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A Simple Method to Generate Chromosomal Mutations in *Lactobacillus plantarum* Strain TF103 to Eliminate Undesired Fermentation Products

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Abstract

Gram-positive bacteria have been explored to convert lignocellulosic biomass to biofuel and bioproducts. Our long-term goal is to create genetically engineered lactic acid bacteria (LAB) strains that convert agricultural biomass into ethanol and other value-added products. The immediate approaches toward this goal involve genetic manipulations by either introducing ethanol production pathway genes or inactivating pathways genes that lead to production of undesired byproducts. The widely studied species *Lactobacillus plantarum* is now considered a model for genetic manipulations of LAB. In this study, *L. plantarum* TF103 strain, in which two of the chromosomal L-*ldh* and D-*ldh* genes are inactivated, was used to introduce additional mutations on the chromosome to eliminate undesired fermentation products. We targeted the acetolactate synthase gene (*als*) that converts pyruvate to acetolactate, to eliminate the production of acetoin and 2,3-butanodial. A pBluescript derivative containing sections of the *als* coding region and an erythromycin resistance gene was directly introduced into *L. plantarum* TF103 cells to create mutations under selection pressure. The resulting erythromycin resistant (Em^r) TF103 strain appears to have chromosomal mutations of both the *als* and the adjacent *lysP* genes as revealed by polymerase chain reaction and Southern blot analyses. Mutations were thus generated via targeted homologous recombination using a Gram-negative cloning vector, eliminating the use of a shuttle vector. This method should facilitate research in targeted inactivation of other genes in LAB.

Index Entries: Lactic acid bacteria; *als* and *lysP* mutant; ethanol; acetoin; 2,3-butanodial; acetolactate synthase.

†Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the names by USDA implies no approval of the product to the exclusion of others that may also be suitable.

Introduction

Metabolic engineering often requires the modification of the bacterial genome by inactivation of undesired genes and products. The creation of a genetic knockout mutant usually involves the replacement of a chromosomal gene by a mutated version via homologous recombination. However, gene inactivation studies in Gram-positive bacteria have been limited as a result of the lack of mature recombinant DNA technology in these rather difficult hosts when compared with the well-developed systems in Gram-negative bacteria.

An example of a genetic knockout study in a Gram-positive Group B *Streptococcus* (GBS) involved a two-step procedure as described by Li *(1)*. The first step is a single crossover, resulting in recombinant Kanamycin and chloromphenical (Km^rCm^r) cells after growth of transformed host cells at a nonpermissive temperature. The second step involved further growth under culture conditions without antibiotics, which allowed the second double crossover, resulting in Km^rCm^s colonies that carry a mutant copy of the targeted gene. This strategy requires multiple cloning and selection steps that lead to the double crossover event. First, the kanamycin gene was inserted into the target gene in a cloning vector. Then, the fusion DNA was subcloned into a specific shuttle vector containing the thermosensitive region (Ts) and chloromphenical resistance gene (Cm^r) and introduced into the GBS host cells. Under the nonpermissive growth temperature, the xeno-plasmid can be forcefully integrated into the host chromosome. The second recombination event, between the chromosomal target gene and its deleted copy, resulted in segregational loss of the excised vector, producing a chlorom phenical-sensitive phenotype. Therefore, the excision step can generate either the wild-type gene or a deletion of the target gene in the chromosome *(1)*. Targeted mutations of L-*ldh* and D-*ldh* genes were created in a similar fashion in the Gram-positive bacterium *Lactobacillus plantarum*, that resulted in Cm^r-Em^s TF103 strain *(2)*.

Another method is to introduce an internal fragment of the target gene into a suicide vector incapable of replicating in the organism of interest and then select for a single homologous recombination event. This results in the insertion of the entire plasmid into the target gene by Campbell-type integration, flanked by two truncated copies of the target gene. A single crossover that led to targeted gene inactivation has been reported in *Lactococcus lactis (3)*, *Lactobacillus helveticus* (*4*), and *Lactobacillus pentosus* (*5*).

