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A generalized transducing thiophage (TPC-1) of a facultative sulfur chemolithotrophic bacterium, *Bosea thiooxidans* CT5, of α-*Proteobacteria*, isolated from Indian soil

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Abstract

We have isolated and characterized a double-stranded DNA bacteriophage (TPC-1) of *Bosea thiooxidans*, a facultative sulfur chemolithotrophic bacterium. The name 'thiophage' is introduced for phage(s) infecting sulfur chemolithotrophic bacteria. Electron micrographs showed the phage particle with an icosahedral head and a very short wedge-like tail. TPC-1 is classified as the C1 morphotype of the *Podoviridae* family. Restriction map and terminal ends detection by end fill labeling of the TPC-1 genomic DNA showed that the genome is linear with 5′ protruding cohesive termini. Contour length mapping of the DNA genome also revealed it to be a linear fragment with size (~44 kb) corresponding with the size estimated from restriction fragment analyses and proved the non-redundant nature of the linear genome topology. In colorless sulfur chemolithotrophic microorganisms, TPC-1 is the first report of a generalized transducing thiophage.

Keywords: Bacteriophage; Thiophage; Sulfur chemolithotroph; Generalized transduction; *Podoviridae*; *Bosea thiooxidans*

1. Introduction

A large variety of sulfur oxidizing bacteria capable of chemolithotrophy or photolithotrophy inhabiting different kinds of ecological niche were isolated [1]. However, knowledge on sulfur lithotrophy has been acquired primarily from the studies of colorless sulfur bacteria [2,3]. Studies in the genetics and molecular biology of chemolithotrophy are limited compared to many other microbial metabolic processes [3]. Despite significant efforts were made in the isolation and characterization including molecular systematics of a large number of unicellular, colorless, sulfur chemolithotrophs, no bacteriophage has yet been reported which can mediate genetic transduction. In fact, among the large number of sulfur chemolithotrophs there was only a single report [4] of a phage, HT-2, isolated from sewage [5], which was a lytic phage specific to *Thiobacillus novellus* (now *Starkeya novella*). In electron microscopic studies, the phage HT-2 was shown with a polyhedral head and a long tail. Neither any further detail characterization of HT-2 was made, nor do we know the availability of that phage.

Usefulness of generalized transduction has been shown to map regions of bacterial chromosome by moving gene mutations between strains and such genetical studies remain important even after the genomics and post genomics era [6]. We report here the isolation and molecular characterization of a thiophage, TPC-1, specific to *Bosea thiooxidans* [7]. In colorless sulfur chemolithotrophic mi-

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croorganisms, TPC-1 is the first report of a generalized transducing thiophage.

2. Materials and methods

2.1. Bacterial strains and media used

The bacterial strains and the phage used in this study are listed in Table 1. The strains were grown in Luria–Bertani broth (LB) and Luria–Bertani agar (LA) media [8]. Mineral salts-succinate (MSS) agar or mineral salts-thiosulfate (MST) agar media [9] were used in transduction experiment or to select auxotrophic mutants of *B. thiooxidans*.

2.2. Isolation of thiophage specific to B. thiooxidans CT5

Enrichment of soil was performed as described [10]. To 20 g of soil in 50 ml of LB, freshly grown 10.0 ml culture (10^9 cells ml^{-1}) of *B. thiooxidans* CT5 was mixed in a 250 ml Erlenmeyer flask and was incubated at 30°C for a period of 15 days. This was centrifuged at 8000 rpm for 10 min, and the supernatant was filtered through a sterile 0.45 μm Millipore membrane filter and finally chloroform (0.5%) was added to the filtrate. Aliquots of the filtrate were mixed with the fresh host culture ($\sim 10^9$ colony forming units (CFU) ml^{-1}) and kept standing for 10 min. Molten soft agar medium (LA containing 0.7% bactoagar) was added to the mixture, swirl mixed and overlaid on a basal agar medium (LA) in a Petri plate and incubated at 30°C for the development of plaques. A single plaque was picked up with an inoculating needle, suspended in 1 ml of SM buffer (NaCl, 5.8 g; $MgSO_4 \cdot 7H_2O$, 2 g; 1 M Tris–Cl, pH 7.5, 50 ml; 2% gelatin solution 5 ml; distilled water to make up the volume to 1 l; [11]), macerated with the needle and plaque assay was done. A few plaques of the same morphology were again picked up and the same procedure was repeated again to purify the plaques. To get a phage stock of high titer as a starter lysate, plates containing confluent lysis were chosen. To each plate 1–2 ml of SM buffer was added and kept for an hour for the phages to come out in the solution. The resultant suspension was centrifuged at 5000 rpm for 10 min at 4°C. The supernatant (lysate) was used to infect the cells (5×10^8 CFU ml^{-1} in LB) at 5–7 multiplicity of infection (MOI), kept in static for 10 min and incubated at 30°C on a rotary shaker for complete lysis. Chloroform (0.5%) was added to the lysate, mixed well by swirling, kept a few minutes at room temperature and refrigerated at 4°C for 3–5 days before use.

