

# Evaluation of lytic activity of staphylococcal bacteriophage Sb-1 against freshly isolated clinical pathogens

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## Summary

In recent decades the increase in antibiotic-resistant bacterial strains has become a serious threat to the treatment of infectious diseases. Drug resistance of *Staphylococcus aureus* has become a major problem in hospitals of many countries, including developed ones. Today the interest in alternative remedies to antibiotics, including bacteriophage treatment, is gaining new ground. Here, we describe the staphylococcal bacteriophage Sb-1 – a key component of therapeutic phage preparation that was successfully used against staphylococcal infections during many years in the Former Soviet Union. This phage still reveals a high spectrum of lytic activity *in vitro* against freshly isolated, genetically different clinical samples (including methicillin-resistant *S. aureus*) obtained from the local hospitals, as well as the clinics from different geographical areas. The sequence analyses of phage genome showed absence of bacterial virulence genes. A case report describes a promising clinical response after phage application in patient with cystic fibrosis and indicates the efficacy of usage of Sb-1 phage against various staphylococcal infections.

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## Introduction

During the last decades, a dramatic worldwide increase of antibiotic-resistant *Staphylococcus aureus* infections was observed (Ayliffe, 1997; Chambers, 1997; Herold *et al.*, 1998; Cosgrove *et al.*, 2003). The CDC estimates that 80 000 hospitalized patients experienced a nosocomial infection with methicillin-resistant *S. aureus* (MRSA) every year. Alternative treatment options to antibiotics are therefore urgently needed. One potential treatment option for antibiotic-resistant *S. aureus* is lytic bacteriophages, which naturally infect *S. aureus* (Merril *et al.*, 1996; Barrow and Soothill, 1997; Carlton, 1999; Chanishvili *et al.*, 2001; Duckworth & Gulig, 2002; Inal, 2003; Bruttin and Brussow, 2005; Merrill *et al.*, 2006) known as phage therapy. Phages, unlike antibiotics, are specifically targeted to a particular bacterial species and thus spare bystander bacteria from other species lytic damage. Phages thus much less affect the balance of normal microflora than antibiotics. The G. Eliava Institute of Bacteriophages, Microbiology and Virology (Tbilisi, Georgia) was the first centre, where therapeutic phage cocktails targeting various bacterial pathogens were developed. Several cocktails were for many decades successfully used against *S. aureus* in Georgian hospitals (Chanishvili and Sharp, 2008; Kutateladze and Adamia, 2008).

In 1977, L. Kvachadze isolated a particularly active phage from an Eliava Institute phage cocktail used to treat staphylococcal infections. The phage isolate was named Sb-1 and its properties were described in Russian/Georgian publications (Andriashvili *et al.*, 1981; Ackermann and Abedon, 2001). Sb-1 is particularly well suited for phage therapy. It is a strictly 'virulent' phage, i.e. it propagates exclusively by lytic infections and does not establish a lysogenic state. 'Virulent' phages in contrast to temperate phages do not carry bacterial pathogenicity genes. Sb-1 has a very broad host range on *S. aureus*, including antibiotic-resistant strains. Its genome was sequenced and no known virulence genes were identified. The sequence confirms also our previous observation that the Sb-1 genome includes no GATC sequences (Andriashvili *et al.*, 1986). which are the target of Sau3A, the major restriction enzyme found in many *S. aureus* strains. This article presents a further characterization of Sb-1, its genome map, biological properties, host range against a

broad panel of clinical isolates including MRSA strains, and sample clinical data including a case report of its successful application against a chronic *S. aureus* infection in a cystic fibrosis patient.

## Results and discussion

### Characterization of the Sb-1 phage

The electron microscopy of the purified Sb-1 phage (Fig. 1) displays a typical SPO1-like phage, a class of *Myoviridae* (phages with contractile tail) that is widely distributed in low GC content Gram-positive bacteria. Its genomic map (Fig. 2, sequence GenBank Accession No. HQ163896) confirmed this analysis and identified Sb-1 as a close relative of *S. aureus* phage K (Rees and Fry, 1981; O'Flaherty *et al.*, 2004). A number of SPO1-like *S. aureus* phage genomes have been sequenced and none showed bacterial virulence genes or any other undesired genes (Stewart, 1993; Yarwood *et al.*, 2002; Chibani-Chenoufi *et al.* 2004; Klumpp *et al.*, 2008; Stewart *et al.*, 2009). This was also the case for the phage Sb-1 genome. A number of specific host-lethal genes have been identified which may contribute to its efficacy; a characterization of the properties and effects of some of these genes will be published separately, along with a more detailed genomic analysis. Comparative protein analysis of SPO1-like phages with different host bacteria confirmed the relatedness of phages on level of structural proteins (Fig. 3) (Klumpp *et al.*, 2008; Stewart *et al.*, 2009).

