Biological characterization of induced phages from *Saccharopolyspora hirsuta* 367 and comparison with phage JHJ-1

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Phages JHJ-2 and JHJ-3 were isolated from *Saccharopolyspora hirsuta* 367 UC 8106 following induction with mitomycin C and amplified on *S. hirsuta* NRRL B-5792. Their properties were compared with those of phage JHJ-1, isolated previously from *S. hirsuta* 367 NRRL 12045. The DNA restriction patterns appeared to be identical. One-step growth experiments showed no differences between the replication cycles. Burst sizes ranged from 100 to 110 p.f.u. per cell. However, the three phages showed some differences in their behaviour in different hosts. The host range of phage JHJ-1, on non-lysogenic strains, was emended to include all of the *Saccharopolyspora* strains tested; the host range of phage JHJ-2 was shown to be identical to JHJ-1. Phage JHJ-3 did not form detectable plaques on strains of *S. rectivirgula* or *S. erythraea* except *S. erythraea* NRRL 2359. Neither phage JHJ-2 nor JHJ-3 formed plaques on any lysogenic strains, while JHJ-1 formed plaques on all such strains except *S. hirsuta* 367 UC 8106. Phage JHJ-3 was characterized as a temperate bacteriophage because it formed turbid, self-limiting plaques and lysogenized *S. hirsuta* NRRL B-5792. It was spontaneously released from UC 8106. Both JHJ-1 and JHJ-2 formed clear and invasive (Inv⁺ phenotype: the property to grow on old mycelium) plaques on some *Saccharopolyspora* strains but clear and self-limiting plaques on others. Thus, the expression of the Inv⁺ phenotype encoded by JHJ-1 and JHJ-2 appears to be modulated by the host cell.

Introduction

The genus *Saccharopolyspora* includes species such as *S. erythraea*, the producer of the clinically important antibiotic erythromycin (Labeda, 1987) and *S. rectivirgula* (formerly *Faenia rectivirgula*; Korn-Wendisch *et al.*, 1989), a causative agent of farmer's lung disease (Pepys *et al.*, 1963; Kurup, 1984). Phages infecting these species have been isolated from lysogenic strains, soils or fermenter cultures and characterized for their potential use as cloning vectors and for studying phage evolution (Brzezinski *et al.*, 1986; Donadio *et al.*, 1986; Grund & Hutchinson, 1987; Schneider *et al.*, 1987; Katz *et al.*, 1988; Smorawinska *et al.*, 1988; Schneider & Kutzner, 1989a, b).

Phage JHJ-1 is a lambdoid phage which has double-stranded DNA with cohesive ends. It was spontaneously released by old mycelial cultures of *S. hirsuta* 367 NRRL 12045 (Haket *et al.*, 1990). This phage forms invasive plaques (the ability to grow on old mycelium), a property not previously described in the literature. Host range studies showed that JHJ-1 forms plaques on only two strains, *S. hirsuta* NRRL B-5792 and *S. hirsuta* 367 IC-

11. The latter strain is a derivative of *S. hirsuta* 367 NRRL 12045 (Haket *et al.*, 1990). The spontaneous release of phages resembling phage JHJ-1 was shown to occur infrequently from *S. hirsuta* 367 IC-11 but did not occur from *S. hirsuta* 367 UC 8106, which is another representative of *S. hirsuta* 367 from a different culture collection.

Both *S. hirsuta* 367 UC 8106 and NRRL 12045 (and IC-11) harbour intracellular DNA homologous to phage JHJ-1 DNA and thus appear to carry a prophage (Haket *et al.*, 1990). However, phage JHJ-1 cannot form plaques on *S. hirsuta* 367 UC 8106 from which *S. hirsuta* 367 NRRL 12045 (and IC-11) was derived earlier (Whaley *et al.*, 1980). One explanation for these data was that JHJ-1 is a virulent mutant of a temperate phage that could infect one lysogen but not the other (Haket *et al.*, 1990). In this paper we describe properties of phages JHJ-2 and JHJ-3 which are released from *S. hirsuta* 367 UC 8106 after induction with mitomycin C. These are compared to phage JHJ-1 at the level of their host range, plaque morphology, burst size and DNA restriction patterns.

