The *Pseudomonas aeruginosa* generalized transducing phage ϕ PA3 is a new member of the ϕ KZ-like group of 'jumbo' phages, and infects model laboratory strains and clinical isolates from cystic fibrosis patients

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Pseudomonas aeruginosa is an important pathogen in cystic fibrosis patients, and a model organism for the study of nosocomially acquired infections, biofilms and intrinsic multidrug resistance. In this study we characterize ϕ PA3, a new generalized transducing bacteriophage for *P. aeruginosa*. ϕ PA3 transduced chromosomal mutations between PAO1 strains, and infected multiple *P. aeruginosa* clinical isolates as well as the *P. aeruginosa* model laboratory strains PAK and PA14. Electron microscopy imaging was used to classify ϕ PA3 in the order *Caudovirales* and the family *Myoviridae*. The genome of ϕ PA3 was sequenced and found to contain 309 208 bp, the second-largest bacteriophage currently deposited in GenBank. The genome contains 378 ORFs and five tRNAs. Many ORF products in the ϕ PA3 genome are similar to proteins encoded by *P. aeruginosa* phage ϕ KZ and *Pseudomonas chlororaphis* phage 201 ϕ 2-1, and so ϕ PA3 was classified genetically as a member of the ϕ KZ-like group of phages. This is the first report of a member of this group of phages acting as a generalized transducer. Given its wide host range, high transduction efficiency and large genome size, the 'jumbo' phage ϕ PA3 could be a powerful tool in functional genomic analysis of diverse *P. aeruginosa* strains of fundamental and clinical importance.

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INTRODUCTION

Pseudomonas aeruginosa is an opportunistic human pathogen and a leading cause of hospital-acquired infections (Trautmann *et al.*, 2005). The organism primarily infects those with compromised immune systems. Individuals are most susceptible to infection when suffering from open wounds and chronic pulmonary conditions, such as cystic fibrosis (Harrison, 2007; Wagner & Iglewski, 2008). *P. aeruginosa* has a large genome, with over 5500 genes, and produces a wide variety of secondary metabolites, allowing it to survive in metabolically diverse environments, including soil, water, plants, insects and animals (Boman *et al.*, 1972; Mahajan-Miklos *et al.*, 2000; Stover *et al.*, 2000). The organism can live as biofilms,

colonies or as planktonic cells (Tolker-Nielsen & Molin, 2004; Wagner & Iglewski, 2008). Due to its metabolic diversity and its importance as a human pathogen, *P. aeruginosa* has become an organism of intense study, so genetic tools for this organism have become increasingly important.

Generalized transducing bacteriophages are useful genetic tools for strain engineering and for functional genomic analysis in strains that are not naturally competent, such as *P. aeruginosa* (Wong *et al.*, 2000). Several *P. aeruginosa* generalized transducing bacteriophages have been described, though the transduction efficiency of these bacteriophages, in particular DMS3, UT1 and F116, can be very low (Budzik *et al.*, 2004; Kidambi *et al.*, 1994; Ripp *et al.*, 1994). Given such low transduction efficiency, occasional background spontaneous mutants can be problematic and strain constructions need to be rigorously policed. Additionally, some mutants affected in cell surface properties can be resistant to specific phage infection, thereby restricting the use of the few transducers available. Furthermore, there have been few studies of the utility of

Abbreviations: EOP, efficiency of plaquing; TEM, transmission electron microscopy.

The GenBank/EMBL/DDBJ accession number for the genome sequence of ϕ PA3 is HQ630627.

Three supplementary figures and two supplementary tables are available with the online version of this paper.

transducing phages isolated from a 'lab' strain for work with clinical isolates, particularly when *P. aeruginosa* clinical isolates can also be hypermutable (Driffield *et al.*, 2008; Oliver *et al.*, 2000; Smith *et al.*, 2006). Therefore, there is a need for new *P. aeruginosa* generalized transducing phages which, ideally, would be able to infect a variety of different strains, both clinical isolates and laboratory model strains, and which would have the capacity to move mutations between strains efficiently.

Here we report the identification of a new *P. aeruginosa* generalized transducing phage, ϕ PA3, which is able to transduce chromosomal markers between *P. aeruginosa* strains with high frequency. ϕ PA3 has a wide host range and is able to infect many laboratory and clinical isolates of *P. aeruginosa*. The genomic sequence of this virus has been determined and it belongs to the ϕ KZ-like family of phages (Mesyanzhinov *et al.*, 2002; Thomas *et al.*, 2008). We present evidence that ϕ PA3, a new member of this growing family of 'jumbo' phages, acts as a generalized transducer with high efficiency.

