Matsuura T^a, Terayama T^b, Usami R^a, Yoshida H^a, Hara H^b,
and Matsumoto K^b

Involvement of YkoN in production of a new phospholipid in *Bacillus subtilis* Marburg

^a Department of Applied Chemistry, Graduate School of Engineering, Toyo University, 2100 Kujirai, Kawagoe, Saitama 350-8585, Japan

^b Department of Biochemistry and Molecular Biology, Graduate School of Science and Engineering, Saitama University, 255 Shimo-ohkubo, Sakura, Saitama, Saitama 338-8570, Japan

Corresponding author : Kouji Matsumoto, koumatsu@molbiol.saitama-u.ac.jp
Phone : 81-48-858-3406, Fax: 81-48-858-3698

Received: November 5, 2007 / Revised: November 12, 2007 / Accepted: November 19, 2007

Abstract To understand the physiological role of the *ykoN* gene of *Bacillus subtilis*, the gene was expressed from the IPTG-inducible *spac* promoter in *B. subtilis* cells. When lipid composition of the cells induced for *ykoN* expression was examined, a new spot of phospholipid was found on the thin layer chromatogram. Induction of *ykoN* in *Escherichia coli* cells produced a similar new spot. The new spot was produced in mutant *E. coli* cells lacking cardiolipin or phosphatidylethanolamine, but not in the cells lacking phosphatidylglycerol. The result suggests an involvement of phosphatidylglycerol in production of the new spot. Examination of the lipid composition during the stages of *B. subtilis* growth revealed that the new phospholipid was produced four hours after cessation of logarithmic growth, consistent with an increase in the promoter activity of *ykoN* in the late stage in sporulation. YkoN has the pentapeptide lipase/esterase motif (Gly-X-Ser-X-Gly), and shows a lipolytic activity (Matsuura *et al.*, in press). The mutant YkoN (S205A) in which the central serine residue of the motif is replaced with alanine produced the new phospholipid in the amount as small as only 1/10 of the wild type, suggesting that the lipolytic activity of YkoN is involved in the new phospholipid production.

Key words : *Bacillus subtilis*, *ykoN*, phospholipids

Introduction

The membrane of *B. subtilis* Marburg cells contains many lipids. In addition to the three major glycerophospholipids,

phospholipids, phosphatidylethanolamine (PE), phosphatidylglycerol (PG), and cardiolipin (CL), which are common with those of *E. coli*, the membrane contains lysylphosphatidylglycerol (Lys-PG), mono-, di-, and triglucosyldiacylglycerols, and glycerophosphoglucolipid¹⁾. In *E. coli* cells each of all the three major phospholipids is shown to be dispensable²⁾. Many of the *B. subtilis* lipids, except PG, are also dispensable^{3,4,5,6)}. We are therefore curious to know why *B. subtilis* has so many lipids and what their physiological roles might be. In order to solve the problems we have started clarifying the pathways for biosynthesis of the lipids by identifying the genes involved. Homology search with the amino acid sequence of known enzymes for lipid synthesis has shown a lot of possible genes and has led us to build up an outline of lipid biosynthesis in *B. subtilis*^{7,8)}. Among them the amino acid sequence of the product (YkoN) of *ykoN* was found to have a weak homology with that of UgtP (YpfP) that is responsible for the synthesis of glucosylated diacylglycerates in this organism⁹⁾. However, disruption of *ykoN* caused no change in the content of glucosylated diacylglycerates in the membrane. We have recently shown that YkoN has a lipolytic activity (Matsuura *et al.*, in press). Induction of *ykoN* in *B. subtilis* and *E. coli* cells produced a new spot of phospholipid on thin layer chromatogram.

This paper describes the finding of the new phospholipid in *B. subtilis* cells in sporulation phase and discusses its possible source in relation to the lipolytic activity of YkoN.

