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Characterization and genome sequencing of a *Citrobacter freundii* phage CfP1 harboring a lysin active against multidrug-resistant isolates

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Abstract *Citrobacter* spp., although frequently ignored, is emerging as an important nosocomial bacterium able to cause various superficial and systemic life-threatening infections. Considered to be hard-to-treat bacterium due to its pattern of high antibiotic resistance, it is important to develop effective measures for early and efficient therapy. In this study, the first myovirus (vB_CfrM_CfP1) lytic for *Citrobacter freundii* was microbiologically and genomically characterized. Its morphology, activity spectrum, burst size, and biophysical stability spectrum were determined. CfP1 specifically infects *C. freundii*, has broad host range (>85 %; 21 strains tested), a burst size of 45 PFU/cell, and is very stable under different temperatures (−20 to 50 °C) and pH (3 to 11) values. CfP1 demonstrated to be highly virulent against multidrug-resistant clinical isolates up to 12 antibiotics, including penicillins, cephalosporins, carbapenems, and fluroquinoles. Genomically, CfP1 has a dsDNA molecule with 180,219 bp with average GC content of 43.1 % and codes for 273 CDSs. The genome architecture is organized into function-specific gene clusters typical for tailed phages, sharing 46 to 94 % nucleotide identity to other *Citrobacter* phages. The lysin gene encoding a predicted D-Ala-D-Ala carboxypeptidase was also cloned and expressed in *Escherichia coli* and its activity evaluated in terms of pH, ionic strength, and temperature. The lysine optimum activity was reached at 20 mM HEPES, pH 7 at 37 °C, and was able to significantly reduce all *C. freundii* (>2 logs) as well as *Citrobacter koseri* (>4 logs) strains tested. Interestingly, the antimicrobial activity of this enzyme was performed without the need of pretreatment with outer membrane-destabilizing agents. These results indicate that CfP1 lysin is a good candidate to control problematic *Citrobacter* infections, for which current antibiotics are no longer effective.

Keywords Bacteriophages · Phage lysins · Therapy · Gram-negative bacteria · Antibiotic resistances

Introduction

Citrobacter spp. are ubiquitous Gram-negative, facultative anaerobic bacteria that belong to the *Enterobacteriaceae* family. Currently divided in several species, *Citrobacter freundii* and *Citrobacter koseri* (formerly named *Citrobacter diversus*) are the most common isolated species in human clinical specimens (Janda et al. 1994; O'Hara et al. 2000). *Citrobacter* infections in humans have a prevalence of 3–6 % among all *Enterobacteriaceae*, and the mortality rate reaches 56 % in cases of bacteremia (Samonis et al. 2009). Considered to be emerging as important nosocomial pathogens, *C. freundii* and *C. koseri* are able to cause broad infections in urinary track and wounds and also invasive diseases such as meningitis, bacteremia, septicemia, and pneumonia (Gupta et al. 2003; Samonis et al. 2009). Neonates, immunocompromised, and elderly people are groups with increased risk of infection (Chen et al. 2002).

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Similarly to other *Enterobacteriaceae*, antibiotic-resistance phenotypes of *Citrobacter* spp. have increased in the last years (Doran 1999; Lockhart et al. 2007), delaying successful outcome after health care treatments, potentiating spread of antibiotic resistance and worsening the patient prognosis. Several international surveillance programs have reported the frequent isolation of multi-resistant strains resistant to beta-lactams, quinolones, and aminoglycosides among *C. freundii* and *C. koseri* species (Jones et al. 2000; Perilli et al. 2005; Kanamori et al. 2011). Particularly for *C. freundii*, strains highly resistant to all beta-lactams (e.g., MIC >256 μg/ml for ceftriaxone and cefotaxime), as well as to aminoglycosides (e.g., MIC >256 μg/ml for gentamycin and amikacin), fluoroquinolones (e.g., MIC >32 μg/ml for ciproflioxacin), sulfonamides, tetracycline, tigecycline, and nitrofurantoin, have been detected (Zhang et al. 2008; Institute CaLS 2010; Poirel et al. 2011). New antibiotics in the development pipeline are mostly oriented against Gram-positive species. For all these reasons, the lack of efficient antimicrobials to control *Citrobacter* infections reinforces the importance of finding novel alternative therapies.

Bacteriophages, more commonly referred as phages, are natural predators of bacteria. Since their genetic identity became known, phages have sparked the interest as alternatives for existing antibiotics, essentially for their ability to kill antibiotic-resistant bacteria, high specificity (minimal disruption of normal flora), and low inherent toxicity. Several studies have demonstrated the efficacy of phages in controlling infectious diseases in animals and humans, including those caused by resistant bacteria, giving credibility to phage therapy (Sulakvelidze et al. 2001; Kropinski 2006; Sillankorva et al. 2012). *Citrobacter* phages remain understudied. To our knowledge, besides several *Citrobacter* phages have been sequenced (Edwards et al. 2015; Hwang et al. 2015; LeSage et al. 2015), only three phages were characterized (Petty et al. 2007; Chaudhry et al. 2014; Zhao et al. 2016). In fact, two *Podoviridae C. freundii*-infecting phages (LK1 and phiCFP-1) were partially characterized, being the third phage described as a transducing *Podoviridae* phage specific for *Citrobacter rodentium*.

To broaden the scope of phage therapy that has been limited for *C. freundii* infections, one novel broad-host-range virulent myovirus targeting specifically *C. freundii* (vB_CfrM_CfP1) is here characterized for the first time. This phage was named according to the recent nomenclature proposed and further referred to as CfP1 (Kropinski et al. 2009). CfP1 was fully characterized in terms of physiological and morphological traits, as well for its genomic content, revealing suitable properties for therapy. We also demonstrate that recombinant CfP1 lysin produced from *Escherichia coli* has strong bactericidal activity against several Gram-negative *C. freundii* and *C. koseri* multi-drug resistant strains, without the need of any outer membrane-destabilizing agents.

