Identification of the *nonA* and *nonB* loci of *Bacillus subtilis* Marburg permitting the growth of SP10 phage

Satoshi Matsuoka¹, Tomomi Arai¹, Rikinori Murayama², Fujio Kawamura²,
Kei Asai¹, and Yoshito Sadaie^{1,*}

¹Department of Biochemistry and Molecular Biology, Faculty of Science, Saitama University
Saitama 338-8570, Japan

²Department of Life Science, College of Science, Rikkyo University
Toshimaku 171-8501, Japan

(Received 7 December 2004, accepted 5 January 2005)

Mutational inactivation of both *nonA* and *nonB* genes are required for the permissiveness of *Bacillus subtilis* Marburg cells to infection by phage SP10. By transformational analysis of the *nonA* strain with DNAs from gently lysed protoplasts carrying the integrative plasmid pMUTIN (*em*) insertions in every 20 kb along the whole chromosome, we have identified the *nonA* to be the cured state of endogenous prophage SP β . Direct DNA sequencing, on the other hand, revealed one nonsense mutation of *nonB* in *ydiR*, which is a component gene of the intrinsic restriction system *BsuMR* of *B. subtilis* Marburg. Introduction of the wild type *ydiR* into the *nonB* strain at *aprE* locus resulted in complementation of *nonB*. Furthermore, as the SP10 genome was found to possess multiple *BsuM* target sites, it is considered that SP10 can infect and multiply in *B. subtilis* cells, which are SP β free and possess a defective *BsuMR* restriction system.

Key word: *BsuMR*, *B. subtilis*, *nonA* and *nonB*, SP β , SP10

INTRODUCTION

Bacillus subtilis is refractory to infection by phage SP10, which can multiply in *Bacillus licheniformis*. Mutational inactivation both of *nonA* and *nonB* (non-permissiveness) genes render *B. subtilis* cell permissive to infection by SP10 (Saito et al., 1977). The *nonA* is located close to *rpoB* by PBS1-mediated transduction, whereas the location of *nonB* is not separable by transformational cross from the intrinsic restriction gene *BsuMR* in the *purB* region (Saito et al., 1977). Although *nonB* is believed to be an allele of *BsuMR*, the precise mutational structure of *nonB* has not been elucidated so far.

The intrinsic restriction and modification system *BsuM* of *B. subtilis* Marburg consists of two sets of genes *BsuMM* and *BsuMR* located in the prophage 3 region (Kasahara et al., 1997; Kunst et al., 1997). *BsuMM* consists of *ydiO* and *ydiP*, and *BsuMR* is composed of *ydiR*, *ydiS* and *ydjA* (Ohshima et al., 2002). Therefore *nonB* must be a mutation(s) in *ydiR*, *ydiS* or *ydjA*. The target DNA sequence of *BsuM* is considered to be the same as that of *XhoI*, CTC*GAG (Bron et al., 1988; Jentsch, 1983), in which the 3' cytosine (indicated by asterisk) is the acceptor for *BsuM* modification (Jentsch, 1983). Multiple presence of the

unmethylated target sequences are required for effective restriction by *BsuMR* (Bron et al., 1988). Therefore the SP10 phage genome is assumed to contain a multiple number of *XhoI* sites.

In this communication, we have determined the mutational sites of *nonA* and *nonB*. All the *nonA* strains were cured of the intrinsic temperate phage SP β (Warner et al., 1977) of *B. subtilis* Marburg, which possibly interferes with infection by SP10 phage. The *nonB* strain on the other hand contained one nonsense mutation in *ydiR*, which is a component gene of the *BsuMR* (Ohshima et al., 2002).

MATERIALS AND METHODS

Bacterial strains, plasmids and phages used Bacterial strains and plasmids used are described in Table 1. *B. subtilis* phage used was SP10 provided by Y. Itoh (Inatsu et al., 2002), which was from H.W. Ackerman (Ackerman et al., 1995).

Transformation Conventional DNA mediated transformation was performed as described elsewhere (Ohshima et al., 2002). NonA⁺ transformants were selected as colonies appearing on Luria-Bertani (LB) broth agar, on which SP10 phages (ca. 10⁹), grown in RM125, were spread before plating the transformed culture. Transfor-

