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Molecular Organization of Intrinsic Restriction and Modification Genes *Bsu*M of *Bacillus subtilis* Marburg

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Transcriptional analysis and disruption of five open reading frames (ORFs), *ydiO*, *ydiP*, *ydiR*, *ydiS*, and *ydjA*, in the prophage 3 region of the chromosome of *Bacillus subtilis* Marburg revealed that they are component genes of the intrinsic *Bsu*M restriction and modification system of this organism. The classical mutant strain RM125, which lacks the restriction and modification system of *B. subtilis* Marburg, lacks the prophage 3 region carrying these five ORFs. These ORFs constitute two operons, the *ydiO-ydiP* operon and the *ydiR-ydiS-ydjA* operon, both of which are expressed during the logarithmic phase of growth. The predicted gene products YdiO and YdiP are the orthologues of cytosine DNA methyltransferases. The predicted YdiS product is an orthologue of restriction nucleases, while the predicted YdiR and YdjA products have no apparent paralogues and orthologues whose functions are known. Disruption of the *ydiR-ydiS-ydjA* operon resulted in enhanced transformation by plasmid DNA carrying multiple *Bsu*M target sequences. Disruption of *ydiO* or *ydiP* function requires disruption of at least one of the following genes on the chromosome: *ydiR*, *ydiS*, and *ydjA*. The degrees of methylation of the *Bsu*M target sequences on chromosomal DNAs were estimated indirectly by determining the susceptibility to digestion with *Xho*I (an isoschizomer of *Bsu*M) of DNAs extracted from the disruptant strains. Six *Xho*I (*Bsu*M) sites were examined. *Xho*I digested at the *Xho*I sites in the DNAs from disruptants with disruptions in both operons, while *Xho*I did not digest at the *Xho*I sites in the DNAs from the wild-type strain or from the disruptants with disruptions in the *ydiR-ydiS-ydjA* operon. Therefore, the *ydiO-ydiP* operon and the *ydiR-ydiS-ydjA* operon are considered operons that are responsible for *Bsu*M modification and *Bsu*M restriction, respectively.

The existence of an inherent *Bsu*M restriction and modification system in *Bacillus subtilis* Marburg 168 was first suggested on the basis of the results of an experiment performed with phage ϕ105 and *B. subtilis* and *Bacillus amyloliquefaciens* host strains (28). The *hsrM1* and *nonB* mutations (24, 36) which made host cells permissive to phage infection were isolated and mapped at around 50° on the *B. subtilis* chromosome (24), and it was thought that these mutations were mutations of the endonuclease gene of *Bsu*M. On the other hand, strain RM125 (34) was constructed by transformation of wild-type *B. subtilis* Marburg 168 (YS11) with DNA from the related strain *B. subtilis* 202-5 (= IAM1169, a *B. amyloliquefaciens* strain), which lacked restriction activity against phage ϕ105. Strain RM125 constructed in this way turned out to be deficient in modification activity, as well as restriction activity. However, it was not known whether RM125 lost the same *Bsu*M restriction activity as *nonB* and *hsrM1* strains.

The target sequence of the *Bsu*M restriction and modification system of *B. subtilis* was first predicted to be PyTCGAPu (7, 13), but in another study researchers determined that the *Bsu*M target sequence was CTCGAG by performing a transformation analysis of plasmids carrying multiple PyTCGAPu target sequences (2). Therefore, *Xho*I is believed to be an isoschizomer of *Bsu*M. Moreover, efficient restriction required the presence of multiple target sites for *Xho*I (*Bsu*M) in plasmid DNA (2). On the other hand, cytosine DNA methyltransferase, which methylates a CTCGAG target, was purified from the *B. subtilis* Marburg strain (8), but its gene was not identified.

DNA sequencing of the *B. subtilis* chromosomal *rrnE* region revealed a 13-kb region with a lower G+C content and lower gene density between the *groESL* operon and the *gut* operon (14). This region was called prophage 3 (17) and contained five open reading frames (ORFs), *ydiO*, *ydiP*, *ydiR*, *ydiS*, and *ydjA*, as well as pseudogenes and five smaller ORFs, *ydiM*, *ydiN*, *ydiQ*, *ydjB*, and *ydjC* (14) (Fig 1). Although there are not any paralogue genes on the *B. subtilis* chromosome except *mtbP* of SPβ prophage, the predicted products of *ydiO* and *ydiP* exhibit high levels of similarity to DNA modification enzymes *Hgi*-DIIM from *Herpetosiphon giganteus* and *Msp*I from members of the family *Moraxellaceae* (4, 18), respectively, and the predicted product of *ydiS* exhibits a high level of similarity to DNA restriction enzyme LlaI-2 of *Lactococcus* plasmid pTR2030 (20).

In this study we tried to determine whether ORFs *ydiO*, *ydiP*, *ydiR*, *ydiS*, and *ydjA* are components of the intrinsic *Bsu*M restriction-modification system of *B. subtilis* Marburg which are not present in the classical RM125 strain. Our results indicated that there are two operons, one consisting of *ydiO* and *ydiP* for DNA methylation and the other consisting of *ydiR*, *ydiS*, and *ydjA* for DNA restriction, in the prophage 3 region of the *B. subtilis* Marburg chromosome and that these two operons are not present in the classical RM125 strain.

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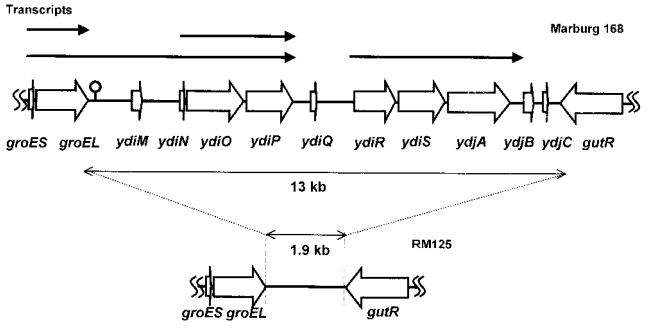

FIG. 1. Transcriptional map of the prophage 3 region of *B. subtilis* Marburg 168 which is not present in the classical RM125 strain deficient in *Bsu*M restriction and modification. The thin arrows indicate the transcripts identified by Northern analysis of this region. The thick arrows indicate the predicted ORFs. The transcription termination signal of the *groESL* operon is indicated by a lollipop.

Furthermore, disruption of the rho-independent termination signal sequence of the *groESL* transcription region resulted in enhanced readthrough and enhanced transcription of the *ydiO-ydiP* operon.