METHODS

Bacterial strains and growth conditions. Bacterial strains used in this study are listed in Table 1. *P. aeruginosa* and *Escherichia coli* strains were grown in Luria Broth (LB) at 37 °C. *Serratia* sp. 39006 and *Erwinia carotovora* subsp. *atroseptica* were grown in LB at 30 °C. LB agar (LBA) plates were used with 1.5% (w/v) agar and soft agar overlays of 0.35% agar, unless otherwise stated. For *P. aeruginosa* strains, final concentrations of antibiotics used were 100 µg gentamicin ml⁻¹, 200 µg tetracycline ml⁻¹ and 200 µg kanamycin ml⁻¹. Bacterial growth was assessed by measuring OD₆₀₀ using a Unicam Heλios spectrophotometer with a 1 cm path length. A full list of the bacterial strains used in this study is included in Table 1.

Phage isolation and preparation. Treated sewage effluent was collected from Milton, Cambridge. Phage isolation and sample preparation were carried out as described by Petty *et al.* (2006). Phages obtained were plaque-purified four times. High-titre lysates were obtained as described by Petty *et al.* (2006), and following removal of the lysate, an additional spin (1 min, 20 000 g) was carried out in order to remove any 'glutinous' precipitate, which often carried over after the initial spin.

DNA manipulations, isolation and genome sequencing. DNA was extracted from phage virions using the Lambda DNA extraction protocol from the Phase Lock Gel (PLG) kit by 5 PRIME. Lysates isolated as described in Petty *et al.* (2006) were used for all phage DNA extractions. Phage DNA digestions were performed for 2 h at 37 °C and assayed by electrophoresis on a 1% agarose gel. DNA extractions from *P. aeruginosa* were performed using the Qiagen DNeasy kit according to the manufacturer's instructions. 454 DNA pyrosequencing was carried out using one region on a Pico Titre Plate for a Roche Genome Sequencer FLX system. Standard DNA sequencing was carried out on an Applied Biosystems 3730xl DNA Analyzer. The three major contigs were generated by 454 pyrosequencing and assembled using Vector NTI (Invitrogen).

Genome annotation and bioinformatics. ORFs and putative tRNAs in ϕ PA3 were identified using Glimmer, RBSFinder and tRNAScanner. Artemis software (Wellcome Trust Sanger Institute) was used to visualize genomic sequences and assemble an annotated ϕ PA3 genome. PSI-BLAST (Müller *et al.*, 1999) was used to identify homologues of ORFs.

Where appropriate (E-value <0.005), multiple iterations were performed. Artemis Comparison Tool (ACT) was used to compare the ϕ PA3, ϕ KZ and 201 ϕ 2-1 annotated genomes. DoubleACT (http://www.hpa-bioinfotools.org.uk/pise/double_act.html) was used to generate ACT-compatible TBLASTX genome comparison files. tRNA secondary structures were predicted using mfold with the default parameters (<http://mfold.bioinfo.rpi.edu/cgi-bin/rna-form1.cgi>). The genomic organization and annotation of ϕ KZ and 201 ϕ 2-1 were used to determine the location of ORF1 in ϕ PA3. Virion structural proteins from ϕ KZ and 201 ϕ 2-1 were annotated, reflecting their classification by N-terminal sequencing or MS (Mesyanzhinov *et al.*, 2002; Thomas *et al.*, 2008).

Host range determination. Top agar overlays containing 100 µl of the strains listed in Table 1 were poured onto a solid LBA plate, allowed to set and dried for 10 min. A 5 µl sample of serial dilutions of ϕ PA3 and a 5 µl LB control were spotted onto the appropriate top lawn and allowed to dry. Plates were incubated overnight and the number of plaques counted, and the efficiency of plaquing (EOP) was determined by dividing the number of plaques for each strain by the number of plaques on the wild-type control strain PAO1.

