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Short Communication

Evaluation of Transferability of R-Plasmid in Bacteriocin-Producing Donors to Bacteriocin-Resistant Recipients

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SUMMARY: Bacteriocin-producing *Escherichia coli* (donors) rapidly kill conventional recipient *E. coli* DH5α in conjugation experiments. To evaluate plasmid transferability of bacteriocin-producing donors, we established 2 different bacteriocin-resistant mutants derived from *E. coli* DH5α and used them as recipients. When the bacteriocin-resistant mutants were used in conjugation experiments, the transconjugant recovery from 20 bacteriocin-producing donors increased from 5% (1/20) to 65% (13/20), and the transfer frequencies increased. These results showed that bacteriocins inhibited the transfer of the R-plasmid from bacteriocin-producing donors. Thus, application of bacteriocin-resistant recipients might aid the evaluation of the potential transferability of plasmids from bacteriocin-producing donors.

Horizontal gene transfer via plasmids causes a rapid spread of antimicrobial resistance among bacteria (1). Transfer of plasmids has been studied by conjugation experiments. The broth-mating, filter-mating, and plate-mating methods have been used in standard conjugation experiments (2,3). Some strains of *Escherichia coli* produce bactericidal proteins known as bacteriocins: colicins and microcins (4). In in vitro conjugation experiments, the bacteriocin sensitivity of some conventional recipients *E. coli* K-12 derivatives may hinder successful plasmid transfer. To reduce the influence of this bactericidal effect, additional rinse steps were added to the filter-mating method (5). Bacteriocin-resistant mutants have been established in a previous study (6). Application of bacteriocin-resistant recipients may eliminate the bactericidal effect in conjugation experiments. In the present study, to evaluate the transferability of plasmids in bacteriocin-producing donors, we performed broth-mating conjugation experiments using bacteriocin-resistant recipients.

Twenty-one of 84 cefazolin-resistant *E. coli* isolates from broilers and layers showed bactericidal activity against an *E. coli* DH5α strain (rifampicin resistant) (Table 1). All the strains were susceptible to rifampicin. To determine the plasmid bearing the antimicrobial resistance genes (R-plasmid), we performed polymerase chain reaction (PCR) and Southern hybridization for the detection of the β-lactamase genes (7) and analysis of the plasmid profiles (8). Southern hybridization was performed by using digoxigenin (DIG)-PCR and DIG Nucleic Acid Detection Kits (Roche Diagnostics, Mannheim, Germany) as per the manufacturer's instructions. The DNA probes were constructed from the purified PCR products of β-lactamase genes ($bla_{\text{CMY-2}}$, $bla_{\text{CTX-M2}}$, and $bla_{\text{CTX-M14}}$). The localization and characterization of the β-lactamase genes are shown in Table 1.

To distinguish between bacteriocins and bacteriophages, we performed a slightly modified version of the classification assay described by Riley et al. (9). Briefly, each strain was grown overnight in Luria-Bertani (LB) broth at 35°C. A 1-mL aliquot of the overnight culture was added to 10 mL of LB broth and incubated at 35°C for 1 h; mitomycin C (6 μg) was then added and the culture was incubated at 35°C for 4 h with vigorous shaking. A 1-mL aliquot of the culture was centrifuged at 10,000 $\times$ *g* for 5 min. The supernatant was transferred to a microcentrifuge tube containing 33 μL of chloroform; this solution was vortexed and centrifuged again at 10,000 $\times$ *g* for 10 min. The supernatants were divided into 2 aliquots. Then, 1 aliquot was treated with trypsin (0.25 mg/mL) and incubated at 37°C for 30 min to inhibit the bacteriocin activity, whilst the other aliquot was frozen at -80°C for 48 h to inhibit the activities of bacteriophages. For the classification assays, *E. coli* DH5α was spread on the surface of an LB agar plate. Once the surface had dried, a 5-μL aliquot of the treated supernatant was spotted onto the same plate, and the plates were incubated at 35°C overnight. The bactericidal activities of the supernatants against *E. coli* DH5α were negated by trypsin digestion and were not affected by freezing. These results indicated that the bactericidal activities of 21 strains were caused by bacteriocins.

