Three New Temperate Phages of *Bacillus subtilis*

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Three temperate bacteriophages of *Bacillus subtilis* were isolated from soil samples and analysed, together with all the other known temperate phages of this organism, with respect to their host range, immunity, serology and DNA restriction pattern, and by other tests. The results show that the newly isolated phages are new members of the immunity sub-group I of the group III of *B. subtilis* temperate bacteriophages. We named these new phages IG1, IG3 and IG4.

INTRODUCTION

Some derivatives of temperate bacteriophages of *Bacillus subtilis* are potentially useful as vectors in molecular cloning (Dean *et al.*, 1978*b*; Kreft & Hughes, 1981). This encouraged us to examine soil for other phages, and in this paper we report the isolation of three new phages. Preliminary data on two of them have been published (Fernandes *et al.*, 1983). We compared the new viruses with the following temperate *B. subtilis* phages: ϕ 3T (Tucker, 1969), ϕ 105 (Birdsell *et al.*, 1969), SPO2 (Boice, 1969), SP16 (Thorne & Mele, 1974), SP β (Warner *et al.*, 1977), ρ 11 (Dean *et al.*, 1976), ρ 6, ρ 10, ρ 14 (Dean *et al.*, 1978*a*), Zeta (Z) (Hemphill *et al.*, 1980) and SPR (Trautner *et al.*, 1980; Noyer-Weidner *et al.*, 1983).

We characterized the new phages with respect to host range, serology, DNA restriction pattern, potential for methylation of specific DNA sequences, immunity of lysogens to super-infection, ability to complement thymine auxotrophy of the host, and production of and resistance to betacin. The newly isolated phages behaved differently from the other known *B. subtilis* temperate bacteriophages.

METHODS

Bacteria and phages. The bacterial strains used are shown in Table 1. Strains TB106, 1L4, 1L6, 1L8, 1L11, RUB830 (ϕ 3T), CU1065 (Z) and CU1547 were used as sources of phages SPR, SP β , SPO2, SP16, ϕ 105, ϕ 3T, Z and ρ 11, respectively. The lysogens were obtained from the area of lysis produced on lawns of indicator strains, using a purified lysate. Bacteria were maintained on plates containing M medium (Yehle & Doi, 1967).

Isolation of new bacteriophages. Soil samples from Parede and Rio Maior (Portugal) were treated by the method described by Dean *et al.* (1978*a*). IGCg100 (SP β sensitive) was used as host strain. Three of the newly isolated phages, called IG1, IG3 and IG4, were studied further.

Media. Modified M medium (Yehle & Doi, 1967) was used for growth of bacteria, for preparation of phage lysates and in plating. GM1, GM2 and selective minimal medium used in the transformation experiments were as described by Yasbin *et al.* (1975).

Production of IG1, IG3 and IG4 lysates. M broth (25 ml) was inoculated with a single colony from the host strain IGCg100. The liquid culture was incubated overnight at 30 °C and used to inoculate 1 litre of M broth to OD₆₀₀ 0.05. The culture was incubated at 37 °C with shaking until OD₆₀₀ 0.3. At this point, the phages were added at an m.o.i. of 3. When the culture density started to decline, lysozyme was added to a final concentration of 30 μ g ml⁻¹. The culture was shaken until lysis was complete, centrifuged for 10 min at 7000 *g* and filtered. At this point the titre was of the order of 10⁸ p.f.u. ml⁻¹.