Table 1. *Actinomycete strains used in this study*

Species	Strain*	Source/reference
<i>Saccharopolyspora</i>		
<i>S. hirsuta</i> 367	UC 8106 (JHJ-3)	The Upjohn Company, Kalamazoo, MI, USA
<i>S. hirsuta</i> 367	IC-11 (JHJ-3)	Haket <i>et al.</i> (1990)
<i>S. hirsuta</i>	NRRL B-5792†	A. J. Lyons, NRRL, Peoria, IL, USA
<i>S. hirsuta</i>	A1143† (=NRRL B-5792†)	J. Lacey, AFRC Institute of Arable Crops Research, Herts, UK
<i>S. hirsuta</i>	B5792LA (JHJ-3)	This study
<i>S. hirsuta</i>	B5792LB (JHJ-3)	This study
<i>S. erythraea</i>	ATCC 11635†	
<i>S. erythraea</i>	NRRL 2338† (=ATCC 11635†)	
<i>S. erythraea</i>	ISP 5517† (=ATCC 11635†)	R. Brzezinski, University of Sherbrooke, Canada
<i>S. erythraea</i>	NRRL 2359	
<i>S. erythraea</i>	NRRL 2360	
<i>S. erythraea</i>	IA 3038	R. Brzezinski
<i>S. rectivirgula</i>	DSM 43787†	J. Schneider, Institut f��r Mikrobiologie, Darmstadt, Germany
<i>S. taberi</i>	NRRL B-16173†	D. P. Labeda, NRRL, Peoria, IL, USA
<i>Streptomyces</i>		
<i>Str. lividans</i>	TK 24	D. Hopwood, John Innes Institute, Norwich, UK
<i>Str. albus</i>	J1074	D. Hopwood
<i>Str. coelicolor</i>	A3(2)	D. Hopwood
<i>Str. labedae</i>	NRRL B-5616	
<i>Str. hygroscopicus</i>	NRRL 2387	
<i>Pseudonocardia thermophila</i>	ATCC 19285	
<i>Amycolatopsis orientalis</i>	NRRL B-3892	

* ATCC, American Type Culture Collection, Rockville, MD 20852, USA; NRRL, Northern Regional Research Laboratory, US Department of Agriculture, Peoria, IL, USA.

† Type culture.

Methods

Bacterial strains and phages. The *Saccharopolyspora* strains used in this work are listed in Table 1. *S. hirsuta* 367 UC 8106 (Whaley *et al.*, 1980) was provided by the Upjohn Company (Kalamazoo, MI, USA). This strain was sent to NRRL, where it was given the designation NRRL 12045. Since the stock of *S. hirsuta* 367 NRRL 12045 spontaneously released phages at a rather high frequency, a subclone of this strain, named *S. hirsuta* 367 IC-11, was isolated for the propagation and plating studies of phage JHJ-1 (Haket *et al.*, 1990). *S. hirsuta* 367 IC-11 was shown to spontaneously release phage at a lower frequency but otherwise behaved as the parent strain *S. hirsuta* 367 NRRL 12045. Although *S. hirsuta* 367 UC 8106 and IC-11 are representatives of the same strain, they behave differently in regard to JHJ-1 phage infection (Haket *et al.*, 1990). *S. hirsuta* NRRL B-5792, the type strain, was used for the propagation of phage JHJ-1 and other phages isolated during this study, since it does not contain DNA homologous to phage JHJ-1.

Since the two strains of *S. hirsuta* 367 (UC 8106 and IC-11) showed differences in sensitivity to JHJ-1 infection (Haket *et al.*, 1990), replicates of the same strains from different culture collections (*S. hirsuta* NRRL B-5792 and A1143 as well as *S. erythraea* ATCC 11635, NRRL 2338 and ISP 5517) were also investigated for their response to phage infections. Phenotypic differences have been observed between *S. erythraea* ATCC 11635 and NRRL 2338 (Paulus *et al.*, 1990).

Media, buffers and chemicals. Medium W and the phage buffer, which were used for phage titration, amplification, host range and one-step growth experiments, were prepared as described previously

(Haket *et al.*, 1990). Medium WM comprised medium W supplemented with 10 mM-MgCl₂. The restriction endonucleases used in this work, *Bam*HI, *Bcl*I, *Bgl*II, *Bst*BI, *Nar*I, *Pst*I, *Pvu*I, *Pvu*II, *Sma*I and *Sph*I, were used according to the manufacturer's recommendations. Mitomycin C was obtained from Boehringer Mannheim.

Induction of the prophages with mitomycin C. Actinophage induction from *S. hirsuta* 367 UC 8106 was performed as described by Ogata *et al.* (1985) with minor modifications. A mycelial culture of *S. hirsuta* 367 UC 8106 was washed in fresh medium W, resuspended in preheated medium (30 °C) and was incubated in the absence or presence of mitomycin C for 1 h. Concentrations up to 50 µg ml⁻¹ were used. The cultures were washed, resuspended in fresh medium W and incubated overnight at 30 °C. The cultures were then centrifuged and the supernatants stored at 4 °C. An aliquot (1 ml) of the supernatants was used to assay the induced phages on plates inoculated with heat-shocked (55 °C, 10 min) spores of *S. hirsuta* NRRL B-5792 and mycelial cultures of *S. hirsuta* 367 IC-11. After 2 d incubation, several turbid plaques were picked from B-5792 infected plates, eluted in phage buffer and then amplified on *S. hirsuta* NRRL B-5792. Two induced phages, named JHJ-2 and JHJ-3, were shown to be phenotypically different after amplification on B-5792 and were therefore characterized further.

Detection of the spontaneous release of phage by *S. hirsuta* 367 UC8106 and IC-11. A double-overlay assay was developed in order to increase the sensitivity of detection of spontaneously released phage. Mycelial cultures were heavily inoculated into 4 ml soft agar in medium WM and incubated for 2 d at 30 °C. A second overlay containing *S. hirsuta* NRRL B-5792, the amplification strain, was then deposited and the

plates were incubated at 30 °C. The phenotype of the plaques was not easy to determine, particularly on the plates inoculated with UC 8106 in the first overlay. Plaques were therefore picked as soon as they appeared, eluted in phage buffer and plated onto strains lysogenized by phage JHJ-3, i.e. *S. hirsuta* 367 IC-11, UC 8106 and B5792LB (a lysogen of B-5792 isolated in this study), and a non-lysogenic strain, *S. hirsuta* NRRL B-5792, to determine both their sensitivity to phage infection and the plaque morphology.

Phage amplification, purification and host range determination. Phage JHJ-1 and one of the mitomycin C induced phages, JHJ-2 were amplified on plates and purified as described by Haket *et al.* (1990) except that the phages were amplified on mycelial cultures of *S. hirsuta* NRRL B-5792 rather than *S. hirsuta* 367 IC-11.

For the propagation of the other mitomycin C induced phage, JHJ-3, particles of one turbid plaque were eluted for 8–16 h at 4 °C in 100 µl of phage buffer and plated on heat-shocked (55 °C, 10 min) spores of *S. hirsuta* NRRL B-5792. After 2 d incubation, ten turbid plaques were picked and eluted in phage buffer (total volume 1 ml). Heat-shocked spores (10^7) of *S. hirsuta* NRRL B-5792 were inoculated into 100 ml of liquid medium W and incubated for 5–6 h to allow germination. The culture was then centrifuged (1000 g), the pellet (germinated spores) resuspended in 10 ml fresh medium WM and the plaque-purified particles of phage JHJ-3 added. After 30 min incubation to allow phage adsorption, 40 ml medium W was added and the culture was incubated for 16 h. After centrifugation (1000 g) the phages were purified from the supernatant by polyethylene glycol 8000 precipitation and CsCl gradient centrifugation (Haket *et al.*, 1990). These conditions preserved the temperate phenotype of phage JHJ-3 and avoided the selective amplification of JHJ-2-type phages.

