JOURNAL OF BACTERIOLOGY, Nov. 1992, p. 7194–7201
0021-9193/92/227194-08$02.00/0

Vol. 174, No. 22

Regulation of the *Bam*HI Restriction-Modification System by a Small Intergenic Open Reading Frame, *bamHIC*, in Both *Escherichia coli* and *Bacillus subtilis*

CATHERINE L. IVES, PETER D. NATHAN, AND JOAN E. BROOKS*

New England Biolabs, 32 Tozer Road, Beverly, Massachusetts 01915

Received 10 June 1992/Accepted 8 September 1992

***Bam*HI, from *Bacillus amyloliquefaciens* H, is a type II restriction-modification system recognizing and cleaving the sequence G^GATCC. The *Bam*HI restriction-modification system contains divergently transcribed endonuclease and methylase genes along with a small open reading frame oriented in the direction of the endonuclease gene. The small open reading frame has been designated *bamHIC* (for *Bam*HI controlling element). It acts as both a positive activator of endonuclease expression and a negative repressor of methylase expression of *Bam*HI clones in *Escherichia coli*. Methylase activity increased 15-fold and endonuclease activity decreased 100-fold when *bamHIC* was inactivated. The normal levels of activity for both methylase and endonuclease were restored by supplying *bamHIC* in *trans*. The *Bam*HI restriction-modification system was transferred into *Bacillus subtilis*, where *bamHIC* also regulated endonuclease expression when present on multicopy plasmid vectors or integrated into the chromosome. In *B. subtilis*, disruption of *bamHIC* caused at least a 1,000-fold decrease in endonuclease activity; activity was partially restored by supplying *bamHIC* in *trans*.**

Restriction-modification (RM) systems are known to protect bacterial cells against invasion by bacteriophage (1). They may also enhance recombination and subsequently be a means to enhance genetic diversity within a population (2). The restriction endonuclease acts to cleave incoming DNA lacking the proper DNA modification. The cognate modification methylase protects the cell's own DNA against degradation by the endonuclease.

Although gene regulation is not yet well understood for any RM system, it is clearly important for a system's effective functioning. The cell's own DNA must be completely protected by the methylase before the endonuclease can be allowed to act on invading DNA. Regulation is of paramount importance when a system is transferred to a new host with unprotected DNA but may also be important during major changes in a cell's physiological state.

Since 1978, when Mann and Smith cloned the *Hha*II RM system, a wide range of RM systems have been cloned and expressed in *Escherichia coli* (22, 38). It was originally anticipated that, in cloning a complete system in one step into a naive *E. coli* host, the endonuclease might act on the new host's DNA before the methylase had the chance to completely protect it (7, 32, 36). That this occurs only rarely suggests that the RM genes are regulated.

Investigators have used cloned RM systems to study the sequence and organization of the genes and properties of the protein components. However, the cloned systems have not proved very useful in illuminating the regulation of these systems. How regulation occurs is difficult to assess, since the RM systems are usually cloned into a heterologous host, maintained on multicopy plasmids, and sometimes transcribed from foreign promoters.

However, RM system control has been demonstrated in cloned systems. Tao et al. described a small open reading frame (designated *pvuIIC*) in the *Pvu*II RM system that has transregulatory properties: its presence is necessary for the expression of the *Pvu*II endonuclease gene (*pvuIIR*) in *E. coli* clones of the system (34). The derived amino acid sequence of *pvuIIC* shows strong similarity to those of small open reading frames found in the *Sma*I and *Eco*RV RM systems (4, 16, 34).

The *Bam*HI RM system was detected in *Bacillus amyloliquefaciens* H, a gram-positive bacterium closely related to the genetically well-characterized species *Bacillus subtilis* (37). *Bam*HI was one of the first RM systems discovered (37), and its endonuclease (R.*Bam*HI) and methylase (M.*Bam*HI) have been purified to apparent homogeneity and characterized (8, 18, 23, 30). The *Bam*HI RM system has been cloned and the nucleotide sequence determined; a small open reading frame encoding 102 amino acids located between the methylase and endonuclease genes was discovered (8). This open reading frame was previously shown to regulate *bamHIM* expression (8, 24) and has a predicted amino acid sequence very similar to that of *pvuIIC* (34). It has been designated *bamHIC* (for *Bam*HI controlling element). In this report we describe the regulation of the *Bam*HI system by *bamHIC* in both *E. coli* and *B. subtilis*.

MATERIALS AND METHODS

Bacterial strains and media. *E. coli* RR1 (*mcrBC*), K802 (*mcrA mcrBC*), ER1451 [*mcrA mcrBC* (Δ*lacZ*)M15 *lacI*^{q}], ER1562 (*mcrA mcrBC*), and ER1563 (*mcrA*) were described in detail previously (26). Strain ADK21, a derivative of K802 carrying the *bamHIM* gene on a λ lysogen, was constructed at New England Biolabs (17b). *B. subtilis* 168*trp*C2, used in *Bacillus* transformation and expression experiments, was provided by Ken Bott.

E. coli strains were grown in Luria broth (7) except during McrB restriction studies, where the cells were grown in Luria-Bertani broth (29). Media were supplemented with 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal, 20 μg/ml; American Bioanalytical), isopropylthio-β-D-galactoside (IPTG, 0.4 mM; Promega), ampicillin (100 μg/ml), or chloramphenicol (37.5 μg/ml) as required.

* Corresponding author.

Endonuclease activity was determined in *B. subtilis* cultures grown in tryptic soy broth (TSB; Difco) or nutrient broth (Difco). Transformed *B. subtilis* cells were selected on tryptone blood agar base plates (Difco). Erythromycin (1 μg/ml) or chloramphenicol (5 μg/ml) was added to the media as required.

For phage restriction assays, *Bacillus* cultures were grown in Penassay broth (Difco); after phage infection, bacterial cultures were plated on M agar as previously described (40).

Plasmids and bacteriophage. Plasmids pBamM1.8 and pBamM2.2 (containing *bamHIM* [7]), pMspI-30 (containing *mspIM* [25]), and pSU11 (containing *bsuRIM* [20]) were described previously. Plasmid pBamRM5.0, a pACYC184 derivative containing the complete *Bam*HI RM system on a 5.0-kb *Hin*dIII fragment, was used in various subcloning experiments (7). Plasmid pACYC184 was used as a control in several complementation experiments (9). Plasmid pBamRM2.7-HP13, containing the complete *Bam*HI system, was described previously (8). pY4Cm, a derivative of pGEM-Blue (Promega Biotec), was used to construct clones that would integrate into the *B. subtilis* chromosome (17).