To establish the bacteriocin-resistant mutants, *E. coli* DH5α was subjected to mutant selection. Briefly, mutants were generated on LB agar plates containing the supernatants of strain 20-C-127, which was random-

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Table 1. Bacteriocin type and *bla* gene characterization of the bacteriocin-producing *E. coli* strains used in this study

Bacteriocin type	Patterns of bacteriocin susceptibility[1)]			Strain no.	Isolation year	*bla* gene	Plasmid size (kbp)[2)]
	DH5α	DH5α-I	DH5α-II				
I	−	+	+	21-C-57	2009	bla_{CMY-2}	110, **80**
				16-C-89	2004	bla_{CMY-2}	160, 150, **90**
				20-C-106	2008	bla_{CMY-2}	110, **80**
				19-C-56	2007	bla_{CMY-2}	120, **80**, 50
				18-L-111	2006	bla_{CMY-2}	160, **90**
				21-C-63	2009	bla_{CMY-2}	150, **100**
				16-L-27	2004	bla_{CMY-2}	120, **90**, 80
				20-C-122	2008	bla_{CMY-2}	**130**, 80, 50
				18-C-9	2006	bla_{CMY-2}	**120**, 50
				21-C-66	2009	bla_{CMY-2}	**80**, 40
				18-L-24	2006	$bla_{CTX-M14}$	**80**, 60
II	−	+	−	20-C-94	2008	bla_{CMY-2}	100, **90**, 70
				20-C-127	2008	bla_{CMY-2}	130, **90**, 50
				21-C-12	2009	bla_{CMY-2}	100, **70**
				21-C-3	2009	bla_{CMY-2}	**110**, 50
				17-C-68	2005	bla_{CTX-M2}	110, **80**
				18-C-24	2006	$bla_{CTX-M14}$	100, **80**
III	−	−	+	16-C-39	2004	bla_{CTX-M2}	90, 70, **40**
				17-C-09	2005	bla_{CMY-2}	100, 80, **70**
				16-L-26	2004	bla_{CMY-2}	**110**, 80
IV	−	−	−	17-L-89	2005	bla_{CMY-2}	**100**, 60

[1)]: + indicates growth with bacteriocins. − indicates not growth with bacteriocins.
[2)]: Bold type indicates *bla* bearing plasmid.

ly selected from the 21 bacteriocin-producing strains. All the colonies on these plates were subcultured, and the mutant resistant to the bacteriocin was designated DH5α-I. The susceptibilities of the DH5α-I to the bacteriocins derived from the 21 strains were determined using the classification assay described above. The DH5α-I was resistant to bacteriocins derived from 17 of the 21 strains (Table 1). Then, we established other mutants by employing the same selection method with the supernatants of strain 16-C-39, which showed bactericidal activity against DH5α-I. The mutant resistant to the bacteriocin derived from strain 16-C-39 was designated DH5α-II, and the bacteriocin susceptibility of this strain was determined. Table 1 shows the susceptibilities of the 2 bacteriocin-resistant mutants against bacteriocins from the 21 bacteriocin-producing strains. These were classified as bacteriocin type (BT)-I (n = 11), BT-II (n = 6), BT-III (n = 3), and BT-IV (n = 1).

Conjugation experiments were carried out using the broth-mating method with bacteriocin-resistant mutants as recipients. The appropriate mating pairs were determined on the basis of the bacteriocin susceptibility patterns. The BT-I- and BT-II-producing donors were mated with the DH5α-I. The BT-I-producing donors, that could not generate transconjugants with the DH5α-I were mated with the DH5α-II. The BT-III-producing donors were mated with the DH5α-II. In the control experiments, *E. coli* DH5α were mated as the recipients for comparing the transconjugant recovery. Overnight cultures of donor (20 μL) and recipient (20 μL) were mixed with 160 μL of fresh LB broth in a 96-well plate and incubated at 35°C overnight. Then, a 2-μL aliquot of this mixture was spotted on transconjugant-selective Mueller-Hinton (MH) agar plates containing rifampicin (50 μg/mL) and cefazolin (32 μg/mL) using a Microplanter inoculator (Sakuma Factory, Tokyo, Japan), and the plates were incubated at 35°C overnight. The conjugation experiments were repeated 3 times. The transfer of R-plasmid was confirmed as described above. When the bacteriocin-resistant mutant DH5α-I was used as a recipient, 73% (8/11) and 50% (3/6) of the BT-I- and BT-II-producing strains, respectively, yielded transconjugants (Table 2). When the bacteriocin-resistant mutant DH5α-II was used the recipient, 0% (0/3) and 67% (2/3) of BT-I- and BT-III-producing strains, respectively, yielded transconjugants. In total, 65% (13/20) of the bacteriocin-producing strains yielded transconjugants. However, when *E. coli* DH5α was used as a recipient, transconjugants were established in only 1 (5%) of the 20 donor strains. Therefore, when bacteriocin-resistant mutants instead of *E. coli* DH5α were used as recipients, the transconjugant recovery in broth-mating conjugation experiments with Microplanter inoculator showed a 13-fold increase.