Materials and Methods

Construction of bacterial strains and plasmids

B. subtilis Marburg and *E. coli* strains and plasmids used in this study are listed in Table 1. The pMutin integration strain TM001 was constructed according to the standard protocol¹⁰⁾ using pTM040 (pMUTIN2 *ykoN*). TM001 has *ykoN* which is controlled by its own promoter and P_{spac} and it also has a disrupted *ykoN* allele fused to *lacZ* for determination of its promoter activity. Construction of pTM040 was carried out by cloning of *ykoN* DNA into pMUTIN2 as follows. The *ykoN* DNA fragment was amplified from *B. subtilis* wild type chromosomal DNA by PCR with Ex *Taq* DNA polymerase using a primer pair *Hind*III-*ykoNS* (5'-CCATATGGAAAGGAAACAAGGcTTTCATC-3') (synthesized by Espec Oligo, Japan), which starts at 117 nucleotide (nt) upstream of the initiation codon with one mismatch (lowercase letter) to create the *Hind*III restriction site (underlined), and *ykoN-EcoRI*A (5'-CCCATTTTgAAtTCCCACC-3'), which starts at 643 nt downstream of the initiation codon with two mismatches (lowercase letter) to create the *EcoRI* restriction site (underlined). The amplified fragment was inserted into *Hind*III-*EcoRI* site of pMUTIN2 to form the recombinant plasmid designated as pTM040.

Cloning of *ykoN* DNA into expression vector pAN18 was performed as follow. The *ykoN* fragment was amplified using a primer pair *Sac*I-*ykoN* (5'-GAACGag-CTCCTCAAATATAG-3'), which starts at 49 nt upstream of the initiation codon, and *ykoN-Xba*I (5'-CCTGCTCtAGAAAGC-3'), which starts at 66 nt downstream of the termination codon. The amplified fragment was inserted into *Sac*I-*Xba*I site of pAN18 to form pTM088.

Cloning of *ykoN* DNA into pSK6 vector was performed as follow. The DNA fragment was amplified by using a primer pair *Nco*I-*ykoN* (5'-GGATCAACC-ACTCTCCATggGAAAGG-3'), which starts at 132 nt upstream of the initiation codon, and pCold-*ykoN* (5'-CTATTCTAgACTCTCTATTTC-3'), which starting at 14 nt downstream of the termination codon. The amplified fragment was inserted into *Nco*I-*Xba*I site of pSK6 to form pTM042.

Cloning of *ykoN* DNA into pET-21(+) to construct pET-*ykoN* plasmid was previously described (Matsuura *et al.*, in press). pET-*ykoNS205A*, which induces the mutant *ykoN* gene whose serine codon at 205 is altered to alanine, was constructed as follows. Two DNA fragments including the altered codon were first prepared by using a primer pair PETykoNS (5'-gccgaAgcTTGCCATA-TTTTTTTAGG-3') and *ykoNmfA* (5'-GtCCCC-ATAgcCCCCCTGTG-3'), which includes three mismatches for the altered codon, and a primer pair

ykoNmfS (5'-CACAGGGGGCgcTATGGGGGaC-3'), which includes three mismatches for the altered codon, and PET-*ykoNA* (5'-ACTATTCTcgAgTC-TCTATTTCAGG-3'). Then the two DNA fragments were mixed and entire *ykoNS205A* DNA fragment was amplified using a primer pair PETykoNS and PETykoNA. The entire *ykoNS205A* DNA amplified was inserted into pET-21(+) to construct pET-*ykoNS205A*.

Cloning of *ykoN* and its *ykoNΔN* allele into expression vector pRSETA was performed as follow. The *ykoN* fragment was amplified using a primer pair *ykoN-S1* (5'-GGAGGgCTagCATGAAAAACATTC-3'), which starts at 11 nt upstream of the initiation codon, and *ykoN-As3* (5'-GAGCGTTTTCAaGctTTTCGCCTG-3'), which starts at 112 nt downstream of the termination codon. The *ykoNΔN* fragment was amplified using a primer pair *ykoN-S2* (5'-CCTTTgctAGCATTTCCA-CCG-3'), which starts at 22 nt downstream of the initiation codon and primer *ykoN-As3*. These amplified fragments were inserted into *Nhe*I-*Hind*III site of pRSETA to form pRSETA-*ykoN* and pRSETA-*ykoNΔN*.

