TWO HIGH-FREQUENCY-TRANSDUCTION PHAGE ISOLATES FROM LYSOGENIC STRAINS OF *PSEUDOMONAS AERUGINOSA* TRANSDUCING ANTIBIOTIC RESISTANCE

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Summary. – Two high frequency transduction (HFT) phage isolates, obtained from seriously ill patients, transducing individual determinants of antibiotic resistance with a frequency of 10^{-5} (phage isolate AP-103) and 10^{-6} (phage isolate AP-343), are described. The frequency of transduction depended on the transduced determinant(s) of resistance used for the detection of transductants and on the individual recipient antibiotic-susceptible strain of *Pseudomonas aeruginosa* (PAO and/or ML series). A multiple-antibiotic resistance was transduced by the phage isolate AP-343 to all tested recipient strains. The appearence of such phages in clinical conditions with an unusually high frequency of transduction might contribute to the dissemination of antibiotic resistance genes among nosocomial strains of *P. aeruginosa*. The existence of HFT phages might reflect an increased efficiency of transduction of antibiotic resistance among *P. aeruginosa* strains, and thus an increased risk of spread of antibiotic resistance even to recently introduced anti-pseudomonadal antibiotics among pseudomonads with unfavourable and unwanted epidemiological consequences in hospital conditions.

Key words: Pseudomonas aeruginosa; antibiotic resistance; transduction

Introduction

Multiple-antibiotic-resistant strains of *Pseudomonas aeruginosa* are dangerous nosocomial pathogens accounting for as much as 5 – 15% of all nosocomial infections (Jones *et al.*, 1996). This situation is strongly aggravated by the fact that these strains are naturaly resistant to many antibiotics. These pathogens rapidly acquire resistance also to most recently introduced antibiotics including ceftazidime (CAZ) and/or imipenem (IMP) (Jarvis *et al.*, 1996). Antibiotic-resistant genes can spread among *P. aeruginosa* strains by (1) conjugal transfer even from other species of Gram-negative organisms, (2) transposition of integrons (Yobe *et al.*, 1996), and (3) transduction, as some strains of *P. aeruginosa* are lysogenic (Blahová *et al.*, 1997).

Wild type phages isolated from lysogenic multiple-antibiotic-resistant strains of *P. aeruginosa* were found to transduce determinants of resistance to various antibiotics including aminoglycoside (Knothe *et al.*, 1981) and ß-lactam antibiotics, i.e. penicillins and cephalosporins (Seginková *et al.*, 1986; Blahová *et al.*, 1993). In addition, generalised transducing phage isolates from *P. aeruginosa*, e.g. F-116 and/or G-101, were found to be able to pick up the resistance genes from the chromosome of multiple-antibiotic-resistant nosocomial strains of this host and transduce them to antibiotic-susceptible recipient strains (Masuda and Ohya, 1992). Nevertheless, the frequency of transduction in

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Abbreviations: ATM = aztreonam; CAR = carbenicillin; CAZ = ceftazidime; CTX = cefotaxime; HFT = high frequency transduction; IMP = imipenem; KAN = kanamycin, STR = streptomycin

laboratory conditions in the two abovementioned studies varied in the range of 10^{-8} –10^{-9}, exceptionally 10^{-7} (Blahová *et al.*, 1997).

In this communication, we report on phages, isolated from seriously ill patients in two hospitals, which apparently transduced individual determinants of antibiotic resistance with a frequency of 10^{-5} – 10^{-6}, depending on the transduced determinant(s) of resistance and on the individual recipient antibiotic-susceptible strain of *P. aeruginosa*. The appearence of such phages in clinical environment with an unusually high frequency of transduction might contribute to the dissemination of antibiotic resistance genes among nosocomial strains of *P. aeruginosa*.

Materials and Methods

Phage isolates AP-103 and AP-343 were obtained from *P. aeruginosa* strains 103/98 and 343/97, respectively.

Bacterial strains. Two donor lysogenic multiple-drug-resistant strains of *P. aeruginosa* were isolated from seriously ill patients under intensive chemotherapy with antibiotics in two large hospitals. The strain 343/97 was isolated from the urine of a septic patient hospitalised in the Regional Hospital in Nové Zámky, Slovak Republic. The strain 103/98 was isolated from the urine of a patient in the Bata's Hospital in Zlín, Czech Republic. Both strains were resistant to a series of classical antibiotics including kanamycin (KAN), streptomycin (STR) and carbenicillin (CAR) (MIC >128 mg/l) and to "advanced" ß-lactam antibiotics like cefotaxime (CTX) and CAZ (MIC = 32 mg/l). The strain 103/98 was resistant also to aztreonam (ATM) (MIC = 16 mg/l). Both strains produced evident plaques of lysis of the bacterial inoculum on the MacConkey Agar (Difco) plates containing the respective antibiotics.

Preparation of phage lysates Bacteria surrounding at least 15 plaques were collected and used for preparation of a wild-type phage lysate by an autolysis procedure (Blahová *et al.*, 1997). The phage lysates were titrated by a standard dilution drop technique, propagated by a standard double-layer, soft-agar technique to a titre of 10^{10} PFU/ml, and used in transduction experiments. Four auxotrophic mutants of *P. aeruginosa* strains, PAO-1670 (*ade- leu- rif+*), ML-1008 (*trp- leu- arg- ile- val- his- rif+*), ML-1292 (*trp- met- ile- val- his-*), and ML-M 88 (*leu- trp- str+*), were obtained from Prof. S. Mitsuhashi, Episome Institute of Maebashi, Japan, