Biosci. Biotechnol. Biochem., **74** (1), 24–30, 2010

Bacillus subtilis Spore Coat Protein LipC Is a Phospholipase B

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Received June 3, 2009; Accepted October 8, 2009; Online Publication, January 7, 2010
[doi:10.1271/bbb.90391]

In *Bacillus subtilis*, the germination-related lipase LipC is located in the spore coat, and mutant spores are defective in L-alanine-stimulated germination. To determine the physiological role of LipC, the recombinant LipC expressed in *Escherichia coli* was purified and characterized. The enzyme hydrolyzes *p*-nitrophenyl ester substrates with various acyl-chain lengths. Thin-layer chromatography and gas chromatography-mass spectrometry analysis indicated that LipC cleaves the fatty acids at the *sn*-1 and *sn*-2 positions of phospholipids as phospholipase B, and that the enzyme shows no selectivity for the polar head groups of lipid molecules. When the amounts of free fatty acids in dormant wild-type and *lipC* mutant (YCSKd) spores were measured, the amount of free fatty acids in the YCSKd spores was about 35% less than in the wild-type spores. These results suggest the possibility that *Bacillus subtilis* LipC plays an important role in the degradation of the outer spore membrane during sporulation.

Key words: bacterial spores; *Bacillus subtilis*; spore germination; sporulation; phospholipase B

Members of the gram-positive *Bacillus* and *Clostridium* genera produce metabolically dormant endospores in response to unfavorable conditions. Bacterial endospores are resistant to severe physical and chemical conditions, including heat, ultraviolet, lytic enzymes, and solvents. These properties are attributed mainly to the unique structure of the spore coat and cortex, as well as to the physical state of the spore core.[1–4] In addition, bacterial spores contain two lipid membrane systems: the inner spore membrane, which surrounds the spore core, and the outer spore membrane, which encases the cortex.[1–4] Despite these resistance properties, spore germination is triggered by specific germinants and leads to an irreversible loss of spore dormancy, followed by outgrowth and the formation of a vegetative cell. Spore germination is controlled by the sequential activation of a set of pre-existing germination-related enzymes but not by protein synthesis,[3,4] and is clearly governed by a new class of sensory and transducer systems that are distinct from the numerous response mechanisms identified to date.[5]

The *Bacillus subtilis* genome-sequencing project revealed about 4,100 protein-encoding genes,[6] and *B. subtilis* has been used as a model organism to understand metabolism, cell division, and macromolecular synthesis, mainly by genetic approaches. Among the many types of cellular development and differentiation, sporulation is one of the best understood. The temporal and spatial control of gene expression, intercellular communication, and various aspects of cell morphogenesis during sporulation have been studied in detail,[7] but the mechanism of germination is still unclear. Many gene products involved in germination have been identified and characterized: receptor-like proteins (defined as Ger family proteins), spore coat proteins, cortex-lytic enzymes, and so on.[8–10] Strains with mutations in these genes are blocked at various stages of spore germination. In a previous study, we found that LipC, a protein component of the spore coat, is a member of a GDSL family of lipolytic enzymes and that *lipC* mutant spores are defective in L-alanine-stimulated spore germination.[11] LipC is expressed in the mother cell compartment at the late stage of sporulation and is localized around forespores in a CotE-, SafA-, and SpoIVD-dependent manner, suggesting that LipC is probably targeted around the outer spore membrane.[11] Kawai *et al.* found that spore membranes of *B. subtilis* Marburg had significantly higher cardiolipin contents than the membranes of exponentially growing cells,[12] but lipid biosynthesis and metabolism in spore formation and/or germination are poorly understood.

In this study, we purified recombinant LipC expressed in *E. coli* and biochemically characterized the enzymatic properties of LipC. In addition, analysis of the free fatty acid contents in dormant spores suggested that LipC hydrolyzes phospholipids during sporulation as a phopholipase B.

Materials and Methods

Materials. Restriction enzymes were purchased from Takara Shuzo (Kyoto, Japan). The *p*-nitrophenyl (*p*NP) esters were from Nacalai Tesque (Kyoto, Japan), Sigma (St. Louis, MO), and Wako Pure Chemicals (Osaka, Japan). His Bind™ Resin was from Novagen (Madison, WI). Phospholipids were from NOF (Tokyo) and Sigma (St. Louis, MO).