Table 1. *B. subtilis* and *E. coli* strains and plasmids.

Strain	Relevant characteristics	Source or reference
<i>B. subtilis</i>		
168	<i>trpC2</i>	Laboratory stock
TM001	168 <i>ykoN</i> ::pMUTIN2(P_{spac} - <i>ykoN</i> Ery ^r)	This work
KP261	168 <i>yppP</i> ::Km ^r	(17), R.Losick
SDB02	168 <i>pss10</i> :: <i>spc</i>	(3)
SDB206	168 <i>ywmE</i> ::pMUTIN4 <i>ywiE</i> :: <i>neo</i> <i>ywjE</i> :: <i>spc</i>	(4)
<i>E. coli</i>		
BL21(DE3)	<i>hsdS gal</i> (λ c1857 <i>ind1 Sam7 nin5 lacUV5-T7-gene1</i>)	F. W. Studier
W3110	F ⁺ λ IN(<i>rrnD-rnmE</i>)1	Laboratory Stock
GN10	W3110 Δ <i>pssA10</i> :: <i>cam</i>	(19)
S330	S301 <i>pgsA30</i> :: <i>kan rcsA</i> ::IS5	(20, 21)
SD11	SD12 <i>cls-1</i>	(22)
Plasmids		
pMUTIN2	P_{spac} <i>lacZ lacI</i> , Ery ^r	(10)
pTM040	pMUTIN2 <i>ykoN</i> , Ery ^r	This work
pET-21(+)	P_{T7} , Amp ^r	Novagen
pET- <i>ykoN</i>	P_{T7} - <i>ykoN</i> , derivative of pET-21(+), Amp ^r	This work
pET- <i>ykoNS205A</i>	P_{T7} - <i>ykoNS205A</i> , derivative of pET-21(+), Amp ^r	This work
pRSETA	P_{T7} , Amp ^r	Invitrogen
pRSETA- <i>ykoN</i>	P_{T7} - <i>ykoN</i> , derivative of pRSETA, Amp ^r	This work
pRSETA- <i>ykoNΔN</i>	P_{T7} - <i>ykoNΔN</i> , derivative of pRSETA, Amp ^r	This work
pSK6	P_{araBAD} , derivative of pSC101, Amp ^r	(23)
pTM042	P_{araBAD} - <i>ykoN</i> , derivative of pSK6, Amp ^r	This work
pAN18	P_{spac} , derivative of pDG148, Km ^r	H. Yoshikawa
pTM088	P_{spac} - <i>ykoN</i> , derivative of pAN18, Km ^r	This work

Culture media and growth of *B. subtilis* and *E. coli*

B. subtilis strains were cultivated in sporulation medium (DSM)¹¹⁾, which contained 0.8% nutrient broth (Difco), 0.1% KCl, 0.025% MgSO₄·7H₂O, 1.0 mM Ca(NO₃)₂, 10 μ M MnCl₂, and 1.0 μ M FeSO₄. Luria-Bertani (LB) broth was used for cultivation of both *B. subtilis* and *E. coli* strains. When required, the following supplements were added to the media (per liter); 20 mg of neomycin (Wako Pure Chem.), 50 mg of spectinomycin (Sigma), 0.3 mg of erythromycin (Sigma), 5 mg of kanamycin, 200 mg ampicillin (Wako Pure Chem.). For induction of plasmid borne *ykoN*, 0.2% L-arabinose (Wako Pure Chem.), or 0.1 mM (*E. coli*) and 1 mM (*B. subtilis*) of isopropyl-1-thio- β -D-galactoside (IPTG) (Wako Pure Chem.) was added.

Lipid analysis

Lipids were extracted by the method of Ames¹²⁾. The lipid fractions were separated by two dimensional thin-layer chromatography (TLC) on silica gel (no. 60, Merck, Darmstadt), first (x-dimension) with chloroform-methanol-water (65:25:4 [vol/vol/vol]) and second (y-dimension) with chloroform-methanol-acetic acid (65:25:10 [vol/vol/vol]). In some cases lipids were separated one-dimensional TLC with the same solvent system as that used for the y-dimension. Phospholipids were visualized by uniformly spraying the molybdenum reagent described by Dittmer and Lester¹³⁾. Detection of carbohydrate moiety and free amino group were conducted with sprays with orcinol-sulphuric acid mixture and ninhydrin in butanol, respectively, as described by Christie¹⁴⁾. The relative intensity of blue spot was quantified by using NIH image (Scion, version 4.0.3.2).