Edited by Hirokazu Inoue

* Corresponding author. E-mail: ysadaie@molbiol.saitama-u.ac.jp

Table1. Bacterial strains and plasmids used

Strain or plasmid	Genotype and/or relevant phenotype ^a	Reference and /or source
<i>B.subtilis</i> strains		
168 Marburg	<i>trpC2</i>	Ohshima et al., 2002
168ΔSPβ <i>cm</i>	<i>trpC2</i> ΔSPβ <i>cm</i>	This study
HLL3g	<i>nonA thr met lys leu his trp ade</i>	Saito et al., 1977,BGSC ^c
PS9	<i>nonA nonB thr met lys leu his trp</i>	Saito et al., 1977,BGSC ^c
RIK135	<i>trpC2 lys-1 nprR2 nprE18 aprEΔ3</i> ΔSPβ <i>cm</i>	This study
RIK136	<i>trpC2 lys-1 leu nprR2 nprE18 aprEΔ3</i> ΔSPβ <i>cm</i>	This study
RIK137	<i>trpC2 lys-1 nprR2 nprE18 aprEΔ3</i> ΔSPβ	This study
RIK1010	<i>trpC2 lys-1 leu nprR2 nprE18 aprEΔ3</i>	This study
RM125	<i>leuA8 argA15</i> ΔSPβ Δ <i>Bsu</i> M	Ohshima et al., 2002
UOT1012 ^b	<i>nonB leu met</i> pBL1	Saito et al., 1977, BGSC ^c
UOT1285	<i>trpC2 lys-1 nprR2 nprE18 aprEΔ3</i>	Saito et al., 1977
UOT1871	<i>trpC2 lys-1 nprR2 nprE18 aprEΔ3 rif1728</i>	Murayama et al., 2004
BSU3	<i>trpC2 ydiR::pMUTIN2(em)</i>	Ohshima et al., 2002
BSU4	<i>trpC2 ydiS::pMUTIN2(em)</i>	Ohshima et al., 2002
BSU5	<i>trpC2 ydjA::pMUTIN2(em)</i>	Ohshima et al., 2002
YS11	<i>nonA leu arg ade</i>	Saito et al., 1977, BGSC ^c
Plasmids		
pAPNC213	<i>sp ap</i>	Morimoto et al., 2002
pMUTIN2	<i>em ap</i>	Vagner et al., 1998
pSB322M	<i>ap</i>	This study
pSB322MCm	<i>ap cm</i>	This study
pCBB31	<i>cm ap</i>	Nanamiya et al., 2004

^a*cm*, *em*, *sp*, and *ap* ; chloramphenicol, erythromycin, spectinomycin and ampicillin genes respectively. ^b*nonB* mutation was introduced from PS9. ^cBGSC, *Bacillus* Genetic Stock Center, Ohio State University, Columbus.

mation with lysed protoplasts as donor DNA developed by Akamatsu and Sekiguchi was performed as described elsewhere (Akamatsu and Sekiguchi, 1987). After serial dilution of the protoplast in SMM buffer, one-tenth volume of the protoplast sample was added to one volume of competent culture. Donor strains for conventional transformation or transformation with DNA from lysed protoplasts carry the integrative plasmid pMUTIN2 (*em*) (Vagner et al., 1998). Disruptants are described in the Table 2 and in the web site of BSORF (<http://bacillus.genome.ad.jp/>).

The experiments with the phage SP10 The number of SP10 phages was counted as the number of plaques in a soft agar (0.8%) containing cells of the indicator strain and SP10 phages. The soft agar was overlaid on nutrient agar (1.5%) and incubated at 37°C. Both agar contained LB broth. SP10 DNA was extracted as follows. The culture of RM125(NonA⁻ NonB⁻) or RIK137(ΔSPβ *ydiS::pMUTIN2*) in LB broth was infected by SP10 phages and cell debris was discarded by centrifugation. After 10% PEG(6000) and 0.5M NaCl precipitation,

phages were suspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) containing 5 mM MgSO₄. The sample was treated with DNase and RNase. EDTA (50 mM) and SDS (1%) were added to the sample to lyse phage. The sample was treated with phenol. DNA solution was made 0.3 M with sodium acetate, and DNA was extracted with glass rod after ethanol addition.

Gene disruption Disruptions of the prophage SPβ genes, *mtbP*, *ligB*, *yonB*, *yopA* and *yomD*, the skin element genes *yqxG* and *yqaP*, and the PBSX gene *xkdK* were carried out as described elsewhere (Ohshima et al., 2002) with plasmid pMUTIN (Vagner et al., 1998) and with DNA fragments synthesized by PCR using primer pairs 5'AAGA-AGCTTAGTGGAATTGGTGCAT/5'GGAGGATCCTCATA-AGTAATTAAAT for *mtbP*, 5'AAGAAGCTTACGACGAGA-ACTATATCACTG/5'GGAGGATCC TAGCTGCAATTGAT-TGACCGT for *ligB*, 5'AAGAAGCTTACTGTGAAAATCAA-AGGCTTG/5'GGAGGATCCTAAATCAACACCAGAGCCT-GT for *yonB*, 5'AGCAAGCTTTTCGAATTTGAA/5'GGAG-GATCCAATAGTCATATCGCTTATCCA for *yopA*, 5'AA-GAAGCTTTAGCTCAGAATAACTACATTC/5'GGAGGAT-

Table 2. Disrupted genes carried by the strains, from which protoplasts and their DNA were prepared for transformation.