Table 1. *Bacterial strains*

Strains	Relevant properties	Origin/reference*
<i>B. amyloliquefaciens</i> RUB501	Ery ^R Str ^R Rfm ^R	Wilson & Young (1972)
<i>B. amyloliquefaciens</i> 10A1	Prototroph	BGSC
<i>B. globigii</i> 11A1	Prototroph	BGSC
<i>B. licheniformis</i> RUB503	Ery ^R Str ^R Rfm ^R Lys ⁻	Wilson & Young (1972)
<i>B. licheniformis</i> 5A2	Prototroph	BGSC
<i>B. niger</i> 12A1	RplV ⁻ Str ⁻	BGSC
<i>B. pumilus</i> 8A1	PBS1 ^S	BGSC
<i>B. pumilus</i> RUB502	Ery ^R Str ^R Rfm ^R Bio ⁻	Wilson & Young (1972)
<i>B. subtilis</i> 168T ⁺	Wild-type	F. E. Young
<i>B. subtilis</i> 12	Trp ⁻ Str ^R	O. E. Landman
<i>B. subtilis</i> W23	Wild-type	F. E. Young
<i>B. subtilis</i> SPβ ^S	<i>thr-5 met-5 leu-8 attSPβ</i>	C. Anagnostopoulos
IGCg100	<i>thr-5 met-5 leu-8 attSPβ</i> Str ^R	Transformant of <i>B. subtilis</i> SPβ ^S with <i>B. subtilis</i> 12 DNA obtained in this laboratory
IGCg115	<i>thr-5 met-5 leu-8 pha-3 attSPβ</i> Str ^R	This work
CU1444	<i>ilvA2 pyrD1 thyA1 thyB1 attSPβ</i>	Hemphill <i>et al.</i> (1980)
QB943	<i>pyrD1 ilvA2 thyA1 thyB1 trpC2</i>	Dedonder <i>et al.</i> (1977)
CU1064	<i>metB5 attSPβ</i>	Zahler <i>et al.</i> (1977)
CU1065	<i>trpC2 attSPβ</i>	Zahler <i>et al.</i> (1977)
IGCg218	<i>metB5</i> (IG1)	This work
IGCg220	<i>metB5</i> (IG3)	This work
IGCg222	<i>metB5</i> (IG4)	This work
IGCg203	<i>thr-5 met-5 leu-8</i> (φ3T)	This work
IGCg251	<i>thr-5 met-5 leu-8</i> (ρ11)	This work
CU1547	<i>trpC2</i> (ρ11)	S. A. Zahler
IGCg204	<i>thr-5 met-5 leu-8</i> (SPβ)	This work
IGCg224	<i>metB5</i> (SPβ)	This work
IGCg249	<i>thr-5 met-5 leu-8</i> (SPR)	This work
CU1065 (Z)	<i>trpC2</i> (Z)	Noyer-Weidner <i>et al.</i> (1983)
IGCg205	<i>thr-5 met-5 leu-8</i> (φ105)	This work
IGCg206	<i>thr-5 met-5 leu-8</i> (SPO2)	This work
TB106	<i>sup-3 Leu⁻ metB5 Thr⁻ Res⁻ Mod⁻</i> (SPR)	Noyer-Weidner <i>et al.</i> (1983)
1L4	<i>leuA8 metB5 thr-5</i> (SPβ)	BGSC
1L6	<i>trpC2</i> (SPO2)	BGSC
1L8	<i>trpC2</i> (SP16)	BGSC
1L11	<i>trpC2</i> (φ105)	BGSC
RUB830 (φ3T)	<i>pheA2 trpC2 ThyA⁻ ThyB⁻</i> (φ3T)	C. Anagnostopoulos

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Concentration and purification of phage lysates. Lysates were concentrated by ultracentrifugation (90 min at 60000 *g*) and then purified by centrifugation in a discontinuous CsCl gradient (1.3, 1.5, and 1.7 *g cm*⁻³) for 4 h at 40000 *g*. The other phages used in this study were obtained from their lysogens by induction with mitomycin C as described by Warner *et al.* (1977), but adding mitomycin C to a final concentration of 1 µg ml⁻¹. The lysates were concentrated and purified by the procedure described above.

Phage assay. The two-layer-plating procedure (Adams, 1959) with M agar was used to assay all the phages.

Preparation and use of antiphage sera. The sera were produced in rabbits. Phages with Freund's incomplete adjuvant were injected into the interscapular space, with one weekly injection for 3 weeks and a booster injection 2 weeks later. The *K* value of the antisera was determined according to Hayes & Clowes (1968). The cross reaction between phage and serum was established following the procedure used to determine the *K* value.

Production and tolerance to betacin. Strain IGCg115 is resistant to infection by IG1, IG3, IG4, SPβ, Z, φ3T and ρ11 and contains the mutation *pha-3* (Estrela *et al.*, 1986). To verify the production of betacin, lysogens of these phages were used. IGCg115 was grown in M broth at 37 °C with shaking to OD₆₀₀ 0.45 and then 0.1 ml was mixed with 3 ml 0.5% (w/v) M agar and poured over the surface of a 1.7% (w/v) M agar plate. After 10 min, spots were made with 10 µl of each lysogen, grown to OD₆₀₀ 0.45. The plates were incubated for 16–18 h at 30 °C.

