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SHORT COMMUNICATION

Characterization of a novel T4-type *Stenotrophomonas maltophilia* virulent phage Smp14

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Abstract *Stenotrophomonas maltophilia* (Sm), with most of the isolates being resistant to multidrugs, is an opportunistic bacterium causing nosocomial infections. In this study, a novel virulent Sm phage, Smp14, was characterized. Electron microscopy showed that Smp14 resembled members of Myoviridae and adsorbed to poles of the host cells during infection. It lysed 37 of 87 clinical Sm isolates in spot test, displayed a latent period of ca. 20 min, and had a burst size of ca. 150. Its genome (estimated to be 160 kb by PFGE), containing m4C and two unknown modified bases other than m5C and m6A as identified by HPLC, resisted to digestion with many restriction endonucleases except MseI. These properties indicate that it is a novel Sm phage distinct from the previously reported phiSMA5 which has a genome of 250 kb digestible with various restriction enzymes. Sequencing of a 16 kb region revealed 12 ORFs encoding structural proteins sharing 15–45% identities with the homologues from T4-type phages. SDS-PAGE displayed 20 virion proteins, with the most abundant one being the 39 kDa major capsid protein (gp23), which had the N-terminal 52 amino acids removed. Phylogenetic analysis based on gp23 classified Smp14 into a novel single-membered T4-type subgroup.

Keywords *S. maltophilia* · Virulent phage · T4-type · Unknown modified bases · Major capsid protein · Phylogenetic analysis

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Introduction

Stenotrophomonas maltophilia (Sm), previously called *Pseudomonas maltophilia* or *Xanthomonas maltophilia* (Palleroni and Bradbury 1993), is an aerobic, gram-negative bacterium found in various environments, including soil, water, sediment, sewage, frozen foods, plants, and animals (Hugh 1981; Juhnke et al. 1987; Aznar and Alcaide 1992; Hauben et al. 1999; Lambert et al. 1999). As an opportunistic human pathogen, Sm has significantly increased the incidence of nosocomial infection in the last decade. This usually occurs in debilitated or immunosuppressed individuals and those involved in postoperative infections (Fisher et al. 1981; Roilides et al. 1992; Ballestero et al. 1995). Long-term intravenous catheter use may be a source of entry (Wang et al. 2004). Despite its relatively low virulence, Sm can cause a wide variety of infections such as bacteremia, endocarditis, peritonitis, urinary tract and gastrointestinal infections, and pneumonia (Muder

et al. 1996; Vartivarian et al. 1996; Kim et al. 2002; Baek et al. 2004; Wang et al. 2004). Furthermore, Sm has intrinsic resistance to many antibiotics such as β-lactams and aminoglycosides (Vartivarian et al. 1994; Spencer et al. 2001; Li et al. 2003). Hence, the antibiotic treatment of Sm has not been effective because of its multidrug resistance. The decline in effectiveness of antibiotic drugs requires different strategies to treat Sm infections. Phage therapy is a promising option for alternative treatments. Although Sm is important in both medicine and the environment, only two bacteriophages infecting this organism have been reported. Previously, eight virulent phages (phiSMA1∼8) were isolated, with one of them (phiSMA5) being characterized (Chang et al. 2005), and a 6,907-base bp plasmid-like prophage (phiSMA9) was sequenced (Hagemann et al. 2006).

In this study, another novel virulent bacteriophage, Smp14, infecting Sm was isolated from the sewage sample of a hospital. Its morphology, phage biology, partial genomic sequenced, virion proteins and phylogenetic relatedness were studied.

Materials and methods

Bacterial strains and growth conditions

Eighty-seven Sm strains used in this study were described previously (Chang et al. 2005). Bacteria were cultivated in Luria-Bertani broth (LB) or LB agar at 37°C. Bacterial growth was monitored turbidimetrically by reading OD_{600}. An OD_{600} of 1.0 corresponded to 1.0×10^9 cells/ml of the Sm strain T14 (SM T14).

