

Genetic Analysis and Overexpression of Lipolytic Activity in *Bacillus subtilis*

V. DARTOIS,* J.-Y. COPPÉE, C. COLSON, AND A. BAULARD†

Laboratoire de Génétique Microbienne, Université Catholique de Louvain, B-1348 Louvain-la-Neuve, Belgium

Received 7 September 1993/Accepted 8 February 1994

The previously cloned *Bacillus subtilis* lipase gene (*lip*) was mapped on the chromosome and used in the construction of a *B. subtilis* derivative totally devoid of any *lip* sequence. Homologous overexpression was performed in this strain by subcloning the *lip* open reading frame on a multicopy plasmid under the control of a strong gram-positive promoter. A 100-fold overproducing strain was obtained, which should facilitate purification of the secreted protein. Furthermore, the Δ *lip* strain BCL1050 constitutes an ideal host for the cloning of heterologous lipase genes.

Lipases (triacylglycerol acylhydrolases; EC 3.1.1.3) are a class of enzymes which are able to hydrolyze ester bonds of triglyceride substrates at oil-water interfaces. This unique feature separates lipases from closely related hydrolytic enzymes such as esterases, although several lipases were shown recently to hydrolyze water-soluble substrates with varying efficiencies (1, 5, 8, 11).

Microbial lipases vary widely in enzymatic properties and substrate specificities. Consequently, they are currently receiving much attention because of their potential applications in various industrial processes. For example, alkaline pH-resistant lipases are currently used as additives in detergents (2, 12). The *Bacillus subtilis* 168 lipase has become an attractive enzyme for industrial applications: (i) expression of the cloned gene in *Escherichia coli* showed that it exhibits a broad substrate range which includes various-chain-length *p*-nitrophenyl esters and triglycerides; and (ii) nucleotide and deduced amino acid sequences predicted a remarkably alkaline pI of 9.73 and rather low M_r s of 22,777 and 19,348 for the native and mature proteins, respectively (3). Thus, the *B. subtilis* enzyme is one of the smallest lipases described so far.

B. subtilis has long been used in industry for the production of secretory proteins. Because of its nonpathogenic nature and high secretion capacity and the existence of a great deal of fermentation technology, *B. subtilis* has been regarded as an attractive host for the secretion of endogenous as well as heterologous proteins (6). Hence, our work has been directed towards genetic analysis and overexpression of lipolytic activity in *B. subtilis* to allow further purification and characterization in a homologous system (11).

The present paper reports the chromosomal disruption, mapping, and deletion of the *B. subtilis* lipase gene. The lipase-deficient strain was used for subsequent overexpression of the lipase gene (*lip*) on a plasmid.

Disruption and mapping of the chromosomal lipase gene. The *lip* gene cloned in *E. coli* on plasmid pLIP1 (3) was first disrupted by Tn5 insertion mutagenesis. The Tn5 kanamycin (Km) resistance marker (*kan*), which is not expressed in *B.*