Materials and methods

Bacterial strains and antibiotic-resistance profiles

Collection strains (including the phage host strain CF#1) were obtained from Salmonella Genetic Stock or the American Type Culture centers. Additional *Citrobacter* clinical isolates were obtained from the Hospital Collection Strain (Braga, Portugal) to study the phage lytic spectrum. This accounts to total of 44 strains used (Table 1), including 39 *Citrobacter* spp. (21 *C. freundii*, 15 *C. koseri*, 1 *Citrobacter amalonaticus*, 1 *Citrobacter youngae*, and 1 *Citrobacter braakii*); 1 *Morganella morganii*; and 1 representative of each *Proteus mirabilis*, *Proteus vulgaris*, *Acinetobacter baumannii*, and *E. coli* species. Clinical isolates typing and antimicrobial susceptibility were performed using Vitek2 (BioMerieux) or Walk Away (Beckman Coulter), according to CLSI guidelines. Beta-lactamases (AmpC)- and extended-spectrum beta-lactamase (ESBL)-producing bacteria were identified using an Etest (BioMerieux) with positive results for cefotetan/cefotetan + cloxacillin and for cefepime/cefepime + clavulanic acid, respectively. All strains were grown at 37 °C in trypticase soy agar (TSA; Oxoid) or trypticase soy broth (TSB; Oxoid) medium.

Phage isolation

The effluent samples used to isolate phages were obtained from a wastewater treatment plant (WWTP) of Frossos, Braga, Portugal. The sample enrichment procedure was performed as follows: 20 ml of centrifuged effluent was mixed with 20 ml double-strength TSB and with 40 μl of each of the exponentially grown *Citrobacter* strains. The resulting suspensions were incubated overnight at 37 °C, 120 rpm (ES-20/60), and centrifuged (10 min, 10,000×*g*, 4 °C). The supernatants were filtered through a 0.22-μm PES membrane (GE Healthcare).

To check the presence of phages, 10 μl of filtered supernatants was spotted in bacterial lawns of all used strains. These plates were incubated overnight at 37 °C and inspected for inhibition halos. When present, they were tested for containing only one single type of phage by repeated picking of the lysate and plaquing onto new bacterial lawns. Plaque picking was repeated until single-plaque morphology was observed. Lysates were stored at 4 °C for further use.

Phage amplification and purification

Concentrated phage solutions were prepared using an adapted method of the plate lysis and elution described by Sambrook and Russel (Sambrook 2001). Briefly, 100 μl phage solution (10^8 plaque-forming unit (PFU)/ml) and 100 μl of the host (CF#1) grown overnight were inoculated in 3 ml on TSA

Table 1 Lytic spectra and efficiency of plating of the *Citrobacter* phage CfP1

Species	Strain	Origin	Patient gender	Antibiotic resistances	Infectivity	EOP[a]
C. freundii	CF#1	EC592 (from Salmonella Genetic Stock Center)			+	High
	CF#2	Feces	Female	Unknown	+	Low
	CF#3	Feces	Female	Unknown	+	High
	CF#4	Urine	Male	AM, CXM, SXT	−	
	CF#5	Urine	Female	AM, AMC, CXM, SXT, MEM, TZP, CT, TZ, CIP, GM, NN, AN, ESBL, AmpC	+	High
	CF#6	Feces	Male	Unknown	+	LFW
	CF#7	Unknown	Unknown	Unknown	+	High
	CF#8	Feces	Female	Unknown	+	LFW
	CF#9	Urine	Female	AM, AMC, CXM, SXT, MEM, TZP, CT, TZ, CIP, GM, NN, AN, ESBL, AmpC	+	High
	CF#10	Urine	Female	AM, AMC, CT, TZ, CXM, TZP	−	
	CF#11	Urine	Male	AM, AMC	+	High
	CF#12	Feces	Female	Unknown	+	LFW
	CF#13	Urine	Female	AM, AMC, CXM	+	High
	CF#14	Urine	Female	Unknown	+	High
	CF#15	Peritoneal fluid	Female	Unknown	−	
	CF#16	Urine	Male	AM, AMC	+	Low
	CF#17	Pus	Male	AM, AMC	+	High
	CF#18	Urine	Female	AM, AMC	+	High
	CF#19	Urine	Male	AM, AMC	+	High
	CF#20	Urine	Male	AM, AMC	+	High
	CF#21	Urine	Male	AM, AMC	+	Low
C. koseri	CK#1	Pus	Male	AM	−	
	CK#2	Urine	Male	AM, AMC, CXM	−	
	CK#3	Feces	Male	Unknown	−	
	CK#4	Pus	Female	AM, CXM	−	
	CK#5	Biopsy material	Male	AM, CXM	−	
	CK#6	Unknown	Unknown	Unknown	−	
	CK#7	Urine	Male	AM, CXM	−	
	CK#8	Urine	Male	AM	−	
	CK#9	Unknown	Unknown	Unknown	−	
	CK#10	Urine	Female	AM	−	
	CK#11	Urine	Male	AM	−	
	CK#12	Ocular Excess	Male	AM	−	
	CK#13	Urine	Male	AM	−	
	CK#14	Urine	Male	AM, AMC, GM, NN, TZP, SXT	−	
	CK#15	Bronchial aspirate	Male	AM	−	
C. amalonaticus	CA	Peritoneal fluid	Female	AM, CXM	−	
C. youngae	CY	Feces	Male	Unknown	−	
C. braakii	CB	Unknown	Unknown	Unknown	−	
M. morganii	MM	CDC 4195–69 (from Salmonella Genetic Stock Center)			−	
P. mirabilis	PM	CECT 4101 (from American Type Culture Center)			−	
P. vulgaris	PV	CECT 174 (from American Type Culture Center)			−	

