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The Alanine Racemase Gene Is Essential for Growth of *Lactobacillus plantarum*

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The *Lactobacillus plantarum alr* gene encoding alanine racemase was cloned by complementation of an *Escherichia coli* Alr$^-$ DadX$^-$ double mutant strain. Knockout of the *alr* gene abolished all measurable alanine racemase activity, and the mutant was shown to be strictly dependent on D-alanine for growth.

Alanine racemases (EC 5.1.1.1) are unique prokaryotic enzymes that interconvert L-alanine and D-alanine (24). They are the sole identified biosynthetic pathway of D-alanine for bacterial cell wall synthesis (24). D-Ala is generally present as a dipeptide, D-alanyl–D-Ala, in the C-terminal position of the UDP–*N*-acetylmuramyl (MurNAc)-pentapeptide precursor (e.g., UDP–*N*-acetylmuramyl–L-Ala–D-Glu–*meso*-diaminopimelic acid–D-Ala–D-Ala) of the peptidoglycan (23). The penultimate D-Ala residue of this precursor is directly involved in the cross-linking of adjacent peptidoglycan strands in cell wall growth (16). Furthermore, the cell wall of gram-positive bacteria contains teichoic acids [poly(alditol phosphate) polymers] which could be of two types: wall teichoic acids, which are covalently linked to peptidoglycan, and lipoteichoic acids (LTA), which are anchored in the cytoplasmic membrane. These teichoic acids contain various substituents, such as D-Ala esters and glycosyl residues (7). *Lactobacillus plantarum* and the phylogenically close species *L. casei* contain LTA which are mainly (90%) or exclusively substituted with D-Ala esters, respectively (1, 9, 18). *L. casei* mutants deficient in LTA D-Ala ester substitutions are characterized by aberrant morphology and defective daughter cell separation (18). Thus, D-Ala is a central molecule in the biosynthesis of the two cell wall polymers peptidoglycan and teichoic acids.

In *Escherichia coli*, two alanine racemases were identified. The *alr*-encoded alanine racemase (named biosynthetic racemase) is constitutively expressed (13, 26, 27), whereas the *dadX*-encoded enzyme (named catabolic racemase) is essential only for L-Ala catabolism, providing a substrate for a D-Ala-specific dehydrogenase encoded by the *dadA* gene. The *dadX* and *dadA* genes constitute an operon positively regulated by L-Ala and repressed by glucose (14, 27). The *dadX* gene product is the major source of alanine racemase activity (85% of total activity) and is probably a secondary source of D-Ala for cell wall biosynthesis (24). Only the Alr$^-$ DadX$^-$ double mutant is dependent on D-Ala for growth (27).

In *Bacillus subtilis*, a single alanine racemase gene (*dal*) has been isolated (6). A Dal$^-$ mutant is dependent on D-Ala for growth only in rich medium and lyses in the absence of supplementation (2, 6, 8). Conversely, growth is not affected in minimal medium, except upon supplementation with L-Ala, which restores the D-Ala dependence for growth (2, 6). Ferrari et al. (6) suggested that a second, L-Ala-repressible racemase is present in *B. subtilis.*

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We have recently characterized the cell wall precursor of *L. plantarum* NCIMB8826: UDP–*N*-acetylmuramyl–L-Ala–D-Glu–*meso*-diaminopimelic acid–D-Ala–D-lactate (5). The terminal depsipeptide D-Ala–D-lactate is specific for a number of vancomycin-resistant species, including *L. plantarum* (3, 5). The origin of the D-lactate terminal residue in *L. plantarum* is largely dependent on the presence of the D-lactate dehydrogenase enzyme (EC 1.1.1.18) (5). The work described here was aimed at understanding the biosynthetic pathway of D-Ala in *L. plantarum* through cloning and disruption of the alanine racemase gene.