The long-term goal of this project is to create genetically engineered LAB strains that convert agricultural biomass into ethanol and other value-added products *(6,7)*. *L. plantarum* is a lactic acid bacterium that ferments glucose to a variety of secondary end products including lactate, ethanol, acetoin, 2,3-butanediol, acetate, and mannitol *(8)*. Extensive genetic studies have been reported using *L. plantarum*, and therefore it is considered a

Table 1
Oligonucleotides Used in This Study

als2306	GA**CTCTAGA**TTCTGGTCAAGTTCAGCGTG
als3423	ACC**AAGCTT**GCCATCATTCCAGATCAAA
als3884	ACC**AAGCTT**GCAGCTAGAATAGTTGCGGG
als4922	CAA**CTCGAG**ACCCAGCCCGTTTAAAGACT
als4467	TAA**CTCGAG**AAAACGAGCGCAATCAGACT
als2669	ACG**AAGCTT**GCTTTCGTCACTTCTGGTG
als3140	ACG**AAGCTT**ACAACGGGTCAGTGATGACA

The bold nucleotides indicate corresponding restriction enzyme sites introduced in the primers.

model system for genetic engineering *(9,10)*. The rationale for this project is based on the L-*ldh and* D-*ldh* double knock-out studies by Ferain *(2,8)*; the mutant strain produced mainly acetoin, as well as other end products including ethanol, 2,3-butanediol, and mannitol *(8)*. The inactivation of the *als* gene, which converts pyruvate to acetolactate, would block the down-stream production of acetoin and 2,3-butanediol (from acetolactate), and therefore might allow pyruvate to be rerouted toward ethanol and/or mannitol production when both L-*ldh* and D-*ldh* are inactivated *(11)*. In this study, we report the generation of targeted mutations on the chromosome of *L. plantarum* TF103 via homologous recombination by using the nonreplicative, Gram-negative pBluescript cloning vector.

Materials and Methods

Bacterial Strains and Growth Conditions

Escherichia coli strain *Dh5α* cells were grown at 37°C in Luna Bertani (LB) medium supplemented with 100 μg/mL ampicillin and/or 35 μg/mL chloramphenicol when necessary. *L. plantarum* NCIMB8826 derivative strain TF103 was provided by Ferain *(2)*. This strain, defective for both D- and L-lactate dehydrogenase activities, was grown in Deman Rogosa Sharpe (MRS) broth with chloramphenicol (10 μg/mL) at 110 rpm and 37°C.

Cloning of Deleted Copy of the als *Gene Into pBluescript*

The preparation of chromosomal DNA from *L. plantarum* strain TF103 was performed using a Bactozol Kit (Molecular Research Center, Inc., Cincinnati, OH) as described in the manufacture's protocol. Polymerase chain reaction (PCR) primers (Table 1) were designed according to the published *L. plantarum* sequences *(10)*. Basic molecular biology techniques were performed as described *(12)*. *L. plantarum* strain TF103 genomic DNA was used as template in following PCR amplifications. PCR primers als2306 and als3423 were used to amplify an 1117-bp fragment. Following

digestion with *Xba*I/*Hin*dIII, the fragment was cloned into *Xba*I/*Hin*dIII sites of pBluescript to generate pBlue2306$_{3423}$. Similarly, the 1038 bp PCR products from primers als3884 and als4922 were cloned into the *Hin*dIII and *Xho*I sites of pBlue2306$_{3423}$ to generate pBlue2306$_{3423}$-3884$_{4922}$, with a 461 bp sequence deleted between als3424 and 3884. The pBlue2306$_{3423}$-3884$_{4922}$ was digested with *Nhe*I (cut at 2606) and *Hin*dIII. The resulting larger vector fragment was ligated with the 1 kb *Xba*I-*Hin*dIII erythromycin fragment from pEI2 *(13)* to generate pBlue2306$_{2606}$Em3884$_{4922}$, which has an erythromycin resistance gene inserted between als2306-2606 and als3884-4922 sequences. All the constructs were confirmed by sequencing using the ABI Prism 310 and the ABI Prism Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer, Foster City, CA). Sequence analyses were performed with the SDSC biology workbench (http://www.sdsc.edu/Research/biology/) and NCBI (http://www.ncbi.nlm.nih.gov/).