Plasmid DNA was prepared by the alkaline lysis method (10) or by the cleared lysate method followed by CsCl-ethidium bromide ultracentrifugation (21).

The *B. subtilis* phage ϕ3Tc, a clear plaque variant of ϕ3T, was used in *B. subtilis* phage restriction experiments; phage lysates were prepared as described previously (12). All phage dilutions were done in 1× Spizizen salts (31).

Cloning enzymes and techniques. DNA manipulations and subcloning procedures were done as described previously (21) unless otherwise stated. All restriction endonucleases, DNA linkers, T4 DNA ligase, and the large (Klenow) fragment of *E. coli* DNA polymerase I were produced at and used as recommended by New England Biolabs. Calf intestine alkaline phosphatase was purchased from Boehringer Mannheim and used as previously described (21).

Individual DNA fragments from restriction digests of plasmids were separated by agarose gel electrophoresis with molecular biology grade agarose (IBI), excised, and then purified with a unidirectional electroelutor (IBI) as previously described (7) or a Prep-A-Gene DNA purification matrix kit (Bio-Rad).

E. coli transformations were done by the $CaCl_2$-heat shock method (11) or by electroporation with a Bio-Rad gene pulser and the manufacturer's recommended procedure. Transformations for the McrBC restriction experiments were done as previously described (7). *B. subtilis* transformations were done by the procedure of Bott and Wilson (3).

***Bam*HI constructs in *E. coli*.** The plasmid pBamC (Fig. 1) carries *bamHIC* on a pACYC vector (9) that is compatible with pUC (39) derivatives. The *Bal*I site was first converted to a *Bgl*II site, and a *Hin*cII-*Bam*HI fragment of pACYC184 containing most of the tetracycline resistance gene was deleted in the construction.

The plasmid pBamRM2.7 (Fig. 2) contains the complete *Bam*HI RM system and was constructed from a 2.7-kb *Hin*dIII-*Bgl*II fragment of pBamRM5.0 and pUC19 cleaved at the *Hin*dIII and *Bam*HI sites. The ligation mix was transformed into ADK21 cells, and the proper construct was isolated.

The plasmid pBamRMdC (Fig. 2) is a derivative of pBamRM2.7 in which the intergenic *bamHIC* has been disrupted by inserting a *Bgl*II linker at an *Xmn*I site within the gene after partial digestion and religation.

***Bam*HI constructs for expression in *B. subtilis*.** The plasmid pBamRM2.7-HP13 (8) contains the complete *Bam*HI system

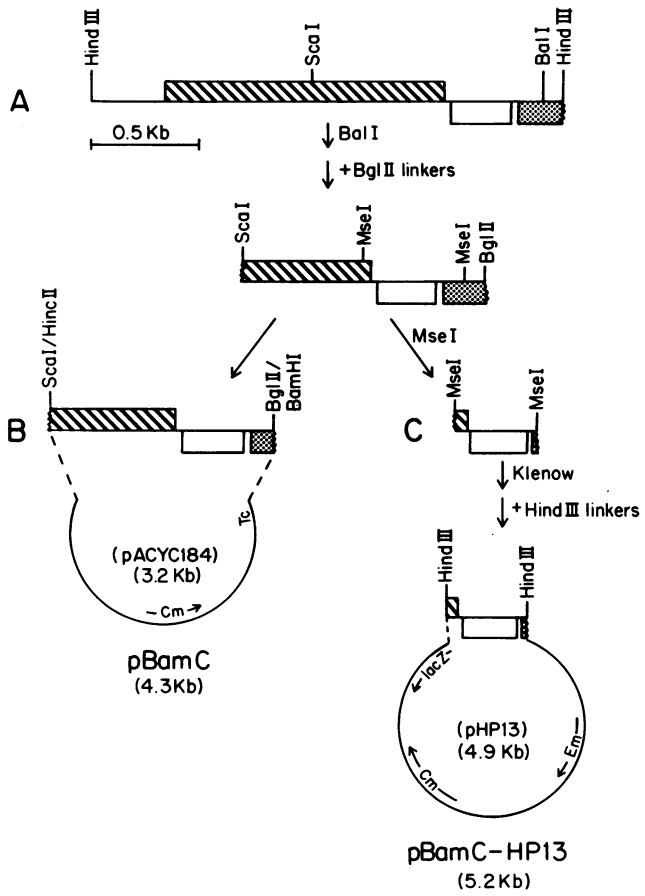
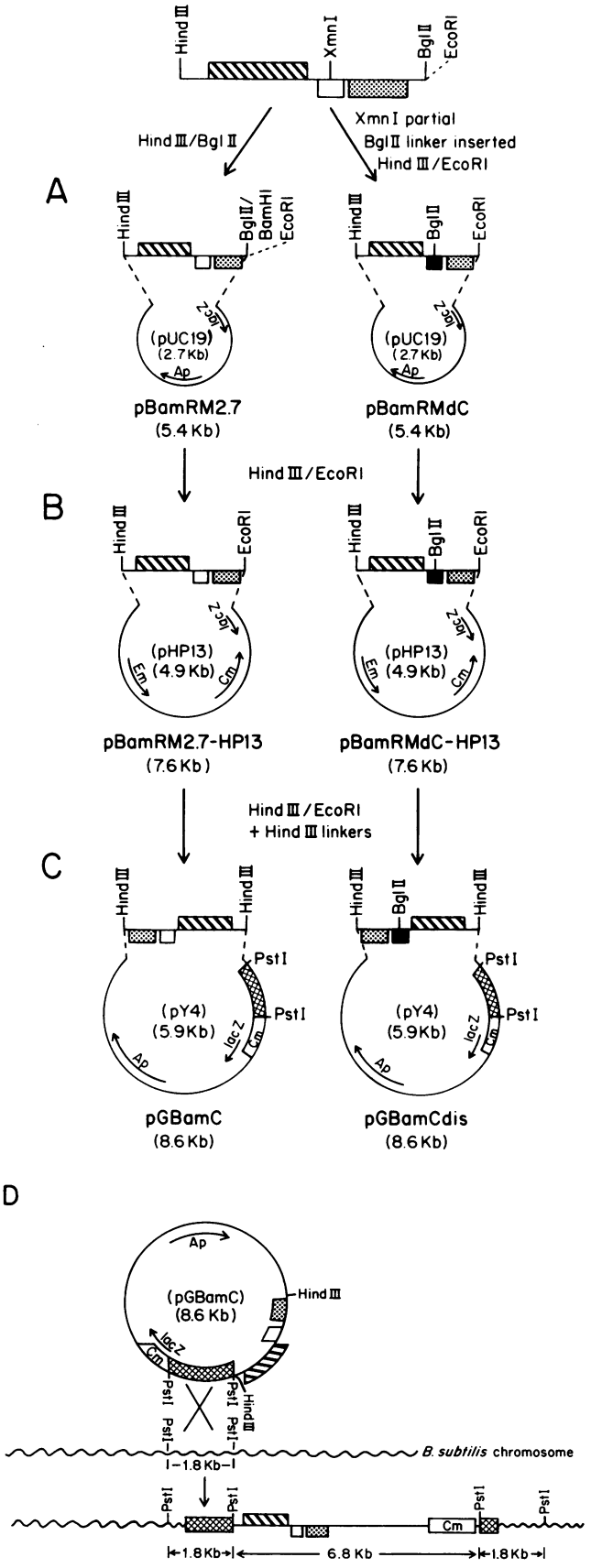


