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ISOLATION AND CHARACTERIZATION OF TWO TYPES OF ACTINOPHAGES INFECTING *STREPTOMYCES SCABIES* MR13

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Two types of actinophages, ϕS and ϕL, were isolated from soil samples by using *Streptomyces scabies* MR13, a potato scab pathogen, as an indicator strain. The phages were partially characterized according to their physicochemical properties, plaques and particles morphology and their host-range. The host-range of these phages was narrow for ϕS and wide for ϕL. The adsorption rate constants of the ϕS and ϕL were 3.44 x 10^{-9} and 3.18 x 10^{-9} ml/min, and their burst sizes were 1.61 and 3.75 virions, respectively. One-step growth indicated that ϕS and ϕL have a latent period of 30 min followed by a rise period of 30 min. The temperate character of these phages was tested in other isolates of *Streptomyces*. Four of the phages (ϕSS3, ϕSS12, ϕSS13 and ϕSS17) were identified as temperate phages, since they were able to lysogenize SS3, SS12, SS13 and SS17. ϕSS3, ϕSS12 and ϕSS13 were homoimmune, and they were heteroimmune with respect to ϕSS17. The restriction barriers of lysogenic isolates (SS12, SS13 and SS17) interfered with the blockage of plaques formation by phages (ϕSS12, ϕSS13 or ϕSS17) propagated on them, about 75% of lysogenic isolates had restriction systems. The exposure of the lysogenic isolates (SS12, SS13 and SS17) to UV-irradiation prevented the possible restriction barriers of these isolates, and these barriers could be overcome.

Keywords: actinophages, *Streptomyces* spp., plaques, lysogeny, restriction barriers

Introduction

The economic importance of streptomycetes have stimulated the construction of cloning systems using plasmids and some suitable phages as vectors [1–3]. Most phages have been isolated from soil in which they are widespread, but some isolates

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were obtained from lysogenic wild-type strains. Viruses which infect members of the actinomycete are called actinophages. Actinophages have been played an important role as taxonomic tools for phage typing of streptomycetes [4–6], and for the examination of host restriction barriers [7–9].

Many *Streptomyces* spp. produce type II restriction endonucleases. The expression of restriction endonucleases in streptomycetes can block infection by bacteriophages that contain the corresponding cleavage sites in their DNA [10–12]. Many streptomycete phages lack cleavage sites for subsets of restriction barriers produced by streptomycetes [13–16]. The strong correlation between the expression of restriction and the blockage of plaque formation for certain bacteriophage suggested that about 80% of *Streptomyces* species express significant restriction barriers [11].

The broad host-range of streptomycete phages means that they form plaques on about 50% of hosts tested [11, 17]. Generally, the actinophages with the wide host-range infected several genera or species of actinomycetes [6, 14, 18, 19]. On the other hand, Anne et al. [13], Diaz et al. [15], Nishiwaki et al. [20] reported that some phages have a relatively narrow host-range. Study of the lysogeny in actinomycetes encounters more difficulties than in other bacterial systems. The occurrence of true lysogeny in members of the genus *Streptomyces* by any phage isolated from natural sources was showed by Welsch [21, 22]. Occurring lysogenic cultures were found to exist when they released phages capable of forming plaques on sensitive indicator strains [23–26].

In this study we report the isolation from soil of two phages having the ability to form plaques on *S. scabies* MR13 and give their preliminary characterization, and we have analysed the interaction of these phages with other isolates of *Streptomyces*. In addition, examinations of cross-immunity of the temperate phages causing lysogenic isolates, as well as production of restriction barriers by these isolates are reported.

Materials and methods

Organisms and culture conditions

S. scabies MR13 was provided from Mircen Culture Collection, Faculty of Agriculture, Ain-Shams University, Egypt. The other pathogenic isolates (*S. scabies* S-3 and S-4) were isolated from potato tubers with scab symptoms and produced thaxtomin A [27]. Nonpathogenic isolates were isolated from an Egyptian soil according to the method of Davis and Williams [28]. The grouping and identification characteristics of the nonpathogenic isolates were done by using the articles of ISP [29] and the key of Bergey's Manual of Systematic Bacteriology [30]. These isolates were identified as *S. sioyaensis* SS1 and SS2, *S. pluricolorescens* SS3 and SS4, *S. plicatus*

SS5, SS6 and SS7, *S. coeruleorubidus* SS8, SS9 and SS10, *S. albus* SS11, *S. nigellus* SS12, *S. fulvoviridis* SS13, SS14, SS15 and SS16 and *S. spheroides* SS17.