Generation and Confirmation of the Em^r TF103 als *Mutant*

The genetic *als* deletional mutant was obtained by electroporating *L. plantarum* TF103 cells with the pBluescript derived plasmid pBlue2306$_{2606}$Em3884$_{4922}$ as described previously *(14)*. The transformants were selected for erythromycin resistance on MRS plates supplemented with 5 μg/mL erythromycin. Five Em^r mutants were selected and genomic DNA was prepared as described *(6)*. Genomic PCR of the mutant strains was performed using two primers (als2306 and als4667) located on each side of the deleted region of the *als* gene. The original pBlue2306$_{2606}$Em3884$_{4922}$ plasmid DNA and parent TF103 genomic DNA were used as templates in control reactions.

Southern Hybridization

Alkaline transfer of *Eco*RI digested genomic DNA on 0.8% agarose gels to nylon membranes was done as described in ref. *(12)*. Probes were labeled by PCR using appropriate primers (*see* Table 1) and digoxigenin-dUTP of Roche Applied Science (Penzberg, Germany). As indicated in Fig. 1, probe 1 was labeled from the intentionally deleted 363 bp fragment amplified from TF103 genomic DNA by using primers als2306 and als2669, probe 2 was labeled from the 283 bp PCR fragment amplified using primers als3140, als3423; and probe3 was labeled from the 783 bp PCR product using primers als3884 and als4667. The DNA sequences of all three probes were confirmed by sequencing. Filters were hybridized and washed using the DIG Easy Hyb kit (Roche) as instructed by the manufacturer. The digoxigenin-labeled DNA molecular weight marker set III was purchased from Roche. Immunological detection of DIG-labeled nucleic acids was carried out using the DIG Luminescent Detection Kit (Roche Applied Science, Penzberg, Germany).

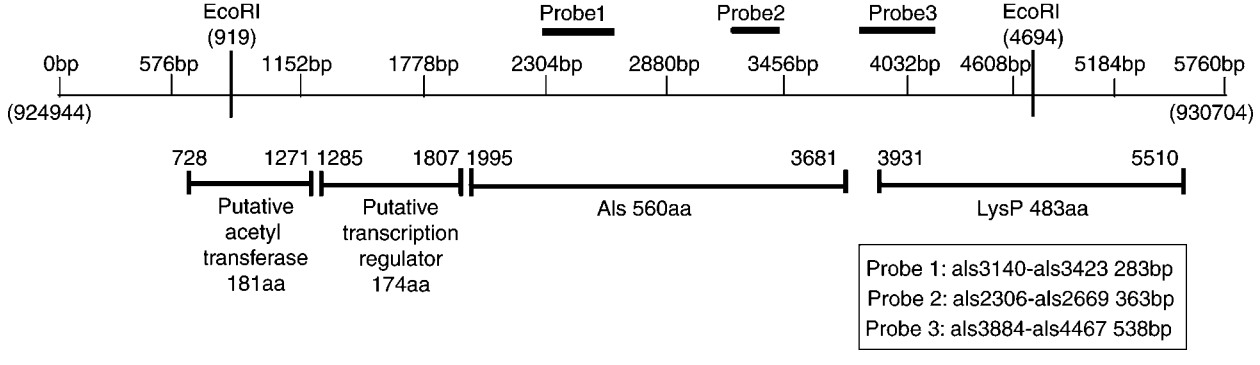

Fig. 1. Schematic diagram of the *L. plantarum* chromosomal regions around the *als* gene and its close vicinity including the *lysP* gene. The nucleotide positions are indicated and the relative positions and sizes of four ORFs including the Als and LysP proteins are drawn in the same scale. The positions of three probes used in Southern hybridization analyses are shown at the top. Probe sizes and PCR primers used to amplify these probes are indicated inside the box at the bottom of the diagram.

