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Plasmid Profiling and Curing of *Lactobacillus* Strains Isolated from the Gastrointestinal Tract of Chicken

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In this study, we assessed the susceptibility of 12 *Lactobacillus* strains, all of which had been isolated from the gastrointestinal tracts of chicken, to three antibiotics (chloramphenicol, erythromycin and tetracycline) used commonly as selective markers in transformation studies of lactic acid bacteria. Among these strains, 17%, 58%, and 25% were found to exhibit a high degree of resistance to 200 μg/ml of tetracycline, erythromycin, and chloramphenicol, respectively. Seven of the 12 *Lactobacillus* strains exhibiting resistance to at least 50 μg/ml of chloramphenicol or erythromycin, and five strains exhibiting resistance to at least 50 μg/ml of tetracycline, were subsequently subjected to plasmid curing with chemical curing agents, such as novobiocin, acriflavin, SDS, and ethidium bromide. In no cases did the antibiotic resistance of these strains prove to be curable, with the exception of the erythromycin resistance exhibited by five *Lactobacillus* strains (*L. acidophilus* I16 and I26, *L. fermentum* I24 and C17, and *L. brevis* C10). Analysis of the plasmid profiles of these five cured derivatives revealed that all of the derivatives, except for *L. acidophilus* I16, possessed profiles similar to those of wild-type strains. The curing of *L. acidophilus* I16 was accompanied by the loss of 4.4 kb, 6.1 kb, and 11.5 kb plasmids.

Key words: antibiotic resistance, curing agents, *Lactobacillus*, plasmid profile, plasmid curing

Lactobacillus is a commercially important bacterium with a wide variety of applications, both in the food industry and as a probiotic agent for the improvement of human health (Cebeci and Gürakan, 2003). The long history of its use in the food industry and its "generally regarded as safe" (GRAS) status render it a promising bacterial strain for genetic modification (Billman-Jacobe, 1996). Genetic modifications to *Lactobacillus* strains are normally targeted toward the improvement or augmentation of specific strain characteristics, such as the production of compounds antagonistic to common food pathogens, the ability to metabolize cholesterol or to tolerate acid or bile, and immune response-enhancing abilities (Kullen and Klaenhammer, 1999). Attempts have also been made to transform *Lactobacillus* strains into delivery vehicles for biological compounds, particularly for the delivery of enzymes into the gut of the host, thereby enhancing digestion (Billman-Jacobe, 1996). Under ideal conditions, these modified strains would benefit the host. However, under natural conditions, the performance of the modified *Lactobacillus* strains is frequently affected by indigenous plasmids, especially in cases in which the manipulations are plasmid-mediated. Incompatibility between the indigenous plasmids and the introduced plasmids is one of the principal factors contributing to plasmid instability within a host (Posno *et al.*, 1991). Most *Lactobacillus* strains, regardless of their source (plants, meat, silage, sourdough or gastrointestinal tract), harbor at least one indigenous plasmid, and often more (Pouwels and Leer, 1993). These plasmids may not only interfere with the stability of the recombinant plasmid, but may also harbor undesirable traits, e.g. antibiotic resistance (Posno *et al.*, 1991). Thus, before genetic modification studies can be considered with such a strain, it becomes necessary to eliminate any such indigenous plasmids. A variety of methods involving chemical and physical agents have been developed to eliminate plasmids, but the effectiveness of these methods has varied according to the strain involved (Ghosh *et al.*, 2000).

In our previous studies, we have isolated 12 *Lactobacillus* strains with probiotic characteristics. These strains have been employed as a probiotic for the improvement of growth performance in chickens (Jin *et al.*, 1998a, 1998b, 2000; Kalavathy *et al.*, 2003). These *Lactobacillus* strains may also constitute excellent candidates for genetic manipulation, and it may be possible to transform them into delivery vehicles for enzymes. However, it remains currently unknown as to whether these strains are, indeed, amenable to genetic manipulation. Hence, a

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preliminary study was conducted in order to determine the potential of these *Lactobacillus* strains as transformation hosts. The objectives of this study were to investigate the resistances of the 12 *Lactobacillus* probiotic strains to three antibiotics used commonly as selective markers in transformation studies, to eliminate any plasmid-mediated antibiotic resistance in the resistant strains, and, after the curing process, to compare the plasmid profiles of the cured derivatives with those of their respective wild-type strains.