To study tolerance to betacin, the lysogens were grown in the same conditions and M agar 0.5%/1.7% was used. But, in this case each lysogen was overlaid and 10 µl of 168T⁺ (lysogenic for SPβ) grown to OD₆₀₀ 0.45 was spotted on the plate. Incubation was as described above.

Transformation. Transformation experiments were done as described by Yasbin *et al.* (1975).

Preparation of DNA. Phage DNAs were extracted according to Maniatis *et al.* (1982).

Endonuclease digestion and agarose gel electrophoresis. Phage DNA (1 µg) was mixed with a site-specific endonuclease in digestion buffer, and incubated at 37 °C for 3 h. Samples were applied to 0.8% agarose gels and electrophoresed in Tris/borate buffer (0.089 M-Tris, 0.089 M-boric acid and 0.002 M-EDTA). Electrophoresis was usually done at 1 V cm⁻¹ for 16 to 18 h. To visualize the DNA, the gels were stained with ethidium bromide (1 µg ml⁻¹) and illuminated with a UV light transilluminator. Photographs were taken with Polaroid film 665.

Recovery of fragments from agarose gels. DNA fragments were recovered from gels by electroelution into dialysis bags as described by Maniatis *et al.* (1982).

RESULTS AND DISCUSSION

Phages IG1, IG3 and IG4 are temperate

The bacteriophages IG1, IG3 and IG4 were isolated from soil samples by the method described. On lawns of *B. subtilis* IGCg100 they produce the turbid plaques typical of temperate phages. In addition, stable cell lines of lysogens for each of the three phages showed both immunity to superinfection and inducibility with mitomycin C. These observations showed that the three viruses were of the temperate type. Consequently, they were further analysed in comparison with the other temperate phages of *B. subtilis*. Most of these phages were catalogued into four groups by Dean *et al.* (1978a): group I constituted by ϕ105, ρ10, ρ6 and ρ14; group II represented by SPO2; group III including ϕ3T and ρ11; and group IV comprising SP16 alone. Later it was shown that phages SPβ, SPR and Z also belonged to group III (Noyer-Weidner *et al.*, 1983).

Host range

We compared IG1, IG3 and IG4 with one phage from each group of temperate phages. The results presented in Table 2, show that IG1, IG3 and IG4 did not infect *B. subtilis* 168, but they did grow on a derivative strain cured of SPβ (for this reason we have included SPβ in these tests). IG1, IG3 and IG4 failed to infect either strain W23 or the other bacilli tested, with the sole exception of IG4, which infected *B. pumilus* with a very low plating efficiency. Taken together, the results on host range show that the new phages are different from ϕ3T, ϕ105, SPO2 and SP16, but are not distinguishable from SPβ.

Immunity properties

Phages IG1, IG3 and IG4 were studied in parallel with one representative of group I, another of group II, and in view of the results on host range, with all the phages belonging to group III. The last group was recently subdivided by Noyer-Weidner *et al.* (1983) into two immunity sub-groups, sub-group I comprising SPβ, SPR and Z, and sub-group II including ϕ3T and ρ11.

All ten phages were plated on each of the corresponding lysogens. The results (Table 3) indicated that IG1, IG3 and IG4 were homoimmune to each other and to SPβ, SPR and Z, while heteroimmune to ϕ3T, ρ11, ϕ105 and SPO2. Lysogens of SPβ supported good growth of ϕ3T and ρ11, showing heteroimmunity, but the reciprocal relationship was not expressed, indicating that the lysogens of ϕ3T and ρ11 were poor indicators. A similar result was obtained by Dean *et al.* (1978a).

Phages IG1, IG3 and IG4 can, therefore, be included in the sub-group I of group III.

Serological relatedness

This was tested with antisera prepared against SPβ and ϕ3T (as representatives of the sub-groups I and II respectively), SPR (which is serologically distinct from the other phages of group III; Noyer-Weidner *et al.*, 1983), ϕ105, SPO2, IG1, IG3 and IG4. The results (Table 4) indicate that IG1, IG3 and IG4 are serologically different from ϕ105, SPO2 and SPR, and are related to ϕ3T and SPβ as well as to each other.

Table 2. *Host range of temperate B. subtilis phages*

The host strains were overlaid onto agar plates; dilutions of a phage lysate (10^6 , 10^4 , 10^2 p.f.u. ml⁻¹) were spotted on the plate. +, Turbid plaques; —, no infection. All the results were confirmed by the two layer plating procedure (Adams, 1959).