Phage isolation

Phages infecting *S. scabies* MR13 were isolated from irrigated soil with sewage water following essentially the enrichment method of Dowding [31]. Samples of soil extract were prepared according to the method of Welsch et al. [32]. A 0.1 ml soil extract was added to YEME broth (10 ml) and spores (about 10^6) of *S. scabies* MR13 were added. After overnight incubation at 30 °C with shaking, samples were centrifuged at 3000 rev/min and supernatants were sterilized by passage through Millipore filters (0.45 μm). Phages were detected by plating in soft agar overlays [33], using *S. scabies* MR13 as an indicator strain. Phages are distinguished by differences in plaque morphology, size and turbidity, and were purified by serial replating from single plaques. Two actinophages isolates were studied.

Propagation of actinophages

To obtain phage suspensions of high titre, confluent plate lysates were prepared according to the method of Anne et al. [13]. On solid medium, lysates could be harvested after 20 hours of cultivation. Shaking flasks' lysates were prepared by addition of 10^5 pfu/ml to 200 ml cultures containing homogenized mycelia of the indicator strain (10^7 cfu/ml). Incubation was carried out at 30 °C and 120 rev/min for 40 h. Bacteria were removed by centrifugation at 5000 rev/min for 10 min, and the supernatants were sterilized by passage through 0.45 μm filters (Millipore).

Morphological studies

Phage particles from high-titre lysates were deposited on copper grids (200 mesh) with carbon coated collodion membrane. The grids were then negatively stained with 2% (w/v) aqueous uranyl acetate and observed under transmission electron microscopy.

Host-range determinations

Plaque formations by serial dilutions of each phage was used rather than qualitative spot tests to determine the host-range of the phages, using *S. scabies* MR13, S-3 and S-4 (scab pathogens), and 17 non-pathogenic *Streptomyces* isolates. Spores were collected from YEME plates (2–3 weeks old) and suspended in 0.5 ml sterile water. These spore suspensions were mixed with YEME soft agar (0.8% agar, 10 ml) and plated in Petri-dishes. Within three hours, suspension of actinophage isolates tested

(20 µl, 10^7 pfu/ml) were dropped on these plates. Infection could be scored by the presence of plaques in lawn of *Streptomyces* isolates after 3 days incubation at 30 °C.

Adsorption rate of phages

Adsorption experiments were carried out with the two isolated phage suspensions added to spores of their host indicator (*S. scabies* MR13). Suspensions of each phage were incubated at 30 °C with gentle shaking. Samples were withdrawn at regular intervals after contact. The mycelial fragments of indicator strain were removed by centrifugation (5 min, 5000 rev/min) and the number of phage remaining in the supernatant was counted as pfu/ml. The adsorption rates of the two phages were determined by measuring residual plaque-forming ability in membrane-filtered samples of an attachment mixture [31] and the adsorption rate constant *K* ml/min was calculated [33]. A one-step experiment was conducted as described by Dowding [31].

Determination of lysogeny

Lysogenization was demonstrated according to the method of Schneider *et al*. [8]. Broth cultures of different growth stages of each phage were filtered through a 0.45 µm pore size membrane filter and 50 µl volumes were spotted onto fresh lawn of possible various indicator strains. When lysed zones appeared after 36 hours of incubation at 30 °C, material of these spots was streaked over an agar plate (base-layer). In order to obtain single plaques, the plates were overlaid with a top layer containing spores of the same strain as was used to detect the phage from the culture supernatant. The phages thus detected usually give rise to turbid plaques with growth of lysogenized indicator strains. The plaques with the growth of indicator strains were streaked over agar plates, incubated for 36 hours at 30 °C and overlaid with spores of the indicator strain. Lysogenized colonies released phages were therefore, surrounded by lysis zones within the indicator lawn. These lysogenic bacteria were immune to superinfection by the same phage.

UV-treatment

The lysogenic isolates (*S. nigellus* SS12, *S. fulvoviridis* SS13 and *S. spheroides* SS17) exhibited considerable restriction-barriers for the temperate phages. In order to overcome these barriers, high phage titres were used. In some cases, however, this did not result in plaque formation, and the UV enrichment procedure had to be applied [8]. Irradiation by exposure to UV lamp with 240 nm and subsequent incubation were either carried out in the dark, or plates were irradiated and left on the bench, exposed to

laboratory illumination for 30 min prior to incubation in the dark at 28 °C. After 48 hours colonies were counted to obtain survival curves.