FIG. 1. Construction of subclones containing the *bamHIC* gene. The *bamHIM* gene (▨) is transcribed from right to left (indicated by its position above the horizontal line), and the *bamHIR* (▩) and *bamHIC* (▭) genes are transcribed from left to right (indicated by their position below the horizontal line). A vertical wavy line indicates truncation of the *bamHIR* and *bamHIM* genes. The name and size of the vector backbone are indicated inside each plasmid. (A) The 2.2-kb *Hin*dIII fragment from pBamM2.2. The *Bal*I site was changed to a *Bgl*II site as described in Materials and Methods. (B) The 1.1-kb *Bgl*II-*Sca*I fragment was ligated to a 2.9-kb *Hin*cII-*Bam*HI fragment of pACYC184 to form pBamC. The position and orientation of the chloramphenicol (Cm) gene is indicated by an arrow. (C) A 357-bp *Mse*I fragment containing the intact *bamHIC* gene was purified from the 1.1-kb *Sca*I-*Bgl*II fragment and inserted into pHP13 to form pBamC-HP13. The positions and orientations of the chloramphenicol (Cm) and erythromycin (Em) genes are indicated by arrows.

on pHP13, an *E. coli-B. subtilis* bifunctional vector (15). The plasmid pBamRMdC-HP13 contains the *Bam*HI system with a disrupted *bamHIC* on pHP13. It was constructed by isolating the 2.7-kb *Hin*dIII-*Eco*RI fragment of pBamRMdC and subcloning it in pHP13 (Fig. 2). The ligation mixture was transformed into K802(pBamM2.2) host cells after a 30-min induction with 0.3 μg of chloramphenicol per ml. For large-scale CsCl plasmid preparations of pBamRM2.7-HP13 and pBamRMdC-HP13, these plasmids were transferred to *E. coli* ADK21 cells. The CsCl-purified plasmids were then used to transform competent *B. subtilis* 168trpC2 cells.

The plasmid pBamC-HP13 carries *bamHIC* on the pHP13 shuttle vector (Fig. 1). The 1.1-kb *Sca*I-to-*Bgl*II fragment containing *bamHIC* was removed from pBamM2.2 (7) and



purified with the Prep-A-Gene kit. The fragment was digested with *Mse*I, and a 357-bp piece containing *bamHIC* was isolated. After *Hin*dIII linkers were added, the fragment was ligated to pHP13. The ligation mix was electroporated into *E. coli* ER1451 cells, and chloramphenicol-resistant transformants were selected on LB medium containing X-Gal and IPTG. Plasmids were isolated from white colonies and screened for the proper insert.

Plasmid pY4Cm is a derivative of pGEM-Blue (Promega) in which a chloramphenicol resistance gene cassette from pMI1101 (13) was cloned into the *Bam*HI restriction site of the vector. In addition, pY4Cm contains a 1.8-kb *Pst*I fragment of *B. subtilis* DNA isolated from near the origin of replication and harboring a cryptic tetracycline resistance gene (17). The 2.7-kb *Hin*dIII-*Eco*RI fragments of pBamRM2.7-HP13 and pBamRMdC-HP13 were cloned into pY4Cm after the *Hin*dIII linkers were added, generating pGBamC and pGBamRMdC (Fig. 2C). Recombinant plasmids were recovered and transformed into *B. subtilis*, generating two strains: 168ΩpGBamC and 168ΩpGBamRMdC. The proposed mechanism of integration of these plasmids is shown schematically in Fig. 2D; proper integration was confirmed by Southern hybridization analysis.

Analysis of M.*Bam*HI and R.*Bam*HI activities. In crude extracts and during methylase purification, aliquots were assayed by using ligated *Bam*HI linkers [d(pCGGATCCG)] as described previously (8). Methylase units were quantified by using a protection assay (35) in which 1 μg of treated λ DNA was challenged with 25 U of R.*Bam*HI for 20 min. One unit of M.*Bam*HI is defined as the amount of enzyme which, in a 50-μl reaction volume after 1 h at 37°C, is sufficient to completely protect 1 μg of λ DNA (containing five *Bam*HI sites) against R.*Bam*HI cleavage (8).

Endonuclease assays with *E. coli* extracts were performed as described previously (7); for *B. subtilis* extracts, the procedure was modified as follows. Fifty milliliters of culture was grown to the mid-log phase in TSB medium and harvested. The cells were resuspended in 5 ml of sonication buffer (10 mM Tris-HCl [pH 8.0], 10 mM 2-mercaptoethanol, 100 mM NaCl) containing lysozyme (10 μg/ml) and 10 mM EDTA and then frozen at −70°C. Before sonication, the cell pellets were subjected to three freeze-thaw cycles to aid lysis.