Table 1 (continued)

Species	Strain	Origin	Patient gender	Antibiotic resistances	Infectivity	EOP[a]
E. coli	EC	CECT 432 (from American Type Culture Center)			–	
A. baumannii	AB	ATCC 19606 (from American Type Culture Center)			–	

Antibiotic resistances of the clinical isolates *AM* ampicillin, *AMC* amoxicillin/clavulanic acid, *CXM* cefuroxime, *SXT* cotrimoxazole, *TZP* piperacillin/tazobactam, *MEM* meropenem, *CT* cefotaxime, *TZ* ceftazidime, *CIP* ciprofloxacin, *GM* gentamicin, *NN* tobramycin, *AN* amikacin, *ESBL* extended-spectrum beta-lactamase-producing bacteria, *AmpC* beta-lactamase-producing bacteria

[a] The EOP was recorded as high and low representing higher and lower than 1 %, respectively, and marked as LFW for lysis from without events

(with 0.5 % agar) and poured into TSA 1.5 % agar plates. Plates were incubated overnight at 37 °C. After 18-h incubation period, 3 ml of SM buffer (100 mM NaCl, 8 mM $MgSO_4$, 50 mM Tris/HCl, pH 7.5) were added to each plate. The plates were agitated at 120 rpm (ES-20/60) at 4 °C for 24 h. Subsequently, the liquid and top agar were collected, centrifuged (10,000×*g*, 10 min, 4 °C), and the supernatant filtered through 0.22-μm PES membrane filters (GE Healthcare). The phage lysate was first concentrated with NaCl (5.84 % (*w*/*v*)) and PEG 6000 (10 % (*w*/*v*)) and then purified with chloroform (1:4 (*v*/*v*)). Samples in SM buffer were stored at 4 °C until further use.

Electron microscopy

Phage particles were first sedimented by centrifugation (25,000×*g*, 60 min, 4 °C) and washed in tap water by repeating the centrifugation step. Afterward, the suspension was deposited on copper grids with a carbon-coated Formvar carbon film on 200 square mesh nickel grid, stained with 2 % uranyl acetate (pH 4.0) and examined using a Jeol JEM 1400 transmission electron microscope (TEM).

Lytic spectra and efficiency of plating

The isolated phages were investigated for host range specificity and lysis efficiency in screening tests against all strains listed in Table 1. Bacterial lawns were propagated on TSA plates by adding 100 μl of exponential-phase cell culture of each strain and subsequently by spotting 10 μl of phage (10^9 PFU/ml and 10-fold dilution series). After an overnight incubation at 37 °C, results were observed and scored. The relative efficiency of plating (EOP) was calculated as the titer of the phage (PFU/ml) for each isolate divided by the titer for the propagating host.

One-step growth curve

One-step growth curve experiments were performed as described previously (Sillankorva et al. 2008). Briefly, 10 ml of a mid-exponential-phase culture (OD_{620} = 0.5) were harvested by centrifugation (7000×*g*, 5 min, 4 °C) and resuspended in 5 ml fresh TSB medium (OD_{620} = 1.0). The multiplicity of infection (MOI) used was 0.001. Accordingly, 5 ml of 10^5 PFU/ml phage solution was added to the prepared host suspensions, and phages were allowed to adsorb for 5 min at 37 °C, 120 rpm (ES-20/60). The mixtures were then centrifuged (7000×*g*, 5 min, 4 °C) and the pellets were resuspended in 10 ml of fresh TSB medium. Samples were taken every 5 min over a period of 30 min and then every 10 min until 1 h of infection. The phage concentration was assessed by plating 100 μl of 10-fold serial dilutions previously mixed with 100 μl of the overnight cultured host and 3 ml TSA (0.4 % agar). Averages ± standard deviations for all experiments are given for n = 3 repeats.

Stability with pH and temperature

Thermal stability tests were carried out by incubating 10^8 PFU/ml of phage at −20, 4 (as a control), 37, 42, 50, and 60 °C for 24 h. Similarly, the effect of pH was also evaluated using a universal pH buffer (150 mM potassium chloride, 10 mM potassium dihydrogen phosphate, 10 mM sodium citrate, 10 mM boric acid) with pH adjusted to 1, 3, 5, 7 (as control), 9, 11, and 13. In both experiments, phage was diluted and plated on *Citrobacter*-sensitive lawns for enumeration. The difference in titration compared to the control experiments allowed us to calculate the relative survival of the phages. Averages ± standard deviations for all experiments are given for n = 3 repeats.

Bacterial challenge test

Overnight cultures were ×1000 diluted in the following day in fresh TSB and grown to an early exponential phase (OD_{620nm} = 0.2). After a ×10 dilution to reach ~10^6 colony-forming unit (CFU)/ml, cultures were split and challenged with phages at MOI of 0.1 (using SM buffer as control). Bacterial growth was monitored by turbidity measurements (OD_{620nm}) over time, taking samples every hour. Averages ± standard deviations for all experiments are given for n = 3 repeats.

DNA isolation and genome sequencing

Phage DNA was extracted from highly concentrated and purified phage stock ($\geq 10^{10}$ PFU/ml) using the methodologies described elsewhere (Sambrook 2001). Afterward, CfP1 was sequenced with an Illumina MiSeq platform conducted in Nucleomics Core (VIB, Belgium). CfP1 genome was mixed with another non-homologous phage (at equimolar ratio) and subjected to quality controls using an Agilent Bioanalyzer. DNA library preparations were made by a custom kit (Nextera XT sample prep) to generate an average insert size of 500 bp. All DNA library preparations were pooled together and sequenced using 150-bp unpaired reads. After processing, reads were trimmed to remove adapters, contaminations, or low-quality sequences. Contigs were assembled with a relatively homogenous coverage with the CLC genomics Workbench version 7 (CLC Bio, Aarhus, Denmark) using the de novo assembly algorithm and manual inspection.