Materials and Methods

Bacterial strains and growth conditions

The 12 *Lactobacillus* strains (wild-type) used in this study, namely, *L. crispatus* I12, *L. acidophilus* I16, *L. brevis* I23, *L. fermentum* I24, *L. fermentum* I25, *L. acidophilus* I26, *L. brevis* I211, *L. brevis* I218, *L. brevis* C1, *L. brevis* C10, *L. fermentum* C16 and *L. fermentum* C17, were isolated from the gastrointestinal tracts of local broiler chickens, and were identical to those described by Jin *et al.* (1996). All of the strains were grown in Man Rogosa Sharpe (MRS) broth (Oxoid, UK), and were incubated under anaerobic conditions in anaerobic jars, with gas-generating kits (Oxoid, UK) at 39°C.

Determination of antibiotic resistance

The susceptibility of the wild-type *Lactobacillus* strains to antibiotics was evaluated using a slightly modified version of the macrodilution broth method developed by Jones *et al.* (1985). Chloramphenicol, erythromycin, and tetracycline were the antibiotics used in this procedure. The concentrations of these tested antibiotics ranged from 3.125 to 200 μg/ml. An appropriate amount of antibiotic solution was separately added to the MRS broth, in order to obtain the desired antibiotic concentrations. Tubes containing MRS broth without antibiotics were used as a control. Each of the tubes were inoculated with a 16-h *Lactobacillus* culture at a 1% concentration (v/v), and were anaerobically incubated for 24 h at 39°C, which represents the optimal growth conditions of these strains. We also determined the minimum inhibitory concentration (MIC), which is the lowest concentration of antibiotic sufficient to completely inhibit the growth of the organism. As recommended by Jones *et al.* (1985), the observation of a faint haziness in the growth medium was not considered to represent growth, whereas the observation of definite turbidity was considered growth, as the drug had clearly failed to completely inhibit growth at that particular concentration. In addition to visual observations, we also evaluated bacterial growth with a spectrophotometer (DU-65, Beckman, USA). Turbidity of less than 0.15 at an OD of 600 nm was considered "no growth", and turbidity of 0.15 or greater was considered "growth". All experiments were conducted three times, each with triplicate.

Curing experiments

The wild-type *Lactobacillus* strains which proved resistant to 50 μg/ml of antibiotics or more were subjected to plasmid curing. Several chemical curing agents, such as novobiocin, SDS, acriflavin, and ethidium bromide, were used to cure the indigenous plasmid(s) of the *Lactobacillus* strains. Prior to curing, we determined the sublethal concentrations of the four curing agents for the *Lactobacillus* strains. The sublethal concentration was defined as the highest concentration allowing for the detectable, albeit reduced, growth of a *Lactobacillus* strain (OD_{600} = 0.15 - 0.4). The sublethal concentrations of novobiocin, SDS, acriflavin, and ethidium bromide for the *Lactobacillus* strains were found to be in the range of 1.8 to 40.0 μg/ml, 5 to 11 mg/ml, 12 to 28 μg/ml, and 2.5 to 6.5 μg/ml, respectively.

The strains were sub-cultured every 24 h in MRS broth containing a sublethal concentration of the respective curing agent. At appropriate intervals (7, 14, and 21 days), the cultures were serially diluted, then plated onto MRS agar (Oxoid, UK). After 48 h of incubation under anaerobic conditions at 39°C, the emergent colonies were duplicated onto fresh MRS agar and MRS agar containing 10 μg/ml of either chloramphenicol, erythromycin, or tetracycline. Colonies that failed to grow on the MRS antibiotic plates after incubation were considered to have been cured, and their duplicates on the MRS agar were extracted and maintained in MRS broth for further analysis. The curing rate (%) was calculated according to the following formula:

$$\text{Curing rate } (\%) = \frac{(A - B)}{A} \times 100$$

where,

A = number of colonies formed on MRS agar
B = number of colonies formed on MRS antibiotic agar