B. subtilis phage restriction tests were done by a method similar to that described previously for coliphage ϕ80 (14). ϕ3Tc, a clear plaque mutant of ϕ3T with six *Bam*HI sites, was the phage employed (12). Indicator cultures were grown

FIG. 2. Construction of *Bam*HI subclones. Details of the constructions are given in Materials and Methods. The *bamHIM* gene (▨) is transcribed from right to left (indicated by its position above the horizontal line), and the *bamHIR* (▩) and *bamHIC* (▭) genes are transcribed from left to right (indicated by their position below the horizontal line). A disrupted *bamHIC* gene is also indicated (■). The ampicillin (Ap), chloramphenicol (Cm), and erythromycin (Em) resistance genes are indicated. The name and size of the vector backbone are indicated inside each plasmid. (A) pBamRM2.7 and pBamRMdC replicate in *E. coli*. (B) pBamRM2.7-HP13 and pBamRMdC-HP13 replicate in both *E. coli* and *B. subtilis*. (C) pGBamC and pGBamCdis integrate into the *B. subtilis* chromosome. The 1.8-kb *Pst*I fragment containing the *B. subtilis* cryptic chromosomal tetracycline resistance gene is indicated (▩). (D) Proposed mechanism of integration of pGBamC and pGBamCdis into the *B. subtilis* chromosome. The wavy line represents *B. subtilis* chromosomal DNA; the sizes of the expected *Pst*I fragments are indicated below the figure.

to the mid-log phase at 37°C. Plaques were scored after 18 h of incubation at 30°C.

M.*Bam*HI purification. M.*Bam*HI was purified in parallel from *E. coli* K802 host cells containing pBamM1.8 and pBamM2.2, respectively, so that the levels of methylase produced could be compared. The purification regimen of M.*Bam*HI from *E. coli* K802(pBamM1.8) was described in detail previously (8). The same purification scheme was used for the K802(pBamM2.2) cells, except that 3.5 liters of cell culture was used, yielding approximately twice as many cells. After phosphocellulose chromatography, the most active column fractions (30 ml for pBamM2.2 and 15 ml for pBamM1.8) were pooled and concentrated fivefold with a Centricon (Amicon) chamber. The concentrate was dialyzed and stored as described previously (8).

RESULTS

Effect of *bamHIC* on McrBC restriction of methylase-containing plasmids. *E. coli* normally contains restriction systems that act on DNA carrying alien sequence-specific methylation. The best characterized of these is the McrBC system (26–28). During the initial cloning of the *Bam*HI system, two subclones of *bamHIM*, pBamM2.2 and pBamM1.8, were generated (7). The former contained intact *bamHIC*, whereas in the latter *bamHIC* was truncated (8). Transformation studies showed that pBamM2.2 could transform McrBC$^+$ or McrBC$^-$ strains with approximately equal efficiency, but pBamM1.8 was strongly restricted in McrBC$^+$ strains (7). It seemed possible that the *bamHIC* gene product was preventing McrBC restriction of pBamM2.2. If so, providing it on a second plasmid in a host should prevent McrBC restriction of pBamM1.8.

To test this, pBamC, a construct containing *bamHIC* on pACYC184, was made (Fig. 1). The plasmid was transformed into ER1562 and ER1563, which are isogenic *E. coli* McrBC$^-$ and McrBC$^+$ strains, respectively. The strains were transformed in a second step with pBamM1.8 or pBamM2.2 plasmids, and the results are shown in Table 1 as McrBC$^+$/McrBC$^-$ transformant ratios. For pBamM2.2, the ratio of the number of transformants on the McrBC$^+$ host to that on the McrBC$^-$ host was approximately 1.0; the addition of *bamHIC* on a second plasmid had no effect on transformation efficiency.

In the case of pBamM1.8, the transformation efficiency for the McrBC$^+$ host was 100-fold lower than that seen for McrBC$^-$ *E. coli* (Table 1). The presence of *bamHIC* on a second plasmid alleviated McrBC restriction; transformation efficiency increased 100-fold. The alleviation was not simply due to the presence of a second plasmid, since pACYC184 by itself did not reduce the McrBC restriction of pBamM1.8.

Other McrBC-sensitive methylase clones were used to test whether the *bamHIC* function interferes directly with McrBC action. The same McrBC$^+$ and McrBC$^-$ isogenic strains were used, and the plasmids pMspI-30 (encoding *mspIM*, methylating ^{me}CCGG [25, 36]) and pSU11 (encoding *bsuRIM*, methylating GG^{me}CC [19, 20]) were selected, since both have been shown to be strongly restricted when transformed into McrBC$^+$ hosts (20, 27). *bamHIC* had no effect on McrBC restriction of the *mspIM* plasmid; in both the presence and absence of *bamHIC*, the plasmid was restricted 10,000-fold (Table 1). It was of particular interest to see whether *bamHIC* would alleviate the McrBC restriction of pSU11. Like *Bam*HI, the *Bsu*RI RM system is isolated from a *Bacillus* strain and contains a small open reading frame located between the *bsuRIM* and *bsuRIR* genes (19, 20). Despite these similarities, the *bamHIC* gene had no effect on McrBC restriction of pSU11; in the presence or absence of *bamHIC*, the plasmid was still restricted more than 100-fold (Table 1). The *bamHIC* effect was specific for the *Bam*HI system.

TABLE 1. Effect of C.*Bam*HI on McrBC restriction of methylase plasmids

M plasmid[a]	Auxiliary plasmid[b]	Normalized efficiency of transformation[c]
pBamM2.2		6×10^{-1}
pBamM2.2	pBamC	4×10^{-1}
pBamM2.2	pACYC184	5×10^{-1}
pBamM1.8		4×10^{-3}
pBamM1.8	pBamC	5×10^{-1}
pBamM1.8	pACYC184	5×10^{-3}
pMspI-30[d]		1×10^{-4}
pMspI-30	pBamC	1×10^{-4}
pSUII[e]		9×10^{-3}
pSUII	pBamC	5×10^{-3}

[a] Plasmid with a methylase gene cloned onto pUC19 or pBR322.
[b] Isogenic strains ER1562 (McrBC$^-$) and ER1563 (McrBC$^+$) were transformed in a first step with the plasmid indicated before transformation by the M plasmid.
[c] Efficiency of transformation (EOT) = [transformants per ml in ER1563(McrBC$^+$)]/[transformants per ml in ER1562(McrBC$^-$)]. Normalized EOT = [EOT of M plasmid]/[EOT of vector (pUC19 or pBR322)]. All platings were done in triplicate, and the average values were used in computations.
[d] pMspI-30 is a pBR322 derivative containing the *mspIM* gene (24).
[e] pSU11 is a pBR322 derivative containing the *bsuRIM* gene (19).