<i>yaaC</i>	<i>yaaL</i>	<i>yabE</i>	<i>yabR</i>	<i>yacF</i>	<i>yacK</i>	<i>rif(rpoB)</i>	<i>ybaC</i>	<i>ybaX</i>	<i>ybaR</i>	<i>ybbE</i>	<i>ybxI</i>	<i>ybdE</i>	
<i>ybgA</i>	<i>ychD</i>	<i>yccK</i>	<i>yceA</i>	<i>yegI</i>	<i>yegQ</i>	<i>yckC</i>	<i>hxlR</i>	<i>yclE</i>	<i>gabD</i>	<i>ycsD</i>	<i>ycsN</i>	<i>mntH</i>	<i>ydbM</i>
<i>ydcN</i>	<i>ydcS</i>	<i>ydeK</i>	<i>ydeM</i>	<u><i>ydeO</i></u>	<u><i>ydfC</i></u>	<u><i>ydfH</i></u>	<u><i>ydfI</i></u>	<u><i>ydfJ</i></u>	<u><i>ydfK</i></u>	<u><i>ydgC</i></u>	<i>ydgH</i>	<i>ydgK</i>	<i>ydiF</i>
<u><i>ydiL</i></u>	<u><i>ydiB</i></u>	<u><i>ycaC</i></u>	<i>yecA</i>	<i>yerI</i>	<i>yesF</i>	<i>yesS</i>	<i>yetN</i>	<i>yfnA</i>	<i>yflP</i>	<i>yflA</i>	<i>yfiP</i>	<i>yfiE</i>	<i>yfiT</i>
<i>yfhG</i>	<i>ygaJ</i>	<i>ygaO</i>	<i>yhcS</i>	<i>yhxh</i>	<i>yhdR</i>	<i>yheG</i>	<i>yhaG</i>	<i>yhgC</i>	<i>yhxC</i>	<i>yhjJ</i>	<i>yisL</i>	<i>yisT</i>	<i>yitZ</i>
<i>yjaU</i>	<i>yjbJ</i>	<i>yjbV</i>	<i>yjdF</i>	<i>yjgB</i>	<i>yjnA</i>	<i>yjgC</i>	<i>xkdK</i>	<i>spoIIISA</i>	<i>ykaA</i>	<i>ykcC</i>	<i>ykoH</i>	<i>ykoP</i>	<i>kinD</i>
<i>ykuI</i>	<i>ykwD</i>	<i>ykuF</i>	<i>yknZ</i>	<i>ykpB</i>	<i>ylaE</i>	<i>pycA</i>	<i>ylbP</i>	<i>ylxX</i>	<i>ylyB</i>	<i>ylnF</i>	<i>yloS</i>	<i>ylgD</i>	<i>ylxF</i>
<i>ylxL</i>	<i>ylxP</i>	<i>ymfD</i>	<i>pbpX</i>	<i>ymcC</i>	<i>pksG</i>	<i>pksR</i>	<i>ymaD</i>	<i>yncB</i>	<i>yncM</i>	<i>yneT</i>	<i>ynfF</i>	<i>ynlG</i>	<i>dacC</i>
<i>yoga</i>	<i>YoaP</i>	<i>yoaW</i>	<i>yobQ</i>	<i>yocA</i>	<i>yojI</i>	<i>yodC</i>	<i>yodN</i>	<i>yodU</i>	<i>mtbP</i>	<i>ligB</i>	<i>yopA</i>	<u><i>yonB</i></u>	<u><i>yomD</i></u>
<i>ypqP</i>	<i>ugtP</i>	<i>ypvA</i>	<i>ypoC</i>	<i>ypiB</i>	<i>yphF</i>	<i>ypbG</i>	<i>ypuF</i>	<i>spoIIM</i>	<i>yqjE</i>	<i>bkdAB</i>	<i>spo0A</i>	<i>efp</i>	
<i>yqhB</i>	<i>yqzC</i>	<i>yqfO</i>	<i>yqfL</i>	<i>yqeT</i>	<i>yqeM</i>	<i>yqeB</i>	<i>yqxG</i>	<i>yqaP</i>	<i>yrkQ</i>	<i>yrkC</i>	<i>yrda</i>	<i>yraO</i>	<i>aapA</i>
<i>yrhA</i>	<i>yrriI</i>	<i>yrvD</i>	<i>yrzF</i>	<i>ysxE</i>	<i>ysxD</i>	<i>yslB</i>	<i>yshE</i>	<i>ysdC</i>	<i>ysaA</i>	<i>ytwI</i>	<i>ytsJ</i>	<i>ytXK</i>	<i>ezrA</i>
<i>ytoP</i>	<i>ythQ</i>	<i>ytaP</i>	<i>ytsD</i>	<i>ytjA</i>	<i>ytXm</i>	<i>yuaJ</i>	<i>yubE</i>	<i>yugO</i>	<i>yugE</i>	<i>yuxH</i>	<i>yueC</i>	<i>yuiD</i>	<i>yutK</i>
<i>pucM</i>	<i>yurM</i>	<i>yusJ</i>	<i>yuxN</i>	<i>yurD</i>	<i>yvgP</i>	<i>yvaA</i>	<i>yubF</i>	<i>yubT</i>	<i>yvfi</i>	<i>yveL</i>	<i>yvdK</i>	<i>yvcT</i>	<i>yvpB</i>
<i>cypX</i>	<i>yvjD</i>	<i>yviA</i>	<i>yvhJ</i>	<i>ywtG</i>	<i>ywsB</i>	<i>ywqC</i>	<i>mseL</i>	<i>ywmF</i>	<i>ywmE</i>	<i>ywmA</i>	<i>ywlG</i>	<u><i>ywkE</i></u>	<i>ywjF</i>
<i>ywiE</i>	<i>albD</i>	<i>ywhD</i>	<i>ywfA</i>	<i>ywdL</i>	<u><i>ywcH</i></u>	<i>ywcD</i>	<i>ywbO</i>	<i>ywbH</i>	<i>ywaF</i>	<i>ywaA</i>	<i>yxLA</i>	<i>yxkI</i>	<u><i>yxkA</i></u>
<i>yxiO</i>	<i>yxxF</i>	<i>yxxB</i>	<i>yxEL</i>	<i>yxCA</i>	<i>yxNB</i>	<i>yxdD</i>	<i>phrG</i>	<i>yybR</i>	<i>yybD</i>	<i>yyaB</i>			

