

Detection of a plasmid containing Cadmium resistance gene for lactic acid bacteria isolated from foods

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Abstract

This study aimed to isolate lactic acid bacteria (LAB) carrying cadmium resistant genes on plasmids from foods. A total of 30 fermented food samples were collected from local markets and supermarkets. Isolation of cadmium resistant LAB colonies was performed by spread plate technique using MRS agar medium supplemented with cadmium (MRS-Cd). One hundred and fifty cadmium resistant isolates were randomly selected and subjected to plasmid extraction. Only one isolate (LAB-V12) from a fermented vegetable harbored a single plasmid of about 9 Kb. 16S rDNA sequencing data showed that LAB-V12 was likely to be *Lactobacillus plantarum*. The results from plasmid curing demonstrated that a cured strain of the LAB-V12 was sensitive to cadmium. Hence, probably cadmium resistant marker is plasmid mediated. The plasmid containing cadmium resistance gene was tested for stability in its host. It was observed that the LAB-V12 could maintain the plasmid after approximately 100 generations of growth without cadmium selection. The plasmid might be further developed to be a food-grade cloning vector that is useful for genetic modification of LAB.

Keywords: Lactic acid bacteria, Fermented food, Cadmium resistance gene, Plasmid

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Introduction

Lactic acid bacteria (LAB) are commonly used in food industries especially in fermented food production. There are 2 major types of LAB associated food fermentation which are natural fermentation and fermentation with LAB starter culture. The natural fermentation is initiated by LAB naturally associated with food materials; therefore, quality of final products is inconsistent and difficult to control. The fermentation with LAB starter culture is a controlled fermentation initiated by specific types and amounts of LAB. Therefore, quality of final products from this type of fermentation is consistent and controllable.

The fermentation with LAB starter culture is, in part, a starting point of the development of LAB by genetic engineering to obtain LAB with desired characteristics to use as starter cultures. Several genetically modified LAB (GM-LAB) starter cultures have been reported

such as LAB capable of producing exopolysaccharide (Papagianni, 2012), low-calorie sugars (Ladero et al., 2007) and bacteriocins (Gasson, 1993). Most of the GM-LAB have been generated by using appropriate DNA vectors to carry desired genes into LAB. However, the DNA vectors commonly used to genetically engineer LAB are considered as non-food-grade DNA vectors because they have antibiotic resistance genes as selectable marker genes. Consequently, GM-LAB generated by this type of DNA vectors are considered as non-food-grade GM-LAB which pose a safety concern to consumers and tend to be banned for use in food industries in many developed countries (de Vos, 1999).

In order to use DNA vectors to genetically generate food-grade GM-LAB, the DNA vectors have to be food-grade and all of their components have to be generally recognized as safe (GRAS). Presently, food-grade DNA vectors are developed from plasmids of



LAB naturally residing in food consumed in everyday life. These plasmids usually contain cadmium resistance gene, copper resistance gene and nisin resistance gene that can be used as selectable markers in food-grade DNA vectors (He et al., 2012).

The objective of this study was to isolate cadmium resistant LAB from food. From these LAB, the strain carrying a plasmid with a cadmium resistance gene was selected. The selected LAB strain was subjected to plasmid stability test and molecular identification. The plasmid obtained from this study may be useful for the development of a food-grade-DNA vector in the future.

Material and Methods

Isolation of cadmium resistant LAB from food

Thirty fermented food samples collected from local markets were used as sources of cadmium resistant LAB. Ten g of each sample were mixed with 90 ml of phosphate buffer solution (pH 7) by a stomacher (Seward stomacher 400) for 1 min. Ten ml of the liquid part of the mixture were subjected to ten-fold serial dilution. Each dilution was spread on to MRS (deMan, Rosaga and Sharpe) agar containing 1 mM cadmium chloride (MRS-CdCl₂). After incubation at 37°C for 24 h, 150 single colonies of cadmium resistant LAB grown on MRS-CdCl₂ were randomly selected and streaked for purification. The purified cadmium resistant LAB were kept in MRS-CdCl₂ broth containing 20% glycerol at -20°C until use.