MATERIALS AND METHODS

Bacterial strains and plasmids used. The bacterial strains and plasmids used in this study are described in Table 1. *B. subtilis* Marburg 168 *trpC2* (35) was used as a wild-type strain in all experiments.

Media, reagents, and enzymes. Cells of *B. subtilis* or *Escherichia coli* were grown in Luria-Bertani broth (25) or a complex sporulation medium (26). Reagents and enzymes were purchased from TaKaRa (Kyoto, Japan), Wako (Osaka, Japan), and Sigma (St. Louis, Mo.). Ampli*Taq* DNA polymerase (Applied Biosystems, Foster City, Calif.) and TaKaRa LA *Taq* DNA polymerase (TaKaRa) were used for PCR.

DNA manipulation. Plasmid DNA purification, PCR amplification of DNA fragments, digestion of DNA with restriction enzyme, and DNA ligation were carried out as described elsewhere (25). Plasmid DNA was purified by polyethylene glycol precipitation of a sample of *E. coli* C600 cells lysed by alkali. The primers used for long accurate PCR amplification of the prophage 3 region were 5′-ATATGGGCGGCATGGGCGGTATGGGTGGAA (groELF30) and 5′-GCATAACAGCCGCTTTCATGTTTTGAGGTA (gutBR30).

For disruption and confirmation of the ORFs, a primer pair with *Hin*dIII or *Bam*HI sites were used for PCR amplification of a portion of each ORF. The following primer pairs were used: for *groES*, 5′-GCCGAAGCTTCTAAAATTACATATTCA and 5′-CGCGGATCCGTATTTTGAGAAGATAA; for *groEL*, 5′-GCCGAAGCTTAAGAAATTAAGTTTAGT and 5′-CGCGGATCCGATTGTGATAACGCCGT; for *ydiO*, 5′-GCCGAAGCTTAACATAGAAAATTTTTA and 5′-CGCGGATCCCACCTAGTTTTTCAGCA; for *ydiP*, 5′-GCCGAAGCTTATCAGTCAGGACATACA and 5′-CGCGGATCCCCTTGTAGGCGCTCAGC; for *ydiR*, 5′-GCCGAAGCTTGCCGGAGTATCTAGTAC and 5′-CGCGGATCCATTTATGTTTTATCTTA; for *ydiS*, 5′-GCCGAAGCTTAGTATTCCTAGGAATAG and 5′-CGCGGATCCAAAGCGGTATCGAGCGG; for *ydjA*, 5′-GCCGAAGCTTAAAGGATTGTACGTGTA and 5′-CGCGGATCCTAATATCGGGTTCTAGT; for *gutR*, 5′-GCCGAAGCTTCCGTATCGCTTCCATAT and 5′-CGCGGATCCATGCTGGCACCTGGCGT; and for *gutB*, 5′-GCCGAAGCTTAAATTGCCGCTGTCGGA and 5′-CGCGGATCCTTTTCCGCCCCGGCGCA. These primers were custom made (Espec Oligo Service, Tsukuba, Japan) and were also used for PCR analysis of strain RM125.

Disruption of ORFs. Disruption of ORFs with the integrative plasmid pMUTIN was carried out as described elsewhere (35, 39). The primer pairs used for PCR synthesis of *ydiO*, *ydiP*, *ydiR*, *ydiS*, and *ydjA* are described above.

Construction of IPTG-inducible *ydiR-ydiS-ydjA* operon on the chromosome. An isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible promoter was introduced into the promoter region of the *ydiR-ydiS-ydjA* operon by integration of plasmid pMUTIN carrying the N-terminal region of the *ydiR* gene with the Shine-Dalgarno sequence, which was synthesized by PCR performed with primers 5′-AAGAAGCTTGCTTGTGTGATTTTATGGGG and 5′-CGCGGATCCATTTATGTTTTATCTTA. The resulting strain carried P*spac-ydiR*.

Transformation. Competent *B. subtilis cells* were prepared in Spizizen's minimal glucose medium, and transformation was carried out as described elsewhere (1, 15). Transformation of *E. coli* was performed as described elsewhere (6).

Construction of a deletion mutant with a mutation in the *groESL* transcription termination signal sequence. A DNA segment lacking the transcription termination signal sequence was constructed by ligating two DNA fragments which were synthesized by PCR performed with primer pairs 5′-CTTCTGAATTCGACAGAG–5′-CGGTTAAAACATTGATGTATAAGGG and 5′-AAATCCCCAGTTGGGTTC–5′-ATCCATCAGGATTGATTCC, cloned into plasmid

TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Genotype and/or relevant phenotype[a]	Reference and/or source
B. subtilis strains		
168	*trpC2*	35
RM125	*leuA8 argA15*	34, M. Itaya
BSU1	*trpC2 ydiO*::pET24b *neo*(Km^r) *ydiS*::pMUTIN2 (*erm*)	This study
BSU2	*trpC2 ydiP*::pET24b *neo*(Km^r) *ydiS*::pMUTIN2 (*erm*)	This study
BSU3	*trpC2 ydiR*::pMUTIN2 (*erm*)	This study
BSU4	*trpC2 ydiS*::pMUTIN2 (*erm*)	This study
BSU5	*trpC2 ydjA*::pMUTIN2 (*erm*)	This study
BSU6	*trpC2 ydiR*::pMUTIN2 (P*spac-ydiR erm*)	This study
BSU7	*trpC2 ydiO*::pET24b *neo*(Km^r) *ydiR*::pMUTIN2 (P*spac-ydiR erm*)	This study
BSU8	*trpC2 ydiP*::pET24b *neo*(Km^r) *ydiR*::pMUTIN2 (P*spac-ydiR erm*)	This study
BSU9	*trpC2* Δ*ter*(*groESL*)	This study
E. coli strains		
C600	*thi-1 thr-1 leuB6 lacY1 tonA21 supE44*	40, S. Yasuda
JM109	*recA1 hsdR17* Δ(*lac-proAB*) *endA1 gyrA96 relA1 thi supE44* F′ [*traD36 proAB*$^+$ *lacI*^{q} *lacZ*Δ*M15*]	37, TaKaRa
Plasmids		
pMUTIN2	Em^r (*B. subtilis*) Ap^r (*E. coli*)	35
pET24b *neo*	Km^r (*B. subtilis*)	5
pHV33	Cm^r (*B. subtilis*) Ap^r (*E. coli*), no *Xho*I site	2, M. Itaya
pHV1401	Cm^r (*B. subtilis*) Ap^r (*E. coli*), three *Xho*I sites	2, M. Itaya
pCP112	Cm^r (*B. subtilis*) Ap^r (*E. coli*)	22

[a] *neo* and *erm*, neomycin and erythromycin genes, respectively; Km^r, Ap^r, Em^r, and Cm^r, resistance to kanamycin, ampicillin, erythromycin, and chloramphenicol, respectively.