Cloning of an alanine racemase gene from *L. plantarum* by complementation in *E. coli*. A genomic library from *L. plantarum* NCIMB8826 (National Collection of Industrial and Marine Bacteria, Aberdeen, Scotland) was constructed as follows. Total DNA was partially digested by *Sau*3A, and DNA fragments between 1.5 and 4.5 kb long were recovered from an agarose gel and ligated to *Bam*HI-restricted plasmid pJDC9 (4). A total of 6,300 recombinant clones of *E. coli* TG1 (19) with a mean insert size of 2.3 kb were obtained. Plasmid DNA extracted from a pool of TG1 recombinants was transferred by electroporation in the TLK10 mutant strain of *E. coli* (5×10^7 transformants/μg). This Alr^{ts} DadX$^-$ (MsuA$^-$ in the original report of Wijsman [26]) double mutant strain is dependent on D-Ala for growth at the nonpermissive temperature (41°C) (26, 27). Thus, complementation was performed at 41°C in the absence of D-Ala on Luria-Bertani (LB) medium (19) supplemented with erythromycin (250 μg/ml), which does not contain enough D-Ala to support growth (25). Five candidates bearing plasmids with inserts between 1.5 and 2.3 kb long were isolated after 24 h of incubation at the nonpermissive temperature. Following back transformation of the five recombinant plasmids in TLK10, we selected one clone which conferred efficient complementation together with alanine racemase activity in crude cell extracts. The corresponding plasmid (named pGIP001) was shown to harbor a 2.1-kb insert whose authenticity was confirmed by Southern blotting analysis, which revealed the presence of a single *Sau*3A genomic fragment of 2.1 kb (data not shown).

Gene sequencing and analysis. The complete nucleotide sequence of the pGIP001 insert is shown in Fig. 1. It spans 2,041 bp and contains a single complete open reading frame (ORF) of 375 codons (Fig. 1, ORF2). The ATG start codon is preceded by a putative Shine-Dalgarno (SD) sequence complementary to the 3′ end of the 16S rRNA of *L. plantarum* (EMBL accession no. M58826) at a canonical 9-nucleotide distance with a calculated free energy of duplex formation (ΔG_f) of −11 kcal/mol (21). Inspection of the 3′ noncoding region downstream of ORF2 shows a putative transcriptional