Plasmid isolation from LAB

For Isolation of plasmids from LAB, the protocol described by Anderson and McKay (1983) was used. However, rather than using cultures grown at 32°C in M-17 broth as recommended in the protocol, cultures grown at 37°C in MRS broth (for cadmium sensitive LAB) or MRS-CdCl₂ broth (for cadmium resistant LAB) were used for plasmid isolation in this study. For positive and negative controls, *Tetragenococcus halophilus* with and without 4.5 kb plasmid pUBU were used, respectively (Phumkhachorn and Rattachaikunsopon, 2016). The plasmid isolation results were checked by agarose gel electrophoresis using 1% of agarose gel.

Plasmid curing

The cadmium resistant LAB isolate with a plasmid was grown in MRS-CdCl₂ broth containing ethidium bromide (final concentration of 150 µg/ml) at 37°C for

24 h. The culture was spread onto MRS agar and incubated at 37°C for 24 h. Each single colony grown on the MRS agar was picked up by a sterile toothpick and inoculated on two different agar, MRS agar and MRS-CdCl₂ agar. After incubation at 37°C for 24 h, the cadmium sensitive LAB colony that could grow on MRS agar but not on MRS-CdCl₂ agar was selected for plasmid purification. If the loss of cadmium resistance ability and the loss of plasmid coincidentally occur, it means that the lost plasmid carries a cadmium resistance gene.

Plasmid stability

The cadmium resistant LAB isolate with a plasmid was grown in MRS-CdCl₂ broth at 37°C for 24 h. The bacterial cells were pelleted by centrifugation and washed 3 times by MRS broth. The washed cells were cultured in MRS-CdCl₂ broth at 37°C for 24 h and subcultured (0.1% inoculum) daily into the same medium for 100 generations. Each round of subculturing was estimated to be about 10 generations. After 100 generations of cultivation, the bacterial culture was plated on MRS agar and incubated at 37°C for 24 h. One hundred single colonies were randomly selected from the MRS agar to test for survival ability on MRS-CdCl₂ agar and for the presence of the indigenous plasmid by plasmid isolation.

Identification of cadmium resistant LAB

For identification of cadmium resistant LAB, the protocol of 16S rDNA sequence analysis described by Butprom et al. (2013) was used. The universal primers used in this study were fD1 (5' AGAGTTTGATCCTGGCTCAG 3') and rP2 (5' ACGGCTACCTTGTTACGACTT 3'). The 16S rDNA obtained from PCR was sequenced by BioDesign (Pathumthani, Thailand) and analyzed by the NCBI Blast program.

Results

Isolation of cadmium resistant LAB from food

From 30 samples of fermented food, cadmium resistant LAB were isolated on MRS-CdCl₂ agar. For each fermented food sample, 5 colonies were randomly selected. Therefore, a total of 150 colonies of cadmium resistant LAB were selected for further study (Table 1).



Table 1. Food samples and LAB isolates

Food samples	LAB isolates
Fermented pork rind (Sample No.1-5)	N1, N2, N3, N4, N5, N6, N7, N8, N9, N10, N11, N12, N13, N14, N15, N16, N17, N18, N19, N20, N21, N22, N23, N24, N25
Fermented pork sausage (Sample No.6-10)	S1, S2, S3, S4, S5, S6, S7, S8, S9, S10, S11, S12, S13, S14, S15, S16, S17, S18, S19, S20, S21, S22, S23, S24, S25
Fermented beef sausage (Sample No.11-15)	M1, M2, M3, M4, M5, M6, M7, M8, M9, M10, M11, M12, M13, M14, M15, M16, M17, M18, M19, M20, M21, M22, M23, M24, M25
Fermented fish (Sample No.16-20)	P1, P2, P3, P4, P5, P6, P7, P8, P9, P10, P11, P12, P13, P14, P15, P16, P17, P18, P19, P20, P21, P22, P23, P24, P25
Fermented vegetable (Sample No.21-25)	V1, V2, V3, V4, V5, V6, V7, V8, V9, V10, V11, V12, V13, V14, V15, V16, V17, V18, V19, V20, V21, V22, V23, V24, V25
Kimchi)Sample No.26-30)	K1, K2, K3, K4, K5, K6, K7, K8, K9, K10, K11, K12, K13, K14, K15, K16, K17, K18, K9, K20, K21, K22, K23, K24, K25

Plasmid isolation from cadmium resistant LAB

When 150 isolates of cadmium resistant LAB were subjected to plasmid isolation, only one isolate, LAB-V12, was shown to have a plasmid. The size of the plasmid was approximately 9 kb (Fig. 1).