Results and discussion

Isolation of phages infecting S. scabies MR13

Phage particles were detected in two out of the nine different soil samples irrigated with sewage water, and the phage plaques were obtained on *S. scabies* MR13 as indicator strain. Using differences in plaque morphology, size and lysogeny criteria, two different phages, termed ϕS and ϕL, were identified. ϕS and ϕL produced clear plaques on lawns of *S. scabies* MR13 (Fig. 1). The uniqueness of each phage was confirmed by morphological studies of the phage particles and the host-range determination. Some *Streptomyces* phages were partially characterized through their physicochemical properties, plaque morphology, host-range and particles' morphology [15, 18, 19, 34, 35].

Fig. 1. Plaques of ϕS (left side) and ϕL (right side) on *S. scabies* MR13 after overnight

Morphology of actinophage virions

Electron microscopic observations (Fig. 2) showed that the two types of actinophage virions had hexagonal heads and apparently non-contractile tails. The phage ϕS had a long tail but the other type (ϕL) had short tail. Like nearly all previously characterized *Streptomyces* phages, the shape of the ϕS (type I) virion was

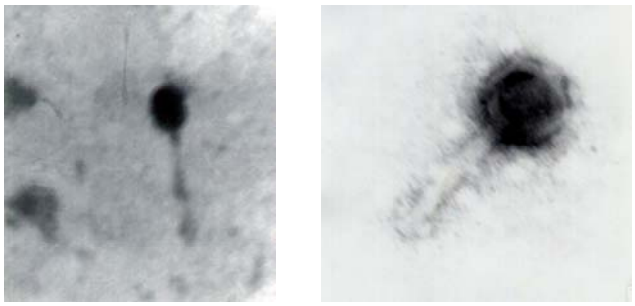

Fig. 2. Electron micrographs of actinophages negatively stained with uranyl acetate. Left side: ϕS, right side: ϕL. The bar represents 30 nm for ϕS and 37.5 nm for ϕL

similar to that of ϕA3 and ϕA8 [15]. The ϕL (type II) virion was similar to that of ϕSH-61 [20] and of ϕNC-4 [19], with a hexagonal head and a relatively short thick tail. The two types of phages had a base-plate at the end of the tail. The dimensions of the two phages are shown in Table I. The small phage (ϕS) had a head of 100 nm from apex to apex and a tail 250 nm long. The head of the largest one (ϕL) recorded 212 nm, but the length of the tail showed 187 nm.

Table I

Morphological characteristics of the isolated *Streptomyces* phages

Criteria			Phage	
			ϕS	ϕL
Plaque morphology		Size (mm)	2–2.5	1–1.5
		Transparency	Clear	Clear
		Edge	Sharp	Sharp
Phage dimension (nm)	Head*	Apex to apex	100±6	212±13
		Side to side	70±5	162±6
	Tail*	Length	250±11	187±5
		Width	8.0	18.0
Base-plate			+	+

* Values are given in nm, and are means ±SD of at least 5 independent measurements.

\+ presence of basal plate.

Host-range of actinophages

The host-range of the isolated phages was examined by plaque formation on 20 strains of pathogenic and nonpathogenic *Streptomyces* belonging to nine species. Each type of phage displayed a different host-range (Table II) which varied from relatively

narrow host-range, as in the case of ϕS (9 out of the 20 strains positive), to wide host as in the case of ϕL (14 hosts). The two actinophage isolates formed clear plaques against scab pathogens on *S. scabies* MR13, *S. scabies* S-3 and *S. scabies* S-4, except *S. scabies* S-4, which was not infected by ϕS. Among the nonpathogenic *Streptomyces* isolates, the two types of phages infected *S. pluricolorescens* SS3, *S. plicatus* SS5, *S. coeruleorubidus* SS10, *S. albus* SS11, *S. nigellus* SS12, *S. fulvoviridis* SS13 and *S. spheroides* SS17. On the other hand, none of the two phages infected *S. sioyaensis* SS1, *S. pluricolorescens* SS4, *S. plicatus* SS6 and SS7, *S. coeruleorubidus* SS9 and *S. fulvoviridis* SS16.