Hosts	Formation of plaques by phage:							
	IG1	IG3	IG4	ϕ 3T	SP β	ϕ 105	SPO2	SP16
<i>B. subtilis</i> 168T ⁺	—	—	—	+	—	+	+	+
<i>B. subtilis</i> IGCg100	+	+	+	+	+	+	+	+
<i>B. subtilis</i> W23	—	—	—	—	—	—	—	+
<i>B. amyloliquefaciens</i> RUB501	—	—	—	—	—	—	—	—*
<i>B. amyloliquefaciens</i> 101A1	—	—	—	—	—	—	—	—
<i>B. licheniformis</i> RUB503	—	—	—	—	—	—	—	+
<i>B. licheniformis</i> 5A2	—	—	—	—	—	—	—	—
<i>B. globigii</i> 11A1	—	—	—	—	—	—	—	—
<i>B. niger</i> 12A1	—	—	—	—	—	—	—	—
<i>B. pumilus</i> 8A1	—	—	+	—	—	—	—	—
<i>B. pumilus</i> RUB502	—	—	—	—	—	—	—	+

* Dean *et al.* (1978a) reported the formation of individual plaques of SP16 on *B. amyloliquefaciens* H, and of ϕ 3T, ϕ 105, SPO2 and SP β on *B. globigii*.

Table 3. *Immunity properties of temperate B. subtilis phages on lysogenic hosts*

The phages were plated on lysogenic strains used as indicators. The values are means of 4 determinations.

Lysogens for:	Strain	Relative e.o.p. of phage:*									
		IG1	IG3	IG4	ϕ 3T	ρ 11	SP β	SPR	Z	ϕ 105	SPO2
IG1	IGCg218	0	0	0	1.05	0.83	0	0	0	1.03	0.49
IG3	IGCg220	0	0	0	1.16	0.21	0	0	0	0.92	0.51
IG4	IGCg222	0	0	0	0.98	0.88	0	0	0	0.67	0.53
ϕ 3T	IGCg203	0.92	0.74	0.59	0	0	0	—	—	0.50	—
ρ 11	IGCg251	0.98	0.21	0.34	0	0	0	—	—	—	—
	CU1547	0.09	0.04	0.19	0	0	0	—	—	—	—
SP β	IGCg204	0	0	0	0.43	0.99	0	0	0	0.04	0.66
	IGCg224	0	0	0	0.56	0.81	0	—	—	—	—
SPR	IGCg249	0	0	0	—	—	0	0	0	—	—
Z	CU1065 (Z)	0	0	0	—	—	0	0	0	—	—
ϕ 105	IGCg205	1.41	0.76	0.97	0.98	—	0.80	—	—	0	0.34
SPO2	IGCg206	1.10	0.38	0.35	0.74	—	1.53	—	—	0.56	0

—, Not done.

* Relative to the e.o.p. on the non-lysogenic strain IGCg100.

Table 4. *Serological relations of temperate B. subtilis phages*

The values are means of 3 determinations.

Serum	Percentage survival after treatment of phage with antiserum							
	IG1	IG3	IG4	ρ 3T	SP β	SPR	ϕ 105	SPO2
Anti-IG1	6	51	6	27	28	>99	>99	>99
Anti-IG3	12	21	23	33	14	>99	>99	>99
Anti-IG4	6	38	25	36	25	>99	>99	>99
Anti- ϕ 3T	21	8	7	4	5	>99	>99	>99
Anti-SP β	7	5	14	31	4	>99	>99	>99
Anti-SPR	>99	>99	>99	—	>99	1	—	—
Anti- ϕ 105	>99	>99	>99	>99	>99	—	1	13
Anti-SPO2	>99	>99	>99	>99	>99	—	21	5

—, Not done.

Conversion to thymine prototrophy

A Thy⁻ strain derived from *B. subtilis* 168 (CU1444) was able to grow in the absence of exogenous thymine, upon lysogenization with IG1, IG3 or IG4. The conversion to thymine prototrophy was stable. In this respect, the new phages are similar to ϕ 3T (Tucker, 1969), ρ 11 (Dean *et al.*, 1976), Z (Hemphill *et al.*, 1980) and SPR (Noyer-Weidner *et al.*, 1983), but not to SP β or the other phages.