Enzyme assay

β -Galactosidase activity of *B. subtilis* TM001 cells cultivated in DSM was assayed according to the method described by Wang and Doi¹⁵⁾.

Results and Discussion

Overexpression of *ykoN* produces a new phospholipid

To understand the physiological role of *ykoN*, we constructed a strain with pMutin-disruption of *ykoN*. The disruptant showed, however, no apparent growth change or noticeable alteration in lipid composition (data not shown). We then induced *ykoN* from the *spac* promoter using multicopy plasmid pTM088. The induction still from the cells in late exponential growth phase in DSM at caused no apparent growth change. Lipid was extracted four hours after addition of 1 mM IPTG and

examined by thin layer chromatography. A new spot of phospholipid, with the Rf value between Lys-PG and PE, was found in the lipid fraction of the induced cells on the thin layer chromatogram (Fig. 1). Since the molybdenum reagent spray¹³⁾ shows up the compounds containing phosphate ester as blue spots, the new spot contains phospholipid, and it does not contain carbohydrate moiety or free amino group as examined with orcinol-sulphuric acid reagent or ninhydrin spray¹⁴⁾, respectively (data not shown). The new spot is thus different from any lipid described for *B. subtilis* cells so far^{1,16)}.

Fig. 1. Thin layer chromatogram showing the production of a new phospholipid in *B. subtilis* 168 cells in which *ykoN* was overexpressed.

Cells of wild type *B. subtilis* and of the strain harboring multicopy plasmid pTM088 were cultivated in DSM at 37°C to late exponential growth phase in the presence of 1 mM IPTG for 4 hours. Lipids were extracted and TLC was carried out as described in materials and methods. Abbreviations: CL, cardio- lipin; PG, phosphatidylglycerol; PE, phosphatidyl-ethanolamine; Lys-PG, lysylphosphatidylglycerol. (1) wild type 168 ; (2) 168/pTM088.

Overexpression of *ykoN* in *E. coli* mutants lacking specific phospholipids

The effect of expression of *ykoN* was then examined in *E. coli* cells. The gene was induced in *E. coli* W3110 cells from *araBAD* promoter of plasmid pTM042. Three hours after addition of 0.2% L-arabinose lipid was extracted and examined by one-dimensional thin layer chromatography. A spot of phospholipid with similar Rf value to that of the new phospholipid in the induced *B. subtilis* cells was also found in the induced *E. coli* cells (Fig. 2, lanes 1 and 2). To examine the origin of the new phospholipid the mutant strains lacking each of three major phospholipids were used to induce *ykoN*. The new phospholipid was produced in the mutant cells lacking

PE (strain GN10; Fig. 2, lanes 3 and 4) or CL (strain SD11; lanes 7 and 8). In the strain S330 that lacks PG, however, the new phospholipid was not produced (lanes 5 and 6). The result suggests an involvement of PG in the production of the new phospholipid. This *ykoN* induction causes a small alteration of growth rate, suggesting that production of the new lipid has a small effect on *E. coli* cells. Since PG is essential for *B. subtilis* cells^{5,6}, we

could not examine the effect of PG-deprivation in *B. subtilis*. Mutant *B. subtilis* cells lacking other lipids, glucosylated diacylglycerides, PE, CL, or Lys-PG^{3,4,5,16,17}, produced the new phospholipid (data not shown). Thus, the production of the new phospholipid in *B. subtilis* cells likely involves PG as well.

Fig. 2. Thin layer chromatogram showing the phospholipid of wild type and mutant *E. coli* cells in which *ykoN* was overexpressed.

