-enterococci shuttle vector pOri253 (B) by inserting the *oriColE1* replicon at the *Xba*I restriction site of its multi cloning sites (MCS). The shuttle vector pOri253 was further modified by inserting lactococcal promoter *P23* at the *Eco*RI/*Bam*HI end of the MCS, resulting in the lactococcal expression vector pOri23 (C). Finally, the

P23-containing plasmid vector was used to clone bacteriocin N15 (*entAI*) gene to be expressed in enterococci (D). Em^r is the erythromycin resistant gene, and *repD* and *repE* are the genes responsible for replication in either enterococci or lactobacilli. The arrows indicate the direction of transcription.

To obtain the expression vector, pOri253 was modified by insertion with the lactococcal promoter *P23* (23) obtained by PCR amplification of the *L. lactis* chromosome using specific primers showed in **Table 2**. The promoter was inserted at the *Eco*RI/*Bam*HI site of pOri253 at one extreme of the cloning site, designated as pOri23 **(Table 1, Figure 1C)**. The constructed plasmid has the ability to replicate both in *E. coli* and enterococci, and also shown erythromycin resistance.

Segregrational Stability of Constructed Plasmids in *E. faecalis* JCM8726

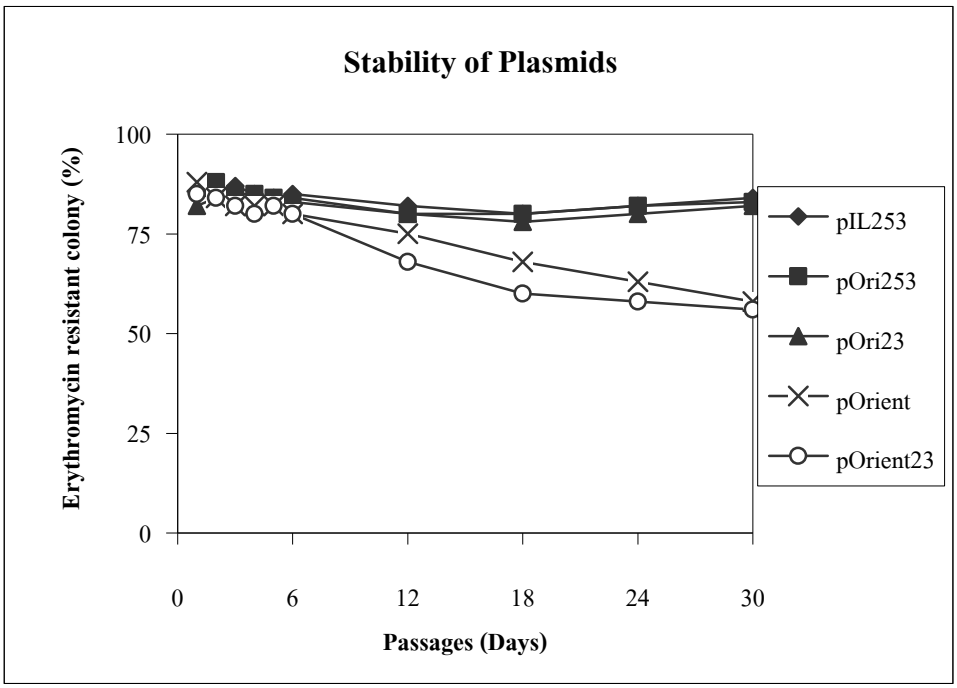

Figure 2. The segregrational stability graph of pIL253, pOri253, pOri23, pOrient and pOrient23 in *E. faecalis* JCM8726. For the first six daily passages and then every sixth passage, cells containing constructed plasmids were subcultured in MRS broth for 30 daily passages. Graph demonstrated the percentage of erythromycin resistant colonies at each point [(Er^r cfu/total cfu) × 100] under nonselective condition.

The stability of constructed plasmids under non-selective pressure (without Er) was shown in **Figure 2**. All of the plasmids were stably maintained in *E. faecalis* for at least 30 passages. However, either pOrient or pOrient23 seems to show segregrational instability. The ratio of the number of Er-resistant cells to the total

number of *E. faecalis* JCM8726 harboring pIL253 or pOri253 was stable, whereas, its clone harboring pOrient or pOrient23 gradually decreased **(Figure 2)**. The decrease in stability may be due to the insertion (24), as the insertion of *entAI* gene gave a selective pressure against cells expressing the bacteriocin N15. The expression of heterologous proteins in bacteria often provokes a toxic effect on the host cell (25) which, usually results in the loss of plasmid from the culture, the reduction in the amount of recombinant protein being expressed or decrease of culture viability. Moreover, the stability of constructed recombinant plasmid can be associated with a decrease in the copy number of plasmids containing inserts (26), influenced by temperature, pH, aeration, composition of the growth medium, on the dilution rate (27).