Transduction assay. Phages were initially tested for their ability to transduce the *lasI*::Gm or *rhlI*::Tc mutations from the appropriate strains listed in Table 1 into wild-type PAO1. Putative transductants were screened by PCR in order to ensure that the resultant strain contained the appropriate disrupted gene. An optimized transduction protocol was produced following characterization of ϕ PA3. An aliquot (100 µl) of a high-titre lysate ($>1 \times 10^9$ p.f.u. ml⁻¹) was used to infect a 10 ml overnight culture of the recipient strain. The mixture was left static at room temperature for 30 min to allow for phage adsorption. Tubes were transferred to a rotary wheel at 37 °C for 20 min. Cells were pelleted by centrifugation (2220 g for 10 min at 4 °C) and the supernatant discarded. Cells were washed with 10 ml LB to remove any remaining lysate, centrifuged again at 2220 g for 10 min at 4 °C, and the supernatant was removed. The cells were then resuspended in 300 µl LB. Aliquots (150 µl) of the cell mixture were spread onto two agar plates containing the appropriate antibiotic. Plates were incubated at 37 °C overnight. In order to estimate the frequency at which spontaneously resistant mutants arose, a 10 ml overnight culture of uninfected recipient strain subjected to a mock transduction procedure was also plated as a control. As a sterility control, 100 µl of the high-titre lysate used was spread on a solid agar plate.

Electron microscopy. All electron microscopy was performed in The Multi-Imaging Centre (University of Cambridge) using an FEI Philips CM100 transmission electron microscope. Carbon-coated, charge-discharged copper grids were placed in 5 µl phage lysate for 45 s and then washed three times in 5 µl water. Phage samples were then stained by placing the grid in 5 µl 1% uranyl acetate for 45 s. The grids were then blotted on filter paper to remove any excess liquid and allowed to air-dry. Digital images obtained by transmission electron microscopy (TEM) were adjusted uniformly to obtain better contrast using Adobe Photoshop.

Phage adsorption. Phage adsorption experiments were performed as described by Petty *et al.* (2006). Briefly, 10 ml overnight cultures of PAO1 and *E. coli* DH5 α were infected with ϕ PA3 (m.o.i. 0.01) and mixed. A 'no-cell' control mixture was created by mixing the same amount of ϕ PA3 lysate with 10 ml LB. Samples were removed at various time points and treated with chloroform, and the number of unadsorbed ϕ PA3 particles was determined by plating on a lawn of PAO1. Adsorption was expressed as the number of free ϕ PA3 particles per millilitre divided by the number of remaining ϕ PA3 particles per millilitre in the no-cell control or the percentage of free ϕ PA3 particles remaining.

One-step growth curve. A ϕ PA3 one-step growth curve was conducted as described by Petty *et al.* (2006). Overnight cultures of

Table 1. Strains and bacteriophage used in this study

Abbreviations: Tc, tetracycline; Gm, gentamicin.

Strain or plasmid	Relevant characteristics	Source or reference
<i>Erwinia carotovora</i> subsp. <i>atroseptica</i> strain		
SCRI1043	Wild-type strain SCRI1043	Laboratory collection
<i>E. coli</i> strain		
DH5 α	<i>endA1</i> , <i>hsdR17</i> (r _K ⁻ m _K ⁻) <i>glnV44</i> <i>thi-1</i> <i>recA1</i> <i>gyrA</i> <i>relA1</i> Δ (<i>lacIZYA-argF</i>) <i>U169</i> <i>deoR</i> [ϕ 80 <i>dlac</i> Δ (<i>lacZ</i>)M15]	Laboratory collection
<i>P. aeruginosa</i> strains		
PAK	Wild-type strain K	Laboratory collection
PA14	Wild-type strain 14	Laboratory collection
PAO1	Wild-type strain ATCC 15692	Laboratory collection
PAO1 <i>lasI</i> ::Gm <i>rhlI</i> ::Tc	PAO1 with Gm cassette inserted into unique <i>EcoRI</i> site of <i>lasI</i> and with Tc cassette inserted into unique <i>EcoRI</i> site of <i>rhlI</i>	Beatson <i>et al.</i> (2002)
PAO1 <i>lasR</i> ::Gm <i>rhlR</i> ::Tc	PAO1 with Gm cassette inserted into unique <i>SalI</i> site of <i>lasR</i> and with Tc cassette inserted into unique <i>BamHI</i> site of <i>rhlR</i>	Beatson <i>et al.</i> (2002)
PAO1 Δ <i>lasIR</i> ::Gm	PAO1 with Gm cassette replacing <i>PstI</i> fragment shared by <i>lasR</i> and <i>lasI</i>	Beatson <i>et al.</i> (2002)
PAO1 Δ <i>rhlIR</i> ::Tc	PAO1 with Tc cassette replacing <i>BamHI</i> – <i>EcoRI</i> fragment shared by <i>rhlR</i> and <i>rhlI</i>	Beatson <i>et al.</i> (2002)
PAO318	Δ (<i>mexAB-oprM</i>) <i>nfxB</i> Δ (<i>mexCD-oprJ</i>) Δ <i>mexL</i> ::FRT	Chuanchuen <i>et al.</i> (2002)
PAO-7H	MexEF–OprN overproducer	Köhler <i>et al.</i> (1997)
<i>Pseudomonas</i> clinical isolates		
7409		Papworth Hospital*
7509a		Papworth Hospital
7509b		Papworth Hospital
10348	Manchester epidemic strain	Papworth Hospital
10563	Liverpool epidemic strain	Papworth Hospital
10937		Papworth Hospital
11118	Manchester epidemic strain	Papworth Hospital
11784		Papworth Hospital
11792		Papworth Hospital
12596		Papworth Hospital
2Jam2a		Papworth Hospital
1824		D. Hocquet strain collection
2030		D. Hocquet strain collection
2151		D. Hocquet strain collection
2152		D. Hocquet strain collection
2156		D. Hocquet strain collection
2169		D. Hocquet strain collection
2439		D. Hocquet strain collection
2988		D. Hocquet strain collection
3308		D. Hocquet strain collection
<i>Serratia</i> sp. strain		
ATCC 39006 LacA	Lac ⁻ derivative of ATCC 39006	Laboratory collection
Phage		
ϕ PA3	<i>P. aeruginosa</i> generalized transducing phage	This study