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Table 1. Bacterial Strains and Plasmids Used in This Study

Strains	Genotype and/or description	Source or reference
B. subtilis		
168	*trpC2*	Bacillus Genetic Stock Center
YCSKd	*trpC2 ycsk* (*lipC*)::pMutin3	11
E. coli		
JM109	*relA supE44 endA1 hsdR17 gyrA96 mcrA mcrB* + *thiΔ(lac-proAB)/F′ (traD36 proAB* + *lacIq lacZΔM15)*	11
BL21 (DE3)	F^- *ompT hsdS*$_B$ (r_B^- m_B^-) *gal dcm* (DE3)	Novagen
Plasmids		
pET22b(+)	*bla*	Novagen
pET-LipC-His	*lipC bla*	This study
pET-LipC(S11A)-His	*lipC* (S11A) *bla*	This study

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* BL21(DE3) was used as host for expression of the *B. subtilis lipC* gene. *E. coli* transformants were grown at 37 °C in Luria-Bertani (LB) broth containing 100 μg/ml of ampicillin (final concentration).

Spore preparation. B. subtilis spores were prepared as described previously.[13] The spores were harvested and purified by washing with sterilized water until the cell debris and vegetative cells could not be observed by microscopy. The purity was confirmed by phase-contrast microscopy. More than 98% of the spores prepared were refractive, and almost no dark or gray spores were visible by phase-contrast microscopy.

Cloning and expression of the B. subtilis lipC *gene in* E. coli. The *B. subtilis lipC* gene was amplified by PCR using *B. subtilis* chromosomal DNA as the template with the following primers: exYCSK1 (5′-TTTTGAATTCATATGGTGCTTCGATATACAGC-3′, *Nde*I site underlined.) and exYCSK3 (5′-ATTTGAATTCTCGAGTGAAACTGATAATTCTTTATAGCC-3′, *Xho*I site underlined.). The resulting PCR product was digested with *Nde*I and *Xho*I and cloned between the same sites of pET22b(+) (Novagen), yielding plasmid pET-LipC-His. *E. coli* BL21(DE3) cells transformed with pET-LipC-His were cultivated in LB medium containing ampicillin. Expression of *lipC* was induced by the addition of 1.0 mM isopropyl β-D-thiogalactopyranoside (IPTG) when the tubidity of culture at 620 nm reached 0.5. After further cultivation for 3 h at 37 °C, the cells were harvested and stored at −20 °C until they were used in enzyme purification.

Site-directed mutagenesis. Site-directed mutagenesis of the cloned *lipC* gene was performed by the strategy of overlap extension using PCR, as described by Higuchi *et al.*[14] The *lipC* gene was amplified by PCR using pET-LipC-His as template with the following primers: exYCSK1, exYCSK3, YCSK(S11A)-FW (5′-GGGCGATGCATTGACGACA-3′), and YCSK(S11A)-RV (5′-TGTCGTCAATGCATCGCCC-3′). The first round of PCR was carried out with primers exYCSK1 and YCSK(S11A)-RV or primers exYCSK3 and YCSK(S11A)-FW. The reaction products from the two PCRs were diluted and used as templates in a second round of PCR. The second PCR was performed using exYCSK1 and exYCSK3 as the primer pair. The resulting PCR product was digested with *Nde*I and *Xho*I and cloned between the same sites of pET22b(+), yielding plasmid pET-LipC(S11A)-His. The presence of the mutation and the fidelity of mutagenesis was confirmed by sequencing.

Purification of LipC. The harvested transformant cells were suspended in 10 ml of 20 mM Tris–HCl buffer (pH 7.9) containing 0.5 M NaCl and 5 mM imidazole (buffer A) and disrupted by ultrasonication. After the cell debris was removed by centrifugation, the supernatant was applied to a column of His Bind™ Resin, which had been equilibrated with buffer A. After the column was washed with 20 mM Tris–HCl buffer (pH 7.9) containing 0.5 M NaCl and 20 mM imidazole, the enzyme was eluted with 20 mM Tris–HCl buffer (pH 7.9) containing 0.5 M NaCl and 0.5 M imidazole. The active fractions were dialyzed against 20 mM Tris–HCl buffer (pH 7.9) containing 10% v/v glycerol.

Enzyme and protein assays. Enzyme activity was measured with *p*NP esters with fatty-acid chain lengths of C4–C18. Stock solutions (20 mM) of *p*NP esters were prepared by dissolving the substrates in acetonitrile. The standard assay mixture (1.0 ml) contained 0.5 mM *p*NP ester, 50 mM Tris–HCl buffer (pH 8.5), 150 mM NaCl, 0.2% Triton X-100, 2.5% acetonitrile (final concentrations), and the enzyme. The reaction mixture without the enzyme was preincubated for 3 min at 37 °C. The reaction was initiated by the addition of the enzyme and terminated after 30 min by the addition of 2.0 ml of acetone. After the addition of 1.0 ml of 2 M Tris–HCl buffer (pH 8.5), the absorbance was measured at 400 nm. One unit of enzyme activity was defined as the formation of 1 μmol *p*-nitrophenol per min. An extinction coefficient, 14,500 M^{-1} cm^{-1} of *p*-nitrophenol at 400 nm, was used in unit calculations. The protein concentration was determined by the method of Bradford[15] with bovine serum albumin as the standard.