Plasmid analysis

Ten ml of 6- to 8-h *Lactobacillus* cultures were used for plasmid extraction. The cells were harvested by 10 minutes of centrifugation at 3,000 × *g* at 4°C, and washed twice in 2 ml of 10 mM Tris-HCl buffer, at a pH of 8.0. After the final wash, the cells were collected by centrifugation, and 200 μl of freshly-prepared lysozyme solution (6 mg/ml in 10 mM Tris HCl, pH 8.0) was added to the cell pellet. The subsequent plasmid extraction procedure was conducted according to the method described by O'Sullivan and Klaenhammer (1993).

Results

Antibiotic susceptibility test

All of the *Lactobacillus* strains exhibited varying degrees of resistance to chloramphenicol, erythromycin, and tetracycline (Table 1). The MICs, which ranged from 12.5 to >200 μg/ml for chloramphenicol and erythromycin, and

50 to >200 μg/ml for tetracycline, were determined to be dependent on the strain involved. The tetracycline MICs were as follows: 50 μg/ml for *L. crispatus* I12, *L. brevis* I23, I211 and I218, and *L. fermentum* I25; 100 μg/ml for *L. fermentum* I24; and 200 μg/ml for *L. acidophilus* I26, *L. brevis* C1 and C10, and *L. fermentum* C17. *Lactobacillus acidophilus* I16 and *L. fermentum* C16 were resistant to tetracycline even at a concentration of 200 μg/ml, the highest concentration employed in the present study. Of the 12 *Lactobacillus* strains, seven strains, namely *L. acidophilus* I16 and I26, *L. fermentum* I24, C16 and C17, and *L. brevis* C1 and C10, were resistant to 200 μg/ml of erythromycin. The other five strains, *L. crispatus* I12, *L. brevis* I23, I211 and I218, and *L. fermentum* I25, were susceptible to erythromycin at a minimum concentration of 12.5 μg/ml. These five strains were also found to be susceptible to chloramphenicol at the same concentration. Three strains, *L. brevis* C1 and C10, and *L. fermentum* C16, exhibited resistance to 200 μg/ml of chloramphenicol. The other four strains, *L. fermentum* I24 and C17 and *L. acidophilus* I16 and I26, exhibited varying degrees of susceptibility to chloramphenicol (MICs of 25, 50, 100 and 200 μg/ml, respectively).

Table 1. MICs (μg/ml) of *Lactobacillus* strains in MRS broth containing various concentrations of tetracycline, erythromycin, and chloramphenicol

Strain	Tetracycline	Erythromycin	Chloramphenicol
L. crispatus I12	50	12.5	12.5
L. acidophilus I16	>200	>200.0	100.0
L. acidophilus I26	200	>200.0	200.0
L. brevis I23	50	12.5	12.5
L. brevis I211	50	12.5	12.5
L. brevis I218	50	12.5	12.5
L. brevis C1	200	>200.0	>200.0
L. brevis C10	200	>200.0	>200.0
L. fermentum I24	100	>200.0	25.0
L. fermentum I25	50	12.5	12.5
L. fermentum C16	>200	>200.0	>200.0
L. fermentum C17	200	>200.0	50.0

Table 2. Curing rates (%) of erythromycin resistance in *Lactobacillus* strains by various curing agents

Strain	Curing rate (%)			
	Novobiocin	Acriflavin	Ethidium bromide	SDS
L. acidophilus I16	3.3	0.0	0	0
L. fermentum I24	64.0	0.0	0	0
L. acidophilus I26	9.0	0.0	0	0
L. brevis C1	0.0	0.0	0	0
L. brevis C10	0.0	1.6	0	0
L. fermentum C16	0.0	0.0	0	0
L. fermentum C17	2.1	0.0	0	0