Results and Discussion

The chromosomal sequences of the *L. plantarum als* gene and vicinity were extracted from the published data of Kleerebezem *(10)*. A total of 5760 bp corresponding to genomic positions 924,944–930,704 were analyzed in this study. As shown in Fig. 1, there are four open reading frames (ORFs) within the 5760 bp DNA fragment (http://www.ncbi.nlm.nih.gov/genomes/framik.cgi? db=genome& gi=276). The relative positions of each ORF are indicated by nucleotide numbers relative to 0 (924,944). The *als* gene, with an ORF of 560 amino acids, is flanked on the left side by a putative transcription regulator (ORF of 174 amino acids) and on the right side by the *lysP* gene encoding a lysine transport protein consisting of 483 amino acids. There are two *Eco*RI sites at positions 919 and 4694. The positions of probes for Southern blotting and PCR primers are all marked by nucleotide numbers relative to the 0 point.

The construction of the pBluescript derivative $pBlue2306_{2606}Em3884_{4922,}$ in which the *ery* gene was inserted into the *als* sequences, was done in *E. coli Dh5a* cells. The plasmid linear map is illustrated in Fig. 2 (the top bar). This plasmid was used to transform TF103 cells and the resulting Em^r colonies were further analyzed for chromosomal integrations.

Two specific primers (als2306 and als4667) located on each side of the deleted region would result in PCR amplification of a 1883 bp fragment from the control plasmid $pBlue2306_{2606}Em3884_{4922}$ (the *ery* gene was inserted at the deleted region, Fig. 2), and of a 2161 bp fragment from the parent TF103 (from 2306 to 4667, Figs. 1 and 2). Total genomic DNA preps from Em^r candidates were subjected to PCR analyses to screen for clones bearing the deleted copy of the *als* gene.

As expected, the 1883 bp and 2161 bp fragments were detected when the control plasmid $pBlue2306_{2606}Em3884_{4922}$ (Fig. 3, lane 1) and parent strain TF103 genomic DNA (Fig. 3, lane2) were used as templates, respectively. However, among five TF103 Em^r genomic DNA samples tested, all yielded a shorter 1683 bp PCR fragment (Fig. 3, lane 3, only one sample shown). The absence of the 2161 bp fragment in the mutant suggested that the mutant strain does not carry the original copy of the *als* gene. Rather, a deleted copy was substituted as reflected by a shorter 1683 bp PCR product. These data suggest that the mutant may result from DNA integration into the chromosome (Fig. 2).

Southern blotting was performed on total DNA from the parent TF103 and an *als* mutant, which was digested by *Eco*RI and hybridized with three different probes. The positions of each probe are indicated in Fig. 1. Probe 1 and probe 2 are located within the *als* coding region, whereas probe 3 is located at the end of *als*, extends to the beginning of *lysP* that encoding the N-terminal part of the LysP protein (Fig. 1).

Positive hybridization of about 3775 bp DNA fragment from *Eco*RI digested genomic DNA, which cuts at position 919 and 4694 (Fig. 1), was