### Antibiotic and phage susceptibility of *S. aureus* isolates

Between 2004 and 2009 we obtained 352 isolates of epidemiologically unrelated *S. aureus* from diagnostic centres and hospitals of Tbilisi. The strains were isolated from patients showing infected wounds, furuncles, otitis

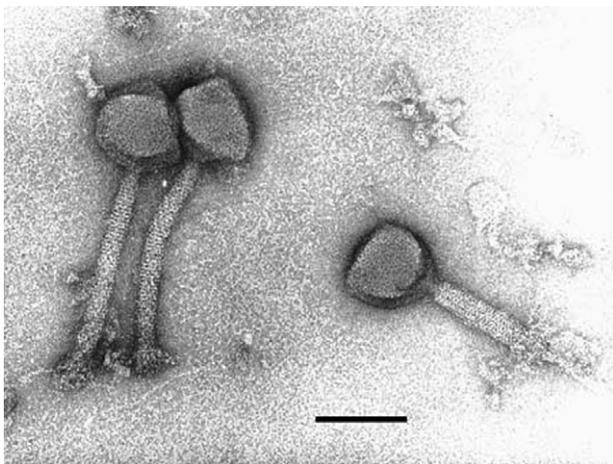


Fig. 1. Electron micrograph of Sb-1 phage (bar = 100 nm).

externa, eye, nose, gum, throat and urinary tract infections. Each isolate was tested for resistance to various antibiotics and against phage Sb-1. Less than 2% of the isolates were resistant to phage Sb-1, while antibiotic resistance levels ranged from 7.2% to 99.7%, respectively, for the different antibiotics.

### Pulsed field gel electrophoresis analysis

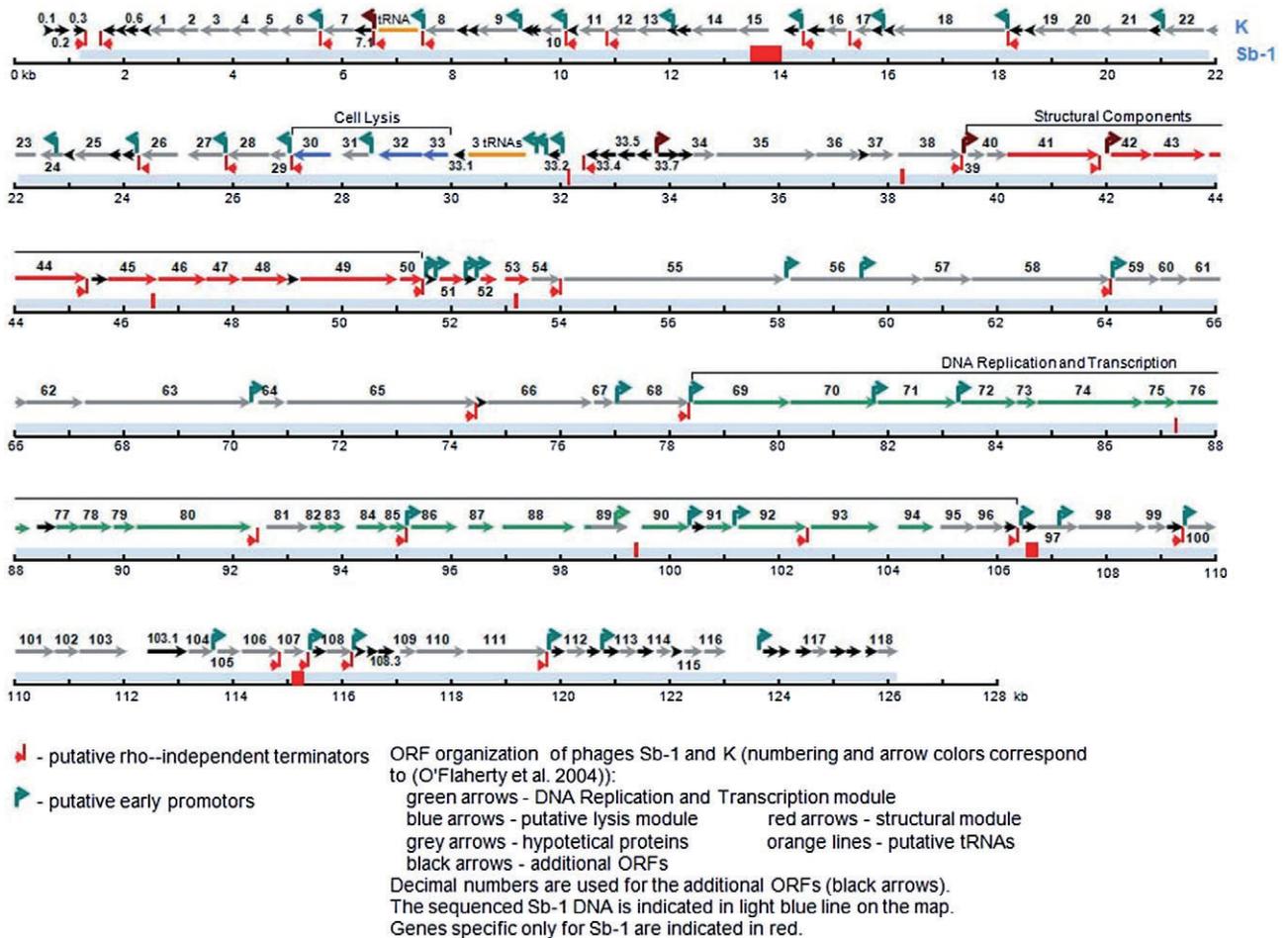
A coverage of greater than 98% is exceptional for a single phage isolate. Therefore we controlled whether the high coverage of Sb-1 phage was the consequence of a collection containing closely related *S. aureus* strains. We explored the diversity of our *S. aureus* strain collection by investigating 54 of the 352 isolated by pulsed field gel electrophoresis (PFGE). Visual analysis of the PFGE results identified 25 distinct restriction pattern. The isolates could be classified into nine multi-strain branches by unweighted-pair group method using average linkages (UPGMA) dendrogram analysis (Fig. 4A). One branch of group 1 was represented by 16 isolates. The other branches were represented by at most four different isolates. Antibiotic resistance pattern of 54, genotyped bacterial strains showed that bacteria reveal high resistance to polypeptides,  $\beta$ -lactames, bacitracin and aminoglycosides (Table 1).