Table II

Actinophage plaque formation on pathogenic and nonpathogenic *Streptomyces* isolates

Streptomyces isolate	Pathogenicity[a] on PMT	Phage[b]	
		ϕS	ϕL
S. scabies MR13	+	+	+
S. scabies S-3	+	+	+
S. scabies S-4	+	–	+
S. sioyaensis SS1	–	–	–
S. sioyaensis SS2	–	–	+
S. pluricolorescens SS3	–	+	+
S. pluricolorescens SS4	–	–	–
S. plicatus SS5	–	+	+
S. plicatus SS6	–	–	–
S. plicatus SS7	–	–	–
S. coeruleorubidus SS8	–	–	+
S. coeruleorubidus SS9	–	–	–
S. coeruleorubidus SS10	–	+	+
S. albus SS11	–	+	+
S. nigellus SS12	–	+	+
S. fulvoviridis SS13	–	+	+
S. fulvoviridis SS14	–	–	+
S. fulvoviridis SS15	–	–	+
S. fulvoviridis SS16	–	–	–
S. spheroides SS17	–	+	+

[a] Presence (+) or absence (–) of scab lesion on potato minitubers (PMT).

[b] +/– plaque/no plaque formation.

The variation of the host-range within the genus and strains of *Streptomyces* phages was demonstrated by many investigations [9, 13, 15, 18, 34, 36]. The relationship between the density of the pathogens in soil and the occurrence of scab disease is not well known because there is no method for estimating the density of pathogens in soil. Ogiso et al. [19] observed that actinophages of *S. scabies* infected both pathogenic scab isolates and nonpathogenic *Streptomyces* spp.

Adsorption rate constant of the two isolated phages

Adsorption of ϕS and ϕL was determined using *S. scabies* MR13 cells grown in phage medium to the early exponential phase of growth (15 h cultures). The two phages were quickly adsorbed to the cells (Table III). About 77% and 74% of all infective ϕS and ϕL particles were adsorbed within 20 min, respectively. The adsorption reached maximum after 32 min for ϕS and ϕL. The adsorption constant (K) was 3.44 x 10^{-9} ml/min for ϕS and 3.18 x 10^{-9} ml/min for ϕL as determined by the formula $K = 2.3/(B)t \times \log (P_0/P)$ [33], where P_0 = phage assay at zero time, P = phage not adsorbed at time t min, (B) = concentration of bacteria as number of cells/ml and K = velocity constant expressed as ml/min.

Table III

Adsorption rate of actinophages ϕS and ϕL

Incubation period (min)	ϕS		ϕL	
	pfu/ml	%	pfu/ml	%
0	1.20×10^9	0	1.65×10^{11}	0
4	0.87×10^9	27.50	1.35×10^{11}	18.18
8	7.30×10^8	39.17	1.15×10^{11}	30.30
12	5.90×10^8	50.83	0.86×10^{11}	47.88
16	4.50×10^8	62.50	6.70×10^{10}	59.39
20	2.80×10^8	76.67	4.30×10^{10}	73.94
24	1.90×10^8	84.17	2.80×10^{10}	83.03
28	9.90×10^7	91.75	1.70×10^{10}	89.70
32	5.60×10^7	95.33	9.80×10^9	94.06

% adsorbed phage particles percentage.

pfu plaque forming unit.

One step growth experiments indicated that ϕS and ϕL have a latent period of 30 min followed by a rise period of 30 min (Fig. 3). The length of these periods is not exceptional compared to many other actinophages [7]. The mean of burst size was

estimated to be 1.61 for ϕS and 3.75 pfu for ϕL. Adsorption was, therefore, one of the restricting factors. Anne *et al*. [26] found that efficient adsorption (more than 90%) of VWB phage to *S. venezuelae* ETH 13640 and to *S. exfoliatus* was probably due to particular phage receptor protein at the cell surface of these strains. El-Tarabily *et al*. [18] found that the adsorption rate constants of the phage, phiS1, phiS2 and phiS3, were 1.58 x 10^{-7}, 1.26 x 10^{-7} and 5.97 x 10^{-9}, respectively. They reported that the latent period values for the three phages were 35, 40 and 40 min, and the rise periods of these phages were 40, 30 and 40 min, respectively.