2.3. Purification and electron microscopy of the phage particles and of the phage DNA

Phage particles were purified by CsCl density gradient

Table 1
Bacterial and bacteriophage strains used in this study

Strains	Description/relevant characteristic(s)	Source/reference
Bacterial strains		
B. thiooxidans CT5	wild-type, Sox^+[a]	DSM 13829; unpublished[b]
CT-6B1	Sox^+, Rif^r (spontaneous mutant of CT5)	this study
CT-7B1	Sox^+, Rif^r Sm^r (spontaneous mutant of CT-6B1)	this study
CT-8B1	Sox^+, Sm^r (spontaneous mutant of CT5)	this study
CTM-1[c]	auxotrophic mutant, *ade101* – adenine	this study
CTM-4	auxotrophic mutant, *met104* – methionine	this study
CTM-7	auxotrophic mutant, *leu107* – leucine	this study
CTM-9	auxotrophic mutant, *val109* – valine	this study
CTM-19	auxotrophic mutant, *met119* – methionine	this study
CTM-20	auxotrophic mutant, *his220* – histidine	this study
KCT001	wild-type, Sox^+[a]	DSM 13826; unpublished[b]
KCT002	wild-type, Sox^+[a]	DSM 13827; unpublished
TCK	wild-type, Sox^+[a]	DSM 13828; unpublished
AS001	wild-type, Sox^+[a]	DSM 13831; unpublished
P. versutus(= *T. versutus*)	Sox^+; type strain	ATCC 25364
S. novella (= *T. novellus*)	Sox^+; type strain	ATCC 8093
B. thiooxidans BI-42	Sox^+; type strain	DSM 9653; [7]
E. coli HB101	*rpsl leu pro thi hsd hsm lacY recA*	[11]
Phage strains		
TPC-1	wild-type, phage of CT5	this study
TPC-1-ts-26	temperature-sensitive mutant of TPC-1	this study

[a]Ability to oxidize reduced sulfur compounds and chemolithotrophic growth is denoted by Sox^+.
[b]These strains are the new isolates and will be reported elsewhere (C. Deb, E. Stackebrandt, S. Pradella, A. Saha, and P. Roy, unpublished observations from this laboratory).
[c]Auxotrophic mutants were selected from CT-7B1.

centrifugation [11]. Three purple white colored bands that were formed after ultracentrifugation at 35 000 rpm for 90 min were dialyzed against SM buffer to eliminate traces of CsCl and other salts if any. The dialyzed stock with chloroform (0.5%) was assayed to determine the titer. The purified phage suspension was adsorbed for 1 min to carbon-coated grids and stained with 2% uranyl acetate, resulting in negative staining of the attached phages [12]. Phage DNA, isolated following the standard protocol [11], was dialyzed against TE (10:1; pH 7.5) to obtain purified phage DNA. The TPC-1 DNA was spread using the basic protein monolayer technique [12]. Samples were stained with uranyl acetate and rotary shadowed with platinum [13]. Grids were examined using a Philips 420T electron microscope. Negatives were enlarged and the length of the DNA measured with fine pointed divider made contour length of the phage genome.

2.4. *Physical characterization of the TPC-1 genome: cohesive termini determination and restriction mapping*

To determine the presence and nature of (3′ or 5′ protruding) cohesive termini, their specific location in restricted fragments of TPC-1 genome, aliquots of phage DNA were heated at 65°C for 10 min, and then subjected to end fill labeling with Sequenase version 2.0 (United States Biochemicals, USA) incorporating one radioactive nucleotide ([α-^{32}P]dATP) along with other three cold dNTPs. After the reaction the DNA was ethanol precipitated, resuspended in sterile water, digested with different restriction endonucleases, separated in agarose gel, vacuum blotted on to nylon membrane and autoradiographed.