Thermal stability and pH activity profiles. In the thermal stability studies, the enzyme was incubated in 50 mM sodium phosphate buffer (pH 7.5) at 4, 20, 30, 40, 50, 55, 60, and 70 °C. At 30-min intervals, the remaining activity was assayed at 37 °C using *p*NP-myristate as the substrate. The effect of pH on enzyme activity was examined by standard assay using *p*NP-myristate as the substrate, except that the reaction mixture (without the substrate) was preincubated at 25 °C for 10 min. The optimal pH for enzyme activity was determined by assaying the enzyme at 37 °C in a pH range from 4.0 to 10.0 (50 mM sodium citrate, pH 4.0 to 6.5; 50 mM sodium phosphate, pH 6.0 to 8.0; 50 mM Tris–HCl, pH 7.5 to 9.0; 50 mM glycine–NaOH, pH 9.0 to 10.0).

Effects of various reagents on enzyme activity. The enzyme was preincubated at 25 °C for 10 min in a buffer containing 50 mM Tris–HCl (pH 8.5), 150 mM NaCl, 0.2% Triton X-100, and one or two of the following additives: phenylmethylsulphonyl fluoride (PMSF), EDTA, DTT, $CaCl_2$, $MgCl_2$, $MnCl_2$, $ZnCl_2$, $FeCl_2$, $CuCl_2$, $HgCl_2$, L-alanine, D-alanine, dipicolinate (DPA), and calcium dipicolinate (Ca-DPA). The additives were incubated with the enzyme at a final concentration of 1 mM or 5 mM. The effects of various reagents on enzyme activity were assayed at 37 °C using *p*NP-myristate as the substrate. The activity of the enzyme without any additives was taken to be 100%.

Phospholipase assays and gas chromatography-mass spectrometry (GC-MS) analysis. Phospholipids were suspended in 50 mM Tris–HCl (pH 8.5) buffer containing 0.15 M NaCl and 0.1% Triton X-100 and dispersed in a bath sonicator until the solution cleared. Phospholipids were mixed with the enzyme in 1.0 ml of buffer solution, and the mixture was incubated overnight at 37 °C with shaking. After extraction with 3.0 ml of a chloroform-methanol solution (4:1), the chloroform layer was dried under nitrogen gas. The products were resuspended in 25 μl of a chloroform-methanol solution (2:1) and spotted on a Silica Gel 60 plate (Whatman, Springfield), along with 100 μg of linoleic acid and 100 μg of phosphatidylcholine (PC). Using the solvent system of Matsuzawa and Hostetler,[16] we developed the plate to a height of 8.5 cm from the origin in a chloroform-methanol-water solution (65:35:5), allowed the plate to dry, and developed it to 15 cm from the origin in a heptane-diethylether-formic acid solution (90:60:4). Sulfuric acid (50%) was used to visualize phospholipids and their digested products.

In GC-MS analysis, 1-palmitoyl-2-myristoyl-*sn*-glycero-3-phosphatidylcholine was used as substrate. The substrate was mixed with the enzyme as described above. In methylation, the reaction products were incubated with 10 vol. of 1.25 M hydrogen chloride methanol solution (Fluka, Buchs) at 90 °C for 2 h. After the products were extracted with hexane, the methylesters were subjected to GC-MS analysis. This was performed with a Hewlett-Packard 6890 gas chromatograph interfaced with an MStation JMS-700 mass spectrometry system (JEOL, Tokyo).