Plasmid curing

After culturing LAB-V12 in MRS-CdCl₂ broth containing ethidium bromide at 37°C for 24 h, the culture was spread onto MRS agar and incubated at 37°C for 24 h. One hundred single colonies were randomly selected and each of them was inoculated on MRS agar and MRS-CdCl₂ agar. Only one colony was shown to be mutated by ethidium bromide and lose its cadmium resistance ability, indicated by inability to grow on MRS-CdCl₂ agar (Fig. 2). The mutated (cadmium sensitive) LAB was designated LAB-V12mt1. The plasmid isolation revealed that LAB-V12mt1 did not have a plasmid.

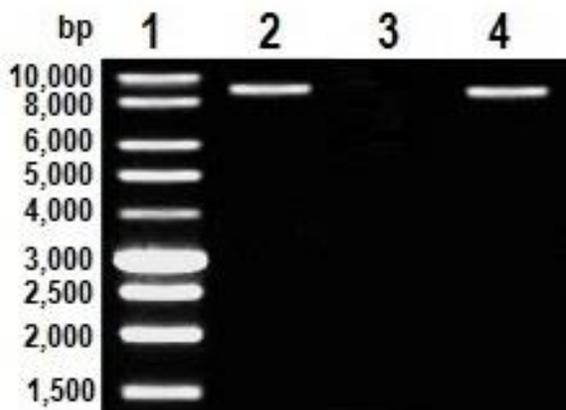


Fig. 1. Agarose gel electrophoresis: Lane 1 = 1 kb DNA ladder; Lane 2 = 9 kb plasmid (approx.) of LAB-V12; Lane 3 = absence of plasmid band indicates complete curing of plasmid of LAB-V12; Lane 4 = stability of plasmid in LAB-V12 without cadmium selection for 100 generations of plasmid in LAB-V12 without cadmium selection for 100 generations

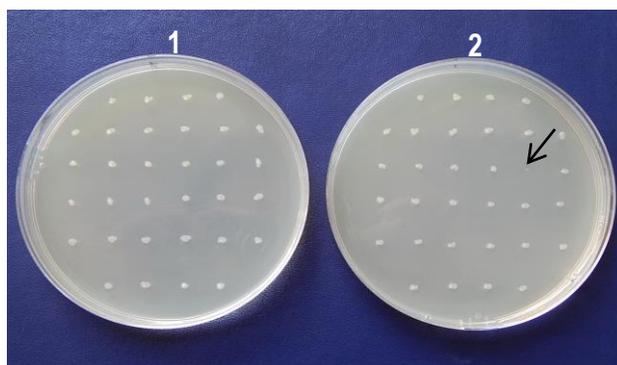


Fig. 2. Isolation of cadmium sensitive mutant strain (LAB-V12mt1) created by treating LAB-V12 with ethidium bromide: 1 = plate without cadmium (MRS agar); 2= plate containing cadmium (MRS-CdCl₂ agar)

Plasmid stability

In to determine the stability of the plasmid in LAB-V12, it was cultured in MRS without cadmium for 100 generations by serial subculturing. The culture was then plated on MRS agar. When 100 colonies randomly selected from the MRS agar were patched on MRS-CdCl₂ agar, all of them were found to be able to grow on the MRS-CdCl₂ agar. By plasmid isolation, all of the selected LAB colonies still carried a plasmid with approximately 9 kb in size (Figure 1), indicating

that the plasmid was highly stable.

Identification of LAB-V12

The PCR product from the amplification of 16S rDNA of LAB-V12 was approximately 1500 bp in size. When its sequence was aligned to the known bacterial 16S rDNA sequences in GenBank database using NCBI Blast program, it showed 99% similarity to 16S rDNA sequence of *Lactobacillus plantarum* strain FM02 (accession number MG913360).