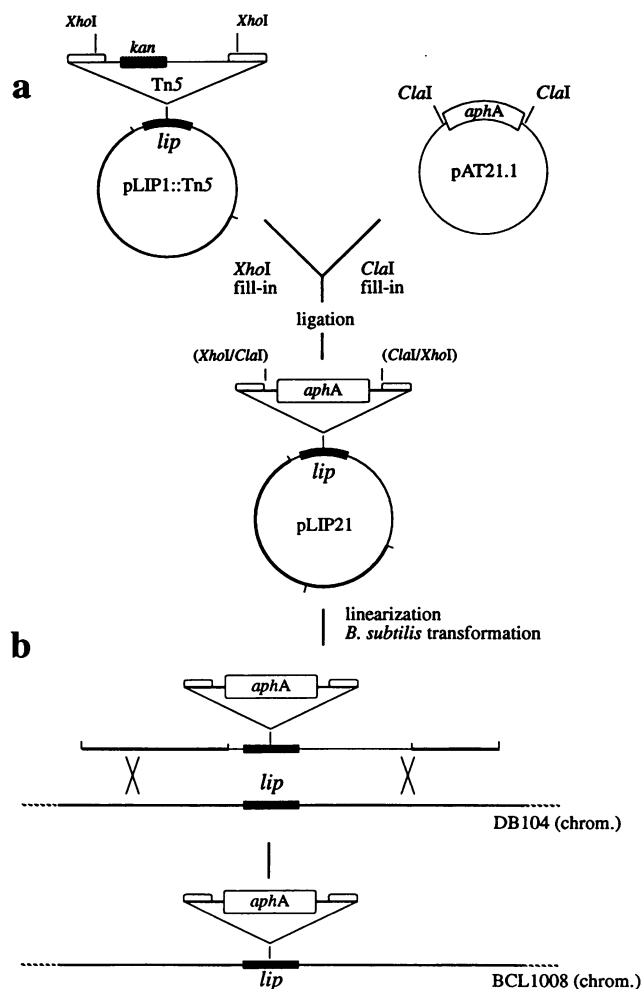


FIG. 1. Strategy for the disruptive replacement of the *lip* gene in the *B. subtilis* protease-deficient strain DB104. (a) The Tn5 internal *Xho*I fragment containing the *kan* gene was replaced with the *Cla*I Km^r (*aphA*) fragment from vector pAT21.1. (b) The linearized recombinant plasmid pLIP21 was introduced into strain DB104. Double homologous recombination at the *lip* locus allowed replacement of the wild-type chromosomal gene with the disrupted *lip*::Km allele.

* Corresponding author. Mailing address: Division of Cellular Biology, Department of Molecular and Experimental Medicine, The Scripps Research Institute, 10666 North Torrey Pines Rd. (NX1), La Jolla, CA 92037. Phone: (619) 554-8366. Fax: (619) 554-6614. Electronic mail address: vdarto@riscsm.scripps.edu.

† Present address: Laboratoire de Microbiologie Génétique et Moléculaire, Institut Pasteur de Lille, 59019 Lille Cedex, France.