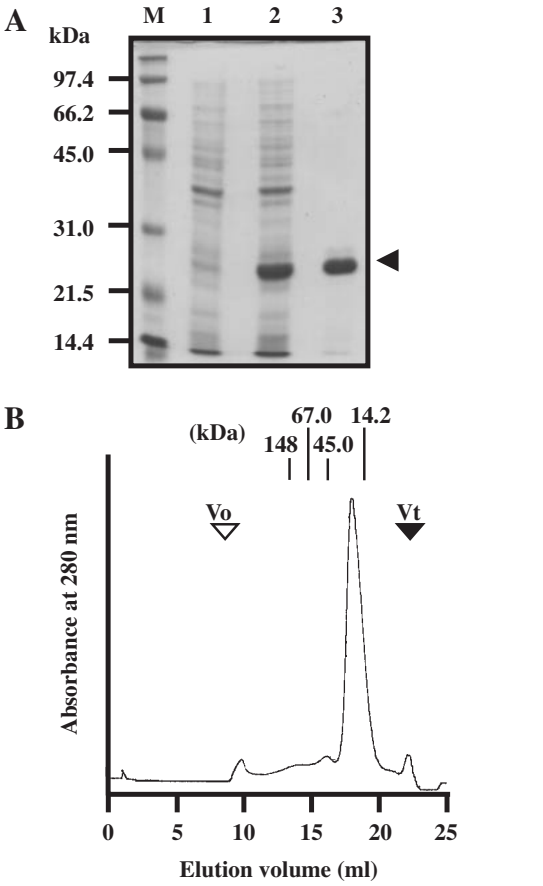

Fig. 1. Purification of Recombinant LipC.

A, SDS–PAGE of the recombinant LipC expressed in *E. coli*. Lane 1, proteins from whole cells of *E. coli* BL21(DE3) harboring pET22b(+); lane 2, proteins from whole cells of *E. coli* BL21(DE3) harboring pET-LipC-His; lane 3, purified LipC. B, Gel filtration analysis of the enzyme. The purified recombinant LipC was subjected to gel filtration chromatography on Superdex 200 HR 10/30.

A J&W DB™-1 capillary column (30 m × 0.25 mm, d.f. = 0.25 μm) was used for the GC. Samples were injected at 80 °C, and the temperature was increased at a rate of 12 °C/min. Electron impact-MS was performed at 70 eV with a mass range from m/z 50–500 and a cycle time of 1 s in the positive ion mode.

Extraction and measurement of free fatty acids from B. subtilis *dormant spores.* The purified *B. subtilis* spores were disrupted in sterilized water at 4 °C with a bead beater, and the suspensions were mixed with a chloroform-methanol-water solution (1:2:0.8) at 4 °C for 1 h. After this, free fatty acids (FFAs) were extracted with a chloroform-methanol-water solution (1:1:1). FFA concentrations were measured using free fatty acids, half-micro test (Roche Diagnostics, Basel) according to the manufacturer's instructions. The data were obtained from at least three independent experiments.

Results

Overexpression of lipC *and purification of the* lipC *gene product*

To determine the lipolytic activity of LipC, we cloned and overexpressed the *lipC* gene in *E. coli*. The overproduced recombinant LipC with a C-terminal hexahistidine-tag was purified by Ni-chelating column chromatography, as described in "Materials and Methods" (Fig. 1A). The molecular weight of 24,669, deduced from the amino acid sequence with the histidine-tag (LEHHHHHH), was in agreement with

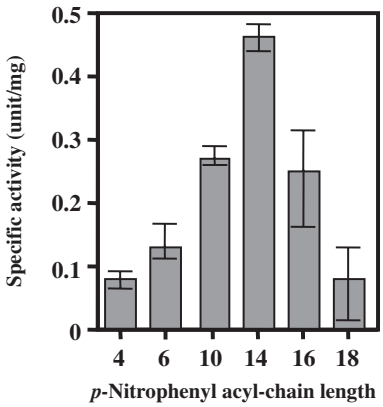

Fig. 2. Substrate Specificity of LipC towards *p*-Nitrophenyl Esters of Various Acyl-Chain Lengths.

Activities were determined by spectrophotometric assay. The data are averages for three independent experiments.

that estimated by SDS–PAGE. When the purified LipC was subjected to gel filtration chromatography on Superdex 200 HR 10/30, it eluted at an elution volume between ovalbumin (45.0 kDa) and cytochrome c (14.2 kDa) (Fig. 1B). This suggests that LipC is a monomer.

Substrate specificity of LipC

Our previous work indicated that LipC is a lipolytic enzyme.[11] To determine the substrate specificity of LipC, we tested the ability of the enzyme to hydrolyze triglycerides and *p*-nitrophenyl esters as substrates. The recombinant enzyme exhibited lipolytic activity towards *p*-nitrophenyl esters, while no activity was observed towards tributyrin (C4), tricaprilin (C8), or triolein (C14) (data not shown). When the substrate specificity of LipC was examined using *p*-nitrophenyl esters of fatty acids of various acyl-chain lengths, the enzyme showed the highest activity towards *p*NP-myristate as the substrate (0.46 units/μg) (Fig. 2). The lipolytic activity of LipC decreased with substrates of shorter and longer chain lengths.