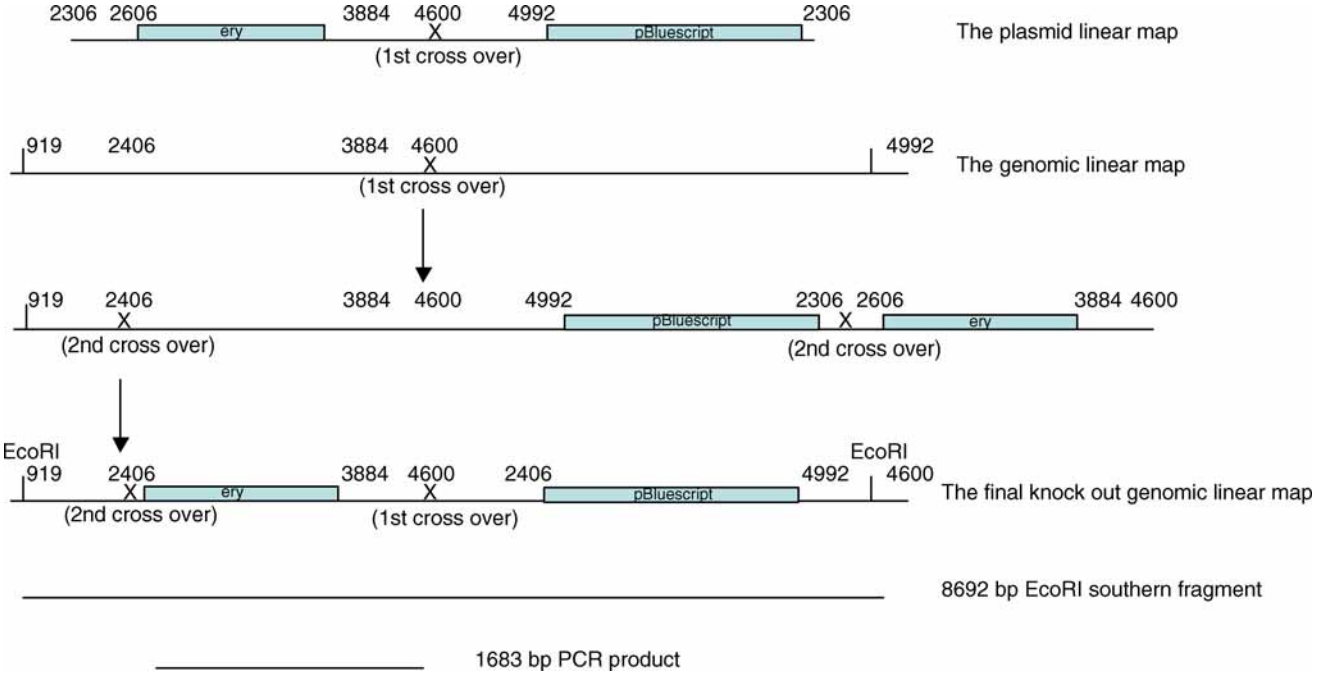

Fig. 2. Schematic representation of a system for generating targeted chromosomal replacement by a two-step integration procedure. Sequential homologous recombinations led to the construction of the chromosomal mutant TF103 Δ *als*Δ *lysP*. The approximate positions of two crossover and *Eco*RI digestion sites are shown. The approximate size of *Eco*RI fragment as detected by Southern and the expected PCR amplicon from the mutant are indicated.

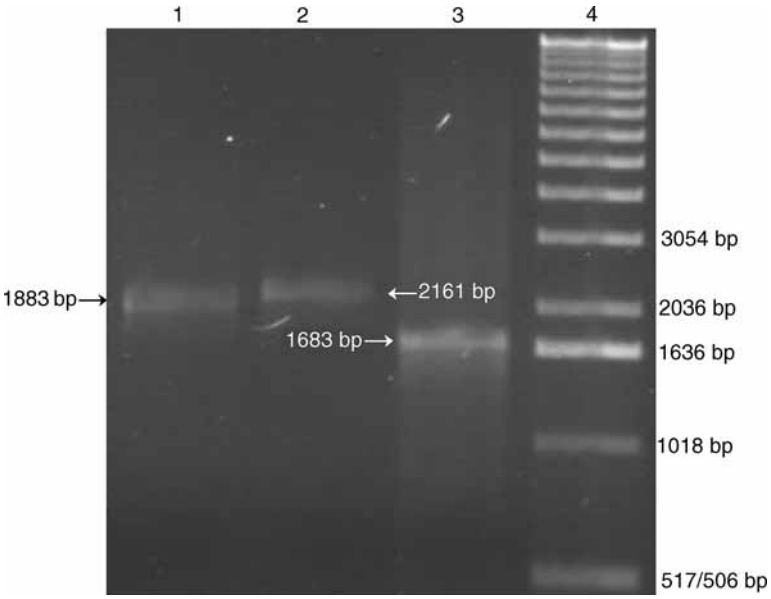

Fig. 3. Agarose (1%) gel electrophoresis of PCR products formed on chromosomal DNA of the new mutant strain TF103 Δ*als*Δ *lysP* using als2306 and als4467 primers. Lane 1, PCR product from control pBlue2306$_{2606}$Em3884$_{4922;}$ Lane 2, PCR product from control genomic DNA of TF103; Lane 3, PCR products from genomic DNA of the TF103 Δ *als*Δ *lysP* mutant; Lane 4, DNA molecular weight markers from invitrogen.