Effect of *bamHIC* on M.*Bam*HI expression in *E. coli*. To determine whether *bamHIC* was exerting its effect at the level of methylase expression, M.*Bam*HI was purified in parallel from *E. coli* K802(pBamM1.8) and K802 (pBamM2.2) cells. The purification was performed as described previously (8) and in Materials and Methods; sodium dodecyl sulfate-polyacrylamide gel electrophoresis of M.*Bam*HI purified from the two sources is shown in Fig. 3. Cells containing pBamM1.8 clearly made more M.*Bam*HI than did cells containing pBamM2.2 (Fig. 3). The methylase yield from cells containing pBamM1.8 was 7.4×10^4 U/mg of cellular protein compared with 4.4×10^3 U/mg of methylase from cells containing pBam2.2. The specific activities of the purified methylase were 1.4×10^5 U/mg of protein when determined on λ DNA from cells containing pBam1.8 and 8.0×10^3 U/mg from cells containing pBam2.2. By visual estimation, approximately 80% of the most active fraction purified from K802(pBam1.8) was M.*Bam*HI protein; less than 10% of the most active fraction purified from K802(pBam2.2) cells was M.*Bam*HI protein. Therefore, *E. coli* hosts with *bamHIM* alone produce approximately 15-fold more M.*Bam*HI than do *E. coli* cells carrying *bamHIM* together with *bamHIC*.

In methylase assays, the use of high concentrations of purified M.*Bam*HI led to methylation of noncanonical sites; this result had been reported previously for M.*Bam*HI (23). Plasmid DNA modified by M.*Bam*HI at noncanonical sites proved to be a good substrate for McrBC cleavage in an in vitro assay; plasmid DNA modified at canonical *Bam*HI sites did not (31a). It is likely that in vivo high methylation levels caused by disruption of *bamHIC* leads to enough noncognate methylation to trigger McrBC restriction.

Effect of *bamHIC* on the complete *Bam*HI system. In the

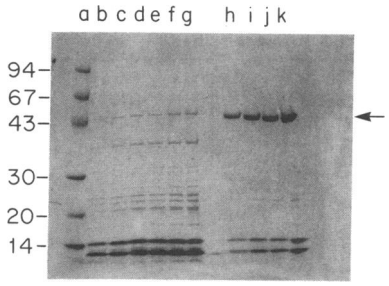


FIG. 3. M.*Bam*HI proteins purified from K802(pBamM1.8) and K802(pBamM2.2). The most active fractions of M.*Bam*HI from each preparation were purified, pooled, and concentrated as described in Materials and Methods. Samples of each were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 10 to 20% polyacrylamide gradient gel (Integrated Separation Systems) and stained with Coomassie blue. Molecular mass markers (lane a) are indicated in kilodaltons on the left side of the figure. Lanes: b through g, 7, 14, 21, 28, 35, and 42 μg of purified protein, respectively, from the K802(pBamM2.2) preparation; h through k, 6, 12, 18, and 24 μg of purified protein, respectively, from the K802(pBamM1.8) preparation. The arrow to the right indicates the 49-kDa M.*Bam*HI protein band. Its identity was confirmed by its N-terminal amino acid sequence (8).

intact *Bam*HI system, *bamHIC* is located between *bamHIR* and *bamHIM* and oriented in the same direction as *bamHIR*. Its position and orientation suggest that the *bamHIC* function could affect both endonuclease and methylase expression. The effect of *bamHIC* on expression of both the methylase and endonuclease was tested by comparison of a plasmid containing *bamHIM* and *bamHIR* with *bamHIC* intact (pBamRM2.7) with a plasmid with *bamHIM*, *bamHIR*, and a disrupted *bamHIC* (pBamRMdC). Both constructs are shown in Fig. 2. In addition, a test was made to determine whether *bamHIC* supplied in *trans* altered methylase or endonuclease expression.

To maintain the intact *Bam*HI system in *E. coli*, it is necessary to have an additional source of *bamHIM* present (7). Therefore, a λ lysogen in which λ carries *bamHIM* fused with the *his* promoter (strain ADK21) was employed (17b) as the host strain. When *bamHIC* was disrupted, methylase activity increased (Table 2). This was similar to the effect on methylase activity when *bamHIR* was absent. However, the level of increase was more modest: a 2- to 3-fold increase rather than the 15-fold increase seen in the methylase clones described above. It is not clear why the difference is smaller in these experiments. The smaller difference could be due to the presence of a second copy of *bamHIM* in ADK21 cells, to the presence of *bamHIR* on the plasmid construct, to the use of a disruption rather than a deletion of *bamHIC*, or to some combination of these factors.

TABLE 2. Effect of *bamHIC* function on R.*Bam*HI and M.*Bam*HI activities[a] in *E. coli* ADK21

Plasmid	Auxiliary plasmid[b]	Enzyme activity, U/g of wet wt (% of wild-type activity)	
		M.*Bam*HI[c]	R.*Bam*HI
pBamRM2.7 (wild type)		7.4×10^4 (100)	8.0×10^5 (100)
pBamRMdC		2.2×10^5 (300)	8.3×10^3 (1)
pBamRMdC	pBamC	8.4×10^4 (110)	2.5×10^6 (310)
pBamRMdC	pACYC184	2.3×10^5 (310)	7.2×10^3 (1)

[a] Cultures (50 ml) containing these plasmids were grown to the mid-log phase and harvested. Endonuclease and methylase activities were assayed from crude extracts as described in Materials and Methods. All experiments were done by growing and assaying three independent colonies; the means of the three assays are reported.

[b] *E. coli* ADK21(pBamRMdC) was transformed in a second step by the plasmids indicated, and survivors were selected for Ap^r and Cm^r.

[c] Values have been adjusted by subtraction of methylation activity found in ADK21 cells. This value is less than 5% of the measured activity shown in each case.

In contrast, the presence of *bamHIC* increased the expression of the endonuclease. The plasmid pBamRM2.7, with *bamHIC* intact, in ADK21 cells directed synthesis of 8×10^5 U of R.*Bam*HI per g; pBamRMdC directed synthesis of only 8.3×10^3 U of R.*Bam*HI per g, a 100-fold drop. As in the case of methylase activity, *bamHIC* complemented in *trans*: when pBamC was present with pBamRMdC, R.*Bam*HI activity increased again to the level found in pBamRM2.7 clones. Once again, pACYC184 in the cells had no effect on activity. Thus, in *E. coli*, *bamHIC* serves to decrease M.*Bam*HI activity while increasing R.*Bam*HI activity, presumably through its gene product.