Effects of temperature, pH, and various reagents on enzyme activity

After the enzyme was incubated at temperatures between 4° and 70 °C for 30 min, the residual activity of LipC was measured. The stability of the enzyme decreased sharply, less than 20% activity remaining when the temperature was over 60 °C, as shown in Fig. 3A. The effect of pH on enzyme activity was measured at 37 °C between pH 4.0 and 10.0. The pH profile indicated that the optimum pH was 8.5 (Fig. 3B). The effects of various reagents on enzyme activity was measured as described in "Materials and Methods." As shown in Table 2, Fe^{2+} and Cu^{2+} significantly activated LipC, whereas the addition of Ca^{2+} inhibited activity to 40%. To determine whether the active site of LipC is a serine residue, activity was measured in the presence of serine-specific inhibitor PMSF. Enzyme activity was not significantly inhibited by PMSF. Other serine hydrolases have shown similar resistance to such inhibitors.[17–19] Additionally, to determine the relationship between LipC activation and general germinants, we assayed enzyme activity in the presence of L-alanine, D-alanine, DPA, and Ca-DPA chelate. The effects of L-alanine and

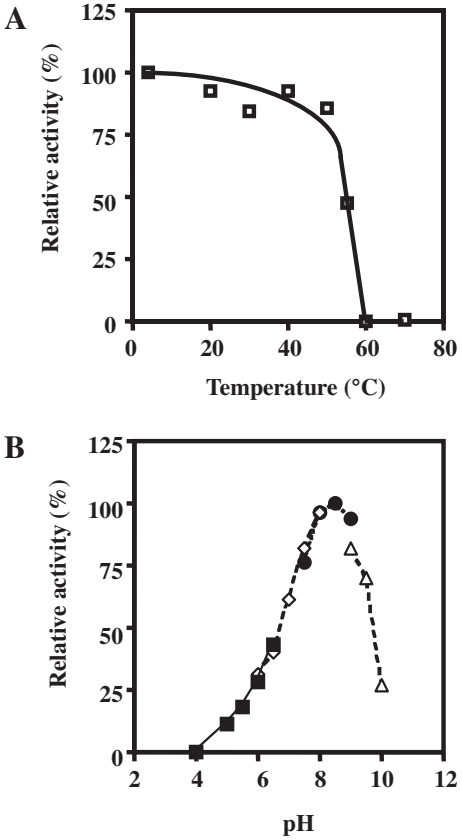

Fig. 3. Effects of Temperature (A) and pH (B) on LipC Activity towards the Substrate *p*NP-Myristate.

A, Thermal stability was carried out by incubating the enzyme in a 50 mM sodium phosphate buffer (pH 7.5) for 30 min at various temperatures before the remaining activity was assayed. B, Optimum pH profile was determined by incubating the enzyme at 25 °C for 10 min at various pHs: 50 mM sodium citrate, pH 4.0 to 6.5 (squares); 50 mM sodium phosphate, pH 6.0 to 8.0 (diamonds); 50 mM Tris–HCl, pH 7.5 to 9.0 (circles); 50 mM glycine–NaOH, pH 9.0 to 10.0 (triangles). The data obtained from three independent experiments are shown.

Table 2. Effects of Various Reagents on LipC Activity

Compound	Final concentration	Relative activity (%)[a]
None		100
$CaCl_2$	1 mM	40
$MgCl_2$	1 mM	98
$MnCl_2$	1 mM	67
$ZnCl_2$	1 mM	84
$FeCl_2$	1 mM	124
$CuCl_2$	1 mM	127
$HgCl_2$	1 mM	61
PMSF	1 mM	72
EDTA	5 mM	81
DTT	5 mM	78
L-Alanine	1 mM	93
D-Alanine	1 mM	94
DPA	1 mM	82
Ca-DPA	1 mM	71

[a]The assay was performed as described in "Materials and Methods." The activity of the enzyme in the absence of additives was taken to be 100%. The average values obtained from three independent experiments are shown.

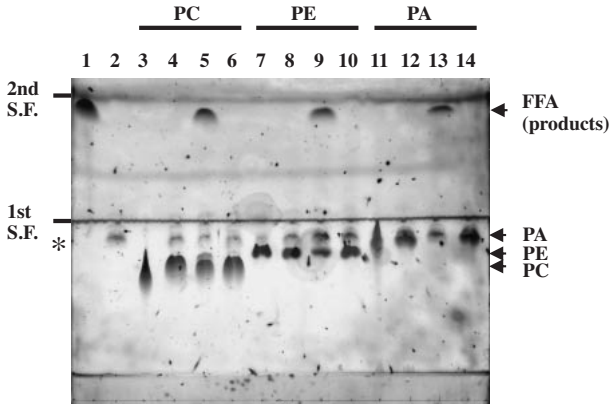

Fig. 4. Thin-Layer Chromatography Analysis of LipC Phospholipase Activity.