obtained with the parent strain TF103 regardless of the probes used (Fig. 4, the first lanes of panel A–C), and this 3775 bp fragment was not detected in the mutant with any of the three probes. These results confirmed that the mutant clone does not bear the original copies of wild type *als* and *lysP*. Instead, both genes were interrupted.

As illustrated in Fig. 1, probe 1 fragment covers the 2306–2669 region that is located in the deleted region of *als* (Fig. 2). Southern blotting results using this specific probe failed to detect any signals in the mutant genomic DNA, indicated that the intented deletion was successful (Fig. 4, panel B, the third lane). Meanwhile, a fragment of about 9 kb was detected with the 283 bp probe 2 specific for the als3140–3423 region and the 587 bp probe 3 specific for the als3884–4667 (Fig. 4, the third lanes of panels B and C).

The combined results of Southern blotting and PCR analyses suggest that the knockout is a result of two crossover events. A schematic diagram of how the mutant was generated is shown in Fig. 2. The first crossover occurred at approx 4600, and the second crossover occurred at approx 2400. After homologous recombination at two positions, both *als* and *lysP* were interrupted. The recombination events led to the hybridization to a 8692 bp fragment when using probes 1 and 3, larger than the 3775-bp wild-type gene fragment. As indicated in Fig. 2, the mutant includes the interruption of the *als* gene with the *ery* insertion in addition to an approx 200 bp

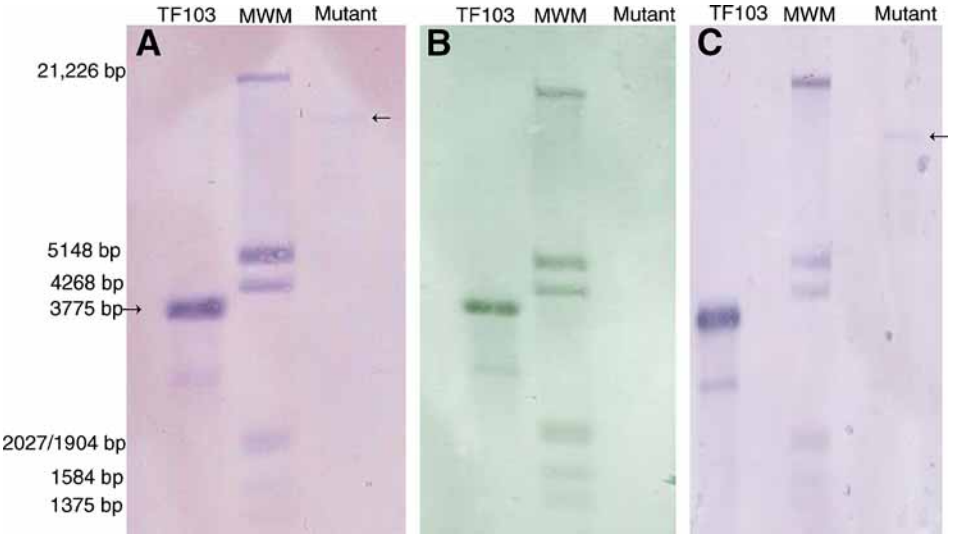

Fig. 4. Southern hybridization analysis of *Eco*RI digested chromosomal DNAs of parent TF103 and the TF103 Δ *als*Δ *lysP* mutant using three gene-specific probes. The relative positions of each probe used are indicated in Fig. 1. MWM: the Molecular Weight Marker III purchased from Roche, with the sizes of the digoxigenin-labeled DNA fragments are indicated on the left.

internal deletion from 2406–2606, and also the interruption of the *lysP* gene by the pBluescript vector insertion. This mutant is therefore designated as TF103 Δ *als*Δ *lysP.*