In silico analysis

Citrobacter phage genomes were annotated using MyRAST (Aziz et al. 2008) and manually inspected for potential alternative start codons. The functions of translated open-reading frames were searched by BLASTP and PSI-BLAST programs (Altschul et al. 1990) (*E* value $\leq 10^{-5}$) and HHPRED (Soding et al. 2005) server, consulted between October and December 2015. Putative transmembrane domains were assigned using Phobius (Kall et al. 2004), TMHMM (Kall and Sonnhammer 2002), and HMMTOP (Tusnady and Simon 2001) software. Signal peptides were predicted by SignalP 4.1 algorithm (Bendtsen et al. 2004). Protein pI, molecular mass, and GC content were calculated using Geneious (Kearse et al. 2012) built-in tools. Transfer RNAs (tRNAs) were scanned using tRNAscan-SE (Schattner et al. 2005) and ARAGORN (Laslett and Canback 2004). The DNA homology comparisons between phages were conducted with progressiveMauve (Darling et al. 2010) and EMBOSS stretcher (Myers and Miller 1988; Rice et al. 2000). The protein homology was assessed using Coregenes (Zafar et al. 2002). Circular map was rendered with CGView (Stothard and Wishart 2005).

Expression plasmid construction

Primers were designed to amplify the CfP1 putative lysin (forward GGCCATGGGCATGTTTAAATTTAGTCAAAAGAGTATCAATAAC and reverse GGCTCGAGTGCAAGTTCAATATGCGG) by PCR with Vent DNA polymerase (Stratagene), with restriction cloning sites of NcoI/XhoI underlined. The PCR product was digested with proper endonucleases (New England Biolabs) and inserted into the same restriction sites of pET28a (Novagen), resulting in the plasmid pET28a–CfP1 lysin with a C-terminal His tag. Digested DNA fragments were purified with DNA Clean & Concentrator™-5 k (Zymo Research) and ligated with T4 DNA ligase (New England Biolabs), according to the manufacturer's protocols. Plasmid was propagated and maintained in *E. coli* TOP10. The sequence of the inserts in the plasmid was confirmed by sequencing (GATC Biotech) with T7 primer pair.

Recombinant protein production and purification

E. coli BL21(DE3) cells harboring each recombinant plasmid was grown at 37 °C in LB medium supplemented with the appropriate antibiotics (50 μg/ml of kanamycin) to an OD_{620nm} of 0.6 and recombinant protein expression induced by IPTG (0.5 mM final concentration) overnight at 18 °C. Cells were recovered by centrifugation, resuspended in lysis buffer (50 mM Tris pH 8.0, 250 mM NaCl, 20 mM imidazole, 5 mM $MgCl_2$, supplemented with 1 mM PMSF), and disrupted by sonication on ice. Soluble cell-free extracts were collected by centrifugation, filtered, and loaded on a 5 ml Niquel Hi-Trap column (GE Healthcare) for recombinant protein purification by immobilized metal ion affinity chromatography (IMAC). Purification was conducted according to the manufacturer's instructions, using 50 mM Tris pH 8.0, 150 mM NaCl with 20 or 40 mM imidazole as running and washing buffer, respectively, and with 300 mM imidazole for the elution buffer (Costa et al. 2013). Purified protein was analyzed by SDS-PAGE using 15 % (*w*/*v*) acrylamide gel, followed by Coomassie Brilliant Blue staining. Protein buffer was exchanged with 10 mM HEPES pH 7.2 (plus 5 % (*v*/*v*) glycerol for recombinant lysin) using PD10 columns (GE Healthcare). The concentration of the recombinant protein was estimated from the absorbance at 280 nm using the respective molar extinction coefficients. Recombinant proteins were maintained at 4 °C until their use in subsequent in vitro studies.

Recombinant protein activity assay

Antibacterial assays were performed access the activity of CfP1 lysin using CF#1 mid-exponentially grown cells (OD_{620nm} of 0.6) suspended to a final concentration of $\approx 1 \times 10^7$ CFU/ml into 20 mM of different buffer systems (sodium citrate, HEPES, or boric acid for pH 6, 7–8, and 9, respectively) and ionic strengths (0–500 mM NaCl). Cultures were incubated for 2 h at 37 °C with the same volume of CfP1 lysin (3 μM final concentration) or buffer. At optimal conditions, the CfP1 lysin was further tested against multidrug-resistant *Citrobacter* isolates (CF#5, CF#9, CK#1, CK#2, and CK#14) in presence and absence of 0.5 mM EDTA. In all cases, CFUs were counted and the antibacterial activity assessed as the relative inactivation in logarithmic units (=log 10 (N_0/N_i) with N_0 = number of untreated cells (in the negative control) and N_i = number of treated cells counted after incubation). Averages ± standard deviations are given for $n = 4$ repeats.

Statistical analysis

Mean and standard deviations were determined for at least three independent experiments, and results were presented as mean ± SD. Statistic analyses were performed using a Student's *t* test with a *P* value <0.05.

Nucleotide sequence accession numbers

CfP1 genome was deposited in the NCBI GenBank database with the accession number KX245890.