Lane 1, linoleic acid (free fatty acid; FFA); lane 2, reaction buffer (50 mM Tris–HCl pH 8.5 buffer containing 0.15 M NaCl and 0.1% Triton X-100); lanes 3 to 6, 7 to 10, and 11 to 14, PC, PE, and PA respectively as substrates; lanes 3, 7, and 11, undigested phospholipids; lanes 4, 8, and 12, reaction buffer containing undigested phospholipids; lanes 5, 9, and 13, reaction products generated by LipC (wild type); lanes 6, 10, and 14, reaction products generated by the LipC mutant (S11A). The asterisk indicates the position of Triton X-100.

D-alanine on enzyme activity were subtle, whereas DPA and Ca-DPA decreased it. These results suggest that LipC is not activated directly by germinants during spore germination.

LipC has phospholipase B activity

To determine the physiological role of LipC, we studied the ability of LipC to cleave phospholipids. After the enzyme was incubated with phospholipids, as described in "Materials and Methods," thin-layer chromatography (TLC) was used to analyze the digested products of phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidic acid (PA). TLC analysis indicated that the digestion of phospholipids by LipC released FFAs (lanes 5, 9, and 13 in Fig. 4), suggesting that the enzymatic activity of LipC is phospholipase A or B. On the other hand, the S11A variant did not release FFAs from all phospholipids (lanes 6, 10, and 14 in Fig. 4), suggesting that part of the putative catalytic triad in LipC, Ser11, is a catalytic residue. To further investigate the enzymatic specificity of LipC, the digested products of phospholipids were methylated and subjected to GC-MS analysis. As shown in Fig. 5A, two main peaks appeared, with retention times of 11.74 min and 13.47 min. The estimated molecular masses of peak 1 and peak 2 were 242 and 270 respectively. These values were identical to the exact molecular weights of methyl myristate and methyl palmitate (Fig. 5B and C). Thus LipC cleaved both ester linkages of the *sn*-1 and *sn*-2 positions of phospholipids, suggesting that LipC has phospholipase B activity.

Physiological role of LipC in B. subtilis *spores*

In a previous study, we noted that the integrity of the outer spore membrane in sporulating cells was doubtful.[20] To determine whether LipC hydrolyzes the phospholipid bilayer during sporulation, we measured the FFA content in dormant spores. Extracts from dormant spores were assayed using free fatty acids by

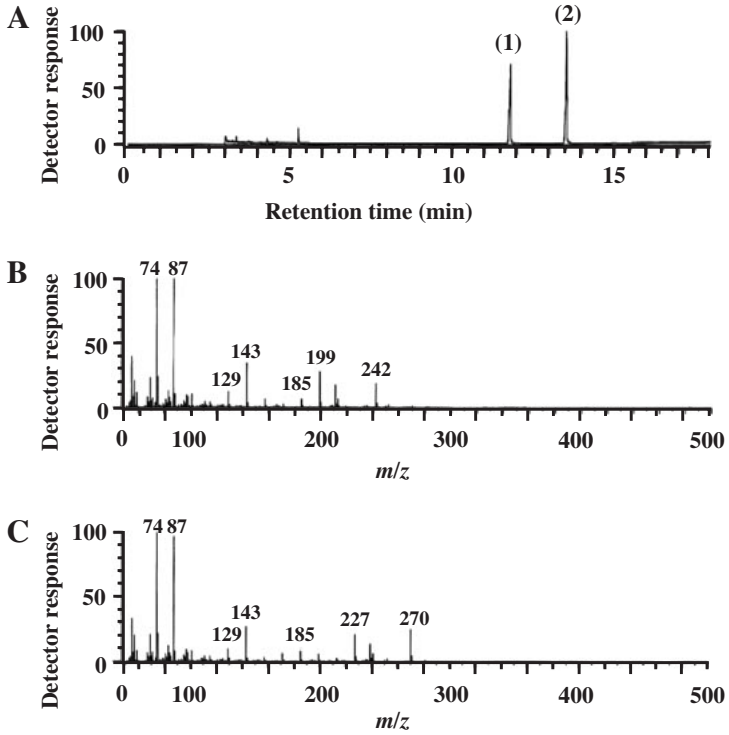

Fig. 5. GC-MS Analysis of Reaction Products Generated by LipC.