Acetolactate synthase (ALS: EC 4.1.3.18) is the first enzyme in the biosynthetic pathway of leucine, valine, and isoleucine. ALS catalyzes the formation of 2-acetolactate or 2-aceto-2-hydroxy-butanoate from pyruvate using thiamine pyrophosphate as a cofactor. Unlike *Lactococcus lactis (15,16)*, which has three *als* genes, two acetolactate synthase large subunit (*ilvB*), and one acetolactate synthase small subunit (*ilvN*), *L. plantarium* WCFS1 genome has only one *als* gene *(10)*, and therefore can not synthesize the branched-chain amino acids valine, leucine, and isoleucine. The *als* in *L. plantarum* is presumably used for the fermentative production of acetoin and 2, 3-butanodial from pyruvate. Detailed biochemical characterization and fermentation product analyses of the mutant are in progress.

The *lysP* gene encodes a membrane protein that is reported to be responsible for lysine transport in *E. coli (17)*. The mutant will be made available for interested researchers in *lysP* related studies.

In summary, we have generated an insertional-deletion mutant TF103 Δ *als*Δ *lysP* with inactivated *als* and also its nearby *lysP* locus. The approach is simple, using only a Gram-negative cloning vector, and eliminating the tedious cloning procedures involved with many Gram-positive shuttle vectors. The results of this study suggest that the pBluescript vector can be

used to introduce engineered DNA into the host chromosome. This opens potential applications of the pBluescript vector in engineering other Gram-positive hosts.

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References

1 Li, J., Kasper, D. L., Ausubel, F. M., Rosner, B., and Michel, J. L. (1997), *Proc. Natl. Acad. Sci. USA* **94,** 13,251–13,256.
2 Ferain, T., Hobbs, J. N., Jr., Richardson, J., et al. (1996), *J. Bacteriol.* **178,** 5431–5437.
3 Leenhouts, K. J., Kok, J., and Venema, G. (1989), *Appl. Environ. Microbiol.* **55,** 394–400.
4 Bhowmik, T. and Steele, J. L. (1993), *J. Gen. Microbiol.* **139,** 1433–1439.
5 Lokman, B. C., Heerikhuisen, M., Leer, R. J., et al. (1997), *J. Bacteriol.* **179,** 5391–5397.
6 Liu, S., Nichols, N. N., Dien, B. S., and Cotta, M. A. (2005), *J. Ind. Microbiol. Biotechnol.*, in press.
7 Bothast, R. J., Nichols, N. N., and Dien, B. S. (1999), *Biotechnol. Prog.* **15,** 867–875.
8 Ferain, T., Schanck, A. N., and Delcour, J. (1996), *J. Bacteriol.* **178,** 7311–7315.
9 Djordjevic, G. M. and Klaenhammer, T. R. (1996), *Plasmid* **35,** 37–45.
10 Kleerebezem, M., Boekhorst, J., van Kranenburg, R., et al. (2003), *Proc. Natl. Acad. Sci. USA* **100,** 1990–1995.
11 Neves, A. R., Ramos, A., Costa, H., et al. (2002), *Appl. Environ. Microbiol.* **68,** 6332–6342.
12 Sambrook, J., Fritsch, E.F., and Maniatis T. (1989), *Cold Spring Harbor Laboratory Press,* Cold Spring Marbor, NY.
13 Posno, M., Leer, R. J., van Luijk, N., et al. (1991), *Appl. Environ. Microbiol.* **57,** 1822–1828.
14 Liu, S., Dien, B. S., and Cotta, M. A. (2005), *Curr. Microbiol.* **50,** 1–6.
15 Godon, J. J., Delorme, C., Bardowski, J., Chopin, M. C., Ehrlich, S. D., and Renault, P. (1993), *J. Bacteriol.* **175,** 4383–4390.
16 Bolotin, A., Wincker, P., Mauger, S., et al. (2001), *Genome Res.* **11,** 731–753.
17 Neely, M. N., Dell, C. L., and Olson, E. R. (1994), *J. Bacteriol.* **176,** 3278–3285.