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PAO1 were diluted to OD₆₀₀ 0.02 in 25 ml LB in a sterile 250 ml conical flask and grown in a shaking waterbath at 37 °C, 300 r.p.m. At OD₆₀₀ 0.1, 10 ml of the culture was removed into a sterile tube and centrifuged at 2220 g for 10 min at 4 °C. Supernatant was discarded and the pellet resuspended in 10 ml fresh LB. Phage samples were added to an m.o.i. of 0.001. The same amount of phage was also added to a control

containing LB alone. Tubes were left static at room temperature for 5 min to allow the phage to adsorb. Cells were pelleted by centrifugation, and the supernatant containing any unadsorbed phages was removed. Pellets were resuspended in 10 ml LB and added to 15 ml LB in a 250 ml conical flask. Cultures were then grown in a shaking waterbath as described above. Samples were taken every 10 min, and the number of

ϕ PA3 particles was determined by serially diluting samples and determining the number of p.f.u. Final growth curve results were expressed as number of phages per initial infectious centre.

RESULTS AND DISCUSSION

Isolation of bacteriophage ϕ PA3 and optimized transduction

Bacteriophage plaques were isolated by direct plating of treated sewage effluent on *P. aeruginosa* strain PAO1. Individual plaques were plaque-purified as described by Petty *et al.* (2006). Each phage was screened for transduction ability and, of seven phages tested, three were found to transduce either a *lasI*::Gm or *rhlI*::Tc mutation between PAO1 strains. Of these, ϕ PA3 was the most efficient transducer and was chosen for further characterization. ϕ PA3 plaque morphology varied depending on the concentration of agar used in the top lawn overlays. Plaques were largest when plated in 0.35 and 0.25 % agar overlays, measuring up to 2.5 mm in diameter (Fig. 1a). Overlays containing 0.35 % agar were used in all subsequent experiments.

To optimize the transduction procedure, ϕ PA3 transduction frequencies were determined for different m.o.i. values and with different numbers of cells (Table 2). Transduction was most efficient when 10^9 cells were used at an m.o.i. of 0.1 (Table 2). To ensure that this was not an artefact of the particular chromosomal marker used, mutations in *lasI*, *rhlI*, *lasR*, *rhlR*, *lasIR* and *rhlIR* were transduced using 10^9 cells and an m.o.i. of 0.1 (Table 2). In each transduction assay, controls containing untreated cells or lysates alone were also plated to ensure that putative transductants were not due to lysate contaminants or spontaneous mutation (data not shown). All such controls were negative. Furthermore, although *P. aeruginosa* is not naturally transformable, phage lysates were treated with DNase (1 mg ml^{-1} , 30 min) to demonstrate that the putative transduction observed was the result of ϕ PA3 and not transformation due to chromosomal DNA. No differences in transduction efficiency were observed (data not shown). Transduction efficiencies were in many cases 100-fold higher than those observed elsewhere with the *P. aeruginosa* transducing phages DMS3, F116 and UT1 (Budzik *et al.*, 2004; Morrison *et al.*, 1978; Ripp *et al.*, 1994). Therefore, for functional genomics programmes, ϕ PA3 could serve as a powerful tool for facile and rigorous *P. aeruginosa* strain construction.