Underlined genes indicate that transformation with DNA from protoplasts carrying these disrupted genes showed higher frequency of NonA⁺ transformation (co-transfer index more than 20) as shown in Fig. 1. Genes are placed in the clockwise order from the chromosome replication origin.

CCTCACCTTTAAAGAAGTTGCTG for *yomD*, 5'AAGAAGCTTTCAACAGATGCAACTGGTAAG/5'GGAGGATCCACTCAGTCTCGCCAAGCGTAT for *yqxG*, 5'AAGAAGCTTATTGGAGTTAGTGTTCGCAC/5'GGAGGATCCTACGCTAATTACGTTCTCATT for *yqaP*, and 5'AAGAAGCTTCGTGCAGGCATTTATTTTAAC/5'GGAGGATCCCGCCATATACTGCAGTTGCTT for *xkdK*.

Overexpression of *ydiR* gene Overexpression of *ydiR* gene was induced by adding IPTG (1 mM) to the culture of the strain carrying, at *aprE* locus, pAPNC213 plasmid (Morimoto et al., 2002) with *ydiR* gene fused to a P_{spac} promoter. P_{spac}-*ydiR* fusion gene on the plasmid pAPNC213 was constructed as follows. Primer pair for PCR synthesis of *ydiR* gene was 5'GTCGTCGACAGATTTGAGGTGATATCTTT/5'GGAGGATCCTTAGTTATTTTCAAAGTCTTC. PCR product was digested with *SalI* and *Bam*HI, and cloned into pAPNC213, which carries split *aprE* gene and contains *SalI*-*Bam*HI cloning site downstream of P_{spac} promoter. pAPNC213-*ydiR* was linearized by *NruI* digestion and was used to transform UOT1012 and then PS9 to spectinomycin resistance.

SP β curing A DNA segment lacking SP β was made by a double-cross over recombination using a linearized recombinant plasmid with two DNA fragments flanking the attachment site of SP β . These DNA fragments were amplified by PCR with UOT1285 genome DNA and

two primer pairs 5'-CGGTCTGAGAATTCAACTTAAATTCACGC/5'-ACACAGCTGTGTACTGATATTAATGACATGC, and 5'-TTTCAGCTGAAAGCTGTATCTCCTGTAAAC/5'-TACGTCGACGTATGTAGACATTGTGTTCAGGC containing *EcoRI*, *PvuII* or *SalI* restriction site. Resulting PCR products were digested with these restriction enzymes, and cloned into the *EcoRI*-*SalI* region of pBR322M (a pBR322- derivative deleted of a DNA fragment by digestion with *PvuII* and *Bst*1107I) to obtain pSB322M. Then pSB322M was digested with *PvuII* and ligated with a Cm^r gene cassette derived from pC194 (Horinouchi et al., 1982) through pCBB31 (Nanamiya et al., 2004; Yamada, 1989). The recombinant plasmid pSB322MCM was linearized with *ScaI* and used to transform UOT1285 (SP β ⁺). A Cm^r transformant was selected and named RIK135 (Δ SP β ::cm). Chromosomal DNA of RIK135 was used to transform Leu⁻ RIK1010 to construct Cm^r Leu⁻ RIK136 (Δ SP β ::cm). To remove the cm gene from RIK136, two DNA fragments (PCR synthesized Leu⁺ DNA and *ScaI*-digested pSB322M linear DNA) were used to transform RIK136, and a Leu⁺ Cm^s transformant was selected by congression and named RIK137 (Δ SP β). The Leu⁺ DNA fragment (about 6.6 kb) was synthesized by PCR with a primer pair 5'-GCTGGAAAAACAGTAGCGGTTATCGGGTAC/5'-CTTGCCTCGACCAGCAATTTTCGCTGGAACG and UOT1285 genome DNA. The deletion of SP β was confirmed by PCR and by sensitivity for SP β C29, a clear mutant of SP β (isolated by F.Kawamura).

168 Δ SP β *cm* (*trpC2* Δ SP β *cm*) was made by transformation of 168 to Cm^r by DNA from RIK135 (Δ SP β *cm*).