Phage techniques

A sewage sample (30 ml) collected from Chung-Ho Memorial Hospital of Kaohsiung Medical University, Kaohsiung, Taiwan was screened for the presence of phages. Phage isolation experiments were carried out as described previously (Chang et al. 2005).

Spot test and titering were performed as described in Chang et al. (2005), while, adsorption test and one-step growth experiments were carried out as described in, Foschino et al. (1995) and Pajunen et al. (2000), respectively.

Phage was purified by loading the phage suspension (5×10^{12} PFU) on the block gradient of CsCl representing 1.30, 1.35, 1.50, and 1.70 g/ml (1 ml for each block), followed by ultracentrifugation at 24,500 rpm for 2 h at 4°C with a TH641 rotor (Sorvall OTD Combi).

Electron microscopic observation of the morphology was performed as previously described (Chang et al. 2005) using the phage particles purified.

Digestion of DNA and chromatography of deoxynucleosides

Isolation of phage DNA was performed as described previously (Chang et al. 2005). Deoxynucleosides of phage DNA were analyzed as described in Ehrlich et al. (1987). The purified phage DNA (500 μg) was treated at 37°C in a mixture of 500 μl containing 500 μg of DNase (Sigma), 200 U of exonuclease III (Promega), 200 U of S1 nuclease (Promega), and 200 U of *Escherichia coli* alkaline phosphatase (NEB). The resultant deoxynucleosides (ca. 50 μg in 100 μl) were separated by high-performance liquid chromatography (HPLC) using a reversed-phase column (Hypersil, GOLD, 250 × 4.6 mm; Thermo) and eluted with the running buffer (0.167 mM sodium hydrogen phosphate, 6.5 mM potassium dihydrogen phosphate, and 5% methanol) at a rate of 0.8 ml/min. To ensure consistency, the column was pretested using a mixture of deoxynucleoside standards. The deoxynucleosides 2′-deoxyadenosine, 2′-deoxyguanosine and 2′-deoxycytidine were purchased from Sigma-Aldrich. The deoxyribonucleotide triphosphates mixture (dNTP) was purchased from TaKaRa, and 4-methyldeoxycytidine triphosphate (m4dCTP), 5-methyldeoxycytidine triphosphate (m5dCTP) and N^6-methyldeoxyadenosine triphosphates (m6dATP) from Fermentas. Before using as standards for HPLC, dNTP, m4dCTP, m5dCTP and m6dATP were treated with alkaline phosphatase. HPLC was performed on a Hitachi model L-7100 Intelligent pump system equipped with a Hitachi model L-7000 interface, attached to a Hitachi model L-7420 UV-VIS detector setting at 256 nm.

Pulsed-field gel electrophoresis (PFGE)

PFGE was performed in a Rotaphor Type V system (Biometra). Phage-containing plugs were prepared as previously described (Chang et al. 2005) and the DNA was digested with 20 U of MseI (NEB) (37°C, 16 h). Electrophoresis was performed for 23 h at 4°C with a pulse time that decreased from 30 to 5 s, an angle that decreased from 120 to 110°, and a voltage that decreased from 180 to 120 V.

Sequence analysis

Fragments of Smp14 DNA (0.7–1.2 kb) obtained by sonication were treated with Taq enzyme and cloned in *E. coli* using pGEM-T (Promega). Both strands of 200 clones were sequenced (ABI Prism 3700, Applied Biosystems). PCR was performed for gap closure. A ca. 16 kb region was sequenced and deposited in the NCBI database under accession number DQ364602.

Open reading frames (ORFs) likely to encode proteins were identified with the program Vector NTI. Multiple

alignments were performed using ClustalX (Jeanmougin et al. 1998), and the phylogenetic tree was constructed using Tree View (Page 1996).

Protein techniques

To identify the virion proteins of Smp14, SDS-polyacrylamide gel electrophoresis (PAGE) and N-terminal amino acid sequencing were conducted as previously described (Chang et al. 2005).

