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Identification of Antibiotic-Resistance Genes from Lactic Acid Bacteria in Indonesian Fermented Foods

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Lactic acid bacteria (LAB) are known to have an important role in food fermentation and are thought to have health-promoting abilities such as probiotic properties. In this study, LAB were isolated from Indonesian fermented foods such as dadih (fermented buffalo milk), tempoyak (fermented durian), bekasam (fermented meat), and tape ketan (fermented glutinous rice). Those isolates were investigated for their resistance to two antibiotics: chloramphenicol and erythromycin. Recent efforts in food science have sought to identify genetic markers for antibiotic resistance within LAB strains, so that these genes can be selected for genetic modification. Such research is currently being directed toward the development of food-grade vectors (plasmid). The aim of this study was to screen LAB isolated from Indonesian traditional fermented foods, for chloramphenicol and erythromycin resistance. In this study, a total of 120 LAB samples were taken from traditional Indonesia fermented foods, and were tested for resistance to chloramphenicol and erythromycin. The results show that three LAB strains remained resistant to doses of up to 5 µg/mL chloramphenicol, while the LAB strain *Lactobacillus plantarum* showed resistance to the antibiotic erythromycin up to a concentration of 15 µg/mL.

Key words: antibiotic resistance, fermented food, food grade vectors, lactic acid bacteria

INTRODUCTION

Antibiotic resistance (AR) has become a global concern as a result of increasing use of antibiotics in humans as well as animals. Compounding this risk is the possibility of horizontal transfer among bacteria in nature so that resistant bacteria may spread zoonotically from animals to humans or between animal species and populations. Studies on selection and dissemination of AR have focused mainly on species with medical clinical relevance (Tjaniadi *et al*. 2003; Deashinta *et al*. 2007). However, interest is lately expanding to include studies on food microbes, with greater attention to probiotic lactic acid bacteria (LAB). LAB are of considerable economic significant because of their use in industrial food fermentation processes, especially those that have probiotic properties and are promoted as a "healthy food". Moreover, interest in antibiotic resistance traits as selectable markers for the genetic modification of LAB, is being directed toward generating food grade vectors (Mathur & Singh 2005; Pan *et al*. 2011).

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Fermented foods may be important vehicles for large amounts of living LAB to enter the human body. Although LAB are generally recognized as safe (GRAS), it is necessary to evaluate the antibiotic resistance of these microbes in different fermented foods (Pan *et al*. 2011). Some LAB strains showed acquired transferable antibiotic resistance traits. They may represent a potential source for the spread of AR genes when added to different kinds of probiotic products (D'Aimmo *et al*. 2007). Several LAB species isolated from different fermented foods have been investigated for their resistances to antibiotics, including erythromycin (Tannock *et al.* 1994; Fons *et al.* 1997; Lin & Chung 1999; Gfeller *et al*. 2003; Sudhamani *et al.* 2007) and chloramphenicol (Biet *et al*. 1999; Park *et al*. 2004; Sudhamani *et al*. 2007).

Fermented foods common in Indonesia include dadih (fermented buffalo milk), tempoyak (fermented durian), bekasam (fermented meat), and tape ketan (fermented glutinous rice) as described in Table 1. These have been consumed for centuries, but no investigation has been conducted yet to assess the genes that determine antibiotic resistance in LAB isolated from those traditional foods. The aim of this study, therefore, is to screen antibiotic resistance

Table 1. Indonesian traditional fermented foods and their processing

Fermented foods	Processing
Bekasam	Flesh was mixed with 10-20% salt (w/v) and ground, roasted rice, then fermented (in sealed container) for 14 days
Tempoyak (Durian meat)	Durian (*Durio zibethinus*) flesh was mixed with 2.5% salt (w/v) and placed in a sealed container to ferment for about 7 days
Dadih	Fermented from fresh raw buffalo milk placed in bamboo tubes capped with banana leaves
Tape ketan	Glutinous rice was steamed, followed by inoculation with ragi tape, then fermented for about 1-2 days until an acid-alcoholic taste was achieved

(in this case erythromycin and chloramphenicol) in LAB strains from Indonesian fermented foods, and to identify the genes that can serve as a selectable marker for such resistance. Our results are not only useful for fermented food safety assessment and evaluation, but will also be advantageous in providing basic information to develop and generate LAB cloning vectors.