ϕ PA3 host range

The host range of ϕ PA3 was determined by plaque assays on various *P. aeruginosa* laboratory and clinical strains. ϕ PA3 was unable to plaque on *E. coli* DH5 α , *Serratia* sp. 39006 and *Erwinia carotovora* subsp. *atroseptica* (data not shown). In addition to PAO1, ϕ PA3 was able to plaque on *P. aeruginosa* laboratory strains PAK and PA14, which are widely used as models in the study of *P. aeruginosa* infection (Table 3). On

Fig. 1. Characterization of ϕ PA3. (a) Plaque morphologies of ϕ PA3 on either 0.35 % (left) or 0.25 % (right) LB agar. (b) Electron microscopy images of ϕ PA3 showing contractile tails, baseplate and tail fibre details. Bars, 100 nm.

all three strains, plaques were clear and were indistinguishable from each other (data not shown). Ten of 27 *P. aeruginosa* clinical isolates were susceptible to ϕ PA3 infection. Unlike the clear plaques observed on PAO1, plaques on several clinical isolates were turbid pinpricks less than 0.5 mm in diameter (data not shown). These results may indicate cell surface variation amongst the different clinical isolates tested in this study. Some strains may not make the receptor, or may make a receptor that is occluded. Alternatively, some strains may have post-infection restriction or abortive infection systems (Fineran *et al.*, 2009; Hoskisson & Smith, 2007; Labrie *et al.*, 2010).

ϕ PA3 morphology

The morphology of ϕ PA3 was determined by TEM. ϕ PA3 virions were observed with both an intact and a contracted

Table 2. Transduction efficiency of ϕ PA3

Abbreviations: Tc, tetracycline; Gm, gentamicin.

Transduction of <i>lasIR</i> ::Gm with ϕ PA3		
Number of cells	m.o.i.	Transduction efficiency
1×10^8	0.1	$<1 \times 10^{-7}$
	1	$<1 \times 10^{-8}$
	10	$<1 \times 10^{-9}$
1×10^9	0.1	7.88×10^{-7}
	1	3.22×10^{-7}
	10	2.55×10^{-9}
1×10^{10}	0.1	1.31×10^{-8}
	1	3.2×10^{-9}
	10	$<1 \times 10^{-11}$
Transduction efficiency of mutations*		
Mutation	Transduction efficiency	
<i>lasIR</i> ::Gm	7.88×10^{-7}	
<i>rhlIR</i> ::Tc	2.83×10^{-7}	
<i>lasI</i> ::Gm	8.94×10^{-7}	
<i>lasR</i> ::Gm	7.24×10^{-8}	
<i>rhlI</i> ::Tc	6.15×10^{-7}	
<i>rhlR</i> ::Tc	3.11×10^{-7}	

* 1×10^9 cells, m.o.i. 0.1.

tail (Fig. 1b). An icosahedral head 100 nm in diameter, a 185 nm tail, a 45 nm baseplate and tail fibres 80 nm in length were all observed (Fig. 1b). A contracted tail, 75 nm in length, was observed in several instances (Fig. 1b). ϕ PA3 was classified in the order *Caudovirales* within the *Myoviridae* family, which also contains phages such as *E. coli* phage T4 and *P. aeruginosa* phage ϕ KZ (Ackermann, 2003; Mesyanzhinov *et al.*, 2002). This classification is similar to that made from electron microscopy images of *P. aeruginosa* phage ϕ KZ and *P. chlororaphis* phage 201 ϕ 2-1, but dissimilar to that of DMS3, a smaller *P. aeruginosa* generalized transducing phage (Budzik *et al.*, 2004; Mesyanzhinov *et al.*, 2002; Thomas *et al.*, 2008).

Characterization of ϕ PA3

Adsorption assays were performed as described by Petty *et al.* (2006). After 5 min, over 95 % of ϕ PA3 particles had adsorbed under these conditions (Fig. 2a). After 10 min, >99 % of ϕ PA3 had adsorbed to the cells (Fig. 2a). The number of adsorbed phage particles reached an apparent maximum after 20 min. Therefore, for subsequent transduction experiments, an adsorption time of 30 min was used. As a negative control, adsorption of ϕ PA3 to *E. coli* strain DH5 α was assayed and ϕ PA3 was unable to adsorb to this strain (Fig. 2a).