PCR Confirmation of the presence or the absence of SP β by PCR was performed with primers, 1(*yodUF*): ATGCCTAAACAGCAAACAGCA, 2(SP β *yotLR*):TGTCTGCGCTCTTTAAAGTTC, 3(SP β *yokAF*):ACGAATCTAAACGCATACGAA, 4(*ypqPR*):TGCTTTTTCTCTTGATGCAA. Their configurations are shown in Fig. 2. *yodU* and *ypqP* are located outside of attachment site of SP β . *yotL* and *yokA* are located at the right and left side ends of SP β (Kunst et al., 1997).

DNA sequencing To find mutated bases of the *nonB* gene, we have sequenced five overlapping DNA fragments of about 1 kb covering the prophage 3 region of *nonA nonB* strain PS9. DNAs were PCR synthesized by using template chromosomal DNA of PS9 with primer pairs 5'GGCTCGCAGTCACAACAA/5'CCCTTTCTTCCC-CGTTAC, 5'CCTTCTGTGGACGATTCG/5'CTCTTCCTC-ACCGCTTAA, 5'GGGAAGTGGGAAAGATCT/5'ACCAC-CGAGTACACGTAC, 5'GATGGCTCGGATAACATT/5'AC-TTCCTCCCATACTCTG, 5'GGCATTGAAAACCGCAAT/5'GAGAGAGAAGCGGTGGAT. Each primer pair contained a SP6 and T7 promoter tag sequence pair 5'atttagtgacactatagaataac/5'taatagcact cactataggg at its 5' ends.

RESULTS AND DISCUSSION

Mapping of *nonA* As *nonA* is reported to be located in the region of *rpoB(rif)* (Saito et al., 1977), conventional transformation of Non⁻ (*nonA nonB*) strain to Rif^r Non⁺ (*nonA*⁺ *nonB*) with biochemically purified DNA from strain UOT1871 carrying *rif1728(rpoB)* (Murayama et al., 2004) was performed. Non⁺ transformants were selected as SP10 phage (grown in RM125) resistant colonies as described in the Materials and Methods section. However no Rif^r Non⁺ transformants were obtained. Next, similar experiments were performed with DNA from strains carrying integrative plasmid pMUTIN2 (*em*) in the 21 orfs located within 20 kb around the *rpoB* gene. However no Em^r Non⁺ transformants were obtained. Therefore with both transformations we could not find linkage of the *nonA* mutation to *rif* (*rpoB*).

Chromosomal DNA from gently lysed protoplast cell carries more genetic markers than biochemically purified DNA (Akamatsu and Sekiguchi, 1987). We performed transformation from Em^s to Em^r of PS9 strain (*nonA nonB*) with chromosomal DNA prepared from protoplasts of 220 disruptant strains carrying integrative plasmid pMUTIN2 (*cm*) (Table 2) described in BSORF (<http://bacillus.genome.ad.jp/>). They are NonA⁺ NonB⁺ strains. Disrupted genes are located at every 20kb along the

Fig. 1. Transformation, with lysed protoplasts as donor DNA, of Non⁻ (*nonA nonB*) strain to Non⁺ (*nonA*⁺ *nonB* or *nonA nonB*⁺). Donor strain (isogenic strain of 168 Marburg carrying *trpC2*) possesses disrupted gene with the integrative plasmid pMUTIN2 (*em*) described in the Table 2. Disrupted genes are placed on abscissa in clockwise order from chromosome replication origin. Co-transfer index on ordinate indicates the ratio, 100 x Em^r Non⁺ / (Em^r Non⁻ + Em^r Non⁺). Recipient strain was PS9 (*nonA nonB*). The locations of *rpoB*, *BsuM* and SP β are shown. O and T indicate the origin and the terminus of chromosome replication respectively.

whole chromosome (Kunst *et al.*, 1997). As shown in Fig. 1, 13 donor strains carrying integrative plasmid pMUTIN2(*em*) in the genes, *ydeO*, *ydfI*, *ydfJ*, *ydfK*, *ydgC*, *ydiL*, *ydjB*, *yeaC*, *yonB*, *yomD*, *ywkE*, *ywcH*, and *yxkA* showed higher transformation frequency (co-transfer index more than 20) of Non⁻ (*nonA nonB*) to Em^r Non⁺ (*nonA⁺ nonB* or *nonA nonB⁺*). Several Em^r Non⁺ colonies from each transformation were taken and examined for their phage resistance. They were used as indicator strains for plaque forming ability of SP10 phage. Transformants obtained in transformation with *ydeO*, *ydfI*, *ydfJ*, *ydfK*, *ydgC*, *ywkE*, *ywcH*, and *yxkA* disruptant DNA were SP10 phage sensitive. On the other hand, transformants with DNAs from donor strains carrying pMUTIN2(*em*) in the genes in the regions of prophages 3 (*ydiL*, *ydjB* and *yeaC*) and SPβ (*yonB* and *yomD*) showed SP10 phage resistance. These results suggested that *nonA* and *nonB* mutations are located in these regions. As described below, since *nonB* is located in the prophage 3 region, *nonA* must be located in the SPβ region.