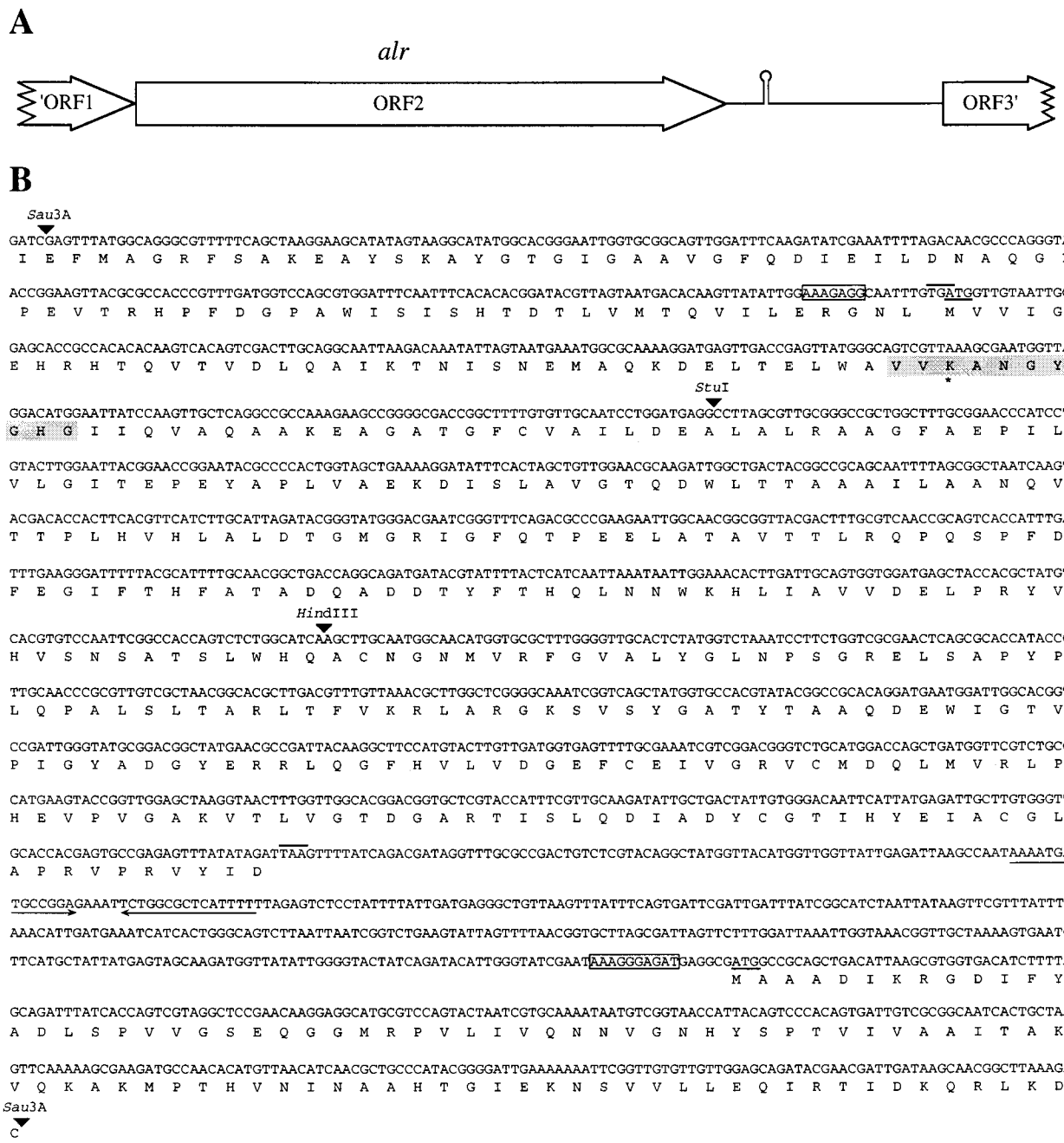

FIG. 1. Genetic organization and sequence of the *alr* locus. (A) Scheme of the insert cloned in pGIP001. The wavy lines in arrow boxes 'ORF1 and ORF3' indicate truncation by the *Sau*3A restriction sites used in cloning. The hairpin shows the location of the putative transcriptional terminator following ORF2 (*alr*). (B) Nucleotide sequence of the *alr* gene from *L. plantarum* (ORF2) and flanking regions including two truncated ORFs ('ORF1 and ORF3'), with the deduced amino acid sequences beneath the nucleotide sequence. The start and stop codons of the different ORFs are underlined and overlined, respectively. The putative SD sequences preceding the *alr* gene and ORF3' are boxed. The opposite arrows downstream of the *alr* gene underline a potential transcription terminator. Also shown are the restriction sites that were used in the cloning of various DNA fragments. The shaded box in the Alr protein indicates a conserved decapeptide motif which includes the active lysyl residue that binds the cofactor pyridoxal-P (marked by an asterisk).

terminator consisting of an inverted repeat followed by a T cluster that could form a stem-and-loop structure in mRNA with a calculated free energy of −17.2 kcal/mol (21). ORF2 codes for a protein (named Alr) which displays homology with the alanine racemase family. The highest level of identity was obtained with Alr proteins from two gram-positive bacteria: *B. stearothermophilus* (46%) and *B. subtilis* (43%) (6, 20). A significant level of identity with the Alr (30%) and DadX (34%) proteins from *E. coli* was also observed (13, 14). A feature common to the known alanine racemases is the presence of a conserved decapeptide motif (VVKANAYGHG) in the N-terminal moiety of the protein, encompassing the active lysyl residue that binds the cofactor pyridoxal-P (20). This motif is also closely conserved in the Alr protein from *L. plantarum* (Fig. 1, shaded box). The 5' region in front of the *alr* gene does not contain any putative promoter but includes a 5'-truncated