One-step growth experiments were conducted to assess the timescale and burst size of ϕ PA3. We observed a latent

Table 3. ϕ PA3 host range

Strain	Origin	EOP	Morphology
PAO1	Laboratory collection	1.0	Clear
PAK	Laboratory collection	0.43	Clear
PA14	Laboratory collection	0.7	Clear
PAO318	D. Hocquet strain collection	0.36	Clear
PAO7H	D. Hocquet strain collection	0.16	Clear
1826	D. Hocquet strain collection	$<1 \times 10^{-10}$	
1949	D. Hocquet strain collection	$<1 \times 10^{-10}$	
1956	D. Hocquet strain collection	$<1 \times 10^{-10}$	
2030	D. Hocquet strain collection	4.1×10^{-3}	Turbid
2151	D. Hocquet strain collection	4.6×10^{-4}	Turbid
2152	D. Hocquet strain collection	$<1 \times 10^{-10}$	
2156	D. Hocquet strain collection	$<1 \times 10^{-10}$	
2169	D. Hocquet strain collection	7.1×10^{-4}	Turbid
2439	D. Hocquet strain collection	4.0×10^{-2}	Clear
2988	D. Hocquet strain collection	$<1 \times 10^{-10}$	
3308	D. Hocquet strain collection	$<1 \times 10^{-10}$	
7409	Papworth Hospital	3.2×10^{-4}	Turbid
10348	Papworth Hospital	$<1 \times 10^{-10}$	
(MES)			
10563	Papworth Hospital	$<1 \times 10^{-10}$	
(LES)			
10937	Papworth Hospital	$<1 \times 10^{-10}$	
11118	Papworth Hospital	$<1 \times 10^{-10}$	
(MES)			
11784	Papworth Hospital	$<1 \times 10^{-10}$	
11792	Papworth Hospital	2.0×10^{-2}	Turbid
12596	Papworth Hospital	$<1 \times 10^{-10}$	
1SF2A	Papworth Hospital	$<1 \times 10^{-10}$	
1SF2B	Papworth Hospital	$<1 \times 10^{-10}$	
7509A	Papworth Hospital	2.4×10^{-4}	Pinprick, turbid
7509B	Papworth Hospital	2.1×10^{-4}	Pinprick, turbid
2Jam1A	Papworth Hospital	$<1 \times 10^{-10}$	
2Jam1B	Papworth Hospital	$<1 \times 10^{-10}$	

replication period of 50–60 min, where the number of phage particles remained low before an exponential increase corresponding to phage release (Fig. 2b). The number of phage particles reached a peak 60 min after phage infection, with a burst size of more than 100 particles per infection centre (Fig. 2b).

ϕ PA3 genome sequencing

Bacteriophages can contain heavily modified nucleic acid in order to evade host restriction and modification systems (Bickle & Krüger, 1993; Dinsmore & Klaenhammer, 1995). ϕ PA3 DNA was isolated and proved readily digestible with *ApaI*, *BamHI*, *BglII*, *EcoRI*, *EcoRV* and *NheI*, while *EagI*, *NotI*, *PmeI*, *SphI* and *XbaI* were unable to digest ϕ PA3 DNA (data not shown). Due to the large number of fragments generated after digestion with the restriction enzymes above, estimates of the ϕ PA3 genome size from the restriction pattern were imprecise. Given the large

Fig. 2. (a) Adsorption of ϕ PA3 to *P. aeruginosa* strain PAO1 (◆) and *E. coli* strain DH5 α (■). Free ϕ PA3 particles were measured at the times indicated and expressed as a percentage of the starting number of phages. (b) One-step growth curve of ϕ PA3. The number of phages present was expressed as the number per initial infection centre at each of the times shown. (a, b) Error bars, SD ($n=3$).

number of fragments and their relative sizes, we suspected that the ϕ PA3 genome was over 100 kb in size.

Sequencing of the ϕ PA3 genome was performed using a combination of 454 pyrosequencing and traditional Sanger sequencing. Initial 454 sequencing generated three contigs, 244.8, 58.9 and 5.5 kb in length. The gaps between contigs were filled using traditional Sanger sequencing across the junctions, and any unknown nucleotides were sequenced a second time using Sanger sequencing. Additionally, 3 kb was randomly selected and sequenced on both strands using Sanger sequencing in order to verify the quality of the 454 sequencing. The final size of the ϕ PA3 genome was 309 208 bp.