### Test against *S. aureus* isolates from a different geographical area

In order to exclude a geographic bias, the lytic activity of phage Sb-1 was also tested against the *S. aureus* strain collection from the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH Germany; German collection of microorganisms and cell cultures). The tested strains included 50 MRSA and 43 toxin-producing MSSA strains. We observed again a variable PFGE pattern with these strains and the German MRSA isolates differed from the Georgian MRSA isolates (Fig. 4B). None of the strains was resistant to the phage used at high titre ( $1 \times 10^{11}$  pfu ml<sup>-1</sup>). Ten strains showed resistance to the phage at a lower titre of  $1 \times 10^9$  pfu ml<sup>-1</sup>. However, after propagation of Sb-1 on these less sensitive bacteria, all 10 strains were subsequently lysed by the low titre of phage.

### Efficiency of plating

One might object that the lytic activity against such a wide range of strains could reflect an enzymatic lysis effect (lysis from without or soluble phage lysis effects) and not true replication of Sb-1 on the test strains. This was not the case as demonstrated by an efficiency of plating (eop) test. On 25 out of 27 distinct strains, the plaque count of



**Fig. 2.** Genome sequence of Sb-1 bacteriophage.

Sb-1 on the test strain was high when compared with the plaque count on the propagating strain (eop of 0.1–1) (Table 2). Test strains for the eop experiments were selected from each PFGE subgroups. The plaque assay demonstrates that the wide host range of Sb-1 on *S. aureus* strains is not an artefact of the spot test.

#### Phage-resistant *S. aureus* strains

Five *S. aureus* strains from Georgian patients were only weakly susceptible towards phage Sb-1. In the spot test only an incomplete 'opaque lysis' phenotype was observed. These strains came from five different patients and three different anatomical sites (N390 was isolated from the vagina; N392 and N396 from urine; N394 and 395 from the eye). In PFGE analysis strains N390, 396 and 395 could not be distinguished while N394 was clearly distinct (data not shown).

On these phage-resistant cells, blind serial passages of phage Sb-1 were performed in liquid and solid media. In

the first passages, no phage plaques were detected. However, after several passages, the titre of the phage rose to  $10^7$ – $10^8$  pfu ml<sup>-1</sup>.

The adsorption of Sb-1 phage was relatively low on the *S. aureus* strains showing the opaque lysis spot phenotype (50–65% input phage adsorbed after 5 min of incubation). When Sb-1 derivative phages finally achieved high titres on the initially phage-resistant cells, 89–95% of the input phages were adsorbed after 5 min (data not shown).