We calculated the R-plasmid transfer frequencies in bacteriocin-producing strains after conjugation, as mentioned above. After 10-fold serial dilutions of the mixture, 100 μL of the mixture was inoculated on MH agar plates containing the appropriate antibiotics (donor-selective plates contained 32 μg/mL cefazolin, and transconjugant-selective plates contained 50 μg/mL rifampicin plus 32 μg/mL cefazolin). The plates were then incubated at 35°C overnight. The plasmid transfer frequencies were calculated by dividing the transconjugant count by the donor count. When *E. coli* DH5α

Table 2. Conjugation experiments involving bacteriocin-producing strains with different bacteriocin-resisitant mutants as recipients

Bacteriocin type	Bacteriocin-producing strain no.	Recipient used in conjugation experiment[1)]		
		DH5α	DH5α-I	DH5α-II
I	21-C-57	−	+	NT
	16-C-89	−	+	NT
	20-C-106	−	+	NT
	19-C-56	−	+	NT
	18-L-111	−	+	NT
	21-C-63	−	+	NT
	16-L-27	−	+	NT
	20-C-122	−	+	NT
	18-C-9	−	−	−
	21-C-66	−	−	−
	18-L-24	−	−	−
	Subtotal	0/11 (0%)	8/11 (73%)	0/3 (0%)
II	20-C-94	+	+	NT
	20-C-127	−	+	NT
	21-C-12	−	+	NT
	21-C-3	−	−	NT
	17-C-68	−	−	NT
	18-C-24	−	−	NT
	Subtotal	1/6 (17%)	3/6 (50%)	NT
III	16-C-39	−	NT	+
	17-C-09	−	NT	+
	16-L-26	−	NT	−
	Subtotal	0/3 (0%)	NT	2/3 (67%)
	Total	1/20 (5%)	11/17 (61%) 13/20 (65%)	2/6 (33%)

1): + indicates a resulting transconjugant. NT indicates not tested.

Table 3. Transfer frequencies of bacteriocin-producing strains

Bacteriocin-producing strain no.	Transfer frequency (transconjugant/donor)		
	Recipient used in conjugation experiment		
	DH5α	DH5α-I	DH5α-II
21-C-57	8.0×10^{-9}	1.8×10^{-1}	NT[1)]
16-C-89	2.3×10^{-8}	6.9×10^{-2}	NT
20-C-106	1.1×10^{-8}	2.0×10^{-2}	NT
19-C-56	4.0×10^{-8}	9.3×10^{-3}	NT
18-L-111	$<1.0\times10^{-10}$	5.8×10^{-3}	NT
21-C-63	9.1×10^{-8}	2.8×10^{-6}	NT
16-L-27	4.3×10^{-8}	5.0×10^{-7}	NT
20-C-122	2.5×10^{-8}	1.3×10^{-7}	NT
18-C-9	$<1.0\times10^{-10}$	6.7×10^{-8}	5.4×10^{-8}
21-C-66	$<1.0\times10^{-10}$	2.5×10^{-8}	1.8×10^{-8}
18-L-24	$<1.0\times10^{-10}$	$<1.0\times10^{-10}$	$<1.0\times10^{-10}$
20-C-94	1.8×10^{-7}	1.6×10^{-3}	NT
20-C-127	$<1.0\times10^{-10}$	2.0×10^{-2}	NT
21-C-12	$<1.0\times10^{-10}$	4.3×10^{-7}	NT
21-C-3	$<1.0\times10^{-10}$	6.3×10^{-8}	NT
17-C-68	$<1.0\times10^{-10}$	$<1.0\times10^{-10}$	NT
18-C-24	$<1.0\times10^{-10}$	$<1.0\times10^{-10}$	NT
16-C-39	$<1.0\times10^{-10}$	NT	5.9×10^{-6}
17-C-09	4.5×10^{-8}	NT	1.4×10^{-7}
16-L-26	$<1.0\times10^{-10}$	NT	8.6×10^{-8}

1): NT indicates not tested.

was used as a recipient, R-plasmid transfer frequency for the 20-C-94 strain was higher than those in other bacteriocin-producing strains (Table 3). The R-plasmid transfer frequencies in 17 of the 20 donors were increased by using the bacteriocin-resistant mutants as recipients. These results suggest that the apparent low frequencies of R-plasmid transfer in bacteriocin-producing donors are affected by the bacteriocins in conjugation experiments with *E. coli* DH5α. The range of R-plasmid transfer frequencies in the bacteriocin-producing donors varied ($<1.0\times10^{-10}$–1.8×10^{-1}). These results suggest that other factors, such as expression of transfer genes and helper plasmids (10), contributed to the transferability of the plasmid.

In conclusion, the applications of bacteriocin-resistant mutants as recipients might aid the evaluation of the potential transferability of R-plasmids in bacteriocin-producing donors.

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Conflict of interest None to declare.

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