Cells of wild type and the mutant *E. coli* strains, GN10, S330, and SD11, harboring plasmid pTM042 were cultivated in LB at 37°C to late exponential growth phase with 0.2% L-arabinose for 3 hours. Lipids were extracted and TLC was carried out as described in materials and methods. Abbreviations: CL, cardiolipin; PA, phosphatidic acid; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; CDP-DG, CDP-diacylglycerol. (1) W3110; (2) W3110/pTM042; (3) GN10; (4) GN10/pTM042; (5) S330; (6) S330/pTM042; (7) SD11; (8) SD11/pTM042.

Fig. 3. Two-dimensional thin layer chromatograms of the phospholipid at different stages of *B. subtilis* growth.

Cells of strain TM001 were grown in DSM at 37°C and growth was followed by measurement of turbidity. IPTG was not added to this DSM culture as this experiment evaluates the activity of *ykoN* expressed by its own promoter, though *ykoN* of TM001 is controllable by P_{spac} . Cells were withdrawn at 1-hour intervals (plates a-h) in the different growth stages (indicated as a-h in Fig. 4) and were subjected to lipid analysis by two-dimensional TLC. The spots of the new phospholipid are circled. In the bottom-right panel positions of phospholipids are indicated. Abbreviations: CL, cardiolipin; PA, phosphatidic acid; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; Lys-PG, lysylphosphatidylglycerol; GPGL, probable glycerophosphoglucolipid; X, another phospholipid not described previously.

Production of the new phospholipid in sporulation phase

As a fairly large amount of the new phospholipid was produced after overexpression of plasmid-borne *ykoN* (Fig. 1), we expected that the new phospholipid would be produced from *ykoN* under its own promoter on the chromosome under normal growth conditions. We examined the change of lipid composition of the strain TM001 at 1-hour intervals from the middle exponential growth phase without IPTG addition (Fig. 3). In exponential and early stationary phases the new phospholipid was not observed (Fig. 3 a-e). In the sporulation phase, at four hours after cessation of exponential growth, however, a faint spot that corresponds to the new lipid was detected (Fig. 3 f). At five and six hours in late sporulation phase the spot became darker (Fig. 3 g and h). The content of the new phospholipid was quite small compared with that produced by the induction of *ykoN* on the multicopy plasmid pTM088 (Fig.1). The presence of this new phospholipid has not been noticed^{1,16}. It is probably because the lipid of the cells in sporulation phase has not been examined. Note that a spot designated as X that has not been described appeared in the sporulation phase.

Fig. 4. Promoter activity of *ykoN* at different stages of *B. subtilis* growth.

Cells of strain TM001 were grown in DSM at 37°C and growth was followed by measurement of turbidity. The culture condition is the same as described in Fig. 3. Cells were withdrawn at 1-hour intervals in the different growth stage (a-h) and the β-galactosidase activity was measured as described in materials and methods. Turbidity in Klett unit (●) and β-galactosidase activity (○) are shown.

The increase of the new phospholipid in late sporulation phase coincides well with a raise in the promoter activity of *ykoN* in sporulation phase as monitored by the activity of promoter fusion *ykoN-lacZ* (Fig. 4). This coincidence suggests that the new phospholipid is produced by the product of *ykoN* in the late sporulation phase and further that this new phospholipid

might play a specific role in the membranes in the late stage of sporulation. An apparent delay in the appearance (at stage f) of the new phospholipid in comparison with the increase in the promoter activity might be due to a requirement for accumulation of YkoN or for certain physiological shift on the membranes for efficient YkoN action.

Involvement of the lipolytic activity of YkoN in production of the new phospholipid