Results and discussion

Bacteriophage Smp14 isolated on Sm resembles the members of Myoviridae in morphology

After enrichment of the sewage sample in cultures of different Sm strains, six of the culture supernatants were found in spot tests to contain phages. Examination of the phages in electron microscopy showed that they were of two different types. Four had an isometric head and a contractile tail in morphology, whereas the remaining two had a small head and a long curly tail. One phage of the former group, showing the best growth inhibition effects on Sm strains, was designated as Smp14 and used for further study.

In CsCl density gradient centrifugation, the phage was found in two layers (1.30–1.35, and 1.35–1.50 g/ml) containing 8×10^9 and 1×10^{12} PFU, respectively, indicating a density of Smp14 between 1.35 and 1.50 g/ml. Electron microscopy of uranyl acetate-stained Smp14 virions (Fig. 1a) revealed particles with a head of 87 × 77 nm, a tail of 129 × 18 nm, a baseplate of 35 × 24 nm, and short tail fibers. The morphology of this phage was thus similar to the members of family Myoviridae or Bradley's group A1 which includes T-even phages (Ackermann 2001, 2003).

Phage Smp14 specifically infects Sm

Smp14 formed clear plaques of 0.5 mm in diameter on the lawns of SM T14. Infection of SM T14 at early-exponential phase (OD_{600} = 0.3, multiplicity of infection = 1.0), the cultures were cleared in less than 2 h, producing 2×10^{10} PFU/ml Smp14. In spot test on 87 *S. maltophilia* strains from our collection, clear and turbid zones were observed in 37 and 12 strains, respectively, whereas no responses were observed for the remaining 38 strains. These results suggest that Smp14 has potential for control of *S. maltophilia*, e.g. phage therapy and treatment of hospital settings, in combination with other Sm phages.

To test for host range of phage Smp14, one strain each of *E. coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Xanthomonas campestris* pv. *campestris*, *Xanthomonas axonopodis* pv. *vesicatoria*, and *Xanthomonas oryzae* pv. *oryzae* were used as indicator hosts for spot test. None of these bacteria was susceptible to Smp14, indicating that this phage has known host range limited to Sm strains.

Phage Smp14 adsorbs to the poles of host cells

The results of electron microscopy from three independent experiments using different samples showed that the phage particles predominantly adsorbed to the poles of SM T14 cells (Fig. 1b, c). Most bacteriophages adsorb evenly to the host surfaces or pili (Koike and Iida 1971; Tosi and Anderson 1973; Ryter et al. 1975; Crowlesmith et al. 1978; Hausmann and Clowes 1971; Kim et al. 1980; Sommer and Newton 1988). So far, only two phages have been found to adsorb to the poles of host cells, *Bacillus subtilis* phage Tg11 (Smirnova et al. 1979) and *Caulobacter crescentus* phage φcbk (Skerker and Shapiro 2000). Thus, the situation of Smp14 adsorption is among the very rare cases.

The adsorption rate of Smp14 to the SM T14 cells is shown in Fig. 2a. Approximately 85% of Smp14 was adsorbed onto the host cell after 5 min, which gradually

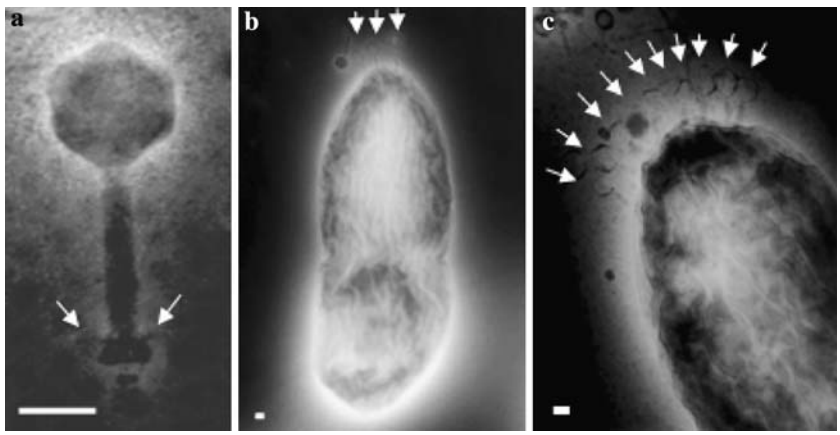

Fig. 1 Transmission electron micrograph of phage Smp14. **a** Phage Smp14 consists of an elongated isometric head, neck, tail, baseplate, and tail fiber structures (*arrows*). **b** and **c** Smp14 particles adsorb to the pole of an SM T14 cell (phage indicated by *arrows*). Scale bar, 50 nm