For a rapid determination of the host range, 1–2 µl phage stock was spotted onto a lawn of either heat-shocked spores (Grund & Hutchinson, 1987; Haket *et al.*, 1990) or with *S. hirsuta* 367 strains UC 8106 and IC-11 on a lawn of mycelial culture. The strains that showed evidence of lysis on the spot tests were assayed with phage dilutions to allow visualization of isolated plaques. Unless otherwise stated, all the incubations were carried out at 30 °C (43 °C for *S. rectivirgula*; Smorawska *et al.*, 1988) and the liquid cultures were agitated at 250 r.p.m. in a gyratory shaker.

Isolation and characterization of strains lysogenized by phage JHJ-3. *S. hirsuta* NRRL B-5792 was infected with JHJ-3 to obtain a confluent infection; two resistant colonies were purified by two cycles of amplification in liquid medium W and streaked onto medium W plates in order to isolate single colonies. The phenotypes of these strains, named B5792LA and B5792LB, were confirmed by plating them with diluted suspensions of the three phages in order to visualize individual plaques. The plates were incubated and examined daily for 5 d.

One-step growth experiment of phages JHJ-1, JHJ-2 and JHJ-3. A one-step growth experiment was performed by a modification of the method described by Dowding (1973). Briefly, 6×10^7 heat-shocked spores of *S. hirsuta* NRRL B-5792 were incubated for 6 h in medium W to allow germination. The culture was centrifuged and the pellets containing the germinated spores were resuspended in 1 ml medium WM to which 6×10^6 p.f.u. of JHJ-1, JHJ-2 or JHJ-3 were added. After 30 min of adsorption, the bacterial cells were washed twice in medium W and resuspended in 6 ml of prewarmed (30 °C) medium W (first growth flask). A 50-fold dilution of the first growth flask was made (second growth flask) and the two flasks were then incubated at 30 °C. Then, 100 µl samples were taken from the two flasks every 30 min for up to 8 h and centrifuged. The supernatants were immediately plated onto heat-shocked spores of *S. hirsuta* NRRL B-5792.

DNA purification and analysis. Phage and bacterial DNA purifications were carried out as previously described (Haket *et al.*, 1990). After

digestion with restriction enzymes, the DNAs were heat treated (90 °C, 10 min) in order to denature the cohesive ends, electrophoresed on agarose gel and blotted onto nitrocellulose membranes, as described previously (Haket *et al.*, 1990). The blots were then hybridized with labelled JHJ-1 DNA (Haket *et al.*, 1990).

Results and Discussion

The prophage of S. hirsuta 367 UC 8106 is inducible with mitomycin C

Saccharopolyspora hirsuta 367 UC 8106 (and IC-11) has previously been shown to contain possible prophage DNA sequences homologous to phage JHJ-1 DNA (Haket *et al.*, 1990). As mitomycin C induces phages from actinomycetes (Ogata *et al.*, 1985), *S. hirsuta* 367 UC 8106 was exposed to various concentrations of mitomycin C and the culture supernatants were plated on the two indicator strains. At mitomycin C concentrations of 25 µg ml⁻¹ or greater, turbid plaques were detected on *S. hirsuta* NRRL B-5792 and clear plaques on IC-11. The ratio of turbid over clear plaques was in the range of $10^5:1$ after correction for the 10^4 -fold restriction on *S. hirsuta* NRRL B-5792 of phage grown on *S. hirsuta* 367 IC-11 (Haket *et al.*, 1990). Phages from two turbid plaques were amplified on *S. hirsuta* NRRL B-5792: the first, named JHJ-2, formed clear and invasive plaques similar to phage JHJ-1 when replated on B-5792 and the second, JHJ-3, retained the turbid plaque phenotype. Thus, the induction experiment proved that strain UC 8106 does contain a prophage.

It was previously reported that strain UC 8106 does not spontaneously release phage (Haket *et al.*, 1990). However, by using a more sensitive assay, it was shown that old mycelial cultures of strain UC 8106 spontaneously released low numbers of phages. The phages produced turbid self-limiting plaques on *S. hirsuta* NRRL B-5792 and thus resembled phage JHJ-3. In the same assay, strain IC-11 released phage resembling JHJ-1.

