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Bacillus subtilis-Phage ϕ1 Overcomes Host-Controlled Restriction by Producing *Bam*Nx Inhibitor Protein

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Summary. *Bacillus amyloliquefaciens* N produces two restriction enzymes, *Bam*NI and *Bam*Nx. *Subtilis*-phage ϕ1 is strongly restricted by *Bam*Nx. We isolated ϕ1rH, a mutant of phage ϕ1, which overcame the *Bam*Nx-restriction by producing inhibitor. This inhibitor inactivated *Bam*Nx specifically and reversibly. The inhibitor directly interacted with *Bam*Nx and the inactivation might be the result of formation of a binary complex. The inhibitory activity was sensitive to treatment with trypsin. The molecular weight of the inhibitor protein was estimated to be approximately 20,000 daltons by gel filtration.

Introduction

Many species of bacteria are known to have a restriction-modification system as a defense mechanism against the invasion of foreign DNA such as that of bacteriophages (Arber 1974; Boyer 1971; Roberts 1976). In these cases, foreign DNA is inactivated by digestion with restriction enzymes while host DNA is protected from these enzymes usually by specific chemical modifications. Recently, several bacteriophages of *Escherichia coli* have been reported which have other types of defense mechanisms against host restriction enzymes (Eskin et al. 1973; Studier and Movva 1976; Spoerel et al. 1979; Brunel et al. 1979; Wagner et al. 1979; Toussaint 1976).

We have found that phage ϕNR2rH overcame *Bam*Nx-restriction of *B. amyloliquefaciens* strain N by producing *Bam*Nx-inhibitor protein after infection (Makino et al. 1979). We noticed that another *subtilis*-phage ϕ1rH, a mutant of ϕ1, also overcomes the restriction-modification system of *Bam*Nx. Phage ϕ1 is a well-known virulent phage (Reilly and Spizizen 1965; Ito and Spizizen 1971; Hemphill and Whiteley 1975) and it can serve as a gene-cloning vehicle in *B. subtilis* host-vector systems (F. Kawamura personal communication). In this paper, we describe the molecular mechanism of inactivation of *Bam*Nx after infection with ϕ1rH.

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Materials and Methods

a) Bacterial Strains and Bacteriophages

Bacillus amyloliquefaciens strain N, *B. amloliquefaciens* strain H, *B. subtilis* 168 (*trpC2*), *B. subtilis* LMAH (*leuA8*, *metB5*, *purA16*, *hisA3*) and *B. subtilis* phage ϕ1 were stocks from our laboratories. *B. subtilis* phage SPP1 was kindly provided by Dr. K. Matsumoto (Life Science Inst., Sophia Univ., Tokyo).

b) Preparation of Phage DNAs

DNA of phage ϕ1 was extracted by phenol from particles (grown on strain N) which had been purified by centrifugation through a CsCl step gradient. ^{3}H-labelled DNA of phage SPP1 (Behrens et al. 1979) was extracted from purified particles grown on *B. subtilis* 168 in L-broth, supplemented with 0.2% glucose and 10 mM $MgSO_4$, containing 5 μCi 6-^{3}H thymidine and 100 μg/deoxyadenosine/ml. The radioactivity of the DNA was 4,600 cpm/μg.

c) Titration of Phage

Exponentially growing cells in L-broth at 37° C with shaking were used as indicators. Titration was carried out according to the method by Rutberg (1969). Phages were grown in L-broth with shaking, and plated after dilution by 50 mM Tris-HCl buffer (pH 7.5) containing 0.1 M NaCl and 0.01 M $MgCl_2$.

d) Enzymes

*Bam*NI and *Bam*Nx were purified by phospho-cellulose (Whatman P11) and hydroxyl-apatite (Seikagaku-kogyo) column chro-

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matography (Greene et al. 1978) and by gel filtration (LKB Ultrogel AcA44). *Bam*HI, *Hae*III, *Hha*I, *Sal*I, and *Sma*I were obtained from Bethesda Research Laboratories, Inc. *Ava*II, *Hph*I and *Mbo*II were obtained from New England Biolabs, Inc. *Eco*RI was obtained from Takara Shuzo Co., Ltd.

