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Abstract
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 delta lip strain BCL1050 constitutes an ideal host for the cloning of heterologous lipase genes.

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

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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*,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.

![Diagram](image)

FIG. 1. Strategy for the disruptive replacement of the *lip* gene in the *B. subtilis* protease-deficient strain DB104. (a) The Tn5 internal XhoI fragment containing the kan gene was replaced with the *ClaI* Km' (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.

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Plasmid pLIP2001, carrying the lip gene and adjacent sequences (A and B), was BsmI digested and self-ligated, allowing removal of the entire lip coding sequence. The resulting plasmid, pLIP2002, was linearized with XmnI and used to transform the B. subtilis lip::Km strain BCL1008 together with the est::Cm selectable marker. Double transformation-integration events of both fragments introduced (congression) were detected by screening for kanamycin sensitivity among Cmr transformants. The relevant genotype of the resulting BCL1050 strain proved to be Alip est::Cm. B. subtilis, was replaced by the pAT21.1 aphA gene (14) conferring kanamycin resistance in gram-positive bacteria (Fig. 1a). After PstI 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 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<sup>+</sup> 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 araI (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 region 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 homologous recombination (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') gene inserted into an arbitrary chromosomal fragment (estB in the present case) from B. subtilis (Fig. 2b). The ratio of selectable (Cm') and nonselectable (Δlip) markers used was 1:5. Transformants were selected for Cm' (3 μg/ml) and then screened for lipase activity. About 0.5% of transformants (Cm'/Δlip) were detected among primary Cm' 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 pMAS E. coli-B. subtilis shuttle vector (16). Recombinant plasmid plIP2030 was isolated following electrotropism 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' 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 n-pentanoyl-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. steatothefophilus are in progress.

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