In order to determine that the induced phages, JHJ-2 and JHJ-3, were related to JHJ-1, DNA from all three phages was digested with restriction endonucleases. No differences between JHJ-1, JHJ-2 and JHJ-3 were detected in any of the restriction patterns (data not shown). Thus, all three phages appear to have originated from the same parent phage. The differences in the phenotypes of the three phages therefore appear to be due to short deletions and/or point mutations and not to major deletions.

Phage JHJ-1 can infect phage JHJ-3 lysogens

To test whether phage JHJ-3 is a true temperate phage or not, two JHJ-3 lysogens of *S. hirsuta* NRRL B-5792,

named *S. hirsuta* B5792LA and B5792LB, were isolated. Digested DNA of these two strains was hybridized with labelled JHJ-1 DNA and was shown to harbour intracellular phage DNA with the same hybridization patterns (data not shown) as in *S. hirsuta* 367 IC-11 and UC 8106 DNA (Haket *et al.*, 1990). *S. hirsuta* NRRL B-5792 DNA did not hybridize with phage JHJ-1 DNA.

Strains B5792LA and B5792LB, as well as the natural JHJ-3 lysogens, *S. hirsuta* 367 UC 8106 and IC-11, were refractory to infection by phage JHJ-3, suggesting that phage JHJ-3 is a temperate phage and that these strains are true lysogens. Phage JHJ-1 produced clear and invasive plaques on both *S. hirsuta* B5792LA and B5792LB as well as in IC-11 but no plaques were detectable in UC 8106. Thus, phage JHJ-1 can infect lysogens, and by analogy to other phage-host systems (Lomovskaya *et al.*, 1980; Johnson *et al.*, 1981), phage JHJ-1 appears to be a virulent mutant insensitive to repression. Since phage JHJ-1 infected all the JHJ-3 lysogens except UC 8106, the lack of sensitivity of UC 8106 may be caused by a block at an early step of infection, possibly at the adsorption level. Attempts to test this hypothesis did not yield reproducible results. The adsorption experiments had to be carried out with mycelial cultures rather than germinated spores since *S. hirsuta* 367 strains did not differentiate sufficiently to yield spore stocks.

Although phage JHJ-2 made invasive plaques on *S. hirsuta* NRRL B-5792, it did not yield plaques on any lysogens and therefore appears to be phenotypically different from phage JHJ-1.

Host range and plaque morphology

The host range and the plaque morphology of all three phages are summarized in Table 2. The phages differed in their behaviour on the different hosts. Phage JHJ-1 made clear invasive plaques on the *S. hirsuta* strains, *S. taberi*, *S. erythraea* NRRL 2359 and, in contrast to some of our previous results (Haket *et al.*, 1990), clear self-limiting (approx. 1 mm) plaques on *S. rectivirgula* and the other strains of *S. erythraea*. The reason for such discrepancies is unknown since the same phage and spore stock were used for both investigations. Phage JHJ-2 did not lyse *S. hirsuta* 367 IC-11 but otherwise behaved like JHJ-1. The broad host range of phage JHJ-1 and JHJ-2 suggests that they could be useful tools in taxonomic investigations of the genus *Saccharopolyspora*. The host range of phage JHJ-3 was more restricted than phages JHJ-1 and JHJ-2 (Table 2). Turbid self-limiting plaques (approx. 3–4 mm 2 d post infection) were only observed on *S. hirsuta* NRRL B-5792, *S. taberi* and *S. erythraea* NRRL 2359. These three phages along with

Table 2. Comparative host range and plaque morphology of phages JHJ-1, JHJ-2 and JHJ-3 on different actinomycete strains

Strain	Phage:		
	JHJ-1	JHJ-2	JHJ-3
<i>S. hirsuta</i> NRRL B-5792	C, I	C, I	T, S
<i>S. hirsuta</i> A1143	C, I	C, I	ND
<i>S. hirsuta</i> 367 IC-11	C, I	NL	NL
<i>S. taberi</i> NRRL B-16173	C, I	C, I	T, S
<i>S. rectivirgula</i> DSM 43787	C, S	C, S	NL
<i>S. erythraea</i> ATCC 11635	C, S	C, S	NL
<i>S. erythraea</i> NRRL 2338	C, S	C, S	NL
<i>S. erythraea</i> ISP 5517	C, S	C, S	NL
<i>S. erythraea</i> NRRL 2359	C, I	C, I	T, S
<i>S. erythraea</i> NRRL 2360	C, S	C, S	NL
<i>S. erythraea</i> IA 3038	C, S	C, S	NL