TABLE 2. Disruption of *ydiO* or *ydiP*[a]

Target gene	No. of kanamycin-resistant transformants (10^2)/μg of DNA with the following recipients:			
	168	BSU3 *ydiR*::pMUTIN2	BSU4 *ydiS*::pMUTIN2	BSU5 *ydjA*::pMUTIN2
ydiO	<3.0	580	570	670
ydiP	<6.0	710	640	580

[a] The pET24b *neo* plasmid carrying a portion of *ydiO* or *ydiP* was used to transform a disruptant strain to resistance to kanamycin (5 μg/ml) in order to disrupt *ydiO* or *ydiP* as described in Materials and Methods.

pCP112 (22), and then introduced into the chromosome of strain 168 by transformation. The inserted plasmid was cured from competent cells. The deletion, Δ*ter*(*groESL*), was confirmed by PCR.

Northern analysis. RNA was extracted with glass beads from cells grown in sporulation medium, separated by electrophoresis, and detected with a digoxigenin (DIG)-labeled RNA probe as described elsewhere (39). A 20-μg RNA sample was used. The RNA probe was constructed with a PCR-synthesized DNA fragment containing the T7 promoter sequence (39). The primer pairs used each consisted of a forward primer and reverse primer T7rev (5′-TAATACGACTC ACTATAGGGCGAAGTGTATCAACAAGCTGG). RNA probes for *ydiO* and *ydiP* were constructed with *ydiO*::pDX-CAT *ydiS*::pMUTIN2 and *ydiP*::pDX-CAT *ydiS*::pMUTIN2 double disruptants, in which the integrative plasmid pDX-CAT, provided by Y. Kasahara, carried the *bgaB* gene (41). A DIG-labeled RNA probe was synthesized with a DIG-UTP labeling kit, and DIG was detected with a DIG detection kit (Roche). The length of RNA detected was estimated by calibrating Northern signals with 16S rRNA, 23S rRNA, and RNA markers that were 9, 6, 5, 4, 3, 2.5, 2, 1.5, 1, and 0.5 kb long (Ambion, Inc., Austin, Tex.).

Southern analysis. The degrees of cytosine methylation were estimated for six fragments containing only one *Xho*I site at nucleotide positions 106975 (9°, AGGCTCGAGTAT), 2633916 (54°, GCGCTCGAGACA), 015718 (172°, GCGCTCGAGCAG), 2800360 (239°, TCACTCGAGATT), 3105700 (265°, CT TCTCGAGGAT), and 3550939 (303°, TTTCTCGAGCTT) on the chromosome (17). Chromosomal DNA from each strain was double digested with *Xho*I and an appropriate restriction endonuclease, separated by agarose electrophoresis, and transferred to a membrane filter as described elsewhere (25). The DNAs were then hybridized to a DNA probe, which was synthesized by PCR and labeled in vitro with alkaline phosphatase (AlkPhos Direct; Amersham), and were detected with a CDP Star detection kit and Hyperfilm ECL (Amersham).

The following primer pairs were used for PCR: for xfrg9, 5′-CGGATCTAC AAACGAAATGG and 5′-CCCTGTTATGGTCTATTCCC; for xfrg54, 5′-TA TAACCACGGAAACTT and 5′-AAAATGTGTATTATGGT; for xfrg172, 5′-GCATCAATCAATCCTGCGAC and 5′-AGCTGTTTTTGGCACACGGC; for xfrg239, 5′-GTGATTAACTGCACTCAGGA and 5′-GGCTGTTCCTGCGAC GGCTG; for xfrg265, 5′-ATGTTCTGGAATGAATTAAA and 5′-ATTCGTC AATGTGCAAAC; and for xfrg303, 5′-GCTGGATCATAGAAACCACC and 5′-GGGCGATTGAAAGTAACT.

RESULTS

Disruption of the ORFs located in the prophage 3 region. Single-gene disruption was carried out with integrative plasmid pMUTIN2 carrying a portion of the target gene, as described elsewhere (35, 39). As the whole *lacZ* gene with the Shine-Dalgarno sequence is joined to the 5′ region of the disrupted gene, transcription of the disrupted gene is easily monitored by monitoring *lacZ* activity. As the inserted plasmid carries an IPTG-inducible P*spac* promoter at the 3′ end of the plasmid, addition of IPTG induces transcription of the genes following the disrupted gene, thus avoiding a polar effect of any operon due to insertional disruption.

Single disruption of *ydiR*, *ydiS*, or *ydjA* was easily performed, while transformation of wild-type strain 168 to erythromycin resistance with pMUTIN2 carrying a portion of the *ydiO* or *ydiP* gene resulted in very tiny colonies on nutrient agar containing antibiotics. Disruption of the gene essential for growth by transformation with an integrative plasmid carrying a cloned target gene often resulted in very tiny transformants. Transformation of the strains in which *ydiR*, *ydiS*, or *ydjA* was already disrupted to kanamycin resistance with the pET24 *neo* plasmid carrying a portion of *ydiO* or *ydiP* yielded transformants (Table 2), indicating that disruption of *ydiO* and *ydiP* function was possible only when the *ydiR*, *ydiS*, or *ydjA* gene or a combination of these genes was already disrupted. Disruption of *ydiO* may have resulted in reduced expression of *ydiP* as pET24 *neo* does not have a P*spac* promoter.