MATERIALS AND METHODS

Bacterial Strain and Growth Condition. A total of 120 samples of LAB isolated from dadih, tempoyak, bekasam, and tape ketan were obtained from stock cultures taken from the Research Center for Biotechnology Indonesian Institute of Sciences (LIPI). Samples were stored at -70 °C in de Mann Rogosa and Sharpe (MRS) broth in the presence of 20% glycerol. All LAB were maintained by subculturing in MRS (Oxoid, UK) broth medium supplemented with 0.02% (w/v) sodium azide from 1% (v/v) inoculum and incubated overnight at 37 °C.

Determination of Antibiotic Resistance and Bacterial Sensitivity. To determine the phenotypic antibiotic resistance, 1% (v/v) inoculum LAB was grown in MRS broth medium containing 0.02% sodium azide and 1 µg/mL erythromycin (Sigma, USA), and/or 2 µg/mL chloramphenicol (Gold Biotechnology, USA). The culture was incubated overnight (16 h) at 37 °C. LAB which acquire antibiotic resistance are able to grow in medium, and the presence of such resistant LAB will cause a change in the color of medium.

Bacterial sensitivity to antibiotics was also determined by the viable cell count technique. MRS broth medium was treated in different dishes with different concentrations of erythromycin (Sigma, USA) and/or chloramphenicol (Gold Biotechnology, USA). Each dish was then inoculated with 1% (v/v) of each sample of LAB and incubated overnight at 37 °C. The LAB cultures were serially diluted in a 1:10 ratio. Next, 100 µL of appropriate dilutions were spread on agar plates and the plates were incubated at 37 °C for 24-48 h. If colonies appeared on an agar plate, that LAB sample was classified as antibiotic resistant.

DNA Extraction. LAB isolates from the strains classified as antibiotic-resistant were cultured in MRS broth (pH 7.0) for 24 h. Bacterial cells were collected by centrifugation at 6,000 rpm for 10 min. The genomic or chromosome DNA was extracted as previously described with modification (Zhu *et al*. 1993). The pellet was resuspended with TE buffer (10 mM Tris–HCl pH 8.0, 1 mM EDTA) containing 60 mg/mL lysozyme and then incubated at 37 °C for 1 h. After incubation, 200 µL of 10% sodium dodecyl sulfate, 100 µL of 5 M NaCl and 80 µL of 10% CTAB were added. The mixture was then incubated at 68 °C for 30 min and an equal amount of chloroform (1:1, v/v) was added. Centrifugation was conducted at 13,000 rpm for 10 min. The supernatant was collected and 1:1 (v/v) ethanol was added. The mixture was inverted and then centrifuged at 13,000 rpm for 10 min. After being air-dried, the DNA was dissolved in TE buffer containing 10 µg/mL RNAse and was stored at -20 °C until use.

Plasmid DNA was extracted from the LAB samples using plasmid MiniKit (Qiagen, USA) according to manufacture's protocol. However, lysis cells where modified using lysozyme as mentioned above.

PCR Amplification for 16S rRNA Identification. For 16 S rRNA sequencing, primers 8F (5-AGAGTTT GATCATGGCTCAG-3; positions 8 to 27) and 15R (5-AAGGAG GTG ATC CAA CCG CA-3; positions 1541 to 1522) were used to amplify the full length of bacterial 16S rRNA (Chao *et al.* 2008). For each 25 uL PCR, we used 400 nM of each primer, 1 U of Taq polymerase, and 10 ng of the genomic DNA template. The PCR conditions were 96 °C for 5 min; 35 cycles consisting of 96 °C for 1 min, 55 °C for 3 min, 72 °C for 1 min, 72 °C for 7 min. The PCR products were subjected to gel electrophoresis in 1% agarose gel, followed by ethidium bromide staining.