Abbreviations: C, clear plaques; I, invasive plaques; T, turbid plaques; S, self-limiting size plaques; NL, no evidence of lysis; ND, not done.

phage SE-6 (Grund & Hutchinson, 1987) are the only phages that produce a lytic infection in *S. erythraea* NRRL 2359 (Donadio *et al.*, 1986; Grund & Hutchinson, 1987; Katz *et al.*, 1988; Smorawinska *et al.*, 1988).

The absence of visible plaques on *S. erythraea* strains (except NRRL 2359) and *S. rectivirgula* infected by JHJ-3 seems unlikely to be due to a lack of phage adsorption since both phages JHJ-1 and JHJ-2 formed plaques on these strains. It is possible that phage JHJ-3 lysogenized all the cells or alternatively that the plaques were not detectable. However, attempts to isolate lysogens of *S. erythraea* strains (except NRRL 2359) were unsuccessful owing to the lack of visible plaques and to the restriction of phages grown in *S. hirsuta* NRRL B-5792 (unpublished results).

The difference in plaque morphology suggests a genetic basis for the invasive properties of phages JHJ-1 and JHJ-2. We define the Inv⁺ (invasive) phenotype as the ability of phages to grow on old mycelium. Consequently, JHJ-3 is the Inv⁻ wild-type phage. The fact that phages JHJ-1 and JHJ-2 both have the Inv⁺ phenotype, but only phage JHJ-1 is insensitive to repression, suggests that these phages have different mutations. Moreover, the results in Table 2 suggest that the Inv⁺ phenotype is strain-dependent and may be modulated by host cellular function(s).

S. hirsuta NRRL B-5792 is a facultatively thermophilic actinomycete as it can grow at temperatures up to 50 °C (Lacey & Goodfellow, 1975). The plaques of phages JHJ-1 and JHJ-2 were more invasive at 43 and 50 °C, respectively, than those observed for incubations at 30 °C. Such behaviour may be due to the higher

Fig. 1. One-step growth curves of phages JHJ-1 (▲), JHJ-2 (●) and JHJ-3 (■) on *S. hirsuta* NRRL B-5792 at 30 °C. These are representative data from one of three experiments.

metabolic rates of cells grown at higher temperatures. At higher temperatures, phage JHJ-3 plaques were still turbid and self-limiting but the diameter of the plaques was larger (approx. 8–9 mm). The host range and plaque morphology on *S. erythraea* strains was the same at 43 °C as at 30 °C.

The reference strains (Table 1), representing genera other than *Saccharopolyspora*, were refractory to infection by the three phages.

The burst size of all three phages on S. hirsuta NRRL B-5792 is similar

Phages JHJ-1 and JHJ-2 were shown to produce Inv^+ and clear plaques on *S. hirsuta* NRRL B-5792 whereas JHJ-3 plaques were turbid and self-limiting. To determine whether the phenotypic properties of the plaques of the three phages were reflected by differences in their replication cycle, one-step growth experiments were performed on germinated spores of *S. hirsuta* NRRL B-5792. There was no significant difference in the respective life cycle of each phage (Fig. 1). The latency and rise periods lasted between 150 and 170 min, and 175 and 235 min, respectively, and the complete cycle lasted approximately 6 h. These periods are rather long compared to those of many actinophages (Lomovskaya *et al.*, 1980). The burst sizes of the three phages ranged from 100–110 p.f.u. per cell. The titre of the three phages decreased slightly after 7 h post infection. This may be

caused by secondary infection by the released phages. Since the invasive property is detectable only on (plated) old cultures, the lack of differences in the burst sizes could be due to the use of young cultures containing germinated spores in the one-step growth experiments.

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