TABLE 3. Transformation of the disruptant strains with plasmids carrying multiple *Xho*I sites

Expt	Recipient strain[a]	No. of Cm^r transformants (10^2)/μg of DNA with:		No. of transformants with pHV1401/ no. of transformants with pHV33	Ratio
		pHV33	pHV1401		
1	168	226	24	0.11	1.0
	BSU1 (*ydiO ydiS*)	75	324	4.32	40.7
	BSU2 (*ydiP ydiS*)	12	110	9.17	86.3
	BSU3 (*ydiR*)	38	128	3.37	31.7
	BSU4 (*ydiS*)	20	46	2.30	21.7
	BSU5 (*ydjA*)	13	54	4.17	39.1
2	168	134	2	0.01	1.0
	BSU9 [Δ*ter* (*groESL*)]	135	82	0.61	61.0
3	168	1.00	0.063	0.063	1.0
	BSU1 (*ydiO ydiS*)	4.75	4.93	1.04	16.5
	BSU2 (*ydiP ydiS*)	0.38	0.38	1.00	15.8
	BSU3 (*ydiR*)	3.50	3.87	1.11	17.6
	BSU4 (*ydiS*)	3.12	3.94	1.26	20.0
	BSU5 (*ydjA*)	8.75	9.69	1.11	17.6
	BSU9 [Δ*ter* (*groESL*)]	4.69	3.94	0.84	13.3

[a] Each strain was transformed to resistance to chloramphenicol (5 μg/ml) with plasmid pHV33 or pHV1401. The genotypes in parentheses or brackets indicate the disrupted genes.

As the predicted products YdiO and YdiP are orthologues of DNA methylases and YdiS is an orthologue of DNA restriction enzymes, disruption of the *ydiO* or *ydiP* gene may result in defective methylation of the chromosome, leading to enhanced susceptibility to a restriction endonuclease which is predicted to be encoded by *ydiS*. Disruption of *ydiS* may result in a defect in the ability to digest the unmethylated chromosome of a *ydiO*- or *ydiP*-disrupted strain. The predicted products YdiR and YdjA have no orthologues; however, both of these proteins seem to be required for the function of YdiS, as disruption of either the *ydiR* gene or the *ydjA* gene was required for disruption of *ydiO* and *ydiP* function. YdiR, YdiS, and YdjA may constitute a restriction endonuclease protein complex that recognizes unmethylated *Bsu*M sites.

Kanamycin-resistant transformation was caused by integration of plasmid pET24b *neo* carrying *ydiO* or *ydiP* at the *ydiO* or *ydiP* site but not at the pMUTIN plasmid site in the disruptant, as transformation resulted in few colonies when an unrelated *ydhC* disruptant with pMUTIN was used as the recipient (data not shown), and pET24b *neo* itself did not give transformants even in a *ydiR-ydiS-ydjA* disruptant (data not shown).

Restriction activity of disruptants with exogenous plasmid DNA. As the restriction and modification system has been