Results

Citrobacter strains are multidrug resistant

A total of 39 *Citrobacter* spp. (21 *C. freundii*, 15 *C. koseri*, 1 *C. amalonaticus*, 1 *C. youngae*, and 1 *C. braakii*) were isolated from the feces, urine, peritoneal fluid, pus, biopsy material, bronchial aspirate, ocular excess, and peritoneal fluid samples from male and female patients admitted to Hospital de Braga (Portugal) (Table 1). The majority of strains (48.7 %) were isolated from urine, indicative of the most common route of infection. Furthermore, *C. freundii* (53.8 %) and *C. koseri* (38.4 %) were the most predominant species confirming their higher clinical significance compared to other *Citrobacter* species. Regarding antibiotic-resistant profiles, most strains were resistant to ampicillin and amoxicillin/clavulanic. *C. freundii* isolates (CF#5 and CF#9) were resistant to 12 antibiotics, including penicillins, cephalosporins, carbapenems, and fluroquinoles. These multidrug-resistant strains were also positive for AmpC and ESBL genes.

CfP1 is a new *C. freundii*-infecting phage resembling *Myoviridae* viruses

Clinical characterized *C. freundii* strains were used to enrich phages from wastewater treatment plant sewage samples. Phage vB_CfrM_CfP1 (CfP1) was isolated and its plaque morphology is characterized by clear and uniform plaques on the host strain (0.8 mm in diameter) with small haloes (0.1 mm in diameter) on 0.4 % agar plates (n = 5). TEM images show a typical morphology of the *Myoviridae* family (Fig. 1) (Ackermann 1996). CfP1 has an icosahedral head with a 94-nm diameter and a contractile tail of 126 × 19 nm with short-tail fibers. Morphological analysis also reveals a neck, conspicuous transverse tail striations, a baseplate, and terminal short spikes with ~15-nm length in both phages. To further characterize the CfP1 infection cycle, one-step growth curve was performed. CfP1 had a latent period of 25 min with average burst size of 45 PFU per infected cell.

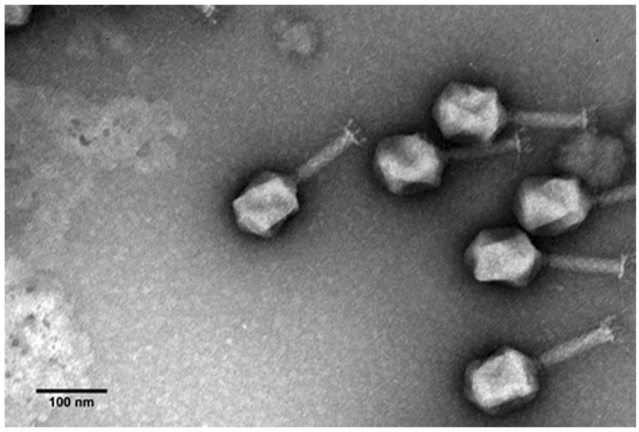

Fig. 1 Electron micrographs of newly isolated *Citrobacter* phage CfP1. TEM image of CfP1-infecting *C. freundii* was negatively stained with 2 % uranyl acetate

CfP1 is species-specific and has a wide host lytic range among clinical isolates

A comprehensive panel of 45 human clinical isolates and reference strains were used to determine the phage lytic range and its efficiency of plating (Table 1). Interestingly, CfP1 has a broad lytic range and is able to infect strains from different origins and antibiotic resistance profiles. CfP1 lyses 18 out of 21 *C. freundii* strains (85.7 %). Clear lysis plaques and broad host ranges suggest that CfP1 is strictly virulent. In addition, CfP1 is specific, unable to lyse close-related (*C. koseri*, *C. amalonaticus*, *C. youngae*, and *C. braakii*) and more distant *Enterobacteriaceae* species. CfP1 is therefore a broad-host-range virus. Regarding EOP experiments, in general, CfP1 displays high to moderate EOP, with some cases of lysis from without events.

CfP1 is able to withstand various pH and temperature conditions

To continue assessing phage potential for therapy, stability tests were made exposing phages to different temperatures and pH for 24 h (Fig. 2). Notably, CfP1 was very stable between −20 and 50 °C, maintaining the titer under these conditions. CfP1 was completely inactivated at 60 °C. No loss of phages were observed in the range of 5 to 11, but its titer dropped by approximately 1 log unit at pH 3 and was completely inactivated at extreme pH values of 1 and 13. Furthermore, CfP1 remained active for 6 months at 4 °C without loss of titer (data not shown).

Antibacterial assays show that CfP1 can inhibit *Citrobacter* growth

To assess the antimicrobial potential, the newly isolated phage was added to cultures containing either CF#1 or CF#9 strains

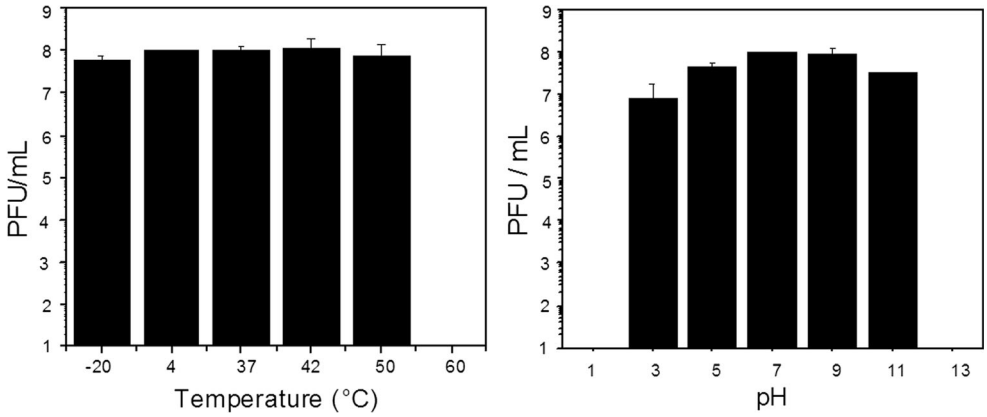

Fig. 2 Biophysical stability. Effect of different temperatures (*left*) and pH (*right*) on CfP1 for 24 h, measured as phage relative survival, i.e., amount of phages enumerated compared with control samples marked with *asterisk*. Averages and standard deviations of three repeated experiments are given