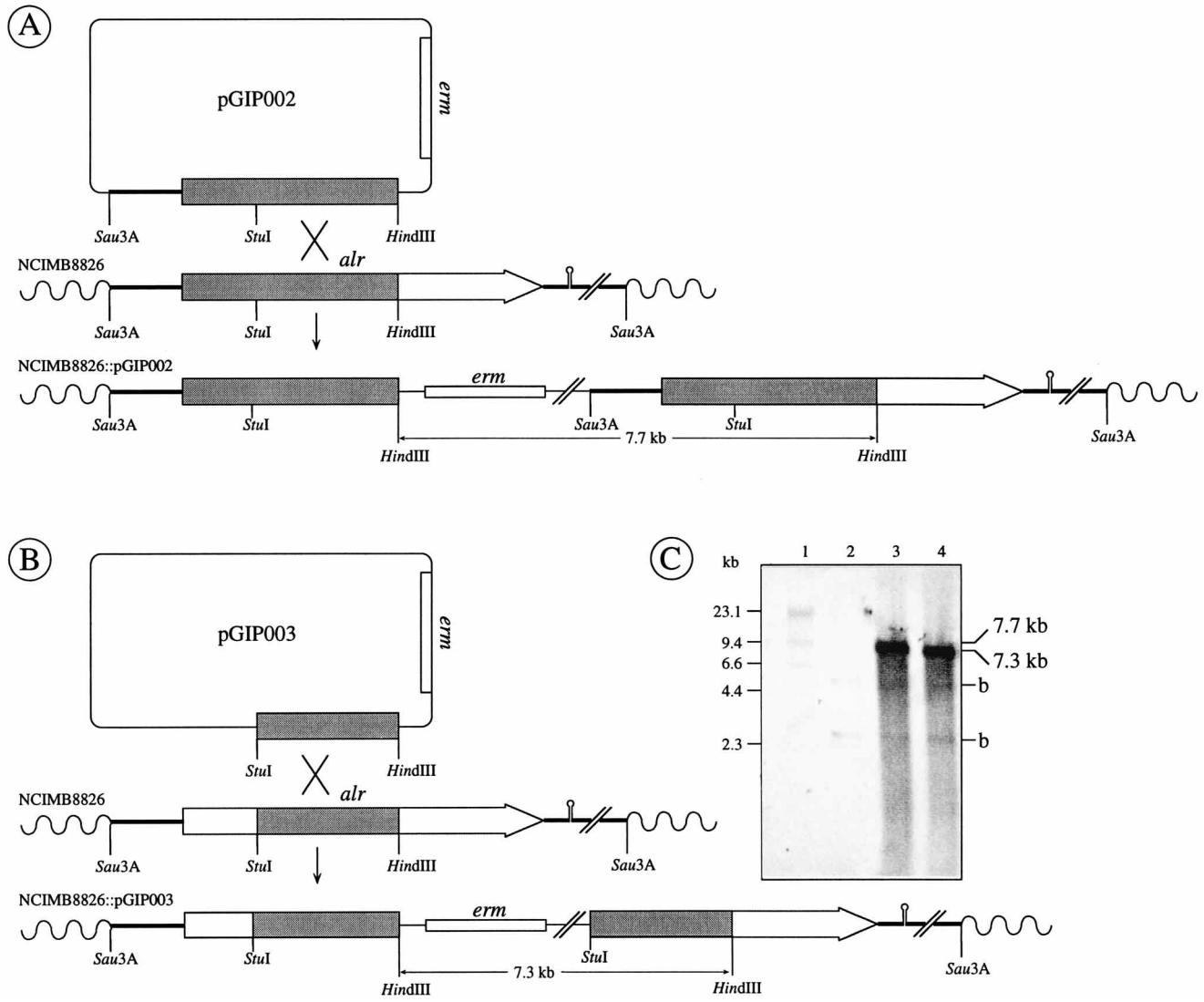

FIG. 2. Campbell-type chromosomal integration of plasmids pGIP002 and pGIP003 in *L. plantarum*. (A) Homologous recombination between suicide plasmid pGIP002 and the NCIMB8826 chromosome taking place in the 5′ moiety of the *alr* gene (shaded box) to yield strain NCIMB8826::pGIP002. (B) Homologous recombination between suicide plasmid pGIP003 and the NCIMB8826 chromosome taking place in the internal *Stu*I-*Hin*dIII fragment of the *alr* gene (shaded box) to yield strain NCIMB8826::pGIP003. The estimated sizes of the *Hin*dIII fragments for NCIMB8826::pGIP002 and NCIMB8826::pGIP003 are indicated below the maps. Thick lines, cloned genomic DNA flanking the *alr* gene; wavy lines, chromosomal borders of the pGIP001 insert; thin lines, framework of the suicide vectors; arrow box, *alr* gene; vertical hairpin, putative transcription terminator; *erm*, erythromycin resistance marker. (C) Southern blot hybridization of the ^{35}S-labelled pGIP001 plasmid to *Hin*dIII digests of chromosomal DNAs from NCIMB8826 (lane 2), NCIMB8826::pGIP002 (lane 3), and NCIMB8826::pGIP003 (lane 4). Lane 1 contains phage λ digested with *Hin*dIII, used as markers (sizes are indicated on the left). The sizes of internal fragments (7.3 and 7.7 kb) and the locations of the border fragments (b) are indicated on the right.