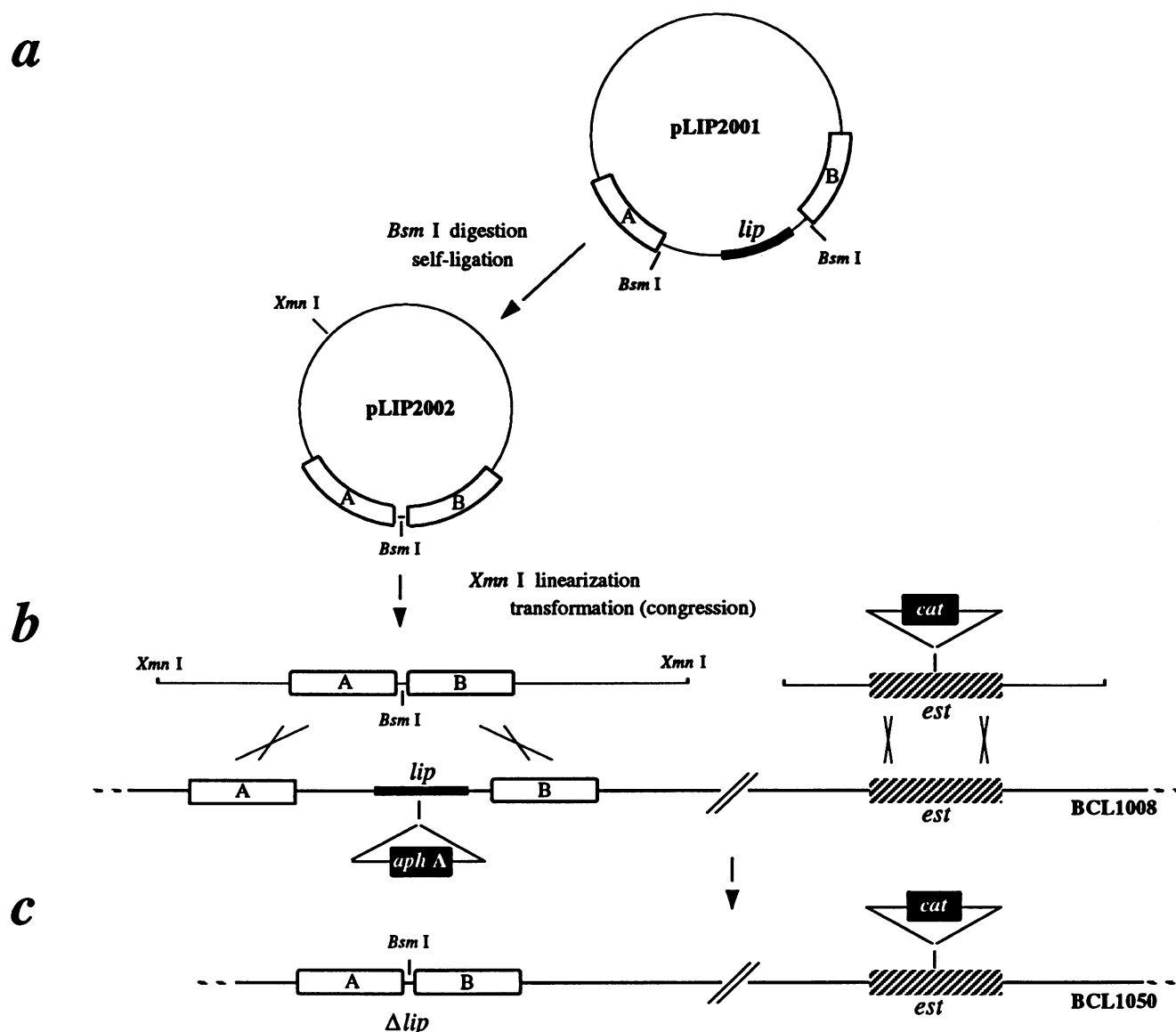


FIG. 2. Congression experiment leading to construction of the lipase-deleted *B. subtilis* strain BCL1050. (a) Plasmid pLIP2001, carrying the *lip* gene and adjacent sequences (A and B), was *Bsm*I digested and self-ligated, allowing removal of the entire *lip* coding sequence. The resulting plasmid, pLIP2002, was linearized with *Xmn*I and used to transform the *B. subtilis* *lip*::Km strain BCL1008 together with the *est*::Cm selectable marker. (b) Double transformation-integration events of both fragments introduced (congression) were detected by screening for kanamycin sensitivity among Cm^r transformants. (c) The relevant genotype of the resulting BCL1050 strain proved to be Δ *lip est*::Cm.

subtilis, was replaced by the pAT21.1 *aphA* gene (14) conferring kanamycin resistance in gram-positive bacteria (Fig. 1a). After *Pst*I linearization of the recombinant plasmid (pLIP21), the DNA was used to transform the *B. subtilis* protease-deficient strain DB104, with selection for kanamycin resistance (Fig. 1b). The resulting strain, designated BCL1008, displayed a lipase-negative phenotype when plated on triolein-rhodamine B medium, which contains the following, per liter of LB agar: 31.25 ml of purified olive oil and 10 ml of a 1-mg/ml rhodamine B solution, emulsified by mixing for 1 min in a domestic mixer (9). This observation strongly suggests the existence of a unique lipase gene in *B. subtilis*. The growth rate of the lipase-deficient strain was comparable to that of wild-type strains, which demonstrates that the *lip* gene is not essential for *B. subtilis* cell development in culture medium. Southern

blotting experiments (data not shown) were carried out to verify that recombination had occurred at the homologous locus.

The inserted Km^r gene was used to map the chromosomal *lip* locus by transduction with bacteriophage PBS1 in which the 300-kb-long genome allows transduction of 10% of the *B. subtilis* chromosome in each single cross. A set of nine strains carrying auxotrophic markers distributed along the chromosome (4) was transduced with a PBS1 lysate made from BCL1008. The kanamycin resistance gene inserted at the *lip* locus was found to be linked to *aroI* (73%), *amyE* (77%), *cys* (16%), and *dal* (4%). By combination of these linkage data according to the method of Wu (15), the *lip* locus was calculated to lie at 22° on the chromosome of *B. subtilis*.