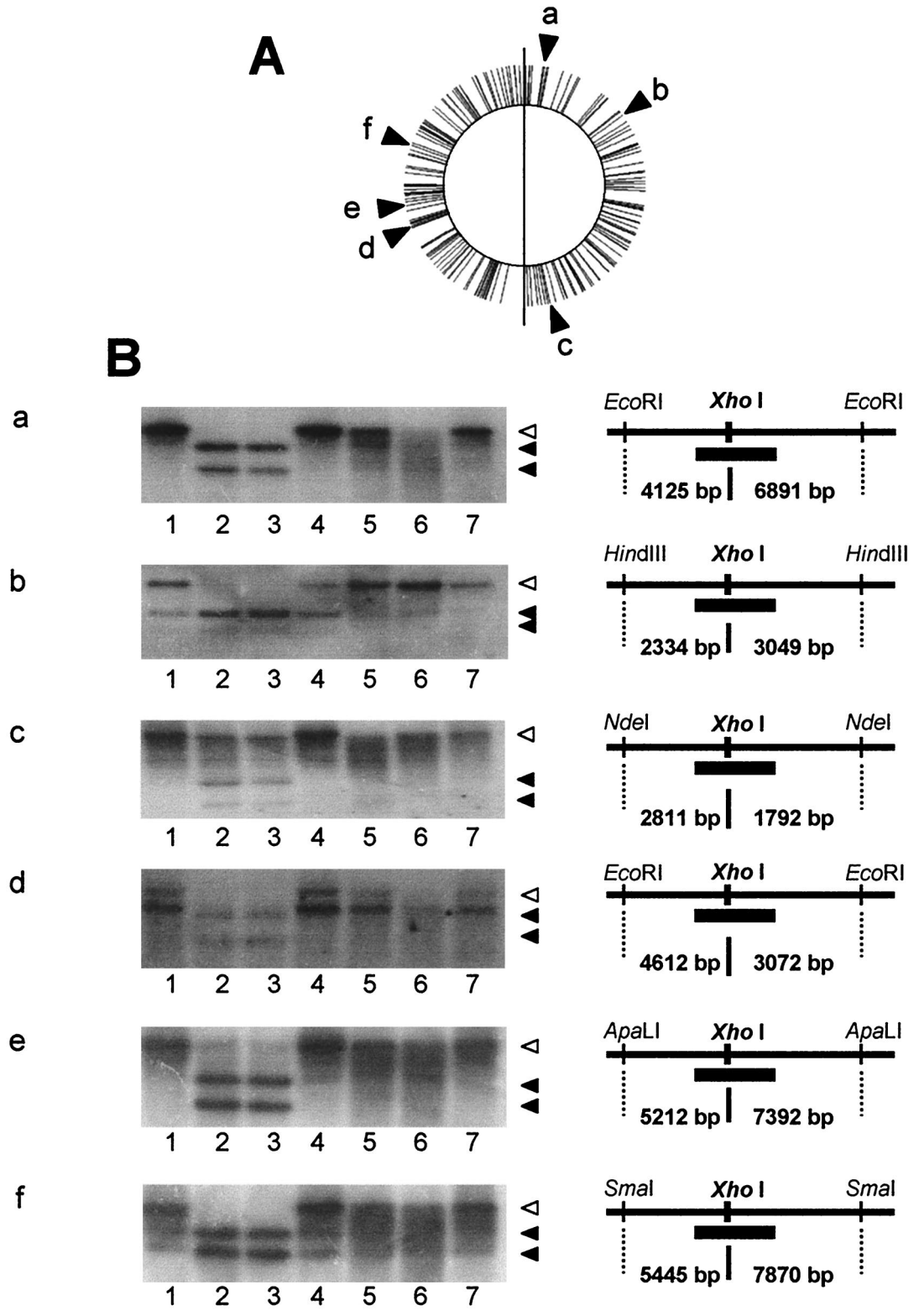

FIG. 2. Southern blot analysis of susceptibility to *Xho*I digestion of chromosomal DNAs extracted from disruptants. Chromosomal DNA was extracted from each disruptant, subjected to double digestion with *Xho*I and an appropriate restriction nuclease, separated by agarose electrophoresis, transferred to a membrane filter, and hybridized with alkaline phosphatase-labeled probe DNA (thick bars in the diagrams in the right portion of panel B). All *Xho*I sites in the chromosome of strain Marburg 168 are indicated by bars in panel A. The results of a Southern analysis for six *Xho*I sites on the chromosomes are shown in panel B. Lane 1, 168 DNA; lane 2, RM125 DNA; lane 3, BSU1 DNA; lane 4, BSU2 DNA; lane 5, BSU3 DNA; lane 6, BSU4 DNA ; lane 7, BSU5 DNA. The positions of *Xho*I sites on the chromosome are as follows: gel a, nucleotide 106975 (9°, AGGCTCGAGTAT); gel b, nucleotide 2633916 (54°, GCGCTCGAGACA); gel c, nucleotide 015718 (172°, GCGCTCGAGCAG); gel d, nucleotide 2800360 (239°, TCACTCGAGATT); gel e, nucleotide 3105700 (265°, CTTCTCGAAGAT); and gel f, nucleotide 3550939 (303°, TTTCTCGAGCTT). The open arrowheads indicate the position of an undigested fragment. The solid arrowheads indicate the positions of digested fragments. The configuration of the restriction sites and the probe binding site for each blot is also shown in panel B.

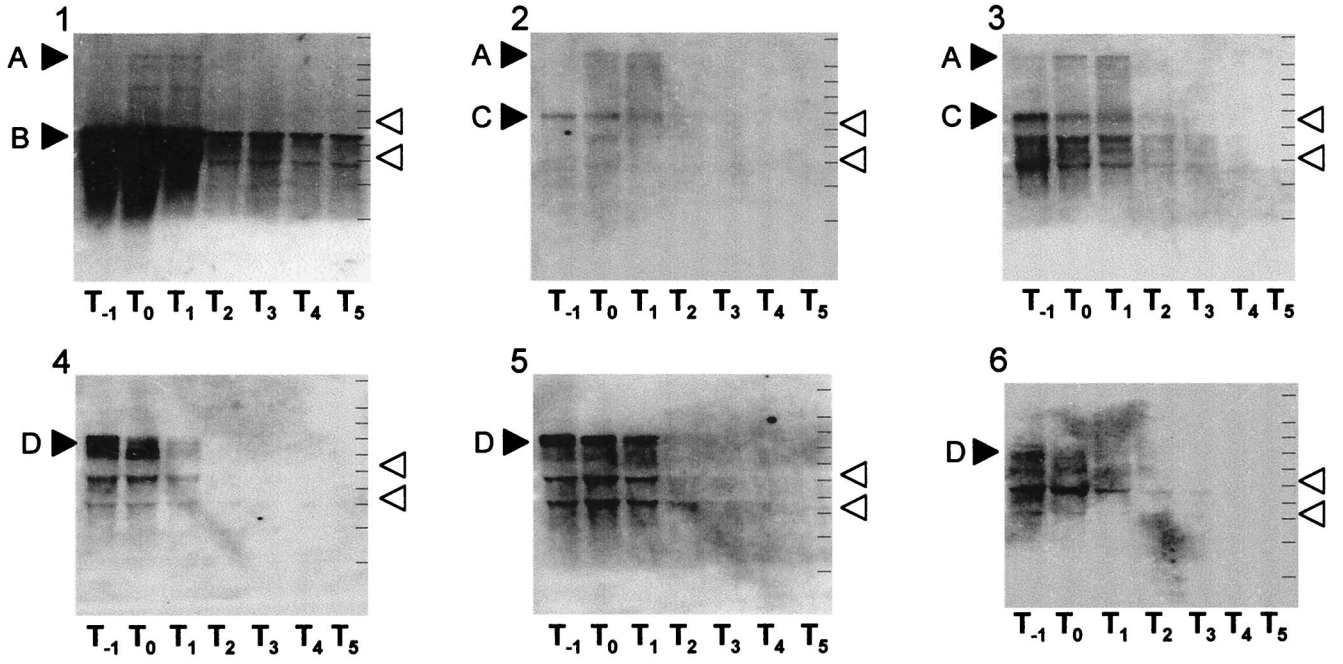

FIG. 3. Northern blot analysis of transcripts of the prophage 3 region. RNAs were prepared from cells grown in sporulation medium at 37°C, separated by electrophoresis, transferred to nitrocellulose membranes, and hybridized with DIG-labeled probe RNA. The RNA samples used were 20-μg samples. The open arrowheads indicate the positions of 16S rRNA (1.55 kb) and 23S rRNA (2.93 kb). The solid arrowheads indicate the positions of the transcripts detected, as follows: band A, 7.6 kb; band B, 2.2 kb; band C, 2.9 kb; and band D, 4.2 kb. Panel 1, *groEL* probe; panel 2, *ydiO* probe; panel 3, *ydiP* probe,; panel 4, *ydiR* probe; panel 5, *ydiS* probe; panel 6, *ydjA* probe. The lines on the right in each membrane indicate the positions of molecular weight markers (9, 6, 5, 4, 3, 2.5, 2, 1.5, 1, and 0.5 kb). The T_n values below each membrane indicate the numbers of hours (n) after the end of the logarithmic phase of growth.

found to be a mechanism for defense against foreign phage DNA, disruption of the restriction and modification system also results in an increase in the ability to be transformed by exogenous plasmid DNA (29). As *Bsu*M of *B. subtilis* has a weak effect on DNAs of phages such as ϕ105 (34), plasmids having multiple *Xho*I sites were used to characterize the *Bsu*M system (2). The frequency of transformation with plasmid pHV1401, which had three *Xho*I sites, was much lower in the wild-type strain, while it was somewhat higher in mutant strains carrying disrupted *ydiO*, *ydiP*, *ydiR*, *ydiS*, or *ydjA*. The frequency of transformation with plasmid pHV33, which did not have a *Xho*I site, was comparable to or somewhat lower than the frequency of transformation with the wild-type recipient (Table 3). The ratio of the frequency of transformation with the plasmid without an *Xho*I site to the frequency of transformation with the plasmid with three *Xho*I sites was more than 10 times higher when the *ydiR-ydiS-ydjA* operon-disrupted strains were used. Plasmid DNAs were isolated from cells of *E. coli* C600 r^+ m^+, and they were susceptible to *Xho*I, indicating that the *Xho*I sites were not methylated. Disruption of only the *ydiR-ydiS-ydjA* operon was enough to result in defective digestion of plasmid DNA with three *Xho*I sites. The *ydiO-ydiP* function must be very weak, and it did not have a strong effect on incoming *Xho*I site methylation, as disruption of *ydiO* or *ydiP* had no effect on the transformation ratio. However, as described below, enhanced transcription of the *ydiO-ydiP* operon by increased readthrough from the *groESL* operon resulted in increased protection of pHV1401.