ϕ PA3 genome annotation

Coding regions were predicted using Glimmer, RBSfinder and tRNAScanner. The genome of ϕ PA3 contained 379 ORFs and five putative tRNAs. Full descriptions of all annotations are included in Supplementary Table S1. Most (89.3%) of the genome was predicted to contain ORFs. The coding regions of ϕ PA3 had a G+C content of 48.0%, which is significantly lower than the 66.6% G+C content of the coding regions in the *P. aeruginosa* PAO1 genome

(Stover *et al.*, 2000). Significant regions of variation in percentage G+C are shown in Fig. 3 (inner ring). No correlations between the areas showing high or low percentage G+C and the coding strand or the predicted function of these regions were observed. None of the regions with significant percentage G+C bias showed characteristics common to recently acquired islands, such as codon bias or tRNA *att* sites (data not shown). Many of the putative products in ϕ PA3 shared high levels of similarity with predicted products from both *P. aeruginosa* phage ϕ KZ and *P. chlororaphis* phage 201 ϕ 2-1, two phages with genomes of 280 and 317 kb, respectively. Along with *P. aeruginosa* phage ϕ EL, they form a group of phages with exceptionally large genomes (Hertveldt *et al.*, 2005).

The putative amino acid sequence of each ORF was annotated using PSI-BLAST. In instances where we identified related proteins, they were very similar to proteins in both ϕ KZ and 201 ϕ 2-1 (Supplementary Table S1). In particular, many virion structural proteins were shared amongst the three highly related phages. For example, the amino acid sequences of ϕ PA3 ORFs 56–64 share on average 55% identity and 71% similarity with the protein products of *gp118–gp126* from 201 ϕ 2-1 and *gp062–gp070* from ϕ KZ (Supplementary Fig. S1). We used the Artemis Comparison Tool to compare ϕ PA3 with ϕ KZ and found regions of significant similarity between the two genomes (Supplementary Fig. S2). Given its genomic and structural similarity to these other *Pseudomonas* phages, ϕ PA3 was classified as a new member of the ϕ KZ-like family of phages.

Like both 201 ϕ 2-1 and ϕ KZ, ϕ PA3 encodes putative tRNAs that are predicted to adjust any codon bias that exists between the phage genome and the host genome (Hertveldt *et al.*, 2005; Mesyanzhinov *et al.*, 2002). From the ϕ PA3 genomic sequence, tRNAScanner predicted five tRNAs. They are shown with their predicted structures in Supplementary Fig. S3. The putative serine tRNA (UCA) may not be functional, as the predicted structure does not resemble a traditional tRNA (Supplementary Fig. S3). The remaining four tRNAs, Asn (AAC), Leu (UUA), Tyr (UAC) and Asp (GAU), all form conventional tRNA structures (Supplementary Fig. S3). The codon usage of each of the tRNAs was examined and compared with that of PAO1 (Supplementary Fig. S3). In each of the five cases, a significant difference existed in codon usage between ϕ PA3 and PAO1 (Supplementary Table S2). By encoding their own tRNAs, rates of translation of the viral transcripts may be unaffected, as found for viral-encoded tRNAs transcribed in other systems (Ventura & Brüssow, 2004).

Gene products encoded by ϕ PA3

Of the ORFs identified in ϕ PA3, 138 are of unknown function (Supplementary Table S1). Like both ϕ KZ and 201 ϕ 2-1, gene products predicted to be involved in nucleotide metabolism were also found in ϕ PA3 (Supplementary Table S1). A

Fig. 3. Genomic organization of ϕ PA3. Representation of the ϕ PA3 genome by DNAPlotter. On the outer ring, the positions of ORFs are shown on the appropriate strand. On the inner ring, percentage G + C is shown relative to the mean G + C content of the genome. A high G + C content is indicated in purple and a low G + C content is indicated in green.

putative dihydrofolate reductase (ORF005), thymidylate kinase (ORF232), deoxycytidine triphosphate deaminase (ORF310), thymidylate synthase (ORF274) and *nrdA/B* (ORF377/378) were all identified in ϕ PA3 as well as ϕ KZ and 201 ϕ 2-1. The biochemical functionality of each of these predicted proteins is not known.

A putative integrase, ORF354, was identified that shares 33 % identity and 53 % similarity with a phage integrase from *Desulfuromonas acetoxidans* DSM 684 (Dace_2830). However, when we examined the amino acid sequence of this putative protein in Pfam we were unable to identify any consensus integrase domains. One possible reason for this may be that the subset of bacteriophage genomes sequenced to date is relatively small, so that conserved domains more prevalent in bacteriophages have yet to be characterized. Alternatively, it is possible that this protein does not function as an integrase. Furthermore, we found no evidence for ϕ PA3 lysogeny (data not shown).