Functional Characterization of the Shuttle Vectors with Bacteriocin N15

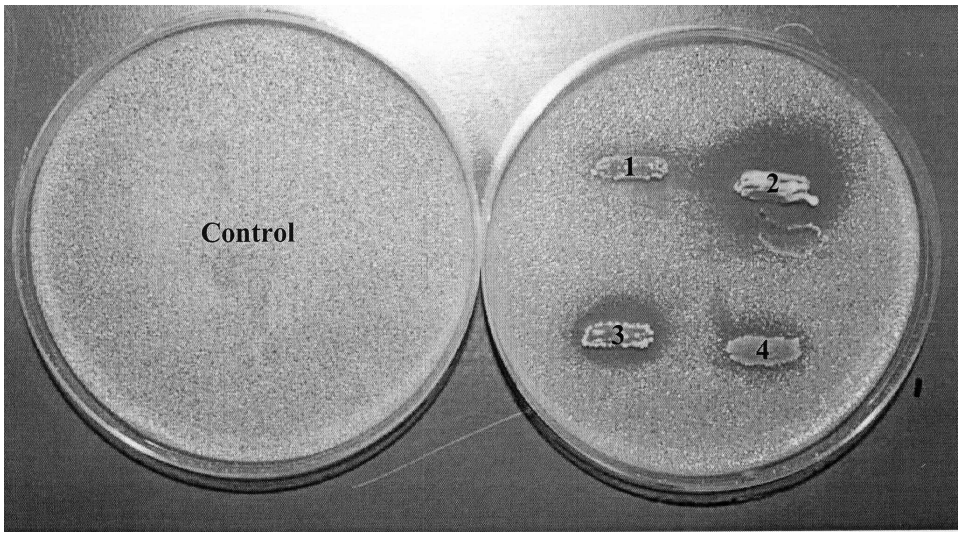

Figure 3. Antibacterial activity *Enterococcus faecalis* JCM 8726 transformants against the indicator bacteria *E. faecali* by the overlay method. Host strain JCM8726 (1, -); *Enterococcus faecium* N15 (2, ++++); JCM8726 harboring pOrient23 (3, ++) and JCM8726 harboring pOrient (4, +).

The *E. faecalis* JCM8726 harboring pOrient23 had higher antibacterial activity than parental *E. faecalis* JCM8726 or its clone harboring pOrient. The result was clearly shown by means of an overlay method in **Figure 3** but not by the agar-well diffusion method (data not shown). It might be affected by the diffusion ability of the bioactive molecule, the temperature or due the amounts of bacteriocin N15 produced. The difference in expression of heterologous genes may not only be caused by the higher copy number of shuttle vectors (28), but also caused by the properties of the lactococcal promoter *P23* (6,7,8,9,10,11,12,13,19,29) which, is composed of pOrient23. Que et al (19) reported the plasmid carrying lactococcal promoter *P23* expressed luciferase constitutively greater than did the non-*P23* containing plasmid. Interestingly, wild type *E. faecium* N15 shows more antibacterial activity than clones **(Figure 3)**. It might be synergistically affected by various types of bacteriocin

proteins, found in strain N15 (18), whereas, the exogenous bacteriocin that produced from strain JCM8726 is a single protein, bacteriocin N15 (pOrient / pOrient23), expressed in *E. faecalis* JCM8726 (bacteriocin non-producing strain). Although, slight activity was detected, the bacteriocin N15 appeared to be responsible for the anti-enterococcal activity of the transformed *E. faecalis* JCM8726. It is possible that a shuttle vector pOri253 may be used as an efficient cloning vector because it contains cloning sites derived from pIL253, which could be useful for insertion of foreign genes. The shuttle plasmid constructed here is useful for construction of various bacteriocin genes which will be stably maintained in enterococci. Moreover, it also can be used for chitinase-encoding genes to create a new strain with improved protective properties (antifungal activity).

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บทคัดย่อ

มณฑล เลิศคณาวนิชกุล

การสร้างพลาสมิดพาหะเพื่อการแสดงออกของจีนที่ควบคุมการผลิตแบคเทอริโอซินเอ็น 15 ที่มีผลในการยับยั้งเชื้อเอ็นเทอโรคอคคัส ฟีคาลิส

พลาสมิดพาหะ pOri253 ขนาด 6.09 กิโลเบส ถูกสร้างขึ้นด้วยการเชื่อมต่อชิ้นส่วน *oriColE1* ขนาด 0.89 กิโลเบสจากพลาสมิด pBluescript II KS เข้ากับพลาสมิด pIL253 ขนาด 5.2 กิโลเบส ที่ตำแหน่ง *Xba*I โดยยังมีตำแหน่งที่สามารถใส่จีนได้คือ *Eco*RI *Bam*HI *Sal*I และ *Pst*I พลาสมิด pOri253 ที่สร้างขึ้นมีคุณสมบัติในการควบคุมให้เชื้อ *Escherichia coli* และ *Enterococcus faecalis* ดื้อยาอีรีโธรมัยซิน นอกจากนี้พลาสมิดดังกล่าวยังสามารถอยู่ได้ใน *E. faecalis* JCM8726 เป็นระยะเวลานานโดยไม่มีการสูญหายไปแม้ว่าจะเลี้ยงในอาหารเลี้ยงเชื้อ Mann Rogosa Sharpe ที่ไม่มียาปฏิชีวนะ และเพื่อทำการศึกษาความสามารถของพลาสมิดในการควบคุมการแสดงออกของจีนจึงได้ใส่ promoter *P23* ของเชื้อแลคโตบาซิลลัสเข้าไปยังตำแหน่งที่เหมาะสมในพลาสมิด pOri253 ได้พลาสมิดชื่อ pOri23 ซึ่งทำให้ชื้อ *E. faecalis* JCM8726 สามารถมีการแสดงออกของจีนแบคเทอริโอซิน (*entAI*) ได้และมีคุณสมบัติในการต่อต้านเชื้อแบคทีเรียได้ดีกว่า *E. faecalis* JCM8726 ที่ใช้เป็นเซลล์เจ้าบ้าน รวมถึง *E. faecalis* ที่มีพลาสมิดชนิดอื่นที่ไม่มี promoter *P23* (pOrient) เมื่อทำการทดสอบด้วยวิธีราดทับด้วยเชื้อที่ใช้ศึกษา

สำนักวิชาสหเวชศาสตร์และสาธารณสุขศาสตร์ มหาวิทยาลัยวลัยลักษณ์ อำเภอท่าศาลา จังหวัดนครศรีธรรมราช 80160