Disruption of the smaller ORFs *ydiM*, *ydiN*, *ydjB*, and *ydjC* in the prophage 3 region did not affect restriction on plasmids (data not shown).

Chromosomal target of the restriction and modification system. Restriction and modification activity against phage or plasmid DNA was weak (34), but a single disruption of *ydiO* and *ydiP* resulted in very tiny colonies on nutrient agar, which indicated that the restriction and modification system works well on the chromosome of *B. subtilis*. To determine whether this system works on the chromosome, methylation of six chromosomal restriction fragments carrying only one *Xho*I target sequence (17) was examined indirectly by digesting chromosomal DNAs with *Xho*I. Susceptibility to the *Xho*I enzyme was evaluated by Southern blot analysis.

As shown in Fig. 2B, gels a and e, at nucleotide positions 106975 (9°) and 3105700 (265°) DNA from the wild-type strain, the *ydiP ydiS* doubly disrupted strain, or the single disruptant with a disruption in the *ydiR-ydiS-ydjA* operon was not susceptible to *Xho*I digestion, while DNAs from RM125 and the *ydiO ydiS* doubly disrupted strain were susceptible. Thus, at least YdiO must be active in methylation of the *Xho*I target sequence, since the *ydiO ydiS* strain might have lost both YdiO and YdiP activities due to the probable polar effect of disruption by the pET24 *neo* plasmid without a P*spac* promoter and the *ydiP ydiS* strain lost only YdiP activity. At nucleotide position 015718 (172°) (Fig. 2B, gel c), the results seem to be similar to those shown in Fig. 2B, gels a and e, although the *Xho*I site on this fragment was somewhat refractory to digestion.

At nucleotide position 2633916 (54°) (Fig. 2B, gel b), the

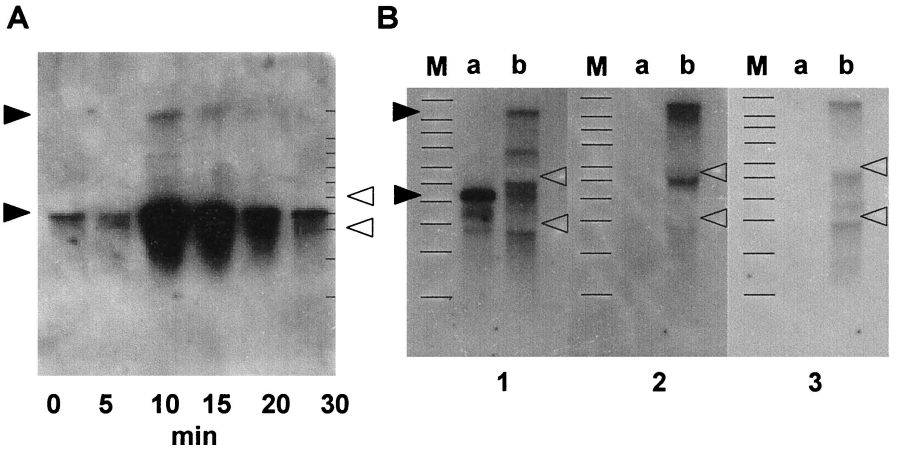

FIG. 4. Heat induction and artificial derivation of readthrough transcript of *groESL* operon. (A) Wild-type cells grown at 37°C in Luria-Bertani broth to the early logarithmic phase were transferred to 48°C. At each of the times indicated, a portion of the culture was removed and RNA was prepared for Northern blot analysis. A DIG-labeled probe for *groEL* was used. Each lane contained 0.3 μg of RNA. The open arrowheads indicate the positions of 16S rRNA (1.55 kb) and 23S rRNA (2.93 kb). The lines on the right indicate the positions of the molecular weight markers described in the legend to Fig. 3. The solid arrowheads indicate the positions of 7.6- and 2.2-kb transcripts. (B) Cells of wild-type strain 168 or mutant strain BSU9, which has a deletion in the rho-independent transcription termination signal sequence of *groESL*, were grown at 37°C in sporulation medium to the end of logarithmic phase. RNA was prepared and subjected to Northern analysis. Each lane contained 5 μg of RNA. The open arrowheads indicate the positions of 16S rRNA (1.55 kb) and 23S rRNA (2.93 kb). The solid arrowheads indicate the positions of 7.6- and 2.2-kb transcripts. In panel 1, the DIG probe was *groEL*, lane a contained wild-type strain 168, and lane b contained a Δ*ter(groESL)* disruptant. In panel 2, the DIG probe was *ydiO*, lane a contained wild-type strain 168, and lane b contained a Δ*ter(groESL)* disruptant. In panel 3, the DIG probe was *ydiP*, lane a contained wild-type strain 168, and lane b contained a Δ*ter(groESL)* disruptant. Lanes M contained molecular weight markers.

*Xho*I enzyme did not digest chromosomal DNAs from the wild-type strain and strains with disruptions in *ydiR*, *ydiS*, or *ydjA*, while DNAs from classical restriction and modification system-deficient strain RM125 and *ydiO ydiS* doubly disrupted strains were digested. DNA from the *ydiP ydiS* doubly disrupted strain was 50% digested. These results suggest that YdiO and YdiP are both active methylases.

For nucleotide position 2800360 (239°) (Fig. 2B, gel d), the results were similar to those shown in Fig. 2B, gels a and e; however, there must be an unknown *Xho*I site very close to the *Xho*I site identified. The former site must be somehow refractory to methylation with active YdiO and YdiP and thus is susceptible to *Xho*I even when active YdiO and YdiP are present. Without YdiO and YdiP or in RM125, the *Xho*I site identified was more susceptible to digestion than the unknown site. At nucleotide position 3550939 (303°) (Fig. 2B, gel f), the *Xho*I site must be partially unmethylated, because DNAs from strains other than RM125 and *ydiO ydiS* strains were partially digested.