Regulation of the cloned *Bam*HI RM system in *B. subtilis*. The above studies were conducted in a heterologous host. To study whether a similar regulation operates during *Bacillus* growth, the cloned system was transferred to *B. subtilis* 168*trp*C2. It had previously been shown that the *Bam*HI RM system could be transferred to *B. subtilis* in one step without the need for premodification of the host, as was the case in *E. coli* (8). In *E. coli*, *bamHIC* regulation of R.*Bam*HI and M.*Bam*HI could be assessed by assaying crude extracts. However, M.*Bam*HI activity could not be reliably assayed in crude extracts of *B. subtilis* because of low activity levels. A similar observation was reported for M.*Bam*HI activity from *B. amyloliquefaciens* extracts (23). Crude *B. subtilis* extracts were assayed for endonuclease activity, as had been done in *E. coli*. The *Bam*HI RM system is known to restrict incoming phage in *B. amyloliquefaciens* (37). Therefore, the clones were also evaluated for the level of phage restriction by using the *B. subtilis* phage ϕ3Tc.

The results of the endonuclease assays and phage tests for the plasmid-borne system in *B. subtilis* are shown in Table 3. Cells containing the complete *Bam*HI system on pBamRM2.7-HP13 produced 4.4×10^4 U of R.*Bam*HI per g. As in *E. coli*, the disruption of *bamHIC* caused reduction of endonuclease activity. However, in *B. subtilis*, the reduction was more dramatic. Instead of the 100-fold drop found in *E. coli*, a drop of greater than 1,000-fold, down to undetectable levels, was observed in *B. subtilis* extracts. Similar results were found in phage restriction tests. Phage ϕ3Tc can infect and form plaques on *B. subtilis* 168 with high efficiency, giving titers of 4.5×10^8 phage per ml. When *B. subtilis* (pBamRM2.7-HP13) was used as a host, the phage were strongly restricted. Disruption of *bamHIC* resulted in total loss of phage restriction.

Next, the *Bam*HI system was studied when integrated in a single copy on the *Bacillus* chromosome, as it is found in *B. amyloliquefaciens*. In this configuration it was also possible to determine whether *bamHIC* could restore restriction endonuclease activity in *trans* in *B. subtilis* as it does in *E. coli*. Therefore, copies of the *Bam*HI RM system, with

TABLE 3. Regulation of the *Bam*HI RM System in *B. subtilis* 168 by the *bamHIC* gene

Plasmid[a]	Second plasmid	Endonuclease activity (U/g)[b]	Efficiency of plating[c]
Control (none)		None detected	1
Autonomous plasmids			
pBamRM2.7-HP13		4.4×10^4	3.6×10^{-6}
pBamRMdC-HP13		$<3.0 \times 10^1$	7.7×10^{-1}
Integrated plasmids			
pGBamC		5.9×10^4	$<3.0 \times 10^{-6}$
pGBamCdis		$<3.0 \times 10^1$	8.3×10^{-1}
pGBamCdis	pHMBamC	1.5×10^4	8.3×10^{-4}

[a] The plasmidless host strain, *B. subtilis* 168*trp*C2, was used as a control. No endogenous endonuclease activity was detected; the detection level of R.*Bam*HI in vitro is <30 U/g. Autonomous plasmids can replicate independently in *B. subtilis* 168*trp*C2; integrated plasmids are established by homologous recombination with the chromosome.

[b] Cultures (25 ml) containing the test plasmids were grown to the mid-log phase and harvested. Endonuclease activity was assayed from crude extracts on pBR322 as described previously (7).

[c] Efficiency of plating is defined as the titer of ϕ3Tc grown on *B. subtilis* 168 containing the test plasmid divided by the titer of ϕ3Tc grown on the control, plasmidless strain *B. subtilis* 168*trp*C2.

bamHIC intact or disrupted, were cloned onto pY4Cm, a nonreplicative vector that integrates into the *B. subtilis* chromosome (Fig. 2). A small fragment containing *bamHIC* was also cloned onto pHP13 to produce pBamC-HP13 (Fig. 1). *B. subtilis* strains containing both forms of the integrated system were then tested for endonuclease activity and phage restriction.

B. subtilis 168ΩpGBamC produced essentially the same amount of R.*Bam*HI as that produced by the plasmid-borne system. With *bamHIC* disrupted (168ΩpGBamdis), no endonuclease was detected in extracts, as is the case with the plasmid-borne system. Introduction of *bamHIC* on pBamC-HP13 restored endonuclease activity to approximately 25% of the level for the complete system.

The results of the phage tests on the integrated system were more complex. When 168ΩpGBamC was used as the host, phage restriction was pronounced: over 6 orders of magnitude. When *bamHIC* was disrupted, phage restriction disappeared. When *bamHIC* was supplied to this mutant on pBamC-HP13, phage restriction was partially restored; but instead of the 4-fold difference in endonuclease activity between 168ΩpGBamC and 168ΩpGBamCdis(pBamC-HP13) seen in crude extracts, the two strains exhibited a 1,000-fold difference in phage restriction. These results show that the in vivo and in vitro activities of the endonuclease are not directly proportional, suggesting that other factors, in addition to the amount of endonuclease in the cell, affect phage restriction. However, the results clearly demonstrate that in both *E. coli* and *B. subtilis*, *bamHIC* positively controls R.*Bam*HI activity.

DISCUSSION

Regulation of *Bam*HI in *E. coli* and *B. subtilis*. It is possible to show *bamHIC* regulation of both endonuclease and methylase expression in *E. coli*. In the presence of *bamHIC*, endonuclease activity increases while methylase activity decreases. The magnitude of the effect is different for the two proteins; while the difference in M.*Bam*HI activity with and without *bamHIC* is 15-fold at most, the difference in R.*Bam*HI activity is 100-fold.