PCR Detection of Antibiotic Resistance Genes. Chromosomes and plasmid DNA were used

as templates to detect the chloramphenicol and erythromycin resistance genes. Primer sequences used in this step are listed in Table 2. The PCR products were then analyzed by electrophoresis gel agarose 2%.

DNA Sequencing and Phylogenetic Analysis. The PCR product was sequenced by 1st BASE company service. Similarity searches with sequences were performed by online BLAST analysis (http://blast.ncbi.nlm.gov/Blast.cgi). For phylogenetic analysis, sequences were aligned by using the CLUSTAL X Software (Thompson *et al.* 1997). A phylogenetic tree was constructed by the neighbor-joining method (Saitou & Nei 1987) and the *Streptococcus pyogenes* strain was used as an outgroup. The stability of the relationship was assessed by bootstrap resampling. Bootstrap analysis was performed for 1000 trials in accordance to clustal X program.

RESULTS

Screening of Antibiotic Resistance of LAB. In the present study, 120 isolates of LAB from four different Indonesian traditional fermented foods: dadih (65 isolates), tape ketan (20 isolates), bekasam (34 isolates), and tempoyak (1 isolate) were screened for their resistance to chloramphenicol (2 µg/mL) and erythromycin (1 µg/mL) as shown in Table 3.

Table 3. Screening of LAB in different fermented foods for resistance to chloramphenicol and erythromycin

Fermented foods	Total isolates	Chloramphenicol	Erythromycin
Dadih	65	30	4
Tape ketan	20	9	9
Bekasam	34	13	3
Tempoyak	1	1	-

Table 2. Oligonucleotide primers used in this study

Primer pairs and their sequences (5-3)	Annealing temperature (ºC)	Amplicon size (bp)	Reference
erm-F : CCG GGC CCA AAA TTT GTT TGA T erm-R : AGT CGG CAG CGA CTC ATA GAA T	55	726	Lee & Morrison 1999
ermB-F : CAT TTA ACG ACG AAA CTG GC ermB-R : GGA ACA TCT GTG GTA TGG CG	52	405	Jensen *et al.* 1999
ermC-F : ATC TTT GAA ATC GGC TCA GG ermC-R : CAA ACC CGT ATT CCA CGA TT	51	294	Jensen *et al.* 1999
Tn554-F : AAG CGG TAA ACC CCT CTG AG Tn554-R : TCA AAG CCT GTC GGA ATT GG	55	440	Jensen *et al.* 1999
cat-F : CCT GCC ACT CAT CGC AGT cat-R : CCA CCG TTG ATA TAT CCC	52	623	Lu Pan *et al.* 2011
catIP501-F : GGA TAT GAA ATT TAT CCC TC catIP501-R : CAA TCA TCT ACC CTA TGA AT	50	486	Guerra *et al.* 2001

Table 4. PCR detection of antibiotic resistance genes in plasmids or chromosomes

Strain	erm		ermB		ermC		Tn554		cat		catIP501	
	P	C	P	C	P	C	P	C	P	C	P	C
Lactobacillus plantarum												
D2	-	-	-	-	-	-	-	-	+	-	-	-
T3	/	-			/	-	/	-	/		/	-
T8	-	-	-	+	-	-	-	-	+	-	-	-
S12	-	-	-	-	-	-	/	-	/	-	/	-
S34			-	-	-	-	-	-	+	+	-	-
Lactobacillus fermentum												
DH1	-	-	-	-	-	-	-	-	-	-	-	-
Pediococcus acidilactici												
S23	-	-	-	-	+	+	+	+	+	-	/	-
DH7	-	-	-	-	-	-	/	+	/	-	/	-

P and C represent "plasmid" and "chromosome", respectively; "+" and "-" represent positive and negative results of PCR detections, respectively. "/" means that PCR was not performed because either no plasmid was isolated from the strain or the strain was not resistant to the relevant antibiotic. Antibiotic resistance genes are labeled erm, ermB, ermC, and Tn554 for erythromycin, and labeled cat and catIP501 for chloramphenicol.