at MOI of 0.1, monitoring changes in the OD over time. In absent of phages, cultures grew exponentially to an OD value of approximately 1.0. In opposite, the presence of CfP1 efficiently inhibited the growth of both CF#1 and CF#9. It took between 8 and 9 h to detect the bacterial regrowth, even when using higher phage doses with a MOI of 10 (data not shown). Therefore, the antibacterial effect of CfP1 proved to be efficient to reduce *Citrobacter* populations to residual numbers up to 8-h incubation period. Inhibition on a longer term or even prevention of phage-resistant phenotypes could be achieved by combining phages (cocktail) that exploit different mechanics of infection (e.g., bacterial receptors) (Gu et al. 2012).

CfP1 genome has similarity to T4-like genomes

CfP1 has a linear genome with a size of 177,970 bp and a GC content of 43.1 %. Based on MyRast algorithm, CfP1 encodes 273 genes, of which 102 with putative function and 171 considered hypothetical/novel (Table S1). Most predicted genes exhibit homology to known proteins of T4virus, mostly to *Citrobacter* phage Miller and *Enterobacteria* phage RB43. Additionally, CfP1 contains a single tRNA coding for Met located between two genes (gp75 and gp76) with unknown function. In terms of genome organization, the core genes were found to encode functions related to DNA replication, recombination and modification, DNA packaging and structural proteins, and cell lysis (Fig. 3). In the left arm of the CfP1, genome is located the majority of proteins related to the DNA replication, recombination, and modification. These proteins resemble those found in the Miller and RB43 phages, which include DNA topoisomerases (gp06 and gp07), DNA helicases/primases (gp25, gp28, gp35, gp38), a RNA polymerase (gp44), and endonuclease or exonuclease a (e.g., gp17, gp49, gp53, gp55, gp66), in contrast with only one endonuclease predicted in Miller genome (Hwang et al. 2015). In the mid-range of the genome, a small group of DNA packaging and structural genes such as baseplates (gp102–103, gp107–109) and tail tubes (gp102–103) proteins are found but is mostly covered by hypothetical proteins. In the right genome arm, there is a larger group of structural proteins coded in a canonical order from gp189 to gp217. CfP1 lytic cassette was also fully annotated and is dispersed throughout the genome, comprising a class III holin (gp265), a lysin (gp153), the Rz and Rz1 subunits of the spanin (gp68–69), and a rI-like antiholin (gp140).

Comparing to other *C. freundii*-infecting phages, CfP1 shares 94.4, 92.2, 76.3, 46.4, and 46.3 % nucleotide sequence identities with Miller (NC_025414.1), IME-CF2 (NC_029013.1), Margaery (NC_028755.1), Merlin (NC_028857.1), and Moon (NC_027331.1), respectively. CfP1 has several collinear regions them, but with Margaery and Merlin, some sequence-block rearrangements were observed, possibly explained by diverging at some points during the course of the evolution (Fig. S1). At protein level, homologies when compared with the same *Citrobacter* phages range from 98, 64, 93, 44, to 43 %, respectively. Interestingly, the CfP1 RNA polymerase (RNPA), which is located at the left arm of the genome, has no identity with any of *Citrobacter* phage proteins. These phages are assigned to the T4 virus genus (Edwards et al. 2015; Hwang et al. 2015; LeSage et al. 2015). However, when CfP1 was compared to other T4 virus, the protein homology is no higher than 84 % for *Klebsiella* phage KP15 (NC_014036.1) or 42 % for *Enterobacteria* phage RB14 (NC_012638.1). All these five *Citrobacter* phages were isolated from sewage samples in USA, and their genomes were recently announced without being characterized in vitro (Edwards et al. 2015; Hwang et al. 2015; LeSage et al. 2015). Overall, CfP1 characteristics fall into the T4 virus major subtype (Grose and Casjens 2014), although having less identity at nucleotide (46.1 %) and protein (41 %) level when compared to *Enterobacteria* phage T4.

CfP1 gp153 encodes a globular lysin with high and broad bactericidal activity

In silico analysis of CfP1 gp153 predicted a 131-amino acid protein (16.0 kDa) with an L-alanoyl-D-glutamate peptidase activity. Surprisingly, despite the presence of a protective outer membrane of the Gram-negative *Citrobacter* cells, the CfP1