It was of interest to see how the system would function when present as a single copy on the *B. subtilis* chromosome, similar to the way it is found in *B. amyloliquefaciens*. In *B. subtilis*, as in *E. coli*, R.*Bam*HI activity increases in the presence of *bamHIC*. However, in *B. subtilis*, the effect of *bamHIC* is significantly stronger, giving a 1,000-fold difference in R.*Bam*HI activity with and without R.*Bam*HI. It is unclear why this should be the case. It is possible that in *B. subtilis* there are other regulatory factors that keep *bamHIR* repressed in the absence of *bamHIC* or perhaps regulatory factors that enhance *bamHIC* activation of *bamHIR*. It is also possible that the difference is simply due to a higher basal level of *bamHIR* expression in *E. coli* relative to that in *B. subtilis* in the absence of *bamHIC*. Whatever the cause, it is likely that the situation in *B. amyloliquefaciens* more closely resembles that in *B. subtilis* than that in *E. coli*. The integrated *B. subtilis* system is currently being used to characterize regulation of R.*Bam*HI activity under different physiological conditions.

It had previously been shown in *B. subtilis* that increasing the copy number of certain chromosomal genes increased their expression level (41). Therefore introducing the *Bam*HI system into *B. subtilis* on a multicopy plasmid seemed like a plausible way to increase R.*Bam*HI expression. However, this did not occur; the same level of expression was found with the system on the plasmid as when it was integrated into the chromosome. The results may be due to the existence of a regulatory mechanism in *Bacillus* strains, limiting the amount of R.*Bam*HI that can be produced in the cell. Or it is possible that since the system is integrated near the *B. subtilis* replication origin and the assays are done on logarithmically growing cells, there may not have been a significant difference in gene copy number in the two types of clones. In any event, the plasmid-borne *Bam*HI system did not provide a better source for production of the R.*Bam*HI enzyme.

Possible mode of action of *bamHIC*. At present there is no direct evidence to show how *bamHIC* is controlling the *Bam*HI system. However, the fact that *bamHIC* can act on both *bamHIR* and *bamHIM* when provided in *trans* suggests it is acting via a protein product, C.*Bam*HI. Since C.*Bam*HI contains a potential helix-turn-helix motif typical of site-specific DNA binding proteins (5, 6), our hypothesis at present is that C.*Bam*HI acts as a transcriptional activator of *bamHIR* and a repressor of *bamHIM*. A similar mode of action has been proposed for C.*Pvu*II (33, 34). Since *bamHIM* and *bamHIR* are divergently oriented, it is possible that by binding at one strategic site on the DNA, C.*Bam*HI could simultaneously repress *bamHIM* transcription while activating that of *bamHIR*. To confirm this hypothesis, it would be helpful to identify the system's promoters.

The hypothesis does not preclude additional control mechanisms operating posttranscriptionally. In fact, preliminary experiments suggest that although *bamHIC* disruption has a strong effect upon transcription, another level of control is also operating (17a). Clarification of the control mechanism awaits overexpression and purification of the C.*Bam*HI protein as well as the development of an in vitro assay. Purification of C.*Bam*HI is currently under way in this laboratory.

Evolutionary aspects of *bamHIC* regulation. As more RM systems are sequenced, more are found to contain C-like open reading frames (38). The C genes are widespread, being

represented in both gram-positive and gram-negative bacteria. The C proteins are more related among themselves than to their respective endonuclease or methylase components (33), which raises interesting questions as to whether they are evolving independently of their restriction systems. Since they are so similar, it is possible that they have retained functional equivalence while shuttling between different hosts. Experiments are being done to determine whether the C genes can cross complement, and the results look promising.

ACKNOWLEDGMENTS

We thank Aaron Marcus and Carla Vaccaro for excellent technical assistance. We also thank Antal Kiss for his gift of pSU11, Mario Noyer-Weidner for his provision of φ3Tc, and Ken Bott for the *B. subtilis* 168*trp*C2 strain. We thank Bob Blumenthal, William Jack, Shuang-Yong Xu, Elisabeth Raleigh, and Ellen Sutherland for critical reading of the manuscript and sharing of unpublished data. Special thanks are due to Don Comb, New England Biolabs, for continued support of the work.

REFERENCES

1. **Arber, W.** 1974. DNA modification and restriction. Prog. Nucleic Acids Res. Mol. Biol. **14**:1–37.
2. **Bickle, T. A.** 1982. The ATP-dependent restriction endonucleases, p. 85–108. *In* S. Linn and R. J. Roberts (ed.), Nucleases. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
3. **Bott, K. F., and G. A. Wilson.** 1968. Metabolic and nutritional factors influencing the development of competence for transfection of *Bacillus subtilis*. Bacteriol. Rev. **32**:370–378.
4. **Bougueleret, L., M. Schwarzstein, A. Tsugita, and M. Zabeau.** 1984. Characterization of the genes coding for the *Eco*RV restriction and modification system of *Escherichia coli*. Nucleic Acids Res. **12**:3659–3676.
5. **Brennan, R. G., and B. W. Mathews.** 1989. The helix-turn-helix DNA binding motif. J. Biol. Chem. **264**:1903–1906.
6. **Brennan, R. G., and B. W. Mathews.** 1989. Structural basis of DNA-protein recognition. Trends Biochem. Sci. **14**:286–290.
7. **Brooks, J. E., J. S. Benner, D. F. Heiter, K. R. Silber, L. A. Sznyter, T. Jager-Quinton, L. S. Moran, B. E. Slatko, G. G. Wilson, and D. O. Nwankwo.** 1989. Cloning the *Bam*HI restriction modification system. Nucleic Acids Res. **17**:979–997.
8. **Brooks, J. E., P. D. Nathan, D. Landry, L. A. Sznyter, P. Waite-Rees, C. L. Ives, L. S. Moran, B. E. Slatko, and J. S. Benner.** 1991. Characterization of the cloned *Bam*HI restriction modification system: its nucleotide sequence, properties of the methylase, and expression in heterologous hosts. Nucleic Acids Res. **19**:841–850.
9. **Chang, A. C. Y., and S. N. Cohen.** 1978. Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the p15A cryptic miniplasmid. J. Bacteriol. **134**: 1141–1156.
10. **Clewell, D. B., and D. R. Helinski.** 1969. Supercoiled circular DNA-protein complex in *Escherichia coli*: purification and induced conversion to an open circular form. Proc. Natl. Acad. Sci. USA **62**:1159–1166.
11. **Cohen, S. N., A. C. Y. Chang, and L. Hsu.** 1972. Nonchromosomal antibiotic resistance in bacteria: genetic transformation of *Escherichia coli* by R-factor DNA. Proc. Natl. Acad. Sci. USA **69**:2110–2114.
12. **Cregg, J. M., and J. Ito.** 1979. A physical map of the genome of the temperate phage φ3T. Gene **6**:199–219.
13. **Donovan, W., L. Zheng, K. Sandman, and R. Losick.** 1987. Genes encoding spore coat polypeptides from *Bacillus subtilis*. J. Mol. Biol. **196**:1–10.
14. **Gingeras, T. R., and J. E. Brooks.** 1983. Cloned restriction/modification system from *Pseudomonas aeruginosa*. Proc. Natl. Acad. Sci. USA **80**:402–406.
15. **Haima, P., S. Bron, and G. Venema.** 1987. The effect of restriction on shotgun cloning and plasmid stability in *Bacillus subtilis* Marburg. Mol. Gen. Genet. **209**:335–342.
16. **Heidmann, S., W. Seifert, C. Kessler, and H. Domdey.** 1989. Cloning, characterization and heterologous expression of the *Sma*I restriction-modification system. Nucleic Acids Res. **17**: 9783–9796.
17. **Ives, C. L., and K. F. Bott.** 1989. Cloned *Bacillus subtilis* chromosomal DNA mediates tetracycline resistance when present in multiple copies. J. Bacteriol. **171**:1801–1810.