***nonA* and curing of SPβ** It is possible that SPβ interferes with multiplication of SP10, simply because of the immunity established by lysogenic SPβ against infecting SP10. The presence or absence of SPβ (Kunst *et al.*, 1997; Warner *et al.*, 1977) in Em^r Non⁺ transformants was confirmed by PCR (Fig. 2). PCR synthesis of DNA fragments covering the outside genes of SPβ and the right or left end genes on SPβ (PCR with primer pairs 1 and 2 in Fig. 2c or 3 and 4 in Fig. 2d), or PCR synthesis of DNA fragment covering outside genes of SPβ (PCR with a primer pairs 1 and 4 in Fig. 2b) were performed by using template DNA from the wild type strain, *nonA⁺* transformant, or *nonA* strains as well as from strains cured of SPβ. The presence of SPβ is shown in Fig. 2C as a DNA band of 1.5 kb long, and in Fig. 2d as a DNA band of 1.3 kb long, and is not shown in Fig. 2b, as SPβ (ca. 134kb) (Lazarevic *et al.*, 1999) is too long to be synthesized by PCR. When the chloramphenicol resistance cassette of pSB322Mcm plasmid was replaced for SPβ, a DNA band of 2.2 kb long was shown in Fig. 2b. A PCR product of 1 kb long is shown in Fig. 2b, when SPβ phage was

Fig. 2. PCR product of SPβ and permissiveness for SP10. (a) Location of SPβ on *B.subtilis* chromosome and configuration of primers used. (b) Agarose electrophoresis of PCR products with primer pairs 1 and 4, and template DNA from wild type strain 168(lane 1), *nonA* strains HLL3g (lane 2) and YS11(lane3), *nonB* strain UOT1012(lane 4), *nonA nonB* strain PS9(lane 5), *em^r nonA⁺ nonB⁻* transformant of PS9 with DNA from *yonB*::pMUTIN2(*em*) (lane 6), *em^r nonA⁺ nonB⁺* transformant of PS9 with DNA from *ydiL*::pMUTIN2(*em*)(lane 7), NonA⁻ Trp⁺ transformant of *ydiS*::*em* strain BSU4 with DNA from YS11(lane 8), NonA⁺ Trp⁺ transformant of *ydiS*::*em* strain BSU4 with DNA from YS11 (lane 9), ΔSPβ strain RIK137 (lane 10), ΔSPβ *cm* strain 168(ΔSPβ *cm*) (lane 11), Trp⁺ ΔSPβ transformant of *ydiS*::*em* strain BSU4 with DNA from RIK137(lane 12), ΔSPβ *cm* transformant of *ydiS*::*em* strain BSU4 with DNA from 168 ΔSPβ *cm* (lane 13). (c) Agarose electrophoresis of PCR products with primer pairs 3 and 4, and template DNA from strains described above. (d) Agarose electrophoresis of PCR products with primer pairs 1 and 2, and template DNA from strains described above. (e) Plaque forming unit per ml of SP10 phage using indicator strains from which template DNAs were extracted for PCR described above.

cured. PCR products indicating the presence of SP β were observed only from *nonA*⁺ strains (Fig. 2, lanes 1, 4, 6, 9) but not from *nonA* strains and strains cured of SP β (Fig. 2, lanes 2, 3, 5, 7, 8, 10, 11, 12, 13). The Trp⁺ NonA[−] transformant of *ydiS* disrupted strain BSU4 (*ydiS::em trpC2*) with protoplast DNA of *nonA* strain YS11 did not show the above PCR product (Fig. 2, lane 8). These results indicate that the wild type strain carries SP β and the *nonA* mutant strains or *nonA* transformants do not carry SP β . Furthermore, all the SP β -free strains carrying the *nonB* mutation were permissive for the infection and growth of SP10 phage (Fig. 2e).

Combination of Δ SP β and *BsuMR*[−] We confirmed the causality of Δ SP β and defective *BsuMR* for a NonA[−] NonB[−] phenotype by using strains cured of SP β . By introducing *ydiR(em)*, *ydiS(em)*, or *ydjA(em)* disrupted genes (Ohshima et al., 2002) into Δ SP β strain RIK137, all the transformant strains became permissive to SP10 infection, while similar transformants of SP β ⁺ UOT1285 were not permissive (Table 3). Permissiveness for SP10 phage therefore requires curing of SP β and the defective function of at least one of the *ydiR*, *ydiS* or *ydjA* genes, whose products constitute *BsuMR*. Furthermore, chloramphenicol resistant (*cm*) transformants of *ydiR*, *ydiS* or *ydjA* strain with DNA from Δ SP β *cm* were permissive for SP10 infection (data not shown).

The frequency of transformation, with DNA from lysed protoplasts, of wild type (SP β ⁺) strain to *nonA* (Δ SP β) was higher than the frequency of similar transformation of *nonA* (Δ SP β) to wild type (SP β ⁺). This is probably due to excision of single strand DNA bearing large (134kb)(Lazarevic et al., 1999) SP β in hetero-duplex intermediate DNA formed during transformation.