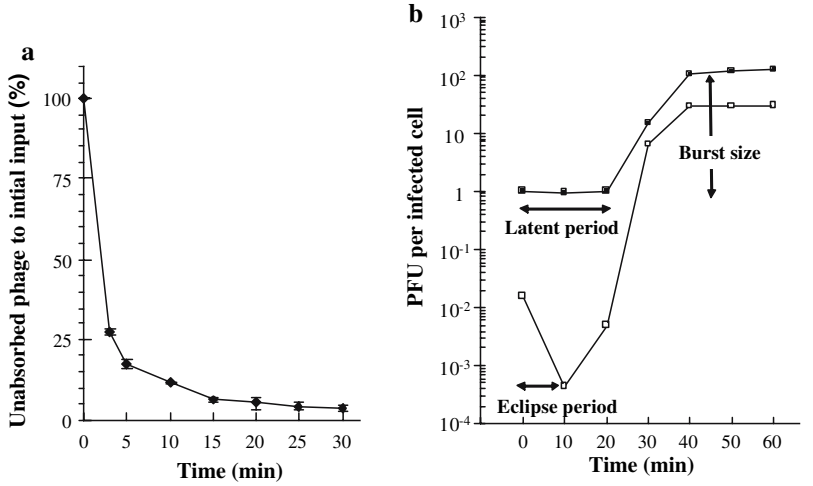

Fig. 2 The adsorption curve and one-step growth curve of Smp14. **a** Time course of Smp14 adsorption to SM T14. **b** One-step growth curve of bacteriophage Smp14. Shown are PFU per infected SM T14 cell in chloroform-treated cultures (*square*) and in untreated cultures (*filled square*) at different time points. Each point represents the mean of three experiments

rose to 95% at 30 min post-infection. This adsorption rate is faster than that of phage phiSMA5, in which 100 min was needed for 80% of the phage to be adsorbed onto the host cell (Chang et al. 2005).

Phage Smp14 has a short latent period and a burst size of ca. 150

The one-step growth curve of Smp14 on SM T14 was determined (Fig. 2b). The latent period was about 20 min. This is only one fourth that (80 min) of phiSMA5, a larger Sm phage with a DNA genome of 250 kb (Chang et al. 2005). An eclipse period of 10 min was observed. The average burst size was about 150 PFU per infected cell, which is 1.5-fold larger than that of phiSMA5. Thus, compared with phiSMA5, Smp14 had a faster adsorption rate, shorter latent period, and a larger burst size.

Phage Smp14 has a genome of about 160 kb refractory to digestion by many restriction endonucleases

The size of the Smp14 genome was estimated by pulse-field gel electrophoresis to be 160 kb (Fig. S1a). To test for the digestibility of Smp14 DNA, 40 type II restriction endonucleases were used for digestion which included *Alu*I, *Apa*I, *Ava*I, *Ase*I, *Bam*HI, *Ban*II, *Bfa*I, *Bgl*II, *Bsp*DI, *Bst*YI, *Cla*I, *Eco*RI, *Eco*RV, *Hae*II, *Hae*III, *Hin*cII, *Hin*dIII, *Hpa*I, *Kpn*I, *Mlu*I, *Mse*I, *Nco*I, *Nde*I, *Nhe*I, *Not*I, *Nsi*I, *Pst*I, *Pvu*II, *Sac*I, *Sac*II, *Sal*I, *Sau*AI, *Sma*I, *Spe*I, *Sph*I, *Stu*I, *Xba*I, *Xho*I, *Xma*I, and *Xmn*I. It was found that the phage DNA could only be cut by *Mse*I (Fig. S1b) whose recognition sequence is 5′TTAA3′ containing only adenine and thymine.