e) Preparation of Crude Extract

Cells of strain N were infected with φ1rH at a multiplicity of five when the number of cells reached approximately 1.5×10^8 per ml in L-broth culture with aeration. The broth was quickly chilled 15 min after infection, then the cells were collected by centrifugation and stored at −80° C. From these cells, we prepared crude extract by the procedure reported previously (Makino et al. 1979) with minor modification.

f) Purification of φ1rH-Mediated BamNx-Inhibitor

φ1rH-mediated *Bam*Nx-inhibitor protein was purified from the crude extract of φ1rH-infected N cells through $(NH_4)_2SO_4$ precipitation, gel filtration, and DEAE-cellulose column chromatography. The details will be published elsewhere. The size of *Bam*Nx-inhibitor was estimated by gel filtration as follows. Ultrogel AcA44(LKB) column (1.5 × 40 cm) was equilibrated with 10 mM Tris-HCl buffer (pH 7.2) containing 0.4 M NaCl, 0.1 mM EDTA and 5 mM 2-mercaptoethanol, and concentrated inhibitor protein was applied to the column: 1.25 ml fractions were collected (flow rate 5 ml/h) and the activity of *Bam*Nx-inhibitor was measured. This column was calibrated by bovine serum albumin, ovalbumin, chymotrypsinogen, myoglobin, and ribonuclease A as molecular weight markers.

g) Unit Definitions and Assay Procedures of BamNx and BamNx-Inhibitor

One unit of *Bam*Nx is the amount required to cleave 1 μg of *subtilis*-phage SPP1 DNA in 60 min incubation at 37° C in 40 μl of reaction mixture. The standard reaction mixture contains 50 mM Tris-HCl (pH 7.2), 15 mM $MgCl_2$, 0.1 mM EDTA, 5 mM 2-mercaptoethanol, DNA and enzyme preparations. One unit of inhibitor is the amount required to inactivate one unit of *Bam*Nx in 15 min incubation without DNA at 37° C in 30 μl of reaction mixture. The remaining activity of *Bam*Nx was measured by prolonged incubation for 60 min after the addition of 1 μg of SPP1 DNA in 10 μl of reaction mixture. The same reaction mixture was used as that mentioned above. In the course of purification of *Bam*Nx-inhibitor, the assay was modified as follows: 10 μl of each fraction was mixed with 0.1 unit of *Bam*Nx in 30 μl of reaction mixture. After 15 min at 37° C, 0.2 μg of φ1rH DNA in 10 μl of reaction mixture was added and incubated for 60 min at 37° C.

After incubation, each sample was applied to a 0.8% horizontal agarose (Sigma type II) slab gel (150 × 150 × 3 mm). Electrophoresis was carried out at room temperature for 1.5 h at a constant voltage of 110 V. The gel was stained with ethidium bromide (0.5 μg per ml) and photographed under UV light. Loening's buffer (Loening, 1968) (0.36 M Tris, 0.3 M NaH_2PO_4, 0.01 M EDTA) was used through electrophoresis and staining.

h) Preincubation Time Course

Six units of inhibitor were preincubated at 30° C with 6 μg of DNA (or 6 units of *Bam*Nx) in a reaction mixture of 120 μl. At 0 min, 1 min, 5 min, 20 min, and 40 min, 20 μl of the reaction mixture was removed and added to 20 μl of reaction buffer containing 1 unit of *Bam*Nx (or 1 μg of DNA). After incubation for 60 min at 37° C, the reaction was stopped by adding sodium dodecyl sulfate (final 0.1%), and each sample was applied to a 0.8% agarose gel. Electrophoresis and staining were carried out as described above. The second largest band (after complete cleavage) was cut out from the gel and heated with one drop of 1 N HCl to solubilize the agarose. After drying on glass filter, the radioactivity was measured in toluene-based scintillator in a liquid scintillation counter (Beckman LS8100). The second largest band was selected for counting because no other fragments or intermediates were observed near it.