#### Phage escape mutants

Next we tested to what extent phage-resistant cells appeared in a culture of phage-sensitive *S. aureus* strains infected with phage Sb-1 in liquid culture. Two propagating strains (N50, N164) and a clinical MRSA isolate (N125) were used for this experiment. We observed only a low level of phage escape mutants: frequency of mutation on *S. aureus* 50 strain was  $2.2 \times 10^{-7}$ , on strains *S. aureus* 125 and 164,  $1.2 \times 10^{-7}$  and  $1.1 \times 10^{-9}$

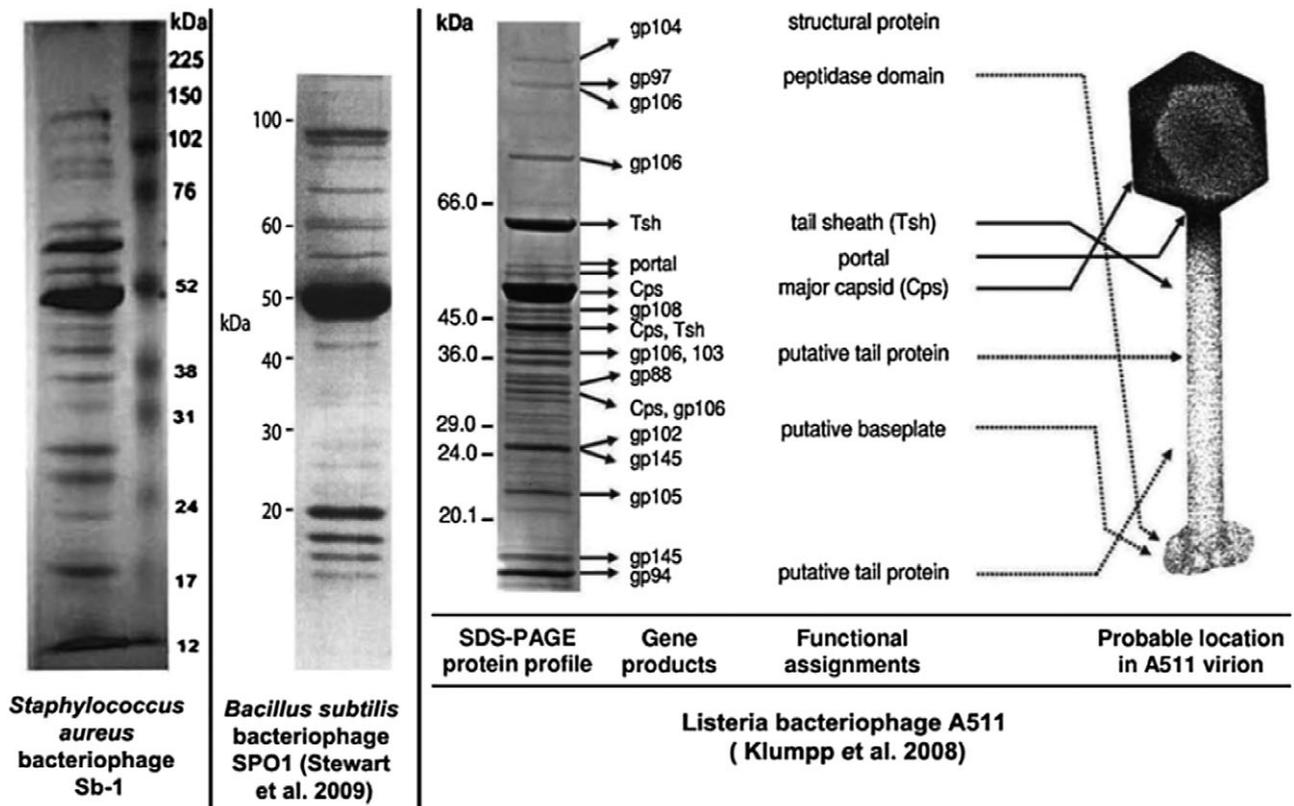


Fig. 3. Comparison of structural proteins of morphologically related bacteriophages.

respectively. Only few of the colonies growing in the presence of Sb-1 could be serially passages: most of them had converted back to the phage-sensitive phenotype.