Since most restriction enzymes with recognition sites containing G and/or C cannot digest the phage DNA, we predicted that the Smp14 genome contains unusual base analogs of G and/or C, and the analog of A or T may also be present.

HPLC analysis suggests that Smp14 genomic DNA contains three modified bases

HPLC analysis results showed that phage DNA contained A, T, G, C, m4C, and two types of unknown modified deoxynucleosides, named unknown deoxynucleoside 1 and unknown deoxynucleoside 2. These two unknown deoynucleosides were neither m5C nor m6A. The levels for A, T, G, C, m4C, unknown deoxynucleoside 1, and unknown deoxynucleoside 2 were about 23.2, 20.1, 20.6, 26.3, 1.2, 3.4, and 5.2% of the total deoxynucleosides, respectively (Fig. S2).

Since base paring requires equimolar A and T as well as G and C, a difference of about 3.1% between A and T (23.2 versus 20.1%) suggests that a T analog is present in Smp14 DNA. Unknown deoxynucleoside 1 was estimated to be 3.4% of the total deoxynucleosides, which is close to the difference between the A and T levels, suggesting that unknown deoxynucleoside 1 might be a T analog. By analogy, unknown deoxynucleoside 2 was about 5.2% of the total deoxynucleosides, which is close to the difference between the C and G contents (6.9%), suggesting that the unknown deoxynucleoside 2 might be a G analog. Numerical analysis results suggested that the Smp14 genomic DNA has a G + C content of about 53.3%. Furthermore, presence of different modified bases (e.g. m6A present in T4 is absent from Smp14) suggests that Smp14 has modification systems different from those of T4 (Miller et al. 2003).

The three types of modified deoxynucleosides, m4C and the two unknown deoxynucleosides, were 9.8% of the total deoxynucleosides. These modified bases should occur about once every 5 bp, assuming that the bases distribute randomly. Therefore the restriction sites with a recognition sequence of more than 5 bp, including the sites for most of the enzymes we used, would be refractory to digestion. In this study, the sum total of the size of

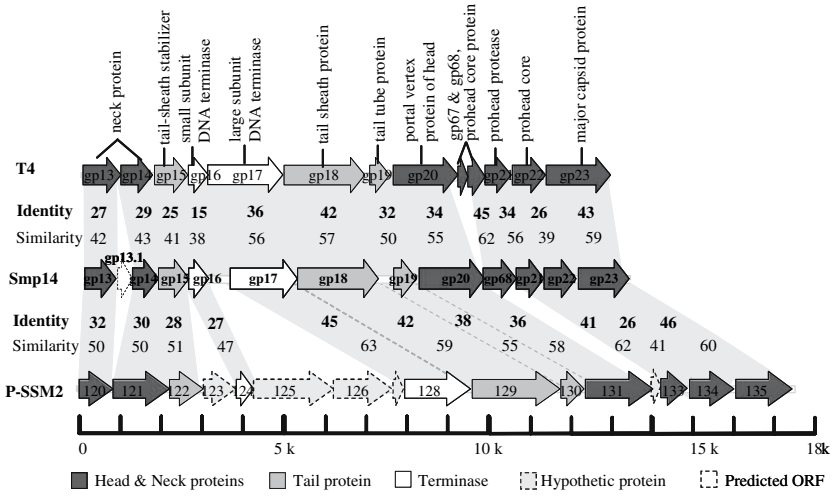

Fig. 3 Organization of the genes coding for mostly virion proteins of bacteriophage T4, Smp14 (coordinates bp 3,359 to 16,651), and P-SSM2. Percent identities and similarities at amino acid sequence level, determined by BLAST (NCBI) and T-COFFEE (EMBnet), are shown between the maps