i) Reversibility of Inhibition

Purified *Bam*Nx (65 units) was incubated at 37° C for 15 min with excess amounts of inhibitor protein (130 units) in 10 mM Tris-HCl buffer (pH 7.2) containing 0.1 mM EDTA, 15 mM $MgCl_2$ and 5 mM 2-mercaptoethanol, and the mixture was applied to a DEAE-cellulose (Whatman DE52) column equilibrated with the same buffer without $MgCl_2$. After washing, adsorbed protein was eluted as batches with buffers containing 0.2 M and 0.5 M NaCl. The eluates were concentrated by dialysis against a 10 mM Tris-HCl buffer (pH 7.2) containing 0.1 mM EDTA, 5 mM 2-mercaptoethanol and 50% (V/V) glycerol, and the activities of both *Bam*Nx and *Bam*Nx-inhibitor were determined.

Results

a) φ1rH Overcomes Host Specific Restriction by BamNx

Bacillus amyloliquefaciens strain N produces two typical type II restriction enzymes, *Bam*NI and *Bam*Nx (Shibata and Ando 1974, 1975, 1976; Ikawa et al. 1979). *Bam*NI $\begin{pmatrix} 3'..G\downarrow GATCC..5' \\ 5'..CCTAG_{\uparrow}G..3' \end{pmatrix}$ is an isoschizomer of *Bam*HI (Wilson and Young 1975) from *B. amyloliquefaciens* strain H, and *Bam*Nx $\begin{pmatrix} 3'..G\downarrow GACC..5' \\ 5'..CCTG_{\uparrow}G..3' \end{pmatrix}$ is an isoschizomer of *Ava*II (Sutcliffe and Church 1978) from *Anabaena variabilis*.

DNA of φ1 phage particles was not at all cleaved by *Bam*NI (also by *Bam*HI) and the strain H which produces only *Bam*HI did not restrict the growth of phage φ1 (Table 1). On the other hand, the DNA was cut into more than 50 pieces by *Bam*Nx (Kawamura et al. 1979) (also by *Ava*II) and the strain N stringently restricted the growth of phage φ1 (Table 1). From plaques which rarely appeared on strain N, we isolated a phage φ1rH as a mutant. φ1rH was insensitive to restriction by *Bam*Nx of strain N (Table 1). In spite of the insensitivity in vivo, the DNA extracted from φ1rH particles grown on strain N was sensitive to *Bam*Nx as was the parental DNA, indicating that φ1rH has the ability to overcome the host-controlled restriction system of strain N. Since the DNA of φ1rH showed the same cleavage patterns by *Eco*RI and *Sal*I as those of the DNA of parental phage φ1, it is certain that φ1rH is a derivative of φ1.

Table 1. Relative plating efficiencies of ϕ1 and ϕ1rH on several *Bacillus* strains

Phage	Grown on	Indicator strains		
		B. subtilis LMAH	*B. amyloliquefaciens*	
			N	H
ϕ1	*B. subtilis* LMAH	1	4.8×10^{-5}	0.37
ϕ1rH	*B. subtilis* LMAH	1	3.09	0.90

The phenomen described above and some of the following observations are similar to those of another *subtilis* phage ϕNR2rH, but we have no evidence suggesting phylogenetic relationships between ϕ1rH and ϕNR2rH. These two phages are different in origin, host range, size of DNA (F. Kawamura personal communication) and DNA cleavage patterns by either *Eco*RI or *Sal*I (data not shown).

b) ϕ1rH Inactivates BamNx by BamNx-Inhibitor

Since ϕ1rH was insensitive to restriction by *Bam*Nx in vivo, we examined the activity of *Bam*Nx in the cells of strain N infected with ϕ1rH. Crude extracts were prepared from N cells and *B. subtilis* (LMAH) cells with or without ϕ1- or ϕ1rH-infection. As shown in Table 2, we could detect *Bam*Nx activity in the extracts from ϕ1-infected and uninfected N cells, but not in the extract from ϕ1rH-infected N cells. We therefore concluded that *Bam*Nx was inactivated by infection with ϕ1rH. We then assayed the inhibitory activity of the crude extracts against *Bam*Nx. The results are summarised in Table 2, showing that the *Bam*Nx-inhibitor appears in cells not only of strain N but also of *B. subtilis* after infection with ϕ1rH and that this *Bam*Nx-inhibitor should be responsible for overcoming the host-controlled restriction-modification system of strain N.