#### Sample clinical applications

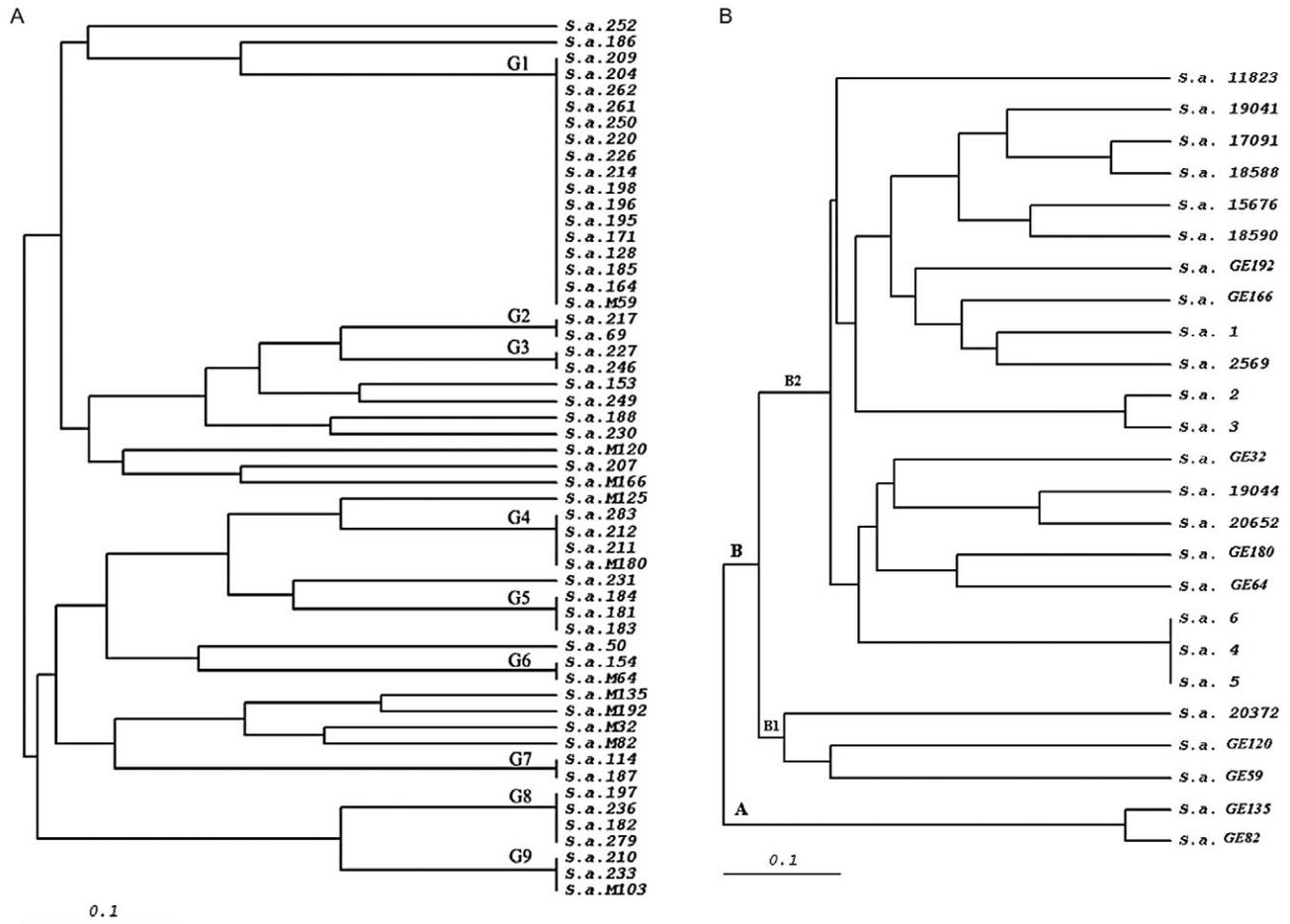
For many years, the Eliava Institute in Tbilisi was producing a staphylococcal phage preparation which was effectively used locally (topically). In late 1970s, the Institute scientists elaborated a method for construction of the phage for intravenous use. In the series of experiments, therapeutic effectiveness was studied to treat different infectious diseases, including acute and chronic sepsis, peritonitis, osteomyelitis, mastitis, purulent arthritis, acute and chronic lung abscesses, chronic pneumonia and bronchitis, bronchoectasis, purulent cysts and others. Sb-1 phage was the key component of the therapeutic preparation.

In some cases when the approved cocktails (commercial preparations) do not work *in vitro* against the pathogen isolated from patient's samples, we isolate specific 'autophage' against patient's specific bacteria and use these phages for treatment of the patient. We present an example of current clinical application of Sb-1 phage with

commercially available Pyophage in patient with cystic fibrosis.