A, Chromatogram of reaction products generated by LipC. In this assay, 1-palmitoyl-2-myristoyl-*sn*-glycero-3-phosphatidylcholine was used as the substrate. B, ESI-MS spectrum of peak 1 from Fig. 5A, corresponding to that of methyl C14:0 (myristate). C, ESI-MS spectrum of peak 2 from Fig. 5A, corresponding to that of methyl C16:0 (palmitate).

the half-micro test (Roche Diagnostics), which can determine the amount of FFAs in serum and plasma. Serum and plasma contain mainly straight-chain acids, while the major components of fatty acids in *Bacillus* species are anteiso- and iso-branched-chain acids.[21,22] We confirmed that the assay was able to measure branched-chain acids. In this study anteiso-C15 was used as the standard. When the extracts from dormant spores were assayed, the amount of FFAs in the wild-type and YCSKd (*lipC* mutant) spores were 120 ± 4 ng/mg spores (wet weight) and 77 ± 2 ng/mg spores (wet weight) respectively. This suggests that LipC hydrolyzes phospholipids and releases FFAs in wild-type spores.

Discussion

LipC is a component of the spore coat in *B. subtilis*, which protects the spore from numerous assaults. In a previous study, we found that LipC is a lipolytic enzyme and that *lipC* mutant spores are defective in L-alanine-stimulated spore germination.[11] In this study, we purified recombinant LipC expressed in *E. coli* and provided the first biochemical characterization of this lipase. The enzyme hydrolyzed *p*NP ester substrates and a variety of phospholipids. The thermal stability and pH-activity profiles of the enzyme indicated that LipC does not possess the extreme resistance properties of spores. Furthermore, calcium ions and germinants did not activate lipase activity, suggesting that the enzyme dose not play a crucial role in spore germination, but comprises part of the spore germination machinery, which includes the spore coat and membranes. It is known that vegetative cells of *B. subtilis* secrete two major lipolytic enzymes, LipA and LipB, both of which belong to the α/β-hydrolase family.[23,24] Unlike LipC, LipA, and LipB are tolerant of alkaline pH. Their optimum pH values are 10 and 12 respectively. LipA has phospholipase (EC 3.1.1.3) activity, and LipB functions as esterase (EC 3.1.1.1). Furthermore, LipA and LipB prefer middle chain (C8) fatty acid esters as substrate. The differences among LipA, LipB, and LipC, in their enzymatic properties and structural features suggest that expression of the *lipA* and/or *lipB* genes during sporulation would not compensate for the deletion of *lipC*.

Based on the speculation that LipC might affect the outer and/or inner spore membranes, we tested to determine whether the enzyme would hydrolyze phospholipids. Phospholipases are hydrolytic enzymes that cleave ester bonds in phospholipids. These enzymes are categorized into four groups: A to D. Phospholipase A cleaves fatty acid residues from the *sn*-1 or *sn*-2 position of the glycerol backbone. Phospholipase B cleaves both fatty acid residues from the *sn*-1 or *sn*-2 position. Phospholipase C and D are phosphodiesterases that release phosphomonoesters and alcohol respectively. In this study, we indicated that LipC hydrolyzed a variety of phospholipids as phospholipase B. *In vivo*, the amount of FFAs in the *lipC* mutant spores was less than that in the wild-type spores, suggesting the possibility that the phospholipase activity of LipC modifies the outer or inner spore membrane and releases FFAs during sporulation. Taking into account the fact that LipC expressed in the mother cell compartment does not contain a signal sequence and is localized around the outer spore membrane,[11] our results suggest that LipC modifies the outer spore membrane. In that case, the outer spore membrane in the *lipC* mutant spores are

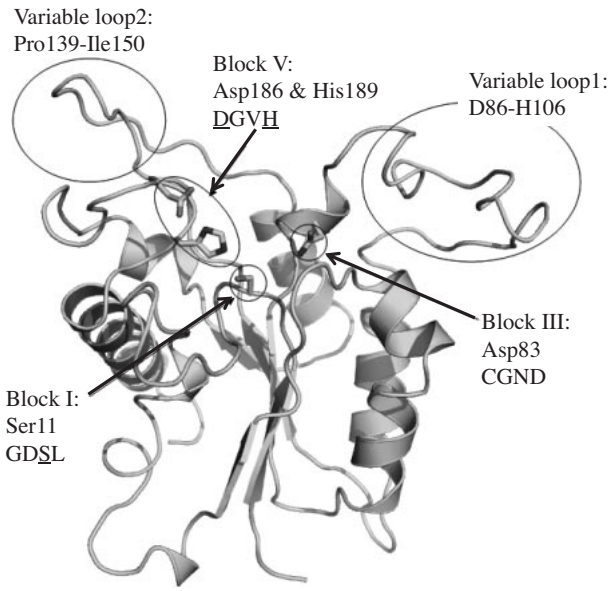

Fig. 6. Homology Modeling of LipC.
The active site is conserved as in all the known structures of SGNH hydrolases. The catalytic triad is underlined. The two loop regions might be involved in the activation of LipC in acting at the lipid-water interface of substrates.