Table 2. Activities of *Bam*Nx and *Bam*Nx-inhibitor in extracts from cells infected with ϕ1 or ϕ1rH. Phage-infected cells were collected and extracted as described in Materials and Methods. About 3 g (wet weight) cells from 4 liter culture were used. Since strong non-specific nuclease activities were observed, the crude extracts from strain LMAH were applied to DEAE-cellulose (Whatman DE52) column and eluted by linear gradient of NaCl (0–0.7 M) in 100 ml of 30 mM Tris-HCl buffer (pH 7.2) containing 0.1 mM EDTA and 5 mM 2-mercaptoethanol. After chromatography, the fractions were assayed for the activity of *Bam*Nx or *Bam*Nx-inhibitor as described. The inhibitor from LMAH cells infected with ϕ1rH showed the same elution patterns from DEAE-cellulose column and gel filtration as those of inhibitor from N cells infected with ϕ1rH

Phage	Strain	Activities of	
		*Bam*Nx	*Bam*Nx-inhibitor
None	N[a]	+	−
ϕ1	N	+	−
ϕ1rH	N	−	+
None	LMAH[b]	−	−
ϕ1rH	LMAH	Not tested	+

[a] *B. amyloliquefaciens* strain N
[b] *B. subtilis* Marburg

c) Molecular Weight of BamNx-Inhibitor

The size of *Bam*Nx-inhibitor protein was estimated to be approximately 20,000 daltons (Fig. 1). The elution pattern did not change when low salt (50 mM NaCl) buffer was used.

d) Sensitivity of BamNx-Inhibitor to Treatment with Trypsin

Three units of *Bam*Nx-inhibitor (about 4 μg protein) were treated with 45 BAEE units of trypsin (Sigma) in 20 μl of reaction mixture containing 30 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 15 mM $MgCl_2$ and 2-mercaptoethanol. After incubation for 30 min at 30° C, excess amounts of trypsin inhibitor (Sigma) was added to the reaction mixture to inactivate trypsin. The residual activity of *Bam*Nx-inhibitor was assayed 10 min after the addition of trypsin inhibitor: as described in Materials and Methods, 1 unit of *Bam*Nx and DNA was used. Since the inhibitory activity of *Bam*Nx-inhibitor was completely disappeared by treatment with trypsin, we tentatively call it 'Inhibitor Protein'.

e) Inhibitor Protein Directly Interacts with BamNx

To determine whether the inhibitor protein interacts with *Bam*Nx or DNA, we carried out a time course experiment of preincubation of these components (see Materials and Methods). As shown in Fig. 2, inactivation of *Bam*Nx was promoted by preincubating *Bam*Nx-inhibitor protein with *Bam*Nx. No promotion of inactivation was observed when the inhibitor protein was preincubated with DNA. This result strongly suggests that the inhibitor protein directly interacts with the restriction enzyme *Bam*Nx, not with the substrate DNA of *Bam*Nx. This conclusion was supported by the fact that *Bam*Nx-inhibitor protein did