**Case report.** Patient M. Kh. was a 7-year-old girl suffering from cystic fibrosis diagnosed in 2002. The disease was confirmed by genetic analyses: she is homozygous for the mutation – **1677 delTA**. The sweat test revealed a strongly elevated chloride concentration of 126–135 mmol l<sup>-1</sup>. She showed a chronic colonization of the lungs with *Pseudomonas aeruginosa* and *S. aureus*. During several years, the patient was treated with broad spectrum antibiotics, but there was no effect on the *P. aeruginosa* and *S. aureus* colonization. The initial bacterial concentration (before phage application) in the patient's sputum was 1 × 10<sup>7</sup> cfu ml<sup>-1</sup> for *S. aureus*, and 8 × 10<sup>6</sup> cfu ml<sup>-1</sup> for *P. aeruginosa*. Phage treatment was provided nine times by nebulizer (with approximately 4- to 6-week interval between phage therapies) and bacteriological analysis was performed at 1-month time intervals.

After the first Pyophage application, the concentration of *P. aeruginosa* drastically decreased – 7 × 10<sup>3</sup> cfu ml<sup>-1</sup>, but the *S. aureus* titre was not so strongly affected, dropping only to 1 × 10<sup>6</sup> cfu ml<sup>-1</sup>. This reflected a weak susceptibility of the strain to Pyophage *in vitro*. During a month when no phage therapy was administered,



**Fig. 4.** UPGMA dendrogram of *S. aureus* strains: (A) strains isolated from the patients from local hospitals, (B) MRSA strains from Georgia (GE) and Germany.

the amount of *P. aeruginosa* remained minimal –  $7 \times 10^4$  cfu ml<sup>-1</sup>.

The physician decided to add use of now well-characterized phage Sb-1, which had high lytic activity against the patient's specific culture *in vitro*. Sb-1 phage was added to the Pyophage cocktail and applied this augmented phage cocktail five times with a nebulizer to the patient. After the joint application (Pyophage with Sb-1 staphylococcal phage), the amount of *S. aureus* drastically decreased and during a medication-free month, the level of *S. aureus* remained fairly low, at about  $10^3$ – $10^5$  cfu ml<sup>-1</sup>; for *P. aeruginosa*, it was 10–100 cfu ml<sup>-1</sup>. No adverse effects were seen in the patient upon application of the Sb-1 phage.

We examined the concentration of phages (staphylococcal and *P. aeruginosa*) in the patient's sputum during phage treatment. Three to four hours after phage application, phage was detected at  $10^2$  pfu ml<sup>-1</sup>. On the second day, before the next phage application, no phage was detected in the sputum (Fig. 5).

Subsequent to phage therapy, the general condition of patient improved. Most notably, the follow-up of the *S. aureus* level in the sputum showed a steady decrease over 2 months until *S. aureus* slipped under the detection level after a month. In contrast *P. aeruginosa* levels showed comparative constancy during 12 months, but at the end of the observation period *P. aeruginosa* titres reached to  $\times 10^5$  pfu ml<sup>-1</sup>, but after therapy, no bacteria was found in the sputum.

The most important fact is that the use of antibiotics in the complex therapy was able to be decreased by 50%. However, no significant improvement has yet been seen in the X-ray and blood analyses. The patient is still under treatment.

## Conclusion

Phage cocktails against pyogenic infections (Pyophage) have a long history of reportedly safe in the former Soviet Union. This phage cocktail is still today sold in Georgia

**Table 1.** Percentage of antibiotic resistance of genetically different clinical *Staphylococcus aureus* strains.

PFGE groups	Bacterial strain	$\beta$ -Lactamase	Aminoglycosides	Tetracycline	Macrolides	Chloramphenicol	Polypeptides	Ciprofloxacin	Methicillin	Bacitracin
G1	16	100	68.7	37.5	68.7	25	100	50	6.25	100
G2	2	100	100	50	100	50	100	50	0	100
G3	2	100	50	0	0	0	100	0	0	100
G4	4	100	100	75	75	50	100	75	25	100
G5	3	100	100	33.3	66.6	0	100	100	0	100
G6	2	100	100	50	50	50	100	100	50	100
G7	2	50	100	100	100	50	100	100	0	100
G8	4	50	100	75	50	75	100	75	0	100
G9	3	33.3	100	66.6	0	33.3	100	33.3	33.3	66.6
Individual	16	93.75	75	37.5	43.75	31.25	100	62.5	43.75	87.5
Total	54	88.8	81.4	44.4	55.5	29.6	100	61.1	20.3	94.4