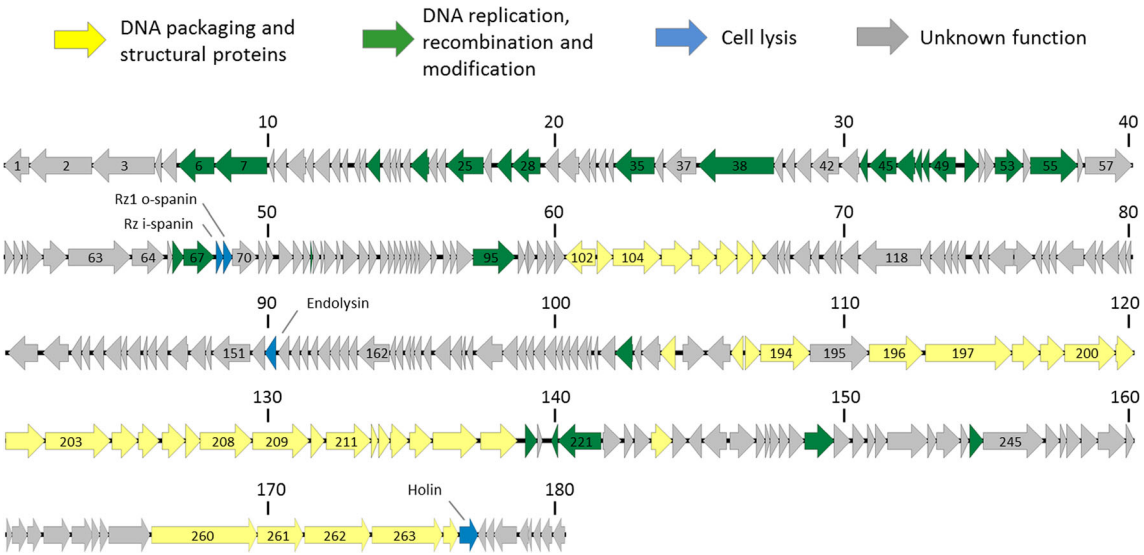

Fig. 3 Genome overview of the *Citrobacter* phage CfP1. Genome map with predicted 273 CDSs numbered and colored (*yellow*, *green*, *blue*, and *gray*) according to their predicted function. Some important CDSs are highlighted. Above genome, the nucleotide position in kb is given

lysin showed intrinsic antibacterial activity and was able to reduce the number of CF#1 cells in a pH-dependent manner (Fig. 4a), achieving maximum reduction (>3 logs) at pH of 7 in 2 h. With the addition of 50 mM NaCl, the antibacterial activity of the lysin at pH 7 was reduced to 1.02 ± 0.28 log and was completely inactivated at higher ionic strengths (Fig. 4b). At optimal conditions (20 mM HEPES, pH 7), the lysin was also able to reduce the number of stationary CF#1 phase cells by 2.93 ± 0.10 logs in 2 h (data not shown). Additional tests also demonstrated the strong antibacterial activity of the lysin against highly resistant *C. freundii* and *C. koseri* isolates (Fig. 4c). Interestingly, the reduction of the cell number of *C. freundii* strains (CF#5, CF#9, and CF#10) between 2 to 3 log units was lower than the >4 log units achieved for *C. koseri* strains (CK#1, CK#2, and CK#14). This means that the lysin has a broader spectrum of activity than CfP1 phage, which is specific to *C. freundii* species. Furthermore, while the addition of the permeabilizing agent 0.5 mM EDTA alone did not affect the viability of *Citrobacter* cells, when it was combined with CfP1 lysin, complete eradication (>5.5 logs) was observed for several strains.

Discussion

Multidrug-resistant bacteria are becoming increasingly prevalent worldwide (Spellberg et al. 2008). The rise of multidrug-resistant strains is especially problematic for Gram-negative pathogens due to the scarce or even inexistence of new active antibiotics, leading to serious life-threatening human infectious diseases (Xu et al. 2014). *Citrobacter* spp. is one pathogen frequently overlooked. The clinical isolates used in this study demonstrated a high resistance to antibiotics. Some isolates from *C. freundii* species are resistant to 12 antibiotics of different classes (CF#5 and CF#9). This resistance levels have been already observed for other known pathogens, such as *Pseudomonas aeruginosa* and *A. baumannii* (Zavascki et al. 2010). Therefore, precaution measures are necessary to manage infections by *Citrobacter* multidrug-resistant strains too.

To find alternative active antimicrobials for the most prevalent *Citrobacter* species, we have isolated and characterized a phage named CfP1, which specially infects *C. freundii*. To our knowledge, only two *Podoviridae C. freundii*-infecting phages (LK1 and phiCFP-1) were characterized so far (Chaudhry et al. 2014; Zhao et al. 2016). Recently, some myovirus genomes infecting *C. freundii* were also announced but without being microbiologically characterized. Therefore, CfP1 is the first myovirus characterized in vitro. Comparatively to CfP1, LK1 and phiCFP-1 presented a similar latent period but with much higher burst size (100 PFU per cell for phiCFP-1 and 801 PFU per cell for LK1), which contributes to their larger plaque sizes (4.5 to 5 mm in diameter) (Chaudhry et al. 2014). Regarding stability, CfP1 was more tolerant to temperature and pH than LK1, the only *C. freundii* phage tested that becomes inactivated at temperatures above 50 °C and pH of 3 after 1 h of incubation (Chaudhry et al. 2014). Therefore, CfP1 seems to hold better characteristics for therapy than phiCFP-1 and LK1. Especially, the ability of CfP1 to infect for example CF#5 and CF#9 strains, which are multidrug-resistant clinical isolates (resistant to 12 antibiotics; isolates producing AmpC and ESLB beta-lactamases), demonstrate its potential use as a alternative antibacterial agent to control hard-to-treat *Citrobacter* infections.

To further characterize the phage antibacterial potential, we have focused our study to analyze the CfP1 lysin antibacterial activity. Therefore, the CfP1 lysin gene was cloned and the recombinant protein was tested against several *Citrobacter*