DNA isolated from IG1, IG3 or IG4 and digested with *Bam*HI retains biological activity to transform strain QB943 to Thy⁺. Furthermore, when the *Bam*HI fragments from such DNAs were isolated by agarose gel electrophoresis and tested in transformation, only the largest of the four (IG1) or five (IG3 and IG4) fragments showed Thy⁺-transforming activity.

Production of and tolerance to betacin

Strains of *B. subtilis* 168 carrying SP β produce a bacteriocin-like substance which inhibits the growth of *B. subtilis* strains that are not lysogenic for this phage (Hemphill *et al.*, 1980). A similar effect was observed for strains of *B. subtilis* lysogenic for phages Z (Hemphill *et al.*, 1980) and SPR (Noyer-Weidner *et al.*, 1983). Production of betacin is associated with tolerance to it. We tested IG1, IG3 and IG4 for (i) production of and (ii) tolerance to a betacin-like substance in the following ways. (i) IGCg115 was overlaid onto agar plates. Lysogens for IG1, IG3, IG4, ϕ 3T, SP β and Z were spotted on plates. On incubation, only the last two lysogens inhibited growth of the bacterial lawn, showing that the new phages are non-producers of betacin-like material. (ii) Lysogens of each of these six phages were overlaid on agar plates and *B. subtilis* 168T⁺ spotted on the plates. On incubation, only the growth of the lysogens of IG1, IG3, IG4 and ϕ 3T was inhibited by the betacin-producing strain *B. subtilis* 168T⁺. This showed that the lysogens of IG1, IG3 and IG4 were not tolerant to betacin.

Restriction analysis

Our objective was to compare the DNAs from all the phages included in group III. This was done by restriction with the site-specific endonuclease *Eco*RI. The fragment patterns obtained for the nine DNA samples are shown in Fig. 1. The results show that the restriction patterns of each of the three new phages are markedly different from each other and from the other phages tested.

It was verified by Noyer-Weidner *et al.* (1983), that all phages of group III with the exception of Z were resistant to *Bsu*RI restriction. However, they can be distinguished on the basis of their potential to methylate target sequences in addition to GGCC. Thus SPR DNA is also resistant to *Msp*I and *Hpa*II degradation, whereas the DNAs of the other three phages (ϕ 3T, ρ 11 and SP β) carry modifications rendering them *Fnu*4HI-resistant (Noyer-Weidner *et al.*, 1983). We compared these phages with IG1, IG3 and IG4 for DNA methylation capacity by analysing the restriction patterns of the phage DNA with the enzymes *Hae*III, *Msp*I, *Hpa*II and *Fnu*4HI. The results obtained when digesting the DNAs with the enzymes *Msp*I and *Hpa*II (Fig. 2) showed that the DNAs of IG1, IG3 and IG4 were sensitive to these enzymes. Similar results were obtained using *Hae*III and *Fnu*4HI (unpublished observations). These phages are in this respect similar to Z (Noyer-Weidner *et al.*, 1983).

Assignment of phages IG1, IG3 and IG4 to group III

According to our results, the newly isolated viruses should be included in the third group of the temperate phages of *B. subtilis*, and within that class they belong to the immunity sub-group I, together with SPR, SP β and Z. The attachment sites (*att*) of IG1, IG3 and IG4 were mapped between *citK* and *metB* (Santos *et al.*, 1985; I. Santos, unpublished observations). This area covers the chromosome segment which includes the *att* sites of SP β and ϕ 3T (Zahler *et al.*, 1977; Zahler, 1982). None of these phages, however, are identical to any of the newly isolated phages. Phages IG1, IG3 and IG4 markedly differ from SPR, SP β or Z in the restriction pattern of their DNA and in their lack of resistance to and production of betacin. In addition, they differ from SPR and SP β by their capacity to convert their host to thymine prototrophy and in their inability

Fig. 1. *Eco*RI restriction patterns of the DNAs of phages IG4, IG3, IG1, SPR, SP β , Z, ρ 11, ϕ 3T, SPP1. *Eco*RI-restricted DNA of phage SPP1 was used as standard.

Fig. 2. Degradation patterns of the DNAs of phages IG4, IG3, IG1, Z, SPR, SPβ, ρ11, ϕ3T after restriction with *MspI* and *HpaII*.

to methylate the target sequences of *Hae*III, *Hpa*II/*Msp*I and *Fnu*4HI. Finally, they are also serologically unrelated to SPR. From these results we conclude that IG1, IG3 and IG4 are new temperate phages belonging to immunity sub-group I of group III.

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