Replacement of the *lip*::Km allele with an in vitro-derived

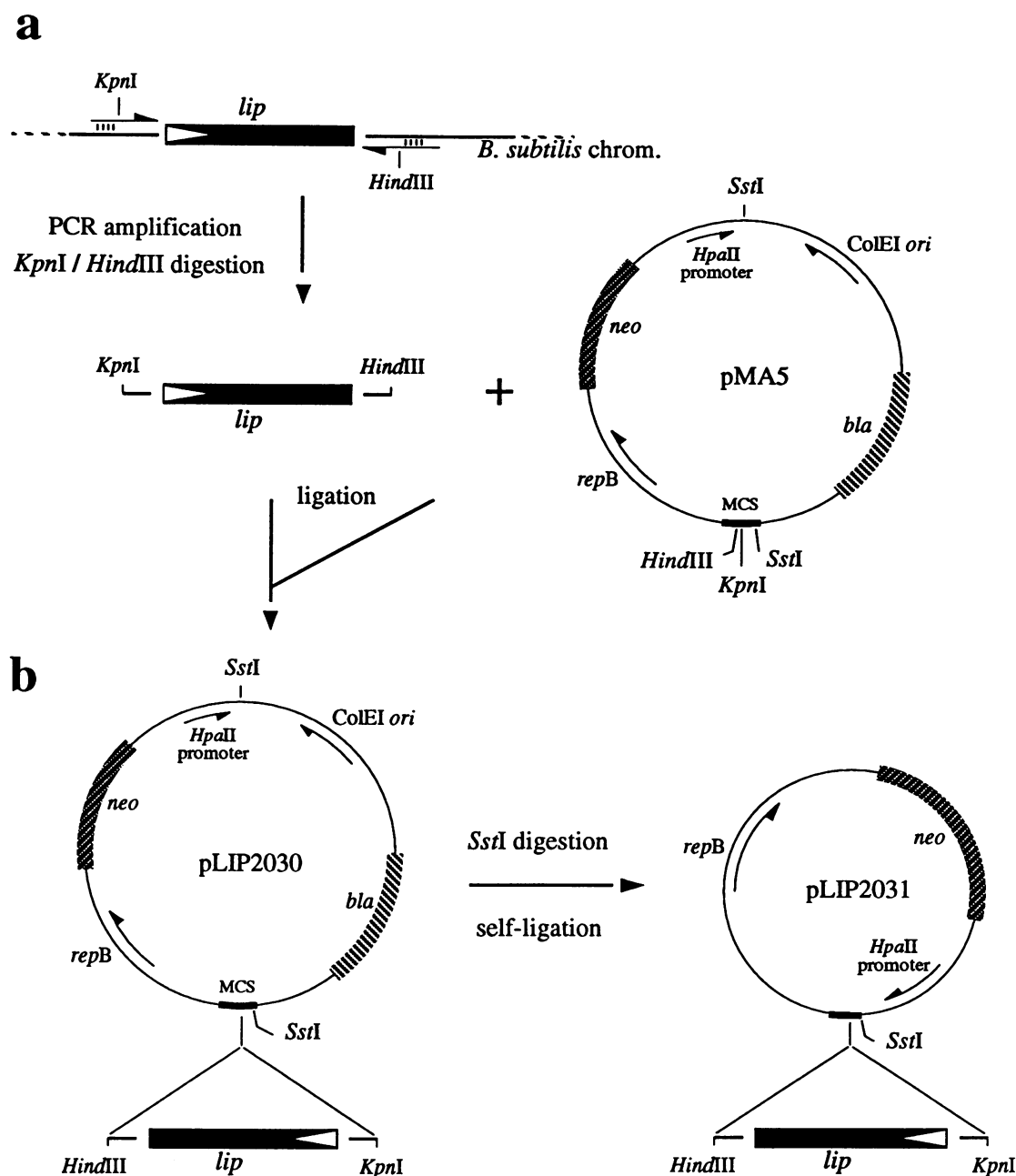


FIG. 3. Overexpression of the endogenous lipase gene in *B. subtilis*. (a) The *lip* coding region was PCR amplified with primers equipped with *KpnI* (sense: 5'-CTT AAT TAA TTT GGT ACC GTA ATA TAA TTG-3') and *HindIII* (antisense: 5'-AAA AAG CTT GCC GAA AGC GGA ATA ACC TTG-3') restriction sites. Amplification products digested with *KpnI* and *HindIII* were subcloned in *E. coli* into the multiple cloning site (MCS) of the pMA5 shuttle vector. (b) *SstI* digestion followed by self-ligation of the *B. subtilis* replicon brought the *lip* ORF under the control of the *HpaII* promoter.

deletion mutation. To avoid recombination between the resident lipase gene and the homologous *lip* allele introduced on a multicopy plasmid, a *B. subtilis* strain with a deletion of the entire *lip* coding sequence was constructed. Since deletion of the *lip* gene was not selectable, the *lip::Km* allele was replaced with a *lip*-deleted fragment by the procedure referred to as congression (7). In the first step, the *EcoRI* insert from pLIP1 was subcloned at the same site of pBluescript SK⁺, devoid of the *BsmI* restriction site, to obtain plasmid pLIP2001 harboring the lipase gene and adjacent sequences. A pLIP2001-derived vector lacking the entire *lip* open reading frame (ORF) was constructed by *BsmI* digestion and self-ligation (Fig. 2a). The resulting plasmid, pLIP2002 (Δlip), retained the chromosomal 600- and 800-bp *lip* flanking regions at the 5' and 3' ends, respectively. Plasmid pLIP2002 was linearized and used to transform *B. subtilis* BCL1008 (*lip::Km*) in combination with a chloramphenicol resistance (*Cm*^r) gene inserted into an arbitrary