Plasmid curing

Seven of the 12 wild-type *Lactobacillus* strains, namely, *L. acidophilus* I16 and I26, *L. fermentum* I24, C16 and C17, and *L. brevis* C1 and C10, exhibited resistance to concentrations of 50 μg/ml and above of all of the tested antibiotics. These seven strains were consequently subjected to a plasmid curing procedure, in order to cure their respective antibiotic resistance properties. Of these seven *Lactobacillus* strains, only five (*L. acidophilus* I16 and I26, *L. fermentum* I24 and C17, and *L. brevis* C10) could be successfully cured of their erythromycin resistance (Table 2). The chemical agents failed to eliminate chloramphenicol and tetracycline resistance, even after prolonged sub-culturing (every 24 h for 28 days) in sublethal concentrations of the individual curing agents. The attempt to use mixtures of the curing agents, such as novobiocin and acriflavin, novobiocin and SDS, novobiocin and ethidium bromide, acriflavin and SDS, acriflavin and ethidium bromide, or SDS and ethidium bromide, also failed to eliminate resistance to chloramphenicol and tetracycline.

Plasmid profile

The plasmid profiles of five wild-type strains and their cured derivatives are shown in Fig. 1. Plasmids ranging in size from 2.5 to 20 kb were detected in all the examined strains. All of the cured derivatives, except for the cured derivative of *L. acidophilus* I16, exhibited plasmid profiles similar to those of their corresponding wild-type strains. For *L. acidophilus* I16, the loss of erythromycin resistance was accompanied by the loss of plasmids with approximate sizes of 4.4 kb, 6.1 kb, and 11.5 kb.

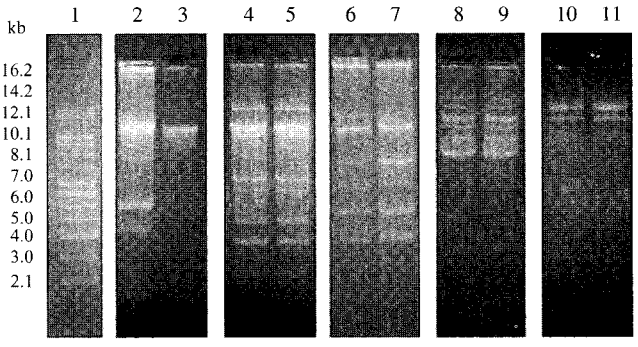

Fig. 1. Plasmid profiles of wild-type strains of *Lactobacillus* and their cured derivatives. Lane 1: supercoiled DNA standard ladder; lane 2: *L. acidophilus* I16WT; lane 3: *L. acidophilus* I16C; lane 4: *L. fermentum* I24WT; lane 5: *L. fermentum* I24C; lane 6: *L. acidophilus* I26WT; lane 7: *L. acidophilus* I26C; lane 8: *L. brevis* C10WT; lane 9: *L. brevis* C10C; lane 10: *L. fermentum* C17WT; lane 11: *L. fermentum* C17C. WT = wild-type strain; C = cured derivative

Discussion

We assayed 12 *Lactobacillus* strains with regard to their susceptibility to three antibiotics, namely chloramphenicol, erythromycin, and tetracycline. This study focused primarily on these three antibiotics, because they serve as selective markers in transformation studies of lactic acid bacteria (Kok *et al.*, 1984; Dao and Ferretti, 1985; Heng *et al.*, 1997). These antibiotics have also been listed as antibiotics which are authorized for veterinary medicine in Europe for the treatment of food-producing animals, including avian, bovine, piscine, and porcine species (Schwarz and Chaslus-Dancla, 2001).