Transcription of the prophage 3 region. To observe transcription of the five ORFs, RNAs were extracted from cells grown at 37°C in sporulation medium. Northern analysis with five RNA probes for each of the five ORFs revealed two transcripts, which were 3 and 4 kb long (Fig. 3, bands C and D). The 3-kb transcript was detected by two probes for *ydiO* and *ydiP*, while the 4-kb transcript was detected by three probes for *ydiR*, *ydiS*, and *ydjA*. Both transcripts were detected in cells in the logarithmic phase of growth. They were not detected in cells collected during sporulation. Therefore, *ydiO* and *ydiP* constitute one operon, and *ydiR*, *ydiS* and *ydjA* constitute the other operon. We identified a very long 7.6-kb transcript (Fig. 3, band A) for the *groESL* operon that included the *groESL* and *ydiO-ydiP* operons, as well as a shorter 2.2-kb transcript (Fig. 3, band B).

Disruption of the terminal signal sequence for transcription of the *groESL* operon. The long transcript must be a readthrough transcript of the *groESL* operon as heat treatment induced this transcript, as well as that of the *groESL* operon (27) (Fig. 4A). In order to determine the effect of readthrough from the *groESL* operon on *ydiO-ydiP* operon expression, we disrupted the terminal signal sequence for transcription of the *groESL* operon. The disrupted strain showed elevated readthrough from the *groESL* operon to the *ydiO-ydiP* operon (Fig. 4B). Enforced readthrough clearly revealed a 3.8-kb transcript, indicating that there is a transcription termination signal sequence between *groESL* and *ydiO*. The disrupted strain became permissive to plasmid transformation, carrying multiple *Xho*I sites that led to easy infection of unmethylated exogenous DNA (Table 2). This strain grew well in minimal glucose medium or sporulation medium and sporulated well (data not shown).

Classical restriction and modification system-deficient mutant RM125 has an altered prophage 3 region. PCR DNA synthesis with the *groEL* and *gutB* primer pairs resulted in a fragment that was 15.7 kb long when wild-type chromosomal DNA was used as the template and in a fragment that was 5 kb long when the template DNA was from RM125 (34) (Fig. 5A). To determine whether the five ORFs of the prophage 3 region are in a 5-kb region in RM125, we performed PCR DNA synthesis with the primer pair for each ORF using chromosomal DNA from the wild-type strain or RM125. As shown in Fig. 5B, when the wild-type strain DNA was used, the PCR synthesized DNA of each ORF, while when RM125 DNA was used, no DNA was synthesized, although with both wild-type

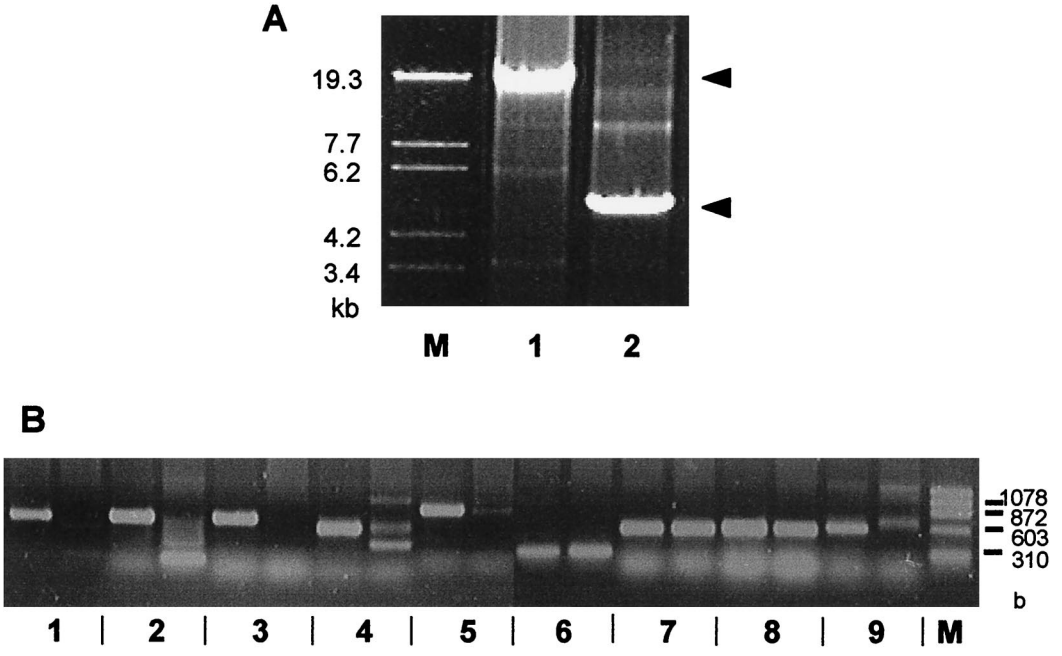

FIG. 5. PCR analysis of prophage 3 region of the RM125 chromosome. (A) Long accurate PCR products obtained with *groEL* and *gutB* primers. Lane1, PCR with 168 DNA; lane 2, PCR with RM125 DNA. Lane M contained molecular weight markers. (B) PCR products obtained with primer pairs for each ORF in the prophage 3 region. For each pair of lanes the left lane contained a PCR mixture with strain 168 DNA, and the right lane contained a PCR mixture with strain RM125 DNA. Lanes 1, primer pair for *ydiO*; lanes 2, primer pair for *ydiP*; lanes 3, primer pair for *ydiR*; lanes 4, primer pair for *ydiS*; lanes 5, primer pair for *ydjA*; lanes 6, primer pair for *groES*; lanes 7, primer pair for *groEL*; lanes 8, primer pair for *gutR*; lanes 9, primer pair for *gutB*. Lane M contained molecular weight markers.

strain DNA and strain RM125 DNA PCR synthesized DNA of *groES* and *groEL* (27), as well as DNA of *gutR* and *gutB* (38). Therefore, the original *B. subtilis*-related strain from which the substituted chromosome was isolated, strain 202-5 (= IAM1169, a *B. amyloliquefaciens* strain), has no genes similar to those of *Bsu*M found in the prophage 3 region of *B. subtilis* Marburg 168 and may have not been infected with the phage carrying *Bsu*M system genes. In short, RM125 has a 1.9-kb insert instead of the 13-kb prophage 3 region. We did not analyze the substituted fragment further.

Effect of induction of the *ydiR-ydiS-ydjA* operon on cell growth. To determine the balance between chromosome methylation and restriction, we placed the *ydiR-ydiS-ydjA* operon under the P*spac* promoter and induced the operon. Addition of IPTG arrested the growth of the cells of only the *ydiO* or *ydiP* disruptant. The viability of the cells was lost rapidly (within 60 min). The concentration decreased from 732×10^6 to less than 1×10^6 CFU/ml for BSU7 and from 456×10^6 to 3×10^6 CFU/ml for BSU8. Even after addition of IPTG, growth was not arrested when the *ydiO-ydiP* operon was active (data not shown). The *ydiO* disruptant might have defective expression of both *ydiO* and *ydiP*, and the *ydiP* disruptant might have defective expression of only *ydiP*. This indicates that even when the *ydiR-ydiS-ydjA* operon is overexpressed, the presumed YdiS nuclease complex does not digest chromosomal DNA if there is expression of the *ydiO* or *ydiP* methylase gene.