Mapping and complementation of *nonB* Since *nonB* is responsible for restriction of phage infection (Saito et al., 1977; Witmer et al., 1981), we directly sequenced five

overlapping DNA fragments of about 1 kb covering the entire *BsuMR* region of the *nonA nonB* strain PS9. We found one nonsense mutation TAA and one synonymous mutation TCA in *ydiR* gene (69th codon CAA and 228th codon TCG respectively) of the *nonA nonB* strain PS9 (Fig. 3). There was no mutation found in the *ydiS* and *ydjA* of the above strain.

We next confirmed whether or not the wild type *ydiR* gene complemented the above *nonB* mutation. Single copy *ydiR* gene was cloned in pAPNC213 (Morimoto et al., 2002) under P_{spac} promoter and introduced at *aprE* locus of *nonA nonB* strain PS9 and induced by IPTG. As shown in Table 4, with 1mM IPTG, PS9 strain carrying pAPNC213-*ydiR* showed NonB⁺ phenotype, i.e., non-permissiveness to SP10 infection. Therefore, these results indicated that above nonsense mutation in *ydiR* is responsible for the *nonB* phenotype.

SP10 genome DNA extracted from SP10 phage grown in the *BsuM* deficient strain RM125(Ohshima et al., 2002), which was also cured of SP β (data not shown), was digested with *XhoI* and we found more than 20 fragments. On the other hand, SP10 DNA extracted from phage lysate derived from a *BsuMM*⁺ strain, which carried disrupted *ydiS* and was cured of SP β , was more refractory to digestion by *XhoI* (data not shown). This is the first indication that the SP10 genome contains multiple *XhoI* sites, which are target sites of the *BsuM* system. Therefore SP10 phage is not able to multiply in

Fig. 3. DNA sequence of *ydiR* gene of the *B.subtilis nonA nonB* strain. DNA sequence of the wild type strain is described above that of the *nonA nonB* strain PS9. The number indicates the number of the codons where mutations were found. The *nonB* mutation possesses one nonsense mutation in the 69th codon (CAA to TAA) of *ydiR* and one synonymus mutation in the 228th codon (TCG to TCA) of *ydiR* gene.

Table 3. Combination of Δ SP β and *ydiR*, *ydiS* or *ydjA* and permissiveness for SP10 growth

Indicator strain ^a	PFU/ml
RIK137 (Δ SP β)	< 5
RIK137 (Δ SP β <i>ydiR::pMUTIN2</i>)	4.6×10^9
RIK137 (Δ SP β <i>ydiS::pMUTIN2</i>)	4.4×10^9
RIK137 (Δ SP β <i>ydjA::pMUTIN2</i>)	4.9×10^9
UOT1285 (<i>ydiR::pMUTIN2</i>)	< 5
UOT1285 (<i>ydiS::pMUTIN2</i>)	< 5
UOT1285 (<i>ydjA::pMUTIN2</i>)	< 5

^aRIK137(Δ SP β) and UOT1285 were transformed with DNA from BSU3, BSU4 or BSU5 to obtain RIK137(Δ SP β *ydiR::pMUTIN2*), RIK137(Δ SP β *ydiS::pMUTIN2*), RIK137(Δ SP β *ydjA::pMUTIN2*), UOT1285 (*ydiR::pMUTIN2*), UOT1285(*ydiS::pMUTIN2*) or UOT1285(*ydjA::pMUTIN2*),

Table 4. Complementation of *nonB* by *ydiR*

Strain	PFU/ml	
	− IPTG	+ IPTG
PS9 (<i>nonA nonB aprE::pAPNC213</i>)	5.4×10^9	6.0×10^9
PS9 (<i>nonA nonB aprE::pAPNC213-ydiR</i>)	6.1×10^9	< 5

Strain PS9 (*nonA nonB*) carrying pAPNC213 with or without *ydiR* was used as an indicator for SP10 phage. 1mM IPTG was used for induction of *ydiR* expression.

B.subtilis cell with an intact *BsuM* system.

An explanation for probable interference of prophage SP β with SP10 multiplication is that wild type SP β repressor may affect expression of the SP10 phage genes leading to non-permissiveness of phage growth. Thermosensitive induction of SP β due to temperature-sensitive mutants of SP β c2 (Rosental et al., 1979) suggested involvement of the repressor YomJ (Lazarevic et al., 1999) in prophage maintenance. Another explanation is that some gene(s) on the prophage SP β is expressed and may interfere with the some process(s) of infection, expression, replication and packaging of SP10 genome. Examples of the expressing genes are the *sspC* gene encoding a small acid-soluble protein integrated in spore, and the genes for the SP β bacteriocin YolG (betacin) and bacteriocin-exporter YokH (Lazarevic et al., 1999).

On the other hand, SP β does not interfere transformation of *B.subtilis* Marburg strain with plasmid pHV1401 harboring multiple *BsuM* target sites. Plasmid-mediated transformation requires only a defect (*nonB*) in *BsuMR* (Bron et al., 1988; Oshima et al., 2002).