All of the tested *Lactobacillus* strains exhibited varying degrees of resistance to chloramphenicol, erythromycin, and tetracycline. The MICs, which ranged from 12.5 - >200 μg/ml for chloramphenicol and erythromycin, and 50 - >200 μg/ml for tetracycline, appeared to be strain dependent. Antibiotic resistance has been frequently found in *Lactobacillus* species isolated from a variety of sources. Chloramphenicol and erythromycin resistances have been found in *L. reuteri* isolated from poultry (Lin *et al.*, 1996), whereas multiple-antibiotic resistances (to cefoxitin, aztreonam, amikacin, gentamicin, kanamycin, streptomycin, sulphamethoxazole, trimethoprim, co-trimoxazole, metronidazole, polymyxin B and colistin sulphate) have been observed in *Lactobacillus* strains isolated from both the human gastrointestinal tract and dairy products (Charteris *et al.*, 1998). The antibiotic resistance profile of microorganisms depends largely on the previous exposure histories of the microorganisms, e.g. to the type of antibiotics, period of exposure, and contact with other resistant microorganisms (Schwarz and Chaslus-Dancla, 2001). Thus, antibiotic resistance profiles differ between microorganisms isolated from different sources. In the present study, 25% of the strains were found to be highly resistant to chloramphenicol (≥200 μg/ml), 58% to erythromycin (≥200 μg/ml) and 17% to tetracycline (≥200 μg/ml). Son *et al.* (1996) reported that 36%, 80%, and 93% of *Lactobacillus* strains isolated from local chickens were resistant to chloramphenicol (≥30 μg/ml), erythromycin (≥30 μg/ml), and tetracycline (≥30 μg/ml), respectively. Teuber *et al.* (1999) also reported that 14.3% and 57.1% of the 14 *Lactobacillus* strains (*L. acidophilus*, *L. fermentum*, *L. crispatus*, *L. casei* and *L. salivarius*) isolated from broiler chicken crops exhibited resistance to erythromycin and tetracycline, respectively. Antibiotic resistance can be attributed to enzymatic inactivation, decreased intracellular drug accumulation, a lack of target sites for antibiotic drugs, or the presence of genes that confer antibiotic resistance (Schwarz and Chaslus-Dancla, 2001). Resistant genes tend to reside either on the self-replicative extrachromosomal plasmids (Fraser *et al.*, 2000), or within the bacterial genome (Endrz *et al.*, 1999).

The multiple-antibiotic resistance exhibited by the 12 *Lactobacillus* strains in the present study suggests that these strains would not make good transformation hosts, unless all or some of the antibiotic resistance exhibited by the strains could be eliminated. This would be necessary to facilitate the transformation process *via* plasmid, one of the most popular and simplest transformation procedures used with *Lactobacillus* species (Gasson and Fitzgerald, 1994). Most of the plasmid vectors constructed for *Lactobacillus* utilize chloramphenicol, erythromycin, and tetracycline as selective markers. The broad host range plasmid, pGK12, harbors both chloramphenicol and erythromycin-resistant genes; pNZ12, pLP825, and pFX3 harbor chloramphenicol-resistant genes; pLP3537 and pNCKH103 contain an erythromycin-resistant gene; and pSA3 carries genes which are resistant to chloramphenicol, tetracycline, and erythromycin (Kok *et al.*, 1984; Xu *et al.*, 1991; Leer *et al.*, 1992). The transformants are generally selected by the use of 10 - 50 μg/ml of antibiotics. Based on the chloramphenicol resistance profiles exhibited by the 12 *Lactobacillus* strains in the present study, seven strains (*L. crispatus* I12; *L. brevis* I23, I211 and I218; *L. fermentum* I24, I25 and C17) qualified to be transformation hosts. Also, on the basis of their erythromycin and tetracycline resistance profiles, five strains (*L. crispatus* I12; *L. brevis* I23, I211 and I218; and *L. fermentum* I25) would make acceptable transformants. The remaining *Lactobacillus* strains would require considerable further treatment in order to eliminate their antibiotic resistance characteristics, were they to be used as transformation hosts.