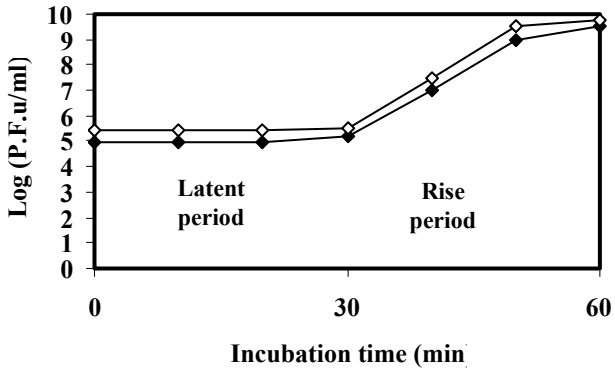

Fig. 3. One-step growth curve of the two phages (◊) ϕS and (♦) ϕL on *Streptomyces scabies* MR13

Determination of temperate phages and lysogenic isolates

Temperate phages could be recognized by the development of lysogenic *Streptomyces* isolates at the center of turbid plaques. The two phages (ϕS and ϕL) isolated from soil on *S. scabies* MR13 formed clear plaques in this host. However, cells growing within the plaques were not immune to superinfection. Therefore, none of the two phages appeared to produce stable lysogenic derivatives in their original host. The temperate character of the two phages was tested in other *Streptomyces* isolates (listed in Table II) in which they produced turbid plaques. The cells growing within these isolates were tested for reinfection of all bacterial lawns. Four phages formed stable lysogens in some of the tested isolates. According to spontaneous induction of phage liberation and immunity to superinfection, these phages were identified as temperate phages. Thus, ϕSS3 was temperate in *S. pluricolorescens* SS3, ϕSS12 in *S. nigellus* SS12 (ϕS derivative), ϕSS13 in *S. fulvoviridis* SS13 and ϕSS17 was temperate in *S. spheroides* SS17 (ϕL derivatives).

Cross-immunity of the temperate phage and restriction barrier of lysogenic isolates

In order to study cross-immunity of the temperate phages, it was necessary to find out at least one or two host strains and/or isolates which could be infected and lysogenized by all temperate phages. The four temperate phages were tested for infection of lysogenic isolates by spotting high-titre phage suspensions (10^5–10^6 phages per spot). Thus, it was possible to use lysogenized isolates (*S. pluricolorescens* SS3, *S. nigellus* SS12, *S. fulvoviridis* SS13 and *S. spheroides* SS17) for cross-immunity studies, revealing that ϕSS3, ϕSS12 and ϕSS13 are homoimmune whereas these phages are heteroimmune with respect to ϕSS17 (Table IV). Lysogenic isolates of SS3, SS12, SS13 or SS17 containing ϕSS3, ϕSS12, ϕSS13 or ϕSS17, respectively were immune to superinfection by ϕSS3, ϕSS12, ϕSS13 or ϕSS17 obtained from their supernatants, respectively, but they were still susceptible to ϕSS3, ϕSS12, ϕSS13 or ϕSS17 propagated on them. Similar result was reported on the immunity and susceptibility of lysogenic isolate of *Streptomyces* by Anne *et al*. [13].

Table IV

Cross-immunity of the four phages and restriction barriers of derivatives lysogenic isolates

Indicator isolates	Phages and isolates on which they were propagated															
	ϕSS3				ϕSS12				ϕSS13				ϕSS17			
	SS3 *	SS1 2#	SS1 3#	SS1 7#	SS3 #	SS1 2*	SS1 3#	SS1 7#	SS3 #	SS1 2#	SS1 3*	SS1 7#	SS3 #	SS1 2#	SS1 3#	SS1 7*
SS3	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
SS12	+	–	+	–	+	–	+	–	+	–	+	–	+	–	+	+
SS13	+	+	–	–	+	+	–	–	+	+	–	–	+	+	–	+
SS17	+	–	+	+	+	–	+	+	+	–	+	+	+	+	+	–

* Phages (ϕSS3, ϕSS12, ϕSS13, or ϕSS17) were obtained from supernatants of the corresponding lysogenic isolates.

Phages (ϕSS3, ϕSS12, ϕSS13, or ϕSS17) were propagated on SS3, SS12, SS13 or SS17.

+/– Plaques/no plaques were observed when phage suspensions were used in spot tests.