ORF (Fig. 1, ′ORF1). The TGA stop codon of ′ORF1 overlaps the ATG start codon of ORF2 (Fig. 1). ′ORF1 codes for the C-terminal part (74 amino acids [aa]) of a putative protein displaying 23% identity with the C-terminal part of Dpj from *E. coli* (holo-acyl carrier protein synthase, 126 aa) which is responsible for the transfer of the 4′ phosphopantetheine group from coenzyme A to the acyl carrier protein, which is itself involved in fatty acid biosynthesis (12). Thus, the sequence analysis suggests that the *alr* gene from *L. plantarum* is the last gene of a transcription unit including at least two apparently unrelated genes. A similar situation has already been observed with the *alr* gene from *E. coli*, which seems to be cotranscribed with the *dnaB* gene (single-strand DNA binding protein) (13).

A third 3′-truncated ORF (Fig. 1, ORF3′) was identified downstream of the putative terminator of the *alr* gene, preceded by a putative SD sequence with a ΔG_f of −13.4 kcal/mol (21). This ORF encodes the N-terminal part (93 aa) of a putative protein which shows 27% identity with protein PemK (123 aa) from *E. coli*, 23% identity with proteins ChpAK (111 aa) and ChpBK (116 aa) from *E. coli*, and 38% identity with the unknown product of the MTCY39.28 gene (114 aa) from *Mycobacterium tuberculosis* (15, 22; EMBL accession no. MTCY39). All of these proteins are members of the PemK family of DNA binding proteins which are involved in inhibition of cell growth in *E. coli* (10, 22).

Disruption of the *alr* gene and phenotype of the mutants. We constructed two pGIP001 derivatives to be used as suicide integration vectors. Plasmid pGIP002 (Fig. 2A) contains a *Sau*3A-*Hin*dIII fragment (884 bp) corresponding to the 5′ end

TABLE 1. Growth of *L. plantarum* NCIMB8826 and the two *alr* mutants on plates

Medium	Growth[c]		
	NCIMB8826	NCIMB8826::pGIP002	NCIMB8826::pGIP003
MRS[a]	+	−	−
MRS + 0.225 mM D-Ala	+	+	+
MRS + 0.225 mM L-Ala	+	−	−
Min. med.[b]	+	−	−
Min. med. + 0.225 mM D-Ala	+	+	+
Min. med. + 0.225 mM L-Ala	+	−	−

[a] MRS, rich medium (Difco).
[b] Min. med., minimal medium (17).
[c] +, growth; −, no growth.

of the insert, and pGIP003 (Fig. 2B) contains an internal *Stu*I-*Hin*dIII fragment (445 bp) of the *alr* gene. These two constructs were electrotransferred into *L. plantarum* NCIMB8826 (11), and 10 to 30 transformants/μg were obtained after 72 h of growth at 37°C on MRS plates (erythromycin at 5 μg/ml and lincomycin at 10 μg/ml) supplemented with 0.225 mM D-Ala. Integration by homologous recombination of the two suicide plasmids was confirmed by Southern blotting analysis (Fig. 2C), which showed internal and border *Hin*dIII fragments of the expected sizes.