Table 5. Identification of potential LAB isolates from Indonesian traditional fermented foods

Isolate	Species	Identity (%)	Acession No.
S12	*Lactobacillus plantarum*	99	JN560843.1
S34	*Lactobacillus plantarum*	100	AL935263.2
D2	*Lactobacillus plantarum* subsp. *plantarum*	99	HQ615669.1
T3	*Lactobacillus plantarum*	99	AL935263.2
T8	*Lactobacillus plantarum*	100	AL932563.2
DH1	*Lactobacillus fermentum*	98	GU213430.1
DH7	*Pediococcus acidilactici*	97	EF059987.1
S23	*Pediococcus acidilactici*	100	FJ844982.1

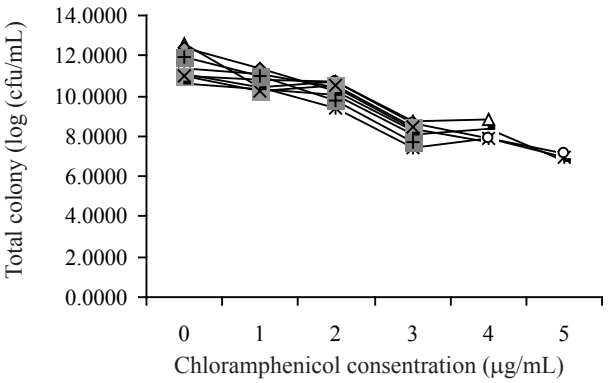

Figure 1. Viability of LAB strains exposed to several concentrations of chloramphenicol. S12, S23, DH7, S34, D2, T3, T8, DH1.

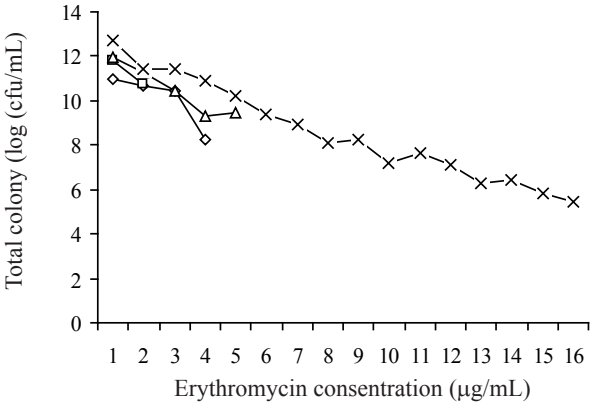

Figure 2. Viability of LAB cells exposed to several concentrations of erythromycin. D2, S23, T3, T8.

Antibiotic resistance tests indicated that 54 isolates were resistant to chloramphenicol and 16 isolates were resistant to erythromycin. The highest number of chloramphenicol resistant isolates came from dadih (30 isolates), while the highest number of erythromycin resistant isolates were found in tape ketan (9 isolates).

Various Levels of Antibiotic Resistance in Different LAB Strains. Among 120 isolates, eight potential isolates were selected and subjected to further molecular identification via 16S rRNA sequence. They were identified as *Lactobacillus plantarum, L. fermentum,* and *Pediococcus acidilactili* (Table 4). In order to screen the isolates further, bacterial sensitivity to chloramphenicol and erythromycin was determined by viable cell method, using agar medium containing various concentrations of these antibiotics. The number of growth colonies indicated their resistance ability. Moreover, antibiotic resistance genes of these eight strains were assessed by PCR detection using specific primers (Table 5).

Antibiotic resistance of these potential strains for chloramphenicol and erythromycin are shown in Figure 1 and 2, respectively. Figure 1 shows that three strains remained resistant up to 5 µg/mL chloramphenicol, while the highest antibiotic resistance for erythromycin remain occur at concentration of 15 µg/mL is *Lactobacillus plantarum* T8 (Figure 2).