Discussion

LAB are a group of bacteria consisting of many genera and species. Generally, they can be found in fermented food due to their importance in food fermentation. Therefore, in this study, local fermented food samples were used as sources of LAB. There are several selective media for selection of LAB such as MRS, M-17 and Rogosa media on which different groups of LAB can grow. For MRS medium, it was normally used to select *Lactobacillus*, *Streptococcus*, *Pediococcus* and *Leuconostoc*. Since in this study we intended to select cadmium resistant LAB, an appropriate amount (1 mM) of cadmium chloride was added into MRS. The cadmium resistance ability of LAB usually relies on cadmium resistance gene that can be located on plasmid or chromosome (Oger et al., 2003). If a plasmid containing a cadmium resistance gene is found in one of the selected cadmium resistant LAB, the plasmid can be further used for food-grade DNA vector development.

Cadmium resistance ability in bacteria was initially found in *Staphylococcus aureus*. Two genes, *cadA* and *cadC*, were found to be responsible for the ability (Nucifora et al., 1989). Further investigation demonstrated that these two genes were also associated with cadmium resistance ability of several species of LAB such as *Lactococcus lactis*, *Streptococcus thermophilus* and *Lactobacillus plantarum*. The mechanism that these bacteria used to protect themselves to cadmium toxicity was found to be dependent on efflux pump. This pump resided on bacterial cell surface and pumped cadmium from intracellular cytoplasm to extracellular environment. This pump was driven by energy from ATP hydrolysis; therefore, it was an ATP-dependent efflux pump (Silver, 1996).

In this study, among 150 cadmium resistant LAB isolated from fermented food, only one isolate, LAB-V12, was found to have a plasmid carrying a cadmium

resistance gene. Further molecular identification revealed that it was likely to be *Lactobacillus plantarum*. Besides LAB-V12, several *L. plantarum* strains were previously found to be cadmium resistant such as *L. plantarum* CCFM8610 (Zhai et al., 2014), *L. plantarum* B-578 and *L. plantarum* S1 (Kirillova et al., 2017). However, cadmium resistance ability is not restricted to species *L. plantarum*, other species of LAB have been found to have this ability as well; for examples, *Streptococcus thermophilus* and *Lactococcus lactis* (Zhai et al. 2017).

Plasmid curing is a commonly used method for examining whether a gene responsible for a specific phenotype is located on a plasmid (Raja and Selvam, 2009). If the loss of a specific phenotype and the loss of a plasmid coincidentally occur, it can be concluded that the lost plasmid carries a gene that is responsible for the specific phenotype. In this study, ethidium bromide was chosen to be used as a plasmid curing agent among other plasmid curing agents such as acridine orange, quinacrine and novobiocin because the plasmid curing experiment in this study was performed according to our protocol that was specifically developed for LAB (Phumkhachorn et al., 2007). Since in this study the loss of cadmium resistance ability and the loss of the 9 kb plasmid coincidentally occurred in LAB-V12mt1, it suggested that a cadmium resistance gene was likely to be located on the 9 kb plasmid.

The stability of the 9 kb plasmid of LAB-V12 was studied and found that it was highly stable in bacterial cells because the loss of the plasmid was not evident after 100 generations of subculturing. This ability of plasmid is an important factor to be considered when it is used for development of a DNA vector. The DNA vector with high stability will favor the introduction of a desired gene into host cells and produce a genetically consistent mutant. Since the 9 kb plasmid of LAB-V12 was highly stable, it has potential for further use in DNA vector development.

Conclusion

From the isolation of cadmium resistant LAB from different fermented food samples, LAB-V12, isolated from fermented vegetable, was the only cadmium resistant LAB isolate that carried a plasmid.

The plasmid was found to be highly stable and approximately 9 kb in size. Plasmid curing experiment demonstrated that the plasmid carried a cadmium resistance gene. Molecular identification revealed that



LAB-V12 was likely to be *Lactobacillus plantarum*. Since the plasmid of LAB-V12 was originated from food and had a cadmium resistance gene that could be used as a selectable marker, it might be further developed to be a food-grade DNA vector that was useful for genetic modification of food-grade GM-LAB.

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