Both integrants displayed a strict dependence on D-Ala for growth in rich MRS medium (Table 1), as observed for growth in rich media of mutants of *E. coli* (Alr$^-$ DadX$^-$) and *B. subtilis* (Dal$^-$) (6, 26, 27). The dependence on D-Ala for integrant NCIMB8826::pGIP002 also confirmed the absence of a genuine promoter in front of the *alr* gene (see above). So, the cloned *alr* gene (in the anti-*lacZ′* orientation) is most probably under the control of one or more promoters present on plasmid pJDC9 which were able to drive enough expression in *E. coli* to ensure complementation but are not functional in *L. plantarum*. Alanine racemase activity was undetectable in crude cell extracts of the two mutants grown in MRS medium supplemented with D-Ala, while significant alanine racemase activity was found in the wild-type strain (data not shown). Further investigation of the growth capacity of the mutants in minimal medium (17) without alanine or supplemented with L-Ala or D-Ala revealed a phenotype different from that observed with a Dal$^-$ mutant of *B. subtilis* (Table 1). The *L. plantarum* mutants are still dependent on D-Ala for growth in minimal medium, indicating that, conversely to *B. subtilis*, L-Ala does not repress another putative alanine racemase (2, 6). Likewise, the absence of growth on minimal medium supplemented with L-Ala rules out the presence of a second alanine racemase inducible by L-Ala, such as DadX in *E. coli* (27). These results strongly suggest that D-Ala production in *L. plantarum* is dependent on a single alanine racemase.

Nucleotide sequence accession number. The nucleotide sequence reported here is available from the GenBank and EMBL databases under accession no. Y08941.