Phylogenetic Relationship. The phylogenetic tree is shown in Figure 3, and the statistical reliability of the tree topology was evaluated by bootstrap analysis. The results showed that the LAB strains had bootstrap values of 990-1000, indicating a close relationship among potential strains based on further Clustal X and NJ Plot analysis.

DISCUSSION

Antibiotics have been widely used to promote growth in livestock for over 60 years, leading to the selection of antibiotic resistant bacteria that spread throughout the environment. These bacteria may reside in or on fruits, vegetables and animal feed and end up in the fermented food (Devirgilis *et al.* 2008). Some investigations have reported the role of LAB as reservoirs for dissemination of AR in some fermented foods (Pan *et al.* 2011). It has been known that traditional fermented foods play an important role in the food systems in Indonesia. However, no investigation has been conducted to assess and evaluate the antibiotic resistance of LAB in Indonesia fermented foods. In this study, a total of 120 LAB samples from traditional Indonesian fermented foods were tested for resistance to chloramphenicol and erythromycin.

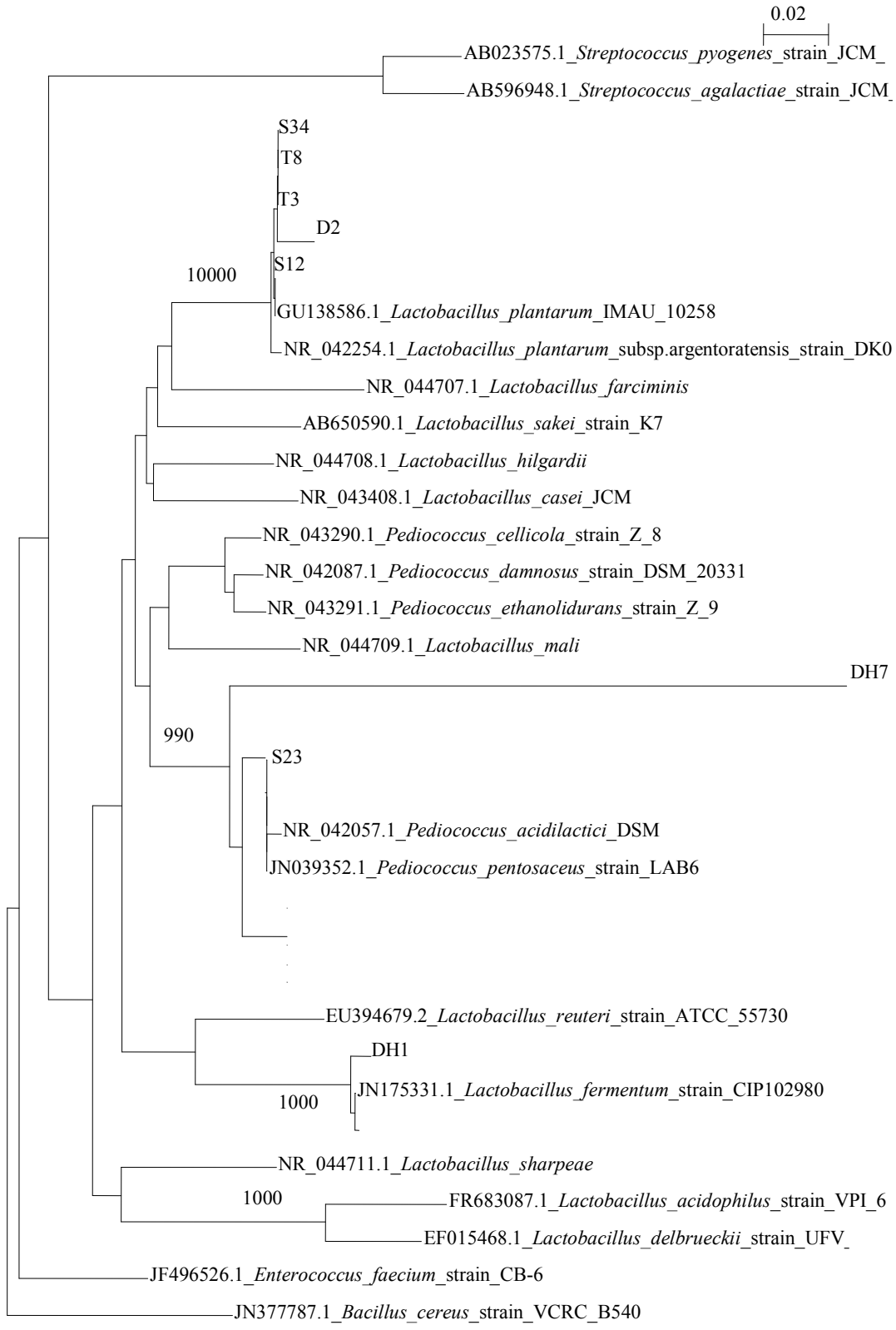

Figure 3. Phylogenetic tree of eight potential isolates LAB tolerant to chloramphenicol and erytromycin.

The presence of antibiotic resistance genes were investigated in eight potential isolates that have been shown previously in Figure 1 and 2, and identified as shown in Table 4. Table 5 shows those LAB strains in which genes for antibiotic resistance have been detected, as well as the viable cell result affected by the presence of these resistance genes. Although it has been shown that *Lactobacillus* spp. is generally susceptible to chloramphenicol, erythromycin, and tetracycline (Zhou *et al.* 2005; Rojo-Bezares *et al.*

2006; D`Aimmo *et al.* 2007), our researchers found strains of *Lactobacillus plantarum* S34, *L. plantarum* DH1, and *Pediococcus acidilactici* DH7 that were resistant to chloramphenicol up to 5 ug/mL, which is in agreement with the presence of *ermB* gene in (*L. plantarum* T8) and *ermB* and *ermC* and *Tn554* genes (in *P. acidilactici* S23 and DH7) encoding erythromycin. Interestingly, the strains with similar resistance traits are closely related, as illustrated in our phylogenetic tree. Moreover, it has been reported that *ermB* gene was also found in *L. plantarum,* another LAB strain resistant to erythromycin, that had been isolated from Chinese fermented food (Pan *et al.