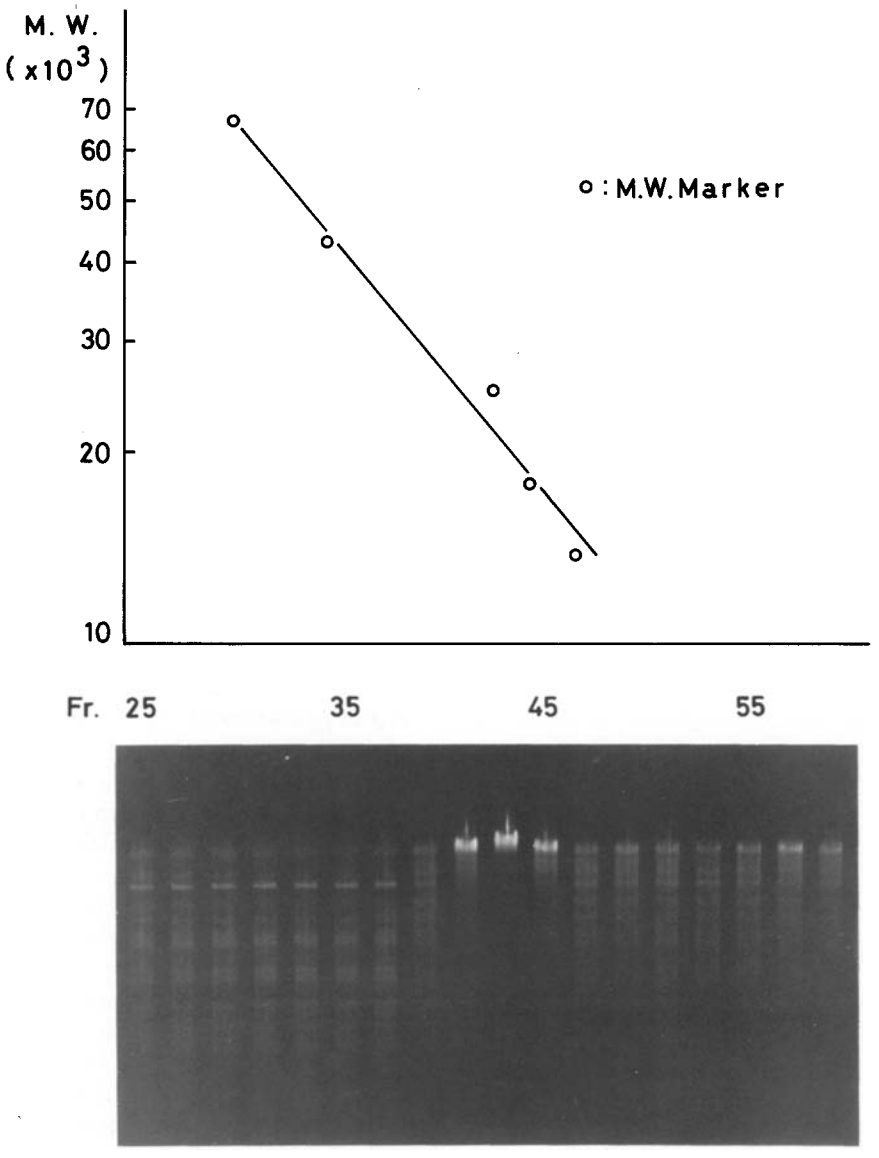

Fig. 1. Elution pattern of inhibitor protein from Ultrogel AcA44 (LKB) column

not reduce the activity of *Ava*II which recognizes and cleaves the same nucleotide sequences as *Bam*Nx (data not shown).

f) Inactivation of BamNx Is Reversible

Since our preliminary experiments suggested the inhibition of *Bam*Nx with the inhibitor protein was reversible, we tried to recover the *Bam*Nx activity from inactive form with the inhibitor protein. *Bam*Nx, inactivated by inhibitor, was applied to a DEAE-cellulose column and eluted with buffers containing 0.2 M and 0.5 M NaCl successively. As shown in Table 3, the activity of *Bam*Nx was not detected at all after preincubation with the inhibitor, but approximately 90% of the initial *Bam*Nx activity was recovered from the 0.2 M NaCl eluate, and more than 80% of the initial inhibitor activity was recovered from the 0.5 M NaCl eluate. The inhibition of *Bam*Nx with the inhibitor protein may be considered as one of the following: (1) inhibitor protein catalyses the modification or proteolysis of the enzyme, resulting in the inactivation of *Bam*Nx, (2) inhibitor protein inactivates *Bam*Nx by forming the complex of inhibitor and *Bam*Nx. Our results fit the idea that *Bam*Nx and the inhibitor