We are grateful to R.H.Doi for critically reading the manuscript and to Y.Itoh for providing SP10 phage. This work was partially supported by a Grant-in-Aid for Scientific Research on Priority Area from the Ministry of Education, Science, Sports and Culture of Japan.

REFERENCES

- Ackermann, H. W., Azizbekyan, R. R., Bernier, R., de Barjac, H., Saindoux, S., Valero, J. R. and Yu, M. X. (1995) Phage typing of *Bacillus subtilis* and *B.thuringiensis*. *Res. Microbiol.* **146**, 643–657.
- Akamatsu, T., and Sekiguchi, J. (1987) Characterization of chromosome and plasmid transformation in *Bacillus subtilis* using gently lysed protoplasts. *Arch. Microbiol.* **146**, 353–357.
- Bron, S., Janniere, L., and Ehrlich, S. D. (1988) Restriction and modification in *Bacillus subtilis* Marburg 168: Target sites and effects on plasmid transformation. *Mol. Gen. Genet.* **211**, 186–189.
- Horinouchi S., and Weisblum, B. (1982) Nucleotide sequence and functional map of pC194, a plasmid that specifies inducible chloramphenicol resistance. *J. Bacteriol.* **150**, 815–825.
- Inatsu, Y., Kimura, K., and Itoh, Y. (2002) Characterization of *Bacillus subtilis* strains isolated from fermented soybean foods in Southeast Asia: Coparison with *B.subtilis* (natto) starter strains. *JARQ* **36**, 169–175.
- Jentsch, S. (1983) Restriction and modification in *Bacillus subtilis*: Sequence specificities of restriction/modification systems *BsuM*, *BsuE*, and *BsuF*. *J. Bacteriol.* **156**, 800–808.
- Kasahara, Y., Nakai, S., Ogasawara, N., Yata, K., and Sadaie, Y. (1997) Sequence analysis of the *groESL-cotA* region of the *Bacillus subtilis* genome containing the restriction/modification system genes. *DNA Res.* **4**, 335–339.
- Kunst, F., Ogasawara, N. et al. (1997) The complete genome sequence of the Gram-positive bacterium *Bacillus subtilis*. *Nature* **390**, 249–256.
- Morimoto, T., Loh, P. C., Hirai, T., Asai, K., Kobayashi, K., Moriya, S., and Ogasawara, N. (2002) Six GTP-binding proteins of the Era/Obg family are essential for cell growth in *Bacillus subtilis*. *Microbiol.* **148**, 3539–3552.
- Murayama, R., Akanuma, G., Makino, Y., Nanamiya, H., and Kawamura, K. (2004) Spontaneous transformation and its use for genetic mapping in *Bacillus subtilis*. *Biosci. Biotechnol. Biochem.* **68**, 1672–1680.
- Nanamiya, H., Akanuma, G., Natori, Y., Murayama, R., Kosono, S., Kudo, T., Kobayashi, K., Ogasawara, N., Park, S.M., Ochi, K., and Kawamura, F. (2004) Zinc is a key factor in controlling alternation of two types of L31 protein in the *Bacillus subtilis* ribosome. *Mol. Microbiol.* **52**, 273–283.
- Lazarevic, V., Dusterhoft, A., Soldo, B., Hilbert, H., Mael, C., and Karamata, D. (1999) Nucleotide sequence of the *Bacillus subtilis* temperate bacteriophage SP β c2. *Microbiol.* **145**, 1055–1067.
- Ohshima, H., Matsuoka, S., Asai, K., and Y. Sadaie. (2002) Molecular organization of intrinsic restriction and modification genes *BsuM* of *Bacillus subtilis* Marburg. *J. Bacteriol.* **184**, 381–389.
- Rosental, R., Toye, P. A., Korman, R. Z., and Zahler, S. A. (1979) The prophage of SP β c2dcitK₁, a defective specialized transducing phage of *Bacillus subtilis*. *Genetics* **92**, 721–739.
- Saito, H., Shibata, T., and Ando, T. (1977) Mapping of genes determining nonpermissiveness and host-specific restriction to bacteriophages in *Bacillus subtilis* Marburg. *Mol. Gen. Genet.* **170**, 117–122.
- Vagner, V., Dervyn, E., and Ehrlich, S. D. (1998) A vector for systematic gene inactivation in *Bacillus subtilis*. *Microbiol.* **144**, 3097–3104.
- Warner, F. D., Kitos, G. A., Roman, M. P., and Hemphill, H. E. (1977) Characterization of SP β : a temperate bacteriophage from *Bacillus subtilis* M168. *Canad. J. Microbiol.* **23**, 45–51.
- Witmer, H., and Franks, M. (1981) Restriction and modification of bacteriophage SP10 DNA by *Bacillus subtilis* Marburg 168: stabilization of SP10 DNA in restricting hosts preinfected with a heterologous phage, SP18. *J. Virol.* **39**, 536–47.
- Yamada, K. (1989) Characterization of *spoII* genes of *Bacillus subtilis*. Ph.D. Thesis. Hiroshima University. Hiroshima. Japan.