intact as a permeability barrier, unlike that in wild-type spores, which is a defective barrier. The germination defect in the *lipC* mutant spores might be caused by a decrease in the passage of germinants across the outer spore membrane to reach the inner spore membrane where the Ger receptors are localized.[25,26] As previously reported, *lipC* mutant spores are not defective in AGFK (L-asparagine, D-glucose, D-fructose, and KCl)-stimulated spore germination.[11] As is well known, the germinant receptors for the AGFK system are totally different from those for the L-alanine, and the permeation pathways of the germinants differ from each other, although the details are not known.[4] Therefore, investigation of the permeability of the *lipC* spores to germinants should provide insight into the spore germination pathways.

Based on the similar sequence position and context of the catalytic triad, *B. subtilis* LipC belongs to the SGNH-hydrolase family.[11] A subgroup of the GDSL family is classified as the SGNH-hydrolase family due to the presence of four strictly conserved residues, Ser-Gly-Asp-His, in four conserved blocks, I, II, III, and V.[27] Rhamnogalacturonan acetylesterase from *Aspergillus aculeatus* (AaRGAE) also belongs to the SGNH-hydrolase family. The structural features of AaRGAE and several other SGNH-hydrolase members have been studied.[27,28] Based on two templates (PDB accession nos. 1k7c_A, and 2o12_A), we created a homology model of LipC using the web server HHpred (http://toolkit.tuebingen.mpg.de/hhpred), which is based on pairwise comparison of profile-hidden Markov models (HMMs) (Fig. 6).[29,30] This structure consists of a central five-stranded parallel β-sheet surrounded by α-helices on both sides. The putative catalytic triad Ser11-Asp186-His189 in LipC is located at a topological switchpoint at the C-terminal end of the central β-sheet. The S11A mutation in LipC resulted in a loss of activity (Fig. 4), suggesting that Ser11 from Block I is a catalytic residue and can serve as the nucleophile. Brumlik and Buckley proposed that Asp83 from Block III is a putative active site,[31] but structural analysis of other SGNH hydrolases suggested that Asp187 from Block V is the acidic residue of the catalytic triad.[28,32,33] Gly50 from Block II and Asn82 from Block III appear to donate H-bonds to the oxyanion hole. In this structural model, we noticed that two surface loops, Asp86-His106 and Pro139-Ile150, might cover the active site in a lid-like fashion. Lipolytic enzymes are characterized by their drastically increased activity when acting at the lipid-water interface of micellar or emulsified substrates, a phenomenon called interfacial activation. In the absence of lipid-water interfaces, the active site of lipases is covered in the closed formation by the lid domain. On the other hand, in the presence of hydrophobic substrates, the lid is opened, making the catalytic residues accessible to the substrate and exposing a hydrophobic surface.[34] Structural analysis showed that *B. subtilis* LipA and LipB lack a lid domain, and that their active sites are solvent exposed.[23,24] Thus, it is possible that the two surface loops of LipC contribute to regulation of switching from the inactive form to the active form during sporulation. There are other possibilities, that the two surface loops of LipC function as domains that have other roles, such as substrate targeting, substrate selectivity, and thermostability. Santarossa *et al.* reported that mutations in lid region affected the substrate preference and thermostability of *Pseudomonas fragi* lipase.[35] Elucidation of the detailed role of those surface loops in the physiological function of LipC is our future task.

E. coli thioesterase I (TAP) of the SGNH-hydrolase family has a single compact domain without a lid and has a broad substrate specificity, including thioesterase, arylesterase, protease, and lysophospholipase activities.[36–38] Since the catalytic triad of lipases is similar to that of serine proteases, the different specificity between TAP and other lipases may be due to the presence of a lid domain like the surface loops of LipC. Although the lipolytic enzymes of the GDSL family play important roles in the bacterial metabolic pathway and virulence factors,[36,39,40] several enzymes in this family show low lipolytic activity, unlike TAP.[37,39–42] Structural comparison between LipC and TAP may provide new insights into the structure-activity relationship in this family. Since many microbial lipases are available for industrial applications as biocatalysts, improving the activity and specificity of the LipC by protein engineering and *in vitro* evolution will also be a focus of our further studies.

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