Single and pairwise double digestions, partial (incomplete) digestions and cloning of *Eco*RI restriction fragments were performed. The restriction analyses and *cos* site determination data were used to construct the restriction map.

2.5. *Temperature-sensitive mutants of TPC-1, auxotrophic mutants of* B. thiooxidans *and transduction experiments*

In 0.2 ml of phage lysate (10^{10} plaque forming units (PFU) ml^{-1}), 0.4 ml phosphate (0.5 M)-ethylenediamine tetraacetic acid (EDTA) (5 mM) buffer (pH 6.0), 20 ml of sterile 1 M $MgSO_4$, 0.5 ml of sterile water and 0.8 ml of hydroxylamine solution (1 M at pH 6.0) were added sequentially, incubated at 25°C for 30 min, plated with host cells and incubated at 25°C to develop plaques. These plaques were examined for selection of temperature-sensitive mutants of TPC-1, considering the permissive temperature 25°C and non-permissive temperature 35°C. Nitrosoguanidine (MNNG)-induced mutagenesis was followed [11] to isolate auxotrophic mutants of strain CT-7B1 (Table 1). The MNNG (50 μg ml^{-1}) treated cells were dilution plated to get isolated colonies to screen for auxotrophs [14].

Transduction experiment was performed following a modified method described earlier [10,15]. Phage (TPC-1 or TPC-1-ts-26) was propagated on the appropriate donor strain. The donor phages were mixed with recipient cells at MOI of 0.1 or 1.0. The infected recipient cells were kept standing for 10 min at room temperature (RT) (for TPC-1) or at 25°C (for TPC-1-ts-26) and then incubated on a rotary shaker for 1 h at 30°C (for TPC-1) or at 25°C (for TPC-1-ts-26). Aliquots were spread on LA containing appropriate antibiotics, mixed with 2.5 ml soft agar and overlaid on LB bottom agar containing the desired antibiotic. Plates were incubated for 2–5 days at 30°C (for TPC-1) or at 35°C (for TPC-1-ts-26) to let grow the transductants. Auxotrophic strains were transduced by infecting with the phages grown on wild-type strain CT5 and transductions to prototrophy were selected on MSS agar plates.

3. Results

3.1. *Isolation of a thiophage, TPC-1, of* B. thiooxidans *CT5*

A lytic phage of the sulfur chemolithotrophic bacterium *B. thiooxidans* CT5 was isolated from natural soils from where the host bacterium was isolated. The phage assay produced approximately 2 mm diameter plaques after 12 h. After prolonged incubation for 2–3 days, the plaque diameter increased to 4–6 mm and few isolated colonies were developed in the center of the plaques. The colonies growing in the plaques were purified and were found resistant to phage TPC-1. These suggest the possibility that the phage TPC-1 could be a low frequency lysogenic phage [6]. The phage TPC-1 failed to infect KCT001, KCT002, TCK, AS001, AS002, *Paracoccus versutus* (formerly *Thiobacillus versutus*), *S. novella*, or *Escherichia coli*, but it was shown to infect *B. thiooxidans* BI-42. High titer, 2×10^{10} to 2×10^{11} PFU ml^{-1}, could be obtained in liquid lysate by infecting log phase culture at 5–7 MOI. Complete lysis, after shaking at 30°C was obtained within 4–5 h. From the one-step growth curve (data not shown) of TPC-1 when propagated on *B. thiooxidans* CT5 or BI-42, the latent period measured was approximately 2 h which was close to the generation time of CT5 in LB medium. Burst size calculated was 50 per adsorbed phage (data not shown).

3.2. *Morphology of TPC-1 and characterization of the TPC-1 genome*

In the CsCl density gradient tube, intact phage particles were concentrated in the bottom band. The middle band contained mostly broken phage heads and the debris