Six putative RNA polymerase beta subunits were identified: ORF54, ORF65, ORF66, ORF67, ORF77 and ORF210. In each instance, similar proteins were also identified in ϕ KZ, 201 ϕ 2-1 and ϕ EL (Supplementary Table S1). It appears that this group of proteins is widely conserved amongst ϕ KZ-like phages and may provide clues as to how this group of phages evolved. For example, one of the proteins described above (ORF67) shared only relatively weak similarity with RNA polymerase beta subunits from multiple organisms (data not shown). Pfam (Wellcome Trust Sanger Institute) identified no known consensus domains in ORF67. In contrast, we used Pfam to identify the RNA polymerase

Rbp1 domain 2 in ORF54. ORF54 also contains the invariant NADFDGD motif required for magnesium binding. Both RNA polymerase beta and beta' subunits were identified. No RNA polymerase alpha subunits were identified (data not shown). By analogy with phage T7 RNA polymerase, these proteins are of particular interest as they could have biotechnological utility.

A recent study identified 31 proteins from the ϕ KZ structural proteome by MS (Thomas *et al.*, 2008). We were able to identify 30 of these in the ϕ PA3 genome. Only gp035 from ϕ KZ appears to have no homologue in ϕ PA3. Another study identified 76 proteins from 201 ϕ 2-1, again using MS (Lecoutere *et al.*, 2009). Of the 76 proteins identified in that study, five were not encoded by the ϕ PA3 genome (Lecoutere *et al.*, 2009).

DISCUSSION

P. aeruginosa is a model organism for studying biofilm formation, secondary metabolites, quorum sensing, bacterial pulmonary infections and antibiotic resistance (Beatson *et al.*, 2002; Harrison, 2007; Stover *et al.*, 2000; Tolker-Nielsen & Molin, 2004; Wagner & Iglewski, 2008). The large genome of *P. aeruginosa* allows its survival in diverse environments such as nematode worms, plants, soil, water and animals (Stover *et al.*, 2000; Trautmann *et al.*, 2005). Additionally, *P. aeruginosa* is hypermutable in the cystic fibrosis lung, making rigorous genetic studies of some clinical isolates problematic (Driffield *et al.*, 2008; Oliver *et al.*, 2000; Smith *et al.*, 2006). Owing to its

importance as a microbial model organism, tools that enable strain engineering and functional genomic analysis in *P. aeruginosa* are essential. Several *P. aeruginosa* generalized transducing phages infecting PAO1 and PA14 (two of the most widely used model strains in the study of *Pseudomonas* infections) have been described (Budzik *et al.*, 2004). However, DMS3, F116 and UT1 transduction efficiencies can be low, and a broad host range has not been established, so additional *P. aeruginosa* transducing phages are needed (Budzik *et al.*, 2004; Morrison *et al.*, 1978; Ripp *et al.*, 1994). This new phage, ϕ PA3, has for some markers a 100-fold higher transducing frequency than those of DMS3, F116 and UT1 (Budzik *et al.*, 2004; Morrison *et al.*, 1978; Ripp *et al.*, 1994). Thus, ϕ PA3 is a powerful tool for the creation and study of chromosomal mutations in this pathogen. In addition to PAO1, ϕ PA3 was able to infect PA14, PAK and a selection of clinical isolates (Table 3). Consequently, homology permitting, it should also be able to transduce between these strains, and this could enable functional genomic studies of both laboratory and clinical *P. aeruginosa* strains.

The ϕ PA3 genome also provides more genetic information about the ϕ KZ family of phages. Its sequence similarity to both ϕ KZ and 201 ϕ 2-1 suggests a common genetic pool for this type of phage. In particular, the region surrounding the late genes of the ϕ PA3 genome diverges from the ϕ KZ sequence (Supplementary Fig. S2). The high levels of similarity among many ORFs from ϕ KZ, 201 ϕ 2-1 and ϕ PA3 mean that further biotechnological applications for this group of phages could be possible. The development of ϕ KZ-like phages as biofilm eradication agents or novel phage therapeutics should be considered carefully because of the theoretical possibility of horizontal gene transfer into different *P. aeruginosa* clinical isolates.

In addition to 'phage' genes, ϕ PA3 contains genes encoding proteins similar to those from a wide variety of organisms. This is not uncommon in the 'jumbo' ϕ KZ-like phages (Hendrix, 2009). It has been proposed that these phages with large genomes have more genomic space in which to 'experiment', and are experiencing higher rates of genetic evolution than their smaller, more genomically compact, counterparts (Hendrix, 2009). As more sequences of large bacteriophages are added to GenBank, this notion may be tested. Finally, the high transduction efficiency of ϕ PA3 means that it should serve as a useful genetic tool for those studying the functional genomics and genetics of *P. aeruginosa* in model laboratory strains, in new environmental isolates and in clinical strains from patients with cystic fibrosis or other pathologies.

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