**Table 2.** Efficiency of plating of phage Sb-1 on different strains of *S. aureus*.

Strain of <i>S. aureus</i>	32	69	103	153	154	164	182	183	184	186	187	188	197	207	212	214	217	220	227	229	230	231	233	236	246	250	279
Efficiency of plating	0.1	1	1	0.5	1	1	1	1	1	1.5	1	1.5	1	1	1	0.5	0.5	1	0	1	1	0.3	0.5	1	0	0.2	1

and Russia as a registered over-the-counter medicine. However, since neither the phage preparation nor the clinical trials conducted with them were published in Western scientific journals, claims on their efficacy were seen with substantial scepticism by Western scientists. Here we demonstrate that *S. aureus* phage Sb-1 is a representative of the SPO1 group of Myoviridae, which can be considered as safe with respect to its genome sequence. The phage can be purified and showed a broad host range against large and representative *S. aureus* strain collections from different geographical areas, including MRSA strains. Phage resistance development is very low and largely reverts to sensitivity. A case study in a cystic fibrosis patient showed no adverse events of phage application in a nebulizer and a promising clinical response. Placebo-controlled double-blinded clinical trials are now warranted to test the value of Sb-1 phage against *S. aureus* infections in defined clinical situations.

## Experimental procedures

### Bacterial strains

A total of 352 hospital strains of *S. aureus*, including our standard host strain, *S. aureus* 50, were obtained from patients in various clinics of Tbilisi between 2004 and 2009.

### Resistance testing

Resistance to antibiotics was determined by the disc diffusion method on Brain–Heart Infusion agar (Difco). Discs of antibiotics with specified concentrations were obtained from 'BBL' (BD BBL Sensi-Disc) and used following the manufacturer's protocol. Resistance to Sb-1 was determined by the spot test and determination of efficiency of plating. A culture was considered resistant if the phage or antibiotic caused no visible decrease in bacterial growth.

### Bacterial growth conditions

*Staphylococcus aureus* 50 was routinely grown at 37°C in Brain–Heart Infusion or in semi-synthetic liquid medium (Kellenberger, 1962) supplemented with 0.1% casein hydrolysate and 0.1% yeast autolysate, to an OD<sub>590</sub> of 0.9–1.0 ( $5 \times 10^8$  cells ml<sup>-1</sup>).

### Phage propagation and purification

Phage was added to exponentially growing bacterial cultures at an moi of 0.1. For large-scale phage cultivation, a modified Frazer fermentor (13 l) (Tikhonenko, 1973) was used and the culture was incubated at 37°C for 4 h. Primary purification and concentration of the phage lysate was performed by chromatography using an ion-exchange DEAE cellulose column and then differential centrifugation (5000 *g*, 18 000 *g*) (Tikhonenko *et al.*, 1963; Tikhonenko, 1973). Purified phages were suspended in SSC buffer (0.15 mM NaCl, 0.015 mM Na