YkoN has the pentapeptide lipase/esterase consensus sequence (Gly-X-Ser-X-Gly) and is shown to have a lipolytic activity (Matsuura *et al.*, in press). Replacement of serine residue in the consensus sequence causes change in the lipolytic activity of LipB¹⁸. And hence, a mutant *ykoN* gene (*ykoNS205A*) of YkoN(S205A), whose central serine residue in the pentapeptide consensus is replaced with alanine, was constructed and was induced in *E. coli* cells. Production of the new phospholipid with the mutant YkoN was reduced to ca. 1/10 of that with the wild type YkoN (Fig. 5A). The replacement of the amino acid residue in the pentapeptide consensus sequence of LipB causes a change in substrate specificity¹⁸, YkoN(S205A) would also have a substrate specificity change. The reduction in the production of the new phospholipid might be caused by this substrate specificity change. This result suggests that the lipolytic activity of YkoN is involved in the production of the new phospholipid. Prediction with Psort program (<http://psort.nibb.ac.jp>) suggests that it is localized on the membrane due to the short hydrophobic residue stretch (ten residues) at its N-terminus, though it is suggested to be a soluble protein by Sosui program (<http://bp.nuap.nagoya-u.ac.jp/sosui>). When a mutant *ykoN* gene (*ykoNΔN*) coding for YkoN that lacks the N-terminal hydrophobic stretch of ten residues was induced in *E. coli* cells, the production of the new phospholipid was much reduced (Fig. 5B). This might suggest that YkoN is localized on the membrane and hydrolyzes an ester bond of phosphatidylglycerol on the cytoplasmic surface of the membrane. This is a likely initial step of YkoN action for the production of the new phospholipid. It should be noted that YkoN is the esterase which cleaves *p*-nitrophenylesters of fatty acids with short chain length preferentially. However, it also cleaves, albeit inefficiently, the esters of long chain length which comprises *B. subtilis* membrane lipids (Matsuura *et al.*, in press). Thus, the lipolytic activity of YkoN is likely responsible for the production of the new phospholipid.

Fig. 5. Two-dimensional thin layer chromatograms showing the effect of replacement of the amino acid in consensus sequence (A) and of the removal of N-terminal hydrophobic residues (B). *E. coli* BL21(DE3) cells harboring pET-*ykoN* (a) pET-*ykoNS205A* (b), pRSETA-*ykoN* (c) or pRSETA-*ykoNΔN* (d) were cultivated in LB at 37°C with 0.1 mM IPTG for 3 hours to late exponential growth phase. Production of the new phospholipid was examined as described in materials and methods. For abbreviations see legend to Fig. 2.

Acknowledgements

We thank Drs. Y. Sadaie, K. Asai, and K. Horikoshi for comments and encouragement. We also thank Dr. H. Yoshikawa for plasmid pAN18 and K. Komori and T. Haraguchi for a part of the experiments with *ykoN* disruptant.