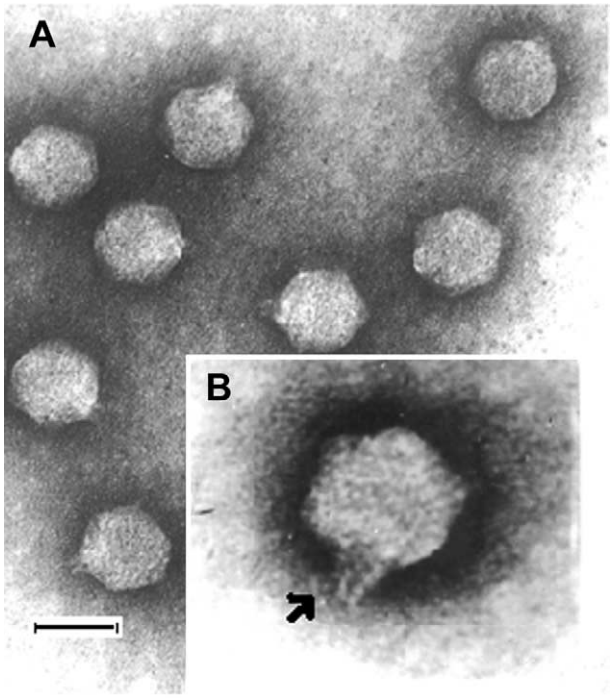

Fig. 1. Electron micrograph of thiophage TPC-1. Polyhedral heads with short tails are observed. Arrow indicates the tail. Bar in A ~60 nm. The insert (B) is the magnified view of a single TPC-1 particle.

formed the top band (data not shown). The phage TPC-1 has an isometric head and a very short wedge-like tail (Fig. 1) which could also be non-contractile [16] with dimensions of about 60 and 12 nm, respectively.

Electron microscopic investigations showed exclusively linear molecules of phage DNA (Fig. 2). The average size of the TPC-1 DNA was determined to be 44 kb by measuring the contour length of single molecules. Two enzymes (viz., *Eco*RI and *Cla*I) were chosen to construct physical map (Fig. 3), as they produced single (Fig. 4A) and double (data not shown) digestion fragments of suitable number and size. Labeling of intact TPC-1 DNA by Sequenase version 2.0 followed by restriction digestions with *Eco*RI or *Cla* I showed two labeled bands (Fig.

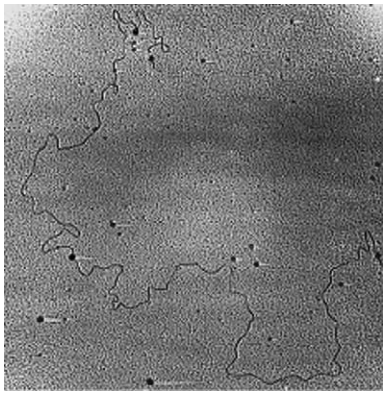

Fig. 2. Electron micrograph of thiophage TPC-1 genomic DNA showing the linear topology. Two free ends of the DNA are indicated by arrows. Bar = ~190 nm.

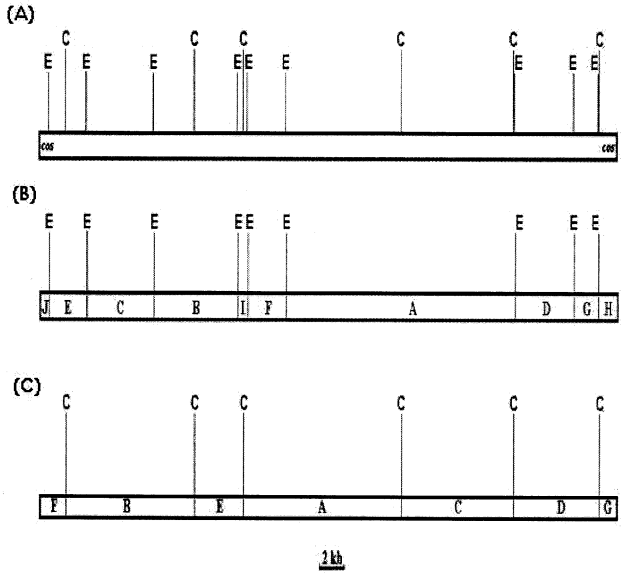

Fig. 3. Restriction map of TPC-1 genomic DNA (44 kb). The double-stranded DNA with cohesive termini and the orientation of restriction fragments is shown for two restriction endonucleases: B: *Eco*RI; and C: *Cla*I. The map is shown in an approximate scale.