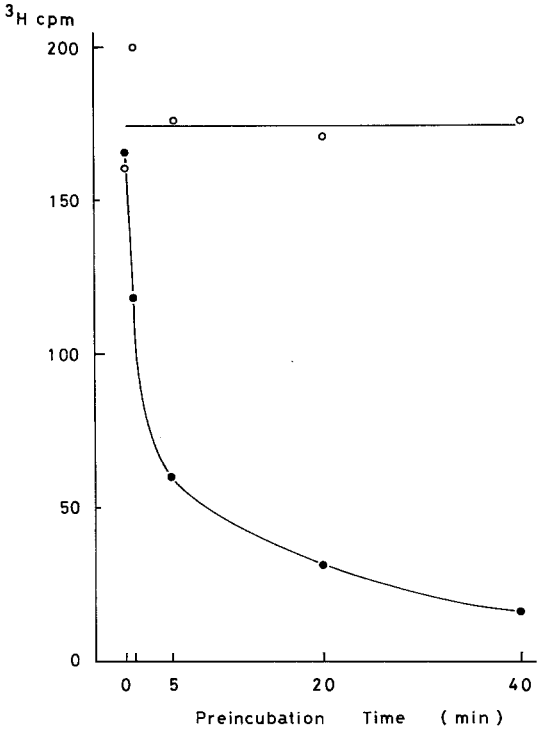

Fig. 2. Preincubation time course of inhibitor protein with DNA or *Bam*Nx. *Open circle,* preincubation of inhibitor protein with DNA. *Closed circle,* preincubation of inhibitor protein with *Bam*Nx

Table 3. Recovery of *Bam*Nx-activity from inhibitor-*Bam*Nx mixture

	*Bam*Nx activity	
	(Unit)	(% of initial)
Initial	65	100
After 15 min incubation at 37° C	none detected	
In 0.2 M NaCl eluate from DEAE-cellulose column	54	83.1

form a reversible complex devoid of cleavage activity. The molecular size of the complex and the ratio of *Bam*Nx and the inhibitor protein in the complex are unknown.

g) Specificity of Inhibition

We tested for the specificity of inhibition by preincubating other typical type II restriction enzymes with inhibitor. φ1rH-mediated *Bam*Nx-inhibitor did not inhibit *Ava*II, *Bam*HI, *Bam*NI, *Eco*RI, *Hae*III, *Hha*I, *Hph*I, *Mbo*II, *Sal*I and *Sma*I. *Bam*Nx was specifically inhibited by the inhibitor protein as far as we tested.

Discussion

Recently, several restriction-modification systems of *Bacillus* species were introduced into *B. subtilis* Marburg strain (Shibata et al. 1979; Saito et al. 1979; Ikawa et al. 1980). We examined the relative plating efficiencies of φ1 and φ1rH on these strains having *Bsu*168, *Bsu*R, *Bsu*1247(I), *Bsu*1247(II), *Bsu*1231(I) or *Bsu*1231(II) system. The strain having *Bsu*1247(I) or *Bsu*1231(II) system restricted φ1, but no significant difference was observed between the plating efficiencies of φ1 and φ1rH on these strains. Therefore, we assumed that φ1rH did not overcome *Bsu*1247(I) and *Bsu*1231(II) systems.

We have already reported that another *subtilis*-phage φNR2rH overcomes host restriction by producing *Bam*Nx-inhibitor protein. The characters in detail of φNR2rH-mediated *Bam*Nx-inhibitor protein are not known at present, but our preliminary data implies that this inhibitor also inactivates *Bam*Nx by forming a reversible complex. It is of interest that two considerably different phages produce proteins which have similar function.

Recently, *coli*-phages T3 and T7 were reported to overcome host restriction by producing proteins which directly interact with *Eco*K (Spoerel et al. 1979). Other *coli*-phages were suggested to escape attacks of host restriction enzyme without modification. It may be possible to explain these phenomena by the existence of an inhibitor like *Bam*Nx-inhibitor proteins or anti-*Eco*K proteins. This kind of host-parasite interaction could play an important role in nature.

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