* 2011). On the other hand, chloramphenicol resistance ability was affected by the presence of *cat* gene in strains *L. plantarum* and *P. acidilactici* encoding resistance to Cm^r. These AR genes were detected in plasmid and/or chromosome.

Genetic engineering of LAB could have a great positive impact on the food and pharmaceutical industries. The genetic engineering techniques can be used to improve product quality or create novel therapeutic strains. The safe use of genetically modified LAB requires the development of food-grade cloning systems composed solely of DNA from the homologous host, or from GRAS organisms, and that do not rely on antibiotic markers. Therefore, the need for new food-grade genetic engineering tools provides a rationale for the development of cloning vectors derived from LAB cryptic plasmids. Cryptic plasmids are extra chromosomal DNA elements that encode no recognizable phenotype besides their replication functions (von Wright & Shibakov 1998; Shareck *et al*. 2004). One strategy to construct vectors is to use the replicons of small cryptic plasmids and incorporate into them selectable markers. In this regard, generating a LAB cloning vector system using vectors especially derived from LAB plasmids, with antibiotic resistant traits would be very promising. It is considered attractive with regard to the safety aspects associated with the use of LAB recombinant.

In conclusion, the present study showed AR of some LAB found in Indonesian fermented food to clinically important antibiotics erythromycin and chloramphenicol. This indicates the high gene transfer frequency of the microbes in Indonesia foods. Such studies would be useful to safety assessment and control of fermented food in Indonesia. Moreover, importantly the information of AR genes will be the basis for generating LAB cloning vector.

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