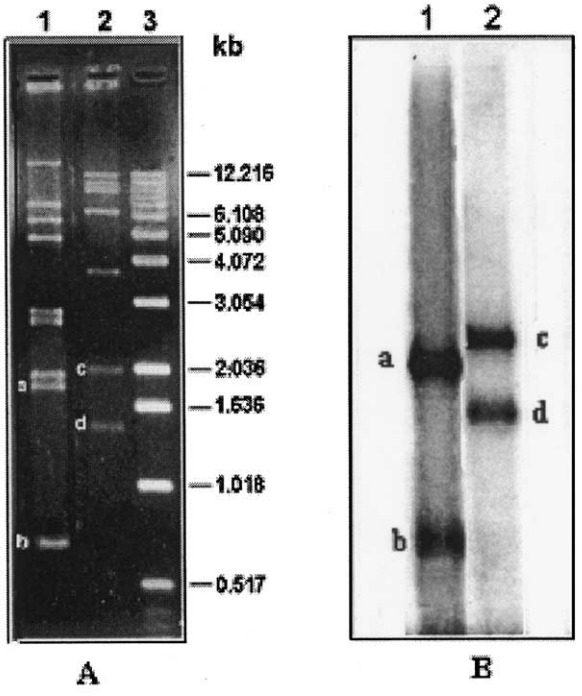

Fig. 4. A: Agarose gel (1%) electrophoresis analysis of restriction endonuclease digested TPC-1 genomic DNA. Lane 1, *Eco*RI; lane 2, *Cla*I; lane 3, 1-kb DNA ladder. B: The autoradiogram showing the end filled TPC-1 DNA by Sequenase version 2.0 DNA polymerase incorporating radioactive nucleotide ([α-^{32}P]dATP) followed by restriction digestion and agarose gel (1%) electrophoresis. Lane 1, *Eco*RI; lane 2, *Cla*I. In both A and B the restriction fragments of TPC-1 DNA [*Eco*RI, 1.7 kb marked as (a) and 0.7 kb marked as (b); and *Cla*I, 2.1 kb marked as (c) and 1.4 kb marked as (d)] having the cohesive ends are indicated.

4B). Restriction of TPC-1 with *Eco*RI generated 10 distinct fragments (Fig. 4A) of which fragments 'a' (~1.7 kb; *Eco*RI-J of Fig. 3) and 'b' (~0.7 kb; *Eco*RI-H of Fig. 3) were detected to be the end fragments (Fig. 4B) bearing *cos* sites. Two fragments of ~0.7 kb following restriction with *Eco*RI migrated at the same position (Fig. 4A) of which one was the terminal fragment. We have been successful to clone all the *Eco*RI restriction fragments except the 1.7-kb and one of the 0.7-kb fragments in the *Eco*RI site of the pBluescript (data not shown). It suggests that those two were the terminal fragments and did not have the compatible *Eco*RI overhangs to be inserted in the *Eco*RI site of the vector. The restriction of end fill labeled genome with *Cla*I (Fig. 4B, lane 2) revealed that the bands 'c' (~2.1 kb, fragment *Cla*I-F of Fig. 3) and 'd' (~1.4 kb, fragment *Cla*I-G of Fig. 3) were the two terminal fragments bearing *cos* sites. As the Sequenase version 2.0 can fill in only 5′ protruding overhang, the labeling of TPC-1 genome ends proved that the *cos* terminal is 5′ protruding. This is also the general property of *cos* ends of phage genome of Gram-negative bacteria [17]. Double digestions with pairwise combination and the determination of *cos* site in the restriction fragments helped to construct and determine the physical map and molecular size (~44 kb) of the genome (Fig. 3) and was in agreement with the size determined by contour length measurement.

3.3. Transduction of chromosomal markers

Wild-type TPC-1 mediated transductions for several markers of *B. thiooxidans* CT5. The auxotrophs were transduced to prototrophy or the antibiotic resistance markers from the resistant strains could be transferred to the wild-type strain (Table 2). Since TPC-1 is a lytic phage it can infect and kill the potential transductants, a temperature-sensitive phage mutant was used and found to increase the transducing efficiency by several folds (Table 2).