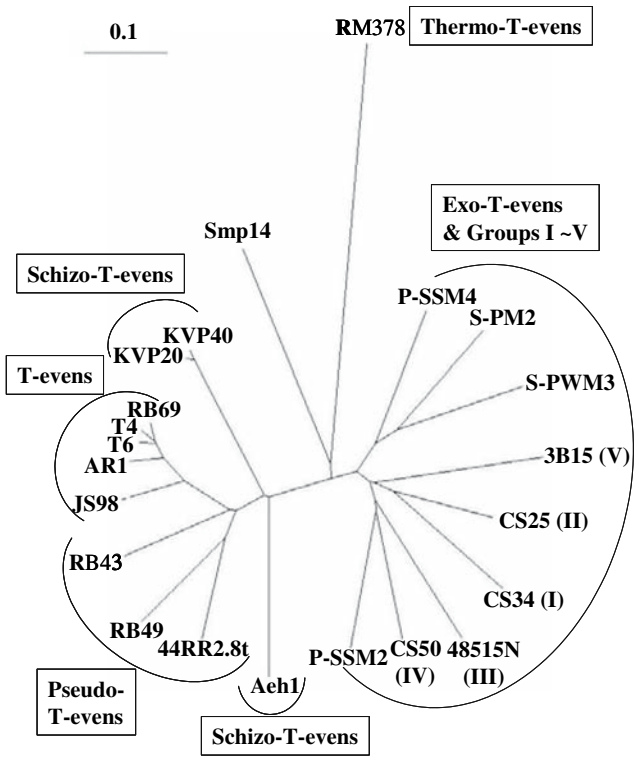

Fig. 4 An unrooted phylogenetic tree based on the central conserved sequences of the major capsid proteins (gp23), corresponding to aa 110-303 of T4 gp23, from 17 T4-type phages and 5 uncultured marine phages. The T4-type phages, with NCBI accession number in parenthesis, are Aeh1 (AAF61695), AR1 (AAD01755), JS98 (AAU29300), KVP20 (BAA25880), KVP40 (NP-899609), nt-1 (AAF61697), PSSM2 (YP-214367), P-SSM4 (YP-214669), RB43 (YP-239203), RB49 (AAQ15480), RB69 (NP-861877), RM 378 (NP-835728), S-PM2 (YP-195142), S-PWM3 (CAC47965), T4 (NP-049787), T6 (CAB01541), 44RR2.8t (AAF61693), and 65 (AAF61694). The clones from the uncultured marine phages are CS25 (DQ105917), CS34 (DQ105922), CS50 (DQ105924), 3B15 (DQ105930), and 48515N (DQ105892)

*Mse*I fragments (Fig. S1b) was very much larger than the phage genome size (160 kb), indicating that the T-modified bases were possibly randomly distributed, rendering the Smp14 genomic DNA only partially digestible by *Mse*I.

A 16 kb region of the Smp14 genome encodes proteins with high degrees of similarity to those of T4 type phages

A 16,703 bp region of the Smp14 genome was determined. The G + C content (54.3%) of this region was close to that calculated by HPLC analysis (~53.3%). This region had 14 predicted ORFs (Table S1), which had the same order as and encoded proteins (mostly structural proteins) with up to 46% identity and 63% similarity to those of the T4-type phages. Figure 3 depicts the genome organization and identity/similarity between those of T4, Smp14, and the T4-type cyanophage P-SSM2 (Chibani-Chennoufi et al. 2004; Sullivan et al. 2005). The Smp14 ORFs were assigned using the numbering system of T4 phage. When an additional gene was present, a two-part number was assigned: the first integer corresponds to the preceding gene and is followed by a period and a second digit (e.g. the ORF between gp13 and gp14 was named gp13.1). As shown in Fig. 3, the order of genes was gp13 (neck protein), gp13.1 (hypothetical gene), gp14 (neck protein), gp15 (proximal tail sheath protein), gp16 (terminase small subunit), gp17 (terminase large subunit), gp18 (contractile protein of the tail sheath), gp19 (tail tube protein), gp20 (portal vertex protein), gp68 (core protein), gp21 (prohead protease), gp22 (prohead core), and gp23 (major capsid protein). In T4, gp20 and gp21 are separated by two small genes (gp67and gp68), both gp67 and gp68 were absent and replaced by a hypothetical gene (orf132) in P-SSM2 and gp67 only was absent from Smp14. In addition, insertion of gp13.1 between gp13 and