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REFERENCES

1. **Archibald, A. R., and J. Baddiley.** 1966. The teichoic acids. Adv. Carbohydr. Chem. **21:**323–375.
2. **Berberich, R., M. Kaback, and E. Freese.** 1968. D-Amino acids as inducers of L-alanine dehydrogenase in *Bacillus subtilis*. J. Biol. Chem. **243:**1006–1011.
3. **Billot-Klein, D., L. Gutmann, S. Sablé, E. Guittet, and J. van Heijenoort.** 1994. Modification of peptidoglycan precursors is a common feature of the low-level vancomycin-resistant VANB-type enterococcus D366 and of the naturally glycopeptide-resistant species *Lactobacillus casei*, *Pediococcus pentosacens*, *Leuconostoc mesenteroides*, and *Enterococcus gallinarum*. J. Bacteriol. **176:**2398–2405.
4. **Chen, J.-D., and D. A. Morrison.** 1988. Construction and properties of a new insertion vector, pJDC9, that is protected by transcriptional terminators and useful for the cloning of DNA from *Streptococcus pneumoniae*. Gene **64:**155–164.
5. **Ferain, T., J. N. Hobbs, Jr., J. Richardson, N. Bernard, D. Garmyn, P. Hols, N. E. Allen, and J. Delcour.** 1996. Knockout of the two *ldh* genes has a major impact on peptidoglycan precursor synthesis in *Lactobacillus plantarum*. J. Bacteriol. **178:**5431–5437.
6. **Ferrari, E., D. J. Henner, and M. Y. Yang.** 1985. Isolation of an alanine gene from *Bacillus subtilis* and its use for plasmid maintenance in *B. subtilis*. Biotechnology **3:**1003–1007.
7. **Fischer, W.** 1988. Physiology of lipoteichoic acids in bacteria. Adv. Microb. Physiol. **29:**233–302.
8. **Heaton, M. P., R. B. Johnson, and T. L. Thomson.** 1988. Controlled lysis of bacterial cells utilizing mutants with defective synthesis of D-alanine. Can. J. Microbiol. **34:**256–261.
9. **Heaton, M. P., and F. C. Neuhaus.** 1994. Role of the D-alanyl carrier protein in the biosynthesis of D-alanyl-lipoteichoic acid. J. Bacteriol. **176:**681–690.
10. **Jensen, R. B., and K. Gerdes.** 1995. Programmed cell death in bacteria: proteic plasmid stabilization systems. Mol. Microbiol. **17:**205–210.
11. **Josson, K., T. Scheirlink, F. Michiels, C. Platteuw, P. Stanssens, H. Joos, P. Dhaese, M. Zabeau, and J. Mahillon.** 1989. Characterization of gram-positive broad-host-range plasmid isolated from *Lactobacillus hilgardii*. Plasmid **21:**9–20.
12. **Lambalot, R. H., and C. T. Walsh.** 1995. Cloning, overexpression, and characterization of the *Escherichia coli* holo-acyl carrier protein synthase. J. Biol. Chem. **270:**24658–24661.
13. **Lilley, P. E., N. P. Stamford, S. G. Vasudevan, and N. E. Dixon.** 1993. The 92-min region of the *Escherichia coli* chromosome: location of the *ubiA* and *alr* genes. Gene **129:**9–16.
14. **Łobocka, M., J. Hennig, J. Wild, and T. Kłopotowski.** 1994. Organization and expression of the *Escherichia coli* K-12 *dad* operon encoding the smaller subunit of the D-amino acid dehydrogenase and the catabolic alanine racemase. J. Bacteriol. **176:**1500–1510.
15. **Masuda, Y., K. Miyakawa, Y. Nishimura, and E. Ohtsubo.** 1993. *chpA* and *chpB*, *Escherichia coli* chromosomal homologs of the *pem* locus responsible for stable maintenance of plasmid R100. J. Bacteriol. **175:**6850–6856.
16. **Matsuhashi, M.** 1994. Utilization of the lipid-linked precursors and the formation of the peptidoglycan in the process of cell growth and division: membrane enzymes involved in the final steps of peptidoglycan synthesis and the mechanism of their regulation, p. 55–71. *In* J.-M. Ghuysen and R. Hakenbeck (ed.), Bacterial cell wall. Elsevier Sciences B. V., Amsterdam, The Netherlands.
17. **Morishita, T., Y. Deguchi, M. Yajima, T. Sakurai, and T. Yura.** 1981. Multiple nutritional requirements of lactobacilli: genetic lesions affecting amino acid biosynthetic pathways. J. Bacteriol. **148:**64–71.
18. **Ntamere, A. S., D. J. Taron, and F. C. Neuhaus.** 1987. Assembly of D-alanyl-lipoteichoic acid in *Lactobacillus casei*: mutants deficient in the D-alanyl ester content of this amphiphile. J. Bacteriol. **169:**1702–1711.
19. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
20. **Tanizawa, K., A. Ohshima, A. Scheidegger, K. Inagaki, H. Tanaka, and K. Soda.** 1988. Thermostable alanine racemase from *Bacillus stearothermophilus*: DNA and protein sequence determination and secondary structure prediction. Biochemistry **27:**1311–1316.
21. **Tinoco, I., Jr., P. N. Borer, B. Dengler, M. D. Levine, O. C. Uhlenbeck, D. M. Crothers, and J. Gralla.** 1973. Improved estimation of secondary structure in ribonucleic acids. Nature (London) New Biol. **246:**40–41.
22. **Tsuchimoto, S., H. Ohtsubo, and E. Ohtsubo.** 1988. Two genes, *pemK* and *pemI*, responsible for stable maintenance of resistance plasmid R100. J. Bacteriol. **170:**1461–1466.
23. **van Heijenoort, J.** 1994. Biosynthesis of the bacterial peptidoglycan unit, p. 39–54. *In* J.-M. Ghuysen and R. Hakenbeck (ed.), Bacterial cell wall. Elsevier Sciences B. V., Amsterdam, The Netherlands.
24. **Wasserman, S. A., E. Daub, P. Grisafi, D. Botstein, and C. T. Walsh.** 1984. Catabolic alanine racemase from *Salmonella typhimurium*: DNA sequence, enzyme purification and characterization. Biochemistry **23:**5182–5187.
25. **Wasserman, S. A., C. T. Walsh, and D. Botstein.** 1983. Two alanine racemase genes in *Salmonella typhimurium* that differ in structure and function. J. Bacteriol. **153:**1439–1450.
26. **Wijsman, H. J. W.** 1972. The characterization of an alanine racemase mutant of *Escherichia coli*. Genet Res. **20:**269–277.
27. **Wild, J., J. Hennig, M. Łobocka, W. Walcsak, and T. Kłopotowski.** 1985. Identification of the *dadX* gene coding for the predominant isoenzyme of alanine racemase in *Escherichia coli* K12. Mol. Gen. Genet. **198:**315–322.