The restriction systems

The restriction barriers of isolates (SS12, SS13 and SS17) have different specificities (Table IV). Some propagations of phages (ϕSS3, ϕSS12, ϕSS13 or ϕSS17) on these isolates did not form plaques, ϕSS3 propagated on SS12, SS13 or SS17 did not form plaques on SS12 and SS17, SS13 or SS12 and SS13, respectively. ϕSS12 propagated on SS13 or SS17 did not form plaques on SS13 or SS12 and SS13, respectively, and ϕSS13 on SS12 or SS17 did not form plaques on SS12 and SS17 or

SS12 and SS13, respectively. ϕSS17 propagated on SS12 or SS13 did not form plaques on SS12 or SS13, respectively. These restriction barriers of SS12, SS13 and SS17 (lysogenic isolates) may interfere with cross-immunity of the temperate phages and, however could be overcome by applying high phage titre. The restriction-modification systems were investigated from other streptomycetes by Schneider *et al*. [8], Chater and Carter [25] and Chater and Wilde [37]. On the other hand, Hahn *et al*. [9] reported that actinophage FP22 appeared to be able to avoid restriction barriers in *Streptomyces* spp, and had a strong cross-immunity.

Photoreactivation system

The three host isolates SS12, SS13 and SS17 exhibited considerable restriction barriers for the temperate phages used in this study. Spontaneous transition from the temperate stage to the lytic cycle occurred at a rate of 1–5% of the viable cells number [13]. Induction of the lytic cycle could be enhanced by exposure of the lysogenic isolates to UV-treatment. As shown by the comparison of the dark and light survival result (Table V), the irradiated isolates exhibited a photoreactivation system.

Table V

Photoreaction after UV-treatment of SS12, SS13 and SS17.

Irradiation time (s)	Surviving cfu%								
	SS12			SS13			SS17		
	Light (a)	Dark (b)	Ratio a/b	Light (a)	Dark (b)	Ratio a/b	Light (a)	Dark (b)	Ratio a/b
0	100	100	1.0	100	100	1.0	100	100	1.0
5	89.46	63.88	1.40	93.33	64.12	1.46	81.85	59.74	1.37
10	43.09	23.70	1.80	56.25	28.17	2.00	61.14	21.54	2.72
15	14.93	2.34	6.38	20.15	4.98	4.05	23.47	3.17	7.47
20	10.91	0.87	12.54	15.42	1.32	11.68	16.62	1.03	16.14
25	3.99	0.05	79.87	6.17	0.088	70.11	7.78	0.085	91.51
30	2.65	0.028	94.64	3.55	0.048	73.96	5.37	0.042	127.86
35	0.83	0.0095	87.36	0.94	0.0418	52.22	2.43	0.024	101.25
40	0.35	0.005	63.63	0.55	0.042	23.80	0.76	0.0089	85.39
45	0.054	0.0093	58.06	0.067	0.0045	17.89	0.065	0.0048	13.54
50	0.015	0.00085	17.65	0.058	0.003	7.78	0.020	0.0032	6.25

Spores of SS12, SS13 or SS17 were irradiated with UV and either (a) exposed to laboratory illumination for 30 min prior to incubation in the dark, or (b) directly incubated in the dark.

Thus 30 s of UV-irradiation in the dark resulted in 0.028, 0.048 and 0.042% survival, whereas 30 min subsequent exposure to laboratory illumination increased survival 94.64, 73.96 and 127.86-folds for SS12, SS13 and SS17, respectively. Photoreactivation systems are widely distributed in bacteria and have also been reported for *Streptomyces* species [8, 38, 39]. Longer irradiation of the lysogenic isolates decreased the number of viable cells. The possible restriction barriers of the lysogenized derivatives of SS12, SS13 and SS17 had to ensure that they would not interfere with the cross-immunity studies and these barriers could be overcome after UV-treatment of these isolates (data not shown). Similar results were obtained on *S. coelicolor* DSM 40622 and DSM 40624 by Schneider *et al*. [8]. Provided that the host strains have typical type II restriction endonucleases and corresponding methylases, as it is very common in *Streptomyces* [11], it should be possible to mutate the endonuclease gene by UV irradiation in some spores without affecting the modification enzyme.

It appears that ϕS and ϕL are good candidates for the development of cloning vectors: (i) they are temperate, a characteristic, which would allow selection of cloned genes during the phage lysogenic cycle, and (ii) they have important infecting scab pathogens.

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