References

- 1) Fischer, W., Nakano, M., Laine, R.A., and Bohrer, W. 1978. On the relationship between glycerophosphoglycolipids and lipoteichoic acids in gram-positive bacteria. I. The occurrence of phosphoglycolipids. *Biochim. Biophys. Acta* 528: 288-297
- 2) Matsumoto, K. 2001. Dispensable nature of phosphatidylglycerol in *Escherichia coli*: dual roles of anionic phospholipids. *Mol. Microbiol.* 396: 1427-1433.
- 3) Matsumoto, K., Okada, M., Horikoshi, Y., Matsuzaki, H., Kishi, T., Itaya, I., and Shibuya, I. 1998. Cloning, sequencing, and disruption of the *Bacillus subtilis* *psd* gene coding for phosphatidylserine decarboxylase. *J. Bacteriol.* 180: 100-106.
- 4) Kawai, F., Shoda, M., Harashima, R., Sadaie, Y., Hara, H., and Matsumoto, K. 2004. Cardiolipin domains in *Bacillus subtilis* Marburg membranes. *J. Bacteriol.* 186: 1475-1483.
- 5) Nishibori, A., Kusaka, J., Hara, H., Umeda, M., and Matsumoto, K. 2005. Phosphatidylethanolamine domains and localization of phospholipid synthases in *Bacillus subtilis* membranes. *J. Bacteriol.* 187: 2163-2174.
- 6) Kobayashi, K., Ehrlich, D.S., *et al.* 2003. Essential *Bacillus subtilis* genes. *Proc. Natl. Acad. Sci. USA* 100: 4678-4683.
- 7) de Mendoza, D., Schujman, G. E., and Aguilar, P. S. 2002. Biosynthesis and function of membrane lipids, p. 43-55. In A. L. Sonenshein, J. A. Hoch, and R. Losick (ed.), *Bacillus subtilis* and its closest relatives. ASM Press, Washington, D.C.
- 8) Matsumoto, K., Kusaka, J., Nishibori, A., and Hara, H. 2006. Lipid domains in bacterial membranes. *Mol. Microbiol.* 61: 1110-1117.
- 9) Jorasz, P., Wolter, F. P., Zahring, U., and Heinz, E. 1998. A UDP glucosyltransferase from *Bacillus subtilis* successively transfers up to four glucose residues to 1,2-diacylglycerol: expression of *ypfP* in *Escherichia coli* and structural analysis of its reaction products. *Mol. Microbiol.* 29: 419-430.
- 10) Vagner, V., Dervyn, E., and Ehrlich, S.D. 1998. A vector for systematic gene inactivation in *Bacillus subtilis*. *Microbiology* 144: 3097-3104.
- 11) Schaeffer, P., Ionesco, H., Rytter, A., and Balassa, G. 1965. La sporulation de *Bacillus subtilis*; étude génétique et physiologique, p.553-563. In *Mécanismes de régulation des activités cellulaires microorganismes*. Centre National de la Recherche Scientifique, édit., Paris, France.
- 12) Ames, G. F. 1968. Lipids of *Salmonella typhimurium* and *Escherichia coli*: structure and metabolism. *J. Bacteriol.* 95: 833-843.
- 13) Dittmer, J.C., and Lester, R.L. 1964. A simple, specific spray for the detection of phospholipids on thin layer chromatograms. *J. Lipid Res.* 5: 126-127.
- 14) Christie, W.W. 1982. Lipid analysis: isolation, separation, identification and structural analysis of lipids. 2nd ed. Pergamon Press, Oxford.
- 15) Wang, P.-Z., and Doi, R.H. 1984. Overlapping promoters transcribed by *Bacillus subtilis* σ^{55} and σ^{37} RNA polymerase holoenzymes during growth and stationary phases. *J. Biol. Chem.* 259: 8619-8625.
- 16) Kawai, F., Hara, H., Takamatsu, H., Watabe, K., and Matsumoto, K. 2006. Cardiolipin enrichment in spore membranes and its involvement in germination of *Bacillus subtilis* Marburg. *Genes Genet. Syst.* 81: 69-76.

- 17) Price, K.D., Roels, S., and Losick, R. 1997. A *Bacillus subtilis* gene encoding a protein similar to nucleotide sugar transferases influences cell shape and viability. *J. Bacteriol.* 179: 4959-4961.
- 18) Eggert, T., Pencreac'h, G., Douchet, I., Verger, R., and Jaeger, K.-E. 2000. A novel extracellular esterase from *Bacillus subtilis* and its conversion to a monoacylglycerol hydrolase. *Eur. J. Biochem.* 267: 6459-6469
- 19) Saha, S.K., Nishijima, S., Matsuzaki, H., Shibuya, I., and Matsumoto, K. 1996. A regulatory mechanism for the balanced synthesis of membrane phospholipid species in *Escherichia coli*. *Biosci. Biotech. Biochem.* 60:111-116.
- 20) Kikuchi, S., Shibuya, I., and Matsumoto, K. 2000. Viability of an *Escherichia coli* *pgsA* null mutant lacking phosphatidylglycerol and cardiolipin. *J. Bacteriol.* 182: 371-376.
- 21) Nagahama, H., Sakamoto, Y., Matsumoto, K., and Hara, H. 2006. RcsA-dependent and -independent growth defects caused by the activated Rcs phosphorelay system in the *Escherichia coli* *pgsA* null mutant. *J. Gen. Appl. Microbiol.* 52: 91-98.
- 22) Shibuya, I., Miyazaki, Ch., and Ohta, A. 1985. Alteration of phospholipid composition by combined defects in phosphatidylserine and cardiolipin synthases and physiological consequences in *Escherichia coli*. *J. Bacteriol.* 161: 1086-1092.
- 23) Suzuki, M., Hara, H., and Matsumoto, K. 2002. Envelope disorder of *Escherichia coli* cells lacking phosphatidylglycerol. *J. Bacteriol.* 184: 5418-5425.