Several chemical curing agents, including novobiocin, acriflavin, ethidium bromide, and SDS, have been successfully used in the curing of other gram-positive bacterial plasmids (Caro *et al.*, 1984). Therefore, these same compounds were employed in the present study, in an attempt to eliminate plasmid-mediated antibiotic resistance. However, these chemicals proved to be fairly ineffective with regard to the curing of plasmids from the *Lactobacillus* strains. Of the seven *Lactobacillus* strains (*L. acidophilus* I16 and I26, *L. fermentum* I24, C16 and C17, *L. brevis* C1 and C10) subjected to plasmid curing, five strains (*L. acidophilus* I16 and I26, *L. fermentum* I24 and C17, and *L. brevis* C10) were successfully cured of their erythromycin resistance. The chemical agents used failed to eliminate chloramphenicol and tetracycline resistance in the tested strains. The effectiveness of curing methods depends on the nature of the bacterial host and/or plasmids, in which some methods work better in a system than others (Ghosh *et al.*, 2000). Bringel *et al.* (1989), Rajini Rani and Mahadevan (1992), and Giles *et al.* (1995) reported a similar ineffectiveness of curing agents in *L. plantarum*, *Aeromonas* sp. and *Pseudomonas* sp. Nevertheless, successes have also been reported, in which some plasmids in *L. fermentum*, *L. reuteri*, and *L. acido-*

philus have been cured successfully with curing agents (Fons *et al.*, 1997; Vescovo *et al.*, 1982). Among the four selected curing agents, novobiocin was the most effective, as it proved capable of curing erythromycin resistance in four *Lactobacillus* strains; evidencing the highest curing rate for *L. fermentum* I24 (64%). It was also noted that the cured derivatives were susceptible to 10 μg/ml of erythromycin, whereas the wild-type strains exhibited resistance at >200 μg/ml of erythromycin. Further attempts to cure the other strains under different conditions, such as by combinations of sublethal concentrations of different curing agents, all also proved ultimately unsuccessful. The effectiveness of novobiocin has also been reported by Ruiz-Barba *et al.* (1991) who observed that novobiocin could induce the loss of extrachromosomal DNA at a higher frequency in *L. plantarum* when compared to SDS or ethidium bromide.

Most *Lactobacillus* species, regardless of their source (plants, meat, silage, sourdough or gastrointestinal tract), harbor at least one indigenous plasmid (Pouwels and Leer, 1993). The functions of these plasmids have classically been correlated with phenotypical properties, including drug resistance, carbohydrate metabolism, amino acid metabolism, and bacteriocin production (Pouwels and Leer, 1993). Although antibiotic resistance is frequently linked to plasmids (Adwan *et al.*, 1998), exceptional cases have also been reported. In the present study, the elimination of erythromycin resistance in *L. fermentum* I24, C10, and C17, and *L. acidophilus* I26, was not associated with any plasmid loss. Nwosu and Ladapo (1999) suggested that more than one antibiotic resistance mechanism might be present, due to their observation that not all antibiotic-resistant strains harbored plasmids. This hypothesis is in agreement with the results of Vescovo *et al.* (1982), in which the loss of two plasmids from *L. acidophilus* strain A274 via the curing process was not found to be related to antibiotic resistance. Wegener and Schwarz (1993) also determined that resistances to penicillin in 44 strains, and kanamycin in 15 strains of *Staphylococcus hyicus,* were not related to plasmids. By way of contrast, the erythromycin resistance of *L. acidophilus* I16 in the present study may have been conferred by one of the three plasmids (4.4, 6.1 or 11.5 kb) that were lost in the cured derivative. However, this will remain unconfirmable pending further analysis with regard to the characteristics of the plasmid. Most antibiotic resistance in *Lactobacillus* strains have ultimately been determined to be plasmid-mediated. Danielsen (2002) has located the gene conferring tetracycline resistance on *L. plantarum* 5057 in one of the four plasmids that are present in the strain. The erythromycin-resistant gene in *L. reuteri* and *L. fermentum*, and the chloramphenicol-resistant gene in *L. reuteri,* are both also associated with plasmids (Lin *et al.*, 1996; Fons *et al.*, 1997; Whitehead and Cotta, 2001).

According to the results of the present study, we are able to conclude that seven wild-type *Lactobacillus* strains (*L. crispatus* I12, *L. brevis* I23, I211 and I218, *L. fermentum* I25, I24 and C17) and five cured derivatives (*L. acidophilus* I16C and I26C, *L. fermentum* I24C and C17C, and *L. brevis* C10C), all of which were found to be susceptible to ≤50 μg/ml of tetracycline, erythromycin, and/or chloramphenicol (the normal concentration used for selection of *Lactobacillus* transformants) appear to harbor potential as transformation hosts.

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