17a. **Ives, C. L., and J. E. Brooks.** Unpublished data.

17b. **Jack, W. E.** Unpublished data.

18. **Jack, W. E., L. Greenough, L. F. Dorner, S. Xu, T. Strzelecka, A. K. Aggarwal, and I. Schildkraut.** 1991. Overexpression, purification, and crystallization of *Bam*HI endonuclease. Nucleic Acids Res. **19**:1825–1829.
19. **Kiss, A., and F. Baldauf.** 1983. Molecular cloning and expression in *Escherichia coli* of two modification methylase genes of *Bacillus subtilis*. Gene **21**:111–119.
20. **Kiss, A., G. Posfai, C. C. Keller, P. Venetianer, and R. J. Roberts.** 1985. Nucleotide sequence of the *Bsu*RI restriction-modification system. Nucleic Acids Res. **13**:6403–6421.
21. **Maniatis, T. E., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
22. **Mann, M. B., R. N. Rao, and H. O. Smith.** 1978. Cloning of restriction modification genes in *E. coli*: the *HhaII* system from *Haemophilus haemolyticus*. Gene **3**:97–112.
23. **Nardone, G., J. George, and J. G. Chirikjian.** 1984. Sequence-specific *Bam*HI methylase: purification and characterization. J. Biol. Chem. **259**:10357–10362.
24. **Nathan, P. D., and J. E. Brooks.** 1988. Characterization of clones of the *Bam*HI methyltransferase gene. Gene **74**:35–36.
25. **Nwankwo, D. O., and G. G. Wilson.** 1988. Cloning and expression of *Msp*I restriction and modification genes. Gene **64**:1–8.
26. **Raleigh, E. A., N. E. Murray, H. Revel, R. M. Blumenthal, D. Westaway, A. D. Reith, P. W. J. Rigby, J. Elhai, and D. Hanahan.** 1988. McrA and McrB restriction phenotypes of some *E. coli* strains and implications for gene cloning. Nucleic Acids Res. **16**:1563–1575.
27. **Raleigh, E. A., and G. Wilson.** 1986. *Escherichia coli* K-12 restricts DNA containing 5-methylcytosine. Proc. Natl. Acad. Sci. USA **83**:9070–9074.
28. **Ross, T. K., E. C. Achberger, and H. D. Braymer.** 1987. Characterization of the *Escherichia coli* modified cytosine restriction (*mcr*B) gene. Gene **61**:277–289.
29. **Silhavy, T. J., M. L. Berman, and L. W. Enquist.** 1984. Experiments with gene fusions. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
30. **Smith, L. A., and J. G. Chirikjian.** 1979. Purification and characterization of the sequence-specific endonuclease *Bam*HI. J. Biol. Chem. **254**:1003–1006.
31. **Spizizen, J.** 1958. Transformation of biochemically deficient strains of *Bacillus subtilis* by deoxyribonucleate. Proc. Natl. Acad. Sci. USA **44**:407–408.

31a. **Sutherland, E., E. Raleigh, J. E. Brooks, and P. D. Nathan.** Unpublished data.

32. **Sznyter, L. A., B. Slatko, L. Moran, K. H. O'Donnell, and J. E. Brooks.** 1987. Nucleotide sequence of the *Dde*I restriction-modification system and characterization of the methylase protein. Nucleic Acids Res. **15**:8249–8266.
33. **Tao, T., and R. M. Blumenthal.** 1992. Sequence and characterization of *pvuIIR*, the *Pvu*II endonuclease gene, and of *pvuIIC*, its regulatory gene. J. Bacteriol. **174**:3395–3398.
34. **Tao, T., J. C. Bourne, and R. M. Blumenthal.** 1991. A family of regulatory genes associated with type II restriction-modification systems. J. Bacteriol. **173**:1367–1375.
35. **Theriault, G., P. H. Roy, K. A. Howard, J. S. Benner, J. E. Brooks, A. F. Waters, and T. R. Gingeras.** 1985. Nucleotide sequence of the *Pae*R7 restriction/modification system and partial characterization of the protein products. Nucleic Acids Res. **13**:8441–8461.

36. **Walder, R. Y., C. J. Langtimm, R. Chatterjee, and J. A. Walder.** 1983. Cloning of the *Msp*I modification enzyme. J. Biol. Chem. **258:**1235–1241.
37. **Wilson, G. A., and F. E. Young.** 1975. Isolation of a sequence-specific endonuclease (*Bam*I) from *Bacillus amyloliquefaciens* H. J. Mol. Biol. **97:**123–125.
38. **Wilson, G. G.** 1991. Organization of restriction-modification systems. Nucleic Acids Res. **19:**2539–2566.
39. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33:**103–119.
40. **Yehle, C. O., and R. H. Doi.** 1967. Differential expression of bacteriophage genomes in vegetative and sporulating cells on *Bacillus subtilis*. J. Virol. **1:**935–947.
41. **Young, M.** 1985. Gene amplification in *Bacillus subtilis*. J. Gen. Microbiol. **130:**1613–1621.