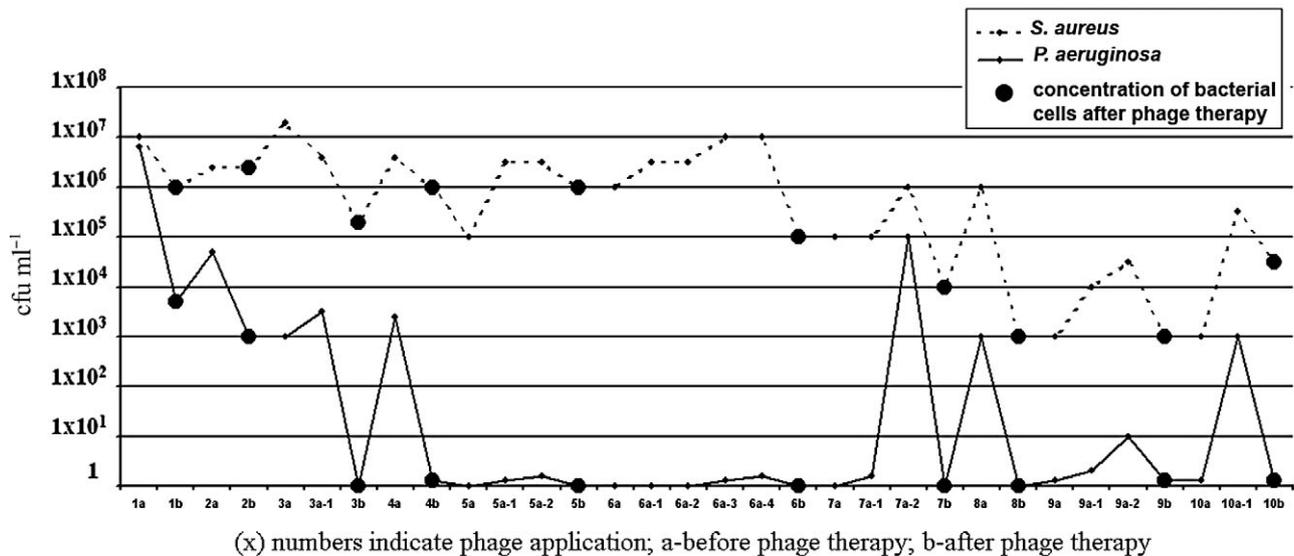


Fig. 5. Concentration of bacterial cells in sputum of the patient before and after phage application.

citrate, pH 7.0). Final purification of native virions was carried out by centrifugation at 18 000  $g$  through a CsCl gradient in an SW25 rotor using a Beckman L2-65B ultracentrifuge at 4°C. Phage fractions were dialysed against SSC. Single-step growth studies were performed as described by Adams (1961, p. 416).

#### Electron microscopy studies

Morphology of phage particles was studied by electron microscope JEM 1200EX (JEOL). Parlodion (Mallinckrodt) plates were overlaid by phage suspensions ( $10^{10}$  pfu ml<sup>-1</sup>) and contrasted by uranylacetate.

#### Study of phage structural proteins

Phage suspensions (60 mkl,  $10^{10}$ – $10^{11}$  pfu ml<sup>-1</sup>) were added in 13.3 mkl of sample buffer [8% of SDS, 2 ml of glycerol, 2 ml of 0.5% bromophenol blue, made up to 10 ml with 0.1 M Tris-HCl (pH 6.5), and 8 mkl of  $\beta$ -mercaptoethanol was added to 100 mkl of this solution prior to use].

Samples then were boiled for 10 min before being loaded onto 10% polyacrylamide gel (Laemmli, 1970).

Electrophoresis was carried out at 60 v, 9 mA during 18 h in running – Tris-glycine buffer (3.02 g of Tris base, 14.4 g of glycine, 1 g of SDS, made up to 1 l with deionized water).

#### Analysis of staphylococcal DNA by PFGE

Overnight cultures were diluted to an OD<sub>540</sub> of 0.8–0.9 and bacteria from 500  $\mu$ l of medium were collected by centrifugation at 5 000 r.p.m. for 15 min, washed with buffer (10 mM Tris base, 1 M NaCl), re-centrifuged in the same conditions, and the bacterial cell pellet resuspended in the same buffer (250  $\mu$ l). Two hundred and fifty microlitres was mixed with an equal volume of (50°C) 1% SeaKem agarose (BioProducts,

USA) and then pipetted into a plug mould. The solid agarose plugs were incubated at 37°C for 24 h in 2 ml of lysis buffer I [6 mM Tris HCl, pH 7.6, 1 M NaCl, 100 mM EDTA, pH 8.0, 0.5% Brij 58, 0.2% Deoxycholate, 0.5% sodium lauryl sarcosine, lysozyme (1 mg ml<sup>-1</sup>), RNase (20 mkg ml<sup>-1</sup>), lysostaphin (60 mkg ml<sup>-1</sup>)]. The plugs were then incubated at 55°C for 24 h in lysis buffer II [0.5 M EDTA, pH 9.0, 1% sodium lauryl sarcosine, proteinase K (50 mkg ml<sup>-1</sup>)]. The plugs were washed three times with TE buffer (Mulvey *et al.*, 2001; Montensinos *et al.*, 2002). The DNA was digested using 25 U of SmaI (BioLab, USA) for 16 h at 25°C. DNA fragments were resolved on a 1.2% agarose gel (SeaKem gold, USB, USA) using a Gene Navigator System apparatus (Amersham Biosciences) for 18 h at 200 V at 14°C with switching times ramped from 5 to 60 s. After electrophoresis, gels were stained with ethidium bromide, rinsed and photographed under UV light. We used SmaI because it had previously been shown to digest the *S. aureus* genome into 15–20 restriction fragments, ranging from 5 to 400 kb, giving different patterns with different strains (Tenover *et al.*, 1995). Macrorestriction patterns were analysed visually. Isolates with identical restriction patterns in size and number of bands were considered the same type; isolates that differed from main types by one to two band shifts were assigned subtypes. Clustering of strains was based on the UPGMA. A dendrogram was generated with standard Freetree and Treeview-based Genetic Distance similarity software.

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