gp14 occurred only in Smp14 but not in the genome of T4-type phages (Desplats et al. 2002; Mesyanzhinov et al. 2004; Sullivan et al. 2005; Nolan et al. 2006). Surprisingly, gp13.1 had significant similarity with the hypothetical protein P45 of *P. aeruginosa* temperate phage F116 (Byrne and Kropinski 2005). Five hypothetical genes in P-SSM2, orf123, orf125, orf126, orf127 and orf132 were absent from the corresponding region of Smp14 and T4 (Fig. 3).

Phylogenetic analysis suggests that Smp14 forms a new subgroup of T4-type phages

According to phylogenetic relatedness of the major head proteins, T4-type phages are divided into at least ten subgroups: T-evens, pseudo-T-evens, schizo-T-evens, thermo-T-evens, exo-T-evens and group I∼V (Filee et al. 2005). To determine the phylogenetic relatedness, the sequence of Smp14 gp23 was aligned with the major capsid proteins from 21 T4-type phages in the database for the construction of a phylogenetic tree. As shown in Fig. 4, all T4-type phages share a common distant ancestor no matter how diverse they are. Although sharing the nearest common ancestor with the thermophilic phage RM378 (Filee et al. 2005) at a very long evolutional distance, phage Smp14 does not belong to any known T4 subgroup. Thus, Smp14 alone forms a new T4-type subgroup.

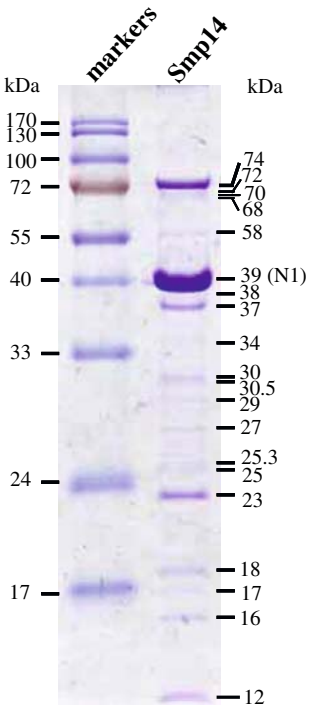

Fig. 5 SDS-polyacrylamide gel (12%) electrophoresis of the Smp14 virion proteins. About 2.5×10^9 PFU of purified phage particles were boiled in sample buffer (20 µl) and loaded onto the well. The 20 bands with estimated molecular masses are shown. Bands N1 was recovered from the gel and subjected to sequence determination of the *N*-terminal ten amino acid residues

The Smp14 virion consists of at least 20 proteins

SDS-PAGE of the Smp14 virion proteins revealed at least 20 bands (Fig. 5). The most abundant one, 39 kDa in size (N1), was recovered from the gels and subjected to *N*-terminal sequencing. The sequence obtained was SSNVTSGVQN, which matched to aa 53–62 of the predicted Smp14 gp23 (the major capsid protein), indicating a cleavage to remove the N-terminal 52 aa. This region is shorter than that in phage T4 where 65 aa is removed to form the mature gp23* (Isobe et al. 1976).

In conclusion, three points are worthy stressing. One, all properties of Smp14 revealed here demonstrate it to be different from phiSMA5. Two, the modification systems in Smp14, which are different from that in T4, deserve further study and genome sequencing will be the first step to take. Three, as one of the problems remain to be solved in phage therapy is the development of phage resistance by the bacterial hosts, it is necessary to use cocktails containing several phages in one preparation to reduce the probability of resistance development and to cover a breadth of host range (Kumer et al. 2007). To achieve this end, more virulent Sm phages will be isolated, which in conjunction with phiSMA5 and Smp14, and probably with antibiotics, can be developed into therapeutic cocktails.

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