chromosomal fragment (*estB* in the present case) from *B. subtilis* (Fig. 2b). The ratio of selectable (*Cm*^r) and nonselectable (Δlip) markers used was 1:5. Transformants were selected for *Cm*^r (3 μ g/ml) and then screened for kanamycin sensitivity. About 0.3% of congressants (*Cm*^r *Km*^s) were detected among primary *Cm*^r transformants. One of these, designated BCL1050 (Fig. 2c), was isolated, and Southern blot analysis and PCR amplification of the Δlip region confirmed the deletion mutation.

Overexpression of the lipase gene in *B. subtilis*. The *lip* coding sequence (3) was PCR amplified with oligonucleotides designed such that *KpnI* and *HindIII* restriction sites were created upstream and downstream of the ORF, respectively (Fig. 3a). Amplification products were first phosphorylated and bulk ligated to avoid problems of cleavage close to the end of the DNA fragments. The ligation mixture was subjected to *KpnI*-*HindIII* digestion, and resulting fragments were subcloned at the same sites of the pMA5 *E. coli*-*B. subtilis* shuttle vector (16). Recombinant plasmid pLIP2030 was isolated following electrotransformation into *E. coli* TG1 (13). *SstI* digestion of pLIP2030 followed by self-ligation removed the *E. coli* replicon and positioned the *lip* ORF just downstream of the strong gram-positive *HpaII* promoter (16) (Fig. 3b). The shortened plasmid was designated pLIP2031 and was used to transform the *B. subtilis* Δlip strain BCL1050 under *Km*^r selection. Lipase activity of late-exponential-phase cultures of the resulting strain, BCL1051, was about 100-fold greater than the wild-type strain activity: 17 U/mg of protein in culture supernatant with *p*-nitrophenyl-palmitate as the substrate, under standard conditions (11).