4. Discussion

We have isolated several colorless sulfur bacteria, viz., KCT001, KCT002, TCK, CT5, AS001 and AS002 in order to study the genetics of chemolithotrophic sulfur compound oxidation [9]. As a part of this program, we attempted to isolate bacteriophage specific for these new isolates along with several existing sulfur chemolithotrophs including *S. novella* and *P. versutus*. However, success was made only for the isolate CT5, which was classified (Deb et al., unpublished) as a new strain of *B. thiooxidans*. According to its morphology, TPC-1 (Fig. 1) can be included into the group C1 of the *Podoviridae* family [4,16,18] of the order *Caudovirales* [16]. No additional structures, such as fibers or collar, were detected, which corresponds with the structural conformation of several phages of the family *Podovirideae*. The size of the genome measured by contour length mapping corresponded with the length calculated from the restriction fragments suggesting the TPC-1 genome to be a linear double-stranded DNA. In restriction digestions with different restriction endonucleases no bands in submolar amounts were obtained with TPC-1 suggesting that the genome is not circularly permuted or terminally redundant. In a similar study with phage e5, the author confirmed that the DNA was not circularly permuted or terminally redundant [17].

To establish the polarity of the single-stranded extensions, Jacobsen et al. [19] took the advantage of the ability of the large fragment of *E. coli* DNA polymerase I (Klenow fragment) to incorporate deoxynucleoside monophosphate residues at recessed terminal 3′-OH groups of Hp1c1 phage DNA. However, we preferred to use Sequenase version 2.0, a DNA polymerase I without any 3′ exonuclease activity, for labeling selectively the terminal ends of TPC-1 DNA precluding the chances of any translation of nicks within the intact genome. In fact, when Klenow enzyme was used to label the TPC-1 phage DNA followed by restriction digestion, all the restriction

Table 2
Results of generalized transduction with TPC-1 or TPC-1-ts-26

Donor strain[a]	Recipient strain[a]	Relevant marker transduced[a]	Average frequency of transduction transductant × PFU^{-1}		
			TPC-1		TPC-1-ts-26[b]
			MOI 0.1	MOI 1	MOI 1
CT-6B1	CT5	Rif^r	10^{-8}	$<10^{-9}$	10^{-6}
CT-7B1	CT5	Rif^r or Str^r	10^{-8}	10^{-9}	10^{-5}
CT-8B1	CT5	Str^r	10^{-8}	$<10^{-9}$	10^{-5}
CT5	CTM-1	*ade101*	10^{-8}	$<10^{-9}$	10^{-6}
CT5	CTM-4	*met104*	10^{-8}	10^{-9}	10^{-6}
CT5	CTM-7	*leu107*	10^{-8}	10^{-9}	10^{-6}
CT5	CTM-9	*val109*	10^{-9}	$<10^{-9}$	10^{-6}
CT5	CTM-19	*met119*	10^{-9}	$<10^{-9}$	10^{-6}
CT5	CTM-20	*his220*	10^{-9}	$<10^{-9}$	10^{-6}

[a]Donor strain, recipient strain, transduced genetic markers are presented in Table 1.
[b]TPC-ts-26 is a temperature-sensitive mutant of TPC-1.

fragments were found labeled (data not shown). This was not unlikely because of nicks of the DNA and 3′ exonuclease activity of Klenow fragment of DNA polymerase I [11]. Results also revealed that *cos* ends are 5′ protruding, because Sequenase version 2.0 would not have filled in the recessed strand if it would have 3′ protruding termini. Though TPC-1 genomic DNA was shown to possess cohesive ends, we failed to detect the joining of terminal ends by cold treatment or by ligation using T4 DNA ligase. Thus, *cos* ends of the TPC-1 genome might be very small in length like HP1c1 where also no in vitro joining of the terminal fragments could be obtained [20]. Generally, the presence of cohesive ends of DNA genome helps in circularization, which is an obligate configuration for rolling circle mode of replication in fulfilling the lytic cycle of a phage. Moreover, the circular form of a temperate phage genome like lambda phage is essential for integration in the host genome. However, at least a few virulent bacteriophages, e.g., k and N1, showed to possess cohesive termini [21].

Though TPC-1 produced almost clear plaques, from other properties it seems to be a low frequency lysogenic phage or a poor lysogen, as has been observed in other bacteriophages of Gram-negative bacteria [6]. To our knowledge, TPC-1 is the first generalized transducing phage specific for a unicellular colorless sulfur chemolithotrophic bacterium. At least eight genetic loci could be transduced between *B. thiooxidans* CT5 strains. Expectedly isolation, characterization of the phage TPC-1 will help and contribute in the genetics of sulfur chemolithotrophy. The phage TPC-1 being a member of *Podoviridae* is important because amongst the three families of bacteriophages, the members in *Podoviridae* are the lowest and constitute only 14% of the total morphotypes of tailed phages [4,18] and thus demands more studies to reveal their common ecological, physiological and genetic implications.

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