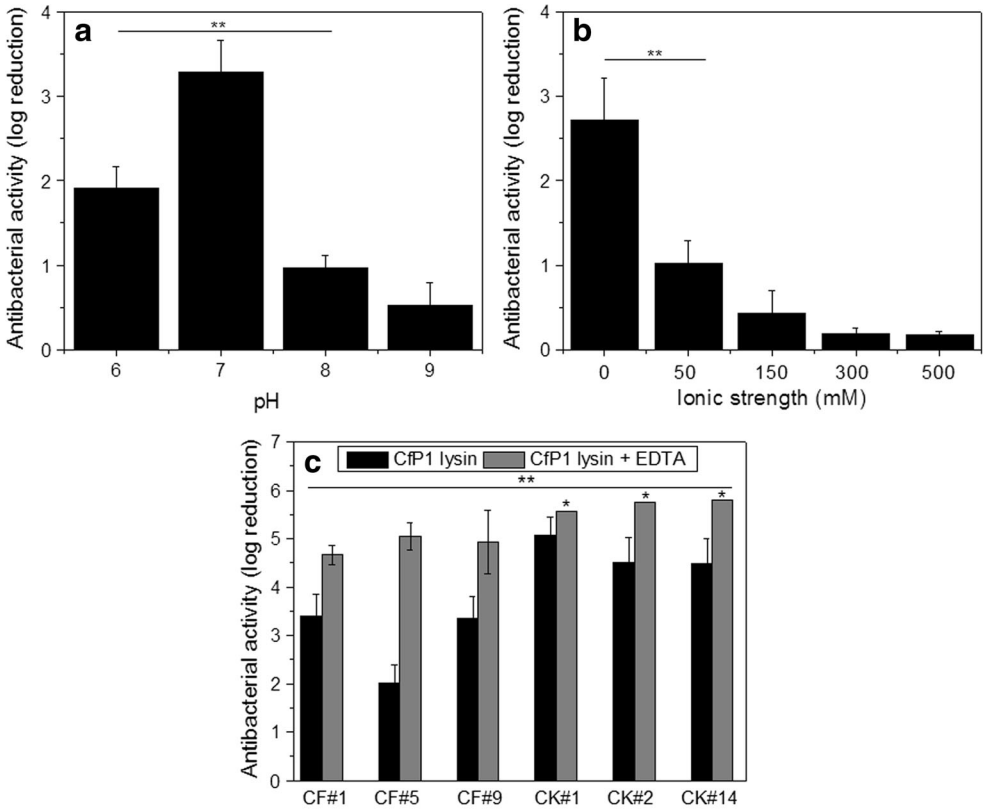

Fig. 4 Recombinant CfP1 lysin antibacterial assays. Lysin activity optimization under different pH values (**a**) and **b** ionic strengths using CF#1 isolate. Exponential growing cells (≈10^7 CFU/ml) were incubated with 3 μM of CfP1 lysin or with buffer (control) for 2 h. Antibacterial activity (**c**) at optimal conditions (20 mM HEPES, pH 7) against six *C. freundii* and *C. koseri* drug-resistant isolates (CF#1, CF#5, CF#9, CK#1, CK#2, and CK#14) challenged with 3 μM of CfP1 lysin in presence or absence of 0.5 mM EDTA. Antibacterial activity was assessed by quantification of the number of CFUs and expressed as relative inactivation in logarithmic units (=$\log_{10}$ (N_0/N_i) with N_0 = number of untreated cells and N_i = number of cells after treatment). Averages ± standard deviations are given for $n = 4$ repeats. *The detection level of 10 CFU/ml has been reached. **Statistically significant (Student's t test and $P < 0.05$) conditions between control and test assays. *CF#* *C. freundii* isolates, *CK#* *C. koseri* isolates

strains. Surprisingly, CfP1 lysin was active against several *Citrobacter* strains, including antibiotic-resistant phenotypes, suggesting that the protein can intrinsically destabilize its outer membrane. The lysin optimum activity was reached at 20 mM HEPES, pH 7 at 37 °C, and interestingly was able to significantly reduce (>3 logs) the number of cells in both *C. freundii* and *C. koseri* multidrug-resistant strains in 2 h. The lysin has therefore a broader spectrum of activity than CfP1 phage. This can be explained because while the lysin degrades the peptidoglycan (A1γ chemotype), which is a common structure shared by among Gram-negative organisms (Schleifer and Kandler 1972), the CfP1 phage activity is limited by its tail fibers that seem to detect only receptors of *C. freundii* strains. On the other hand, the activity of the CfP1 phage seems to tolerate higher pH values better than the recombinant lysin, which indicates that other lytic accessory proteins might have an important role in phage antibacterial activity. The ability of a Gram-negative-specific phage lysin to kill cells "from without" has been solely attributed to few enzymes (e.g., *Enterobacter* phage T4, *Bacillus* phage PlyL, *Acinetobacter* phage PlyF307, and *Pseudmonas* phage OBP lysins) (During et al. 1999; Morita et al. 2001; Walmagh et al. 2012; Lood et al. 2015). This effect was associated to the positively charged amino acids located at the enzyme's C-termini, which is able to disrupt or mediate lysin access to the peptidoglycan, causing subsequent bacteriolysis. We have not found such residues in CfP1 lysin, and therefore, its antibacterial mechanism remains unknown. Covalent modification of lysins with LPS-destabilizing peptides has been also a recent approach to increase the lysin activity against Gram-negative pathogens (Briers et al. 2014a, b; Thandar et al. 2016). These chimeric proteins have additional peptides of polycationic, hydrophobic, or amphipathic nature, known to interfere with the lipopolysaccharide-stabilizing forces, assisting the lysins to spontaneously penetrate the Gram-negative outer membrane barrier and become active antimicrobials. Therefore, the CfP1 lysin's intrinsic activity against Gram-negative pathogens can be further enhanced by tagging such lipopolysaccharide-destabilizing peptides. Taken together, CfP1 lysin seems to be a promising globular protein with stronger and wider spectrum of activity, compared to CfP1 phage.

In summary, we have microbiologically and genomically characterized the first myovirus *C. freundii*-infecting phage, named CfP1. CfP1 is species specific and has broad lytic spectrum, being able to kill multidrug-resistant strains that pose serious threat to human health. CfP1 lysin distinguishes from most phage lysins for its high antibacterial activity against *Citrobacter* pathogens in absence of membrane-destabilizing factors. Both phage and lysin should be further exploited to combat multidrug-resistant *Citrobacter* infections.

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Compliance with ethical standards

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Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants performed by any of the authors. The clinical strains used in this study were collected from patients admitted to the hospital during their treatments and belong to the Hospital Collection Strain.

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