DISCUSSION

As described above, the classical *B. subtilis* Marburg mutant strain RM125 (34) lacks the 13-kb prophage 3 region, which contains the genes of the intrinsic *Bsu*M restriction-modification system of this organism. The classical *hsrM1* and *nonB* (24, 36) mutations were not mapped in this study, but they may be mutations in the *ydiR-ydiS-ydjA* operon as there are no paralogue genes on the *B. subtilis* Marburg chromosome (17).

Instead, the RM125 strain carried a 1.9-kb chromosomal fragment derived from *B. subtilis*-related strain 202-5 (= IAM1169, a *B. amyloliquefaciens* strain), which did not have the restriction-modification system genes of *B. subtilis* Marburg *Bsu*M. On the other hand, five restriction-modification systems have been found in *B. subtilis* Marburg-related bacteria (32). These systems have been introduced into the *B. subtilis* Marburg chromosome by transformation (10, 11, 12, 33). They are *Bsu*B, *Bsu*C, *Bsu*E, *Bsu*F, and *Bsu*R, and *Bsu*C, *Bsu*F, and *Bsu*R are allelic. *Bsu*B, *Bsu*F, and *Bsu*R are known to have one restriction gene and one modification gene. These restriction-modification system genes must be introduced into each *B. subtilis* Marburg-related strain by horizontal transfer, such as *E. coli* P4 phage-mediated transfer of the *Eco*O109I restriction-modification system (16), or by transfer with plasmids such as *Lactococcus lactis* plasmid pTR2030 (20), which carries an LlaI restriction-modification system operon composed of *llaIM*, *llaIC*, *llaI-1*, *llaI-2*, *llaI-3,* and *llaI-4.* LlaIM is a DNA methyltransferase, and LlaI-2 is a homologue of the YdiS protein described above.

Chromosomal DNA from the *ydiO*-disrupted strain, which may have a defective *ydiP* gene due to a probable polar effect, was susceptible to *Xho*I digestion, suggesting that MtbP (17), a prophage SPβ-encoded modification methylase, recognizes a target sequence other than the *Xho*I site if the *mtbP* gene is

active in the *ydiO* disruptant. Parental strain 168 harbors SPβ and the *metbP* gene.

YdiO and YdiP have many common conserved motif sequences (14, 21), suggesting that these genes had the same origin. However, YdiP has a higher level of similarity to MtbP than to YdiO, suggesting that YdiP may have originated from MtbP. Some *B. subtilis* phages carry a tandem pair of methyltransferase genes (reviewed in reference 32), and the *L. lactis* plasmid pTR2030 described above carries an operon consisting of a regulator gene, a methyltransferase gene, a restriction endonuclease gene, and other genes of unknown function that are necessary for endonuclease activity (20). It should be noted that YdiS and YdjA exhibit some homology with LlaI-2 and LlaI-3, respectively. Thus, it is not surprising that *Bsu*M is composed of two operons, one operon consisting of duplicated methyltransferase genes and the other consisting of an endonuclease gene and its associated genes. *Helicobacter pylori* has multiple restriction-modification system genes (31); *jhp0164* and *jhp0165* are located in tandem and are homologous to *ydiS* and *ydjA*, respectively, and *jhp0435*, which is located far from *jhp0164* and *jhp0165*, is homologous to *ydiO*. *E. coli mcrB* is homologous to *ydiS* and constitutes an operon with *mcrC* (3, 23); its product is presumed to be similar to YdjA.

Target sequence CTCGAG of *Bsu*M is symmetrical. Predicted methyltransferases YdiO and YdiP seem to be active, and they are not identical. It is not known how a symmetrical target is recognized by two homologous but nonidentical methyltransferase genes. Determination of the site of methylation will require biochemical study with target DNA and purified YdiO and YdiP proteins, as well as with YdiR, YdiS, and YdjA proteins, which may form a restriction nuclease complex.

Transcriptional analysis of the restriction-modification system genes revealed that the *ydiO-ydiP* operon can be transcribed by readthrough from the *groESL* operon (27) upon heating. Heat treatment of *B. subtilis* Marburg conferred permissiveness to phage SP10 infection (9). This might be because of enhanced methyltransferase activity due to heat induction of the *ydiO-ydiP* operon. Disruption of a transcription termination signal sequence resulted in enhanced synthesis of long transcripts, which led to permissive infection of plasmid DNA with multiple *Xho*I sites. This clearly indicates that methylation of unmethylated incoming DNA is enhanced to protect it from attack by a restriction endonuclease.

The genes controlling the restriction-modification system genes, such as the C gene (30), are not found in the prophage 3 region, but there are some small genes (*ydiM*, *ydiN*, *ydiQ*, *ydjB*, and *ydjC*), and these small genes might be regulatory genes. However, they seem not to contribute to restriction-modification activity, as disruptants with disruptions in these genes showed wild-type transformation by plasmid DNAs.

Pseudogenes (14) are reminiscent of phage and exhibit homology to genes such as the integrase gene of bacteriophage T270 of *Streptococcus pyogenes* and the small subunit of the terminase gene of bacteriophage LL-H (19). There is another pseudogene of *B. subtilis* encoding the YhxB paralogue (17), a glycolysis phosphomannomutase, on the strand opposite *ydiQ*. *B. subtilis* Marburg harbors 10 prophages or regions reminiscent of phages. The prophage 3 region is the smallest, has the smallest number of ORFs, and has the lowest gene density. Therefore, the prophage 3 region may be the oldest region, where restriction-modification genes remain undestroyed. It may be reasonable to assume that the prophage 3 region, which has a lower G+C content, was integrated into the *B. subtilis* chromosome with a lot of active genes and lost most of them by mutational pressure. Only five ORFs coding for restriction and modification enzymes remain active. Inactivation of genes for restriction makes a cell permissive to invasion by foreign DNA, while inactivation of genes for modification leads to autodigestion of the chromosome by the remaining restriction activity. Thus, a set of restriction-modification system genes survived in the prophage 3 region of the *B. subtilis* chromosome.

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