The *B. subtilis* overexpressing strain BCL1051 currently is being used to purify lipase from culture supernatants. Such a homologous system offers the following advantages for optimal purification, characterization, or industrial purposes: (i) efficient protein secretion, (ii) correct maturation of the enzyme, (iii) improved stability of the lipase due to the lack of two major proteases in the culture supernatant of the DB104 host strain, and (iv) the generally regarded as safe (GRAS) status of the *B. subtilis* species. In addition, *B. subtilis* may represent the only potential host for endogenous lipase overproduction since

it possesses a membrane-bound inhibitor which protects the membrane from lipolytic activity (10). It has been suggested that expression of the *B. subtilis* lipase gene inhibits the development and growth of *E. coli* cells (3). Consequently, the Δlip strain BCL1050 could stimulate an interest in the cloning of heterologous lipase genes for industrial applications. Cloning and overexpression of the thermostable lipase from *B. stearothermophilus* are in progress.

We thank Philip Boucher for critical review of the manuscript.

REFERENCES

1. Anguita, J., L. B. Rodriguez Aparicio, and G. Naharro. 1993. Purification, gene cloning, amino acid sequence analysis, and expression of an extracellular lipase from an *Aeromonas hydrophila* human isolate. *Appl. Environ. Microbiol.* **59**:2411-2417.
2. Aunstrup, K. 1990. Novo Nordisk foresees bright future for genetically engineered industrial enzymes. *Eur. Biotechnol. Lett.* **94**:3.
3. Dartois, V., A. Baulard, K. Schanck, and C. Colson. 1992. Cloning, nucleotide sequence and expression in *Escherichia coli* of a lipase gene from *Bacillus subtilis* 168. *Biochim. Biophys. Acta* **1131**:253-260.
4. Dedonder, R. A., J. A. Lepesant, and F. Kunst. 1977. Construction of a kit of reference strains for rapid genetic mapping in *Bacillus subtilis* 168. *Appl. Environ. Microbiol.* **33**:989-993.
5. Fulton, J. E., N. L. Noble, S. Bradley, and W. M. Awad, Jr. 1974. The glycerol ester hydrolase from *Corynebacterium acnes*: a serine lipase. *Biochemistry* **13**:2320-2327.
6. Harwood, C. R. 1992. *Bacillus subtilis* and its relatives: molecular biological and industrial workhorses. *Trends Biotechnol.* **10**:247-256.
7. Hoch, J. A. 1991. Genetic analysis in *Bacillus subtilis*. *Methods Enzymol.* **204**:305-320.
8. Ihara, F., Y. Kageyama, M. Hirata, T. Nihira, and Y. Yamada. 1991. Purification, characterization and molecular cloning of lactonizing lipase from *Pseudomonas* species. *J. Biol. Chem.* **266**:18135-18140.
9. Kouker, G., and K.-E. Jaeger. 1987. Specific and sensitive plate assay for bacterial lipases. *Appl. Environ. Microbiol.* **53**:211-213.
10. Krag, S. S., and W. J. Lennarz. 1975. Purification and characterization of an inhibitor of the phospholipase A1 in *Bacillus subtilis*. *J. Biol. Chem.* **250**:2813-2822.
11. Lesuisse, E., K. Schanck, and C. Colson. 1993. Purification and preliminary characterization of the extracellular lipase of *Bacillus subtilis* 168, an extreme basic pH tolerant enzyme. *Eur. J. Biochem.* **216**:155-160.
12. Newmark, P. 1988. Two European companies market lipases. *Bio/Technology* **6**:369.
13. 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.
14. Trieu-Cuot, P., A. Klier, and P. Courvalin. 1985. DNA sequences specifying the transcription of the streptococcal kanamycin resistance gene in *Escherichia coli* and *Bacillus subtilis*. *Mol. Gen. Genet.* **198**:348-352.
15. Wu, T. T. 1966. A model for three point analysis of random general transduction. *Genetics* **54**:405-410.
16. Zyprian, E., and H. Matzura. 1986. Characterization of signals promoting gene expression on the *Staphylococcus aureus* plasmid pUB110 and development of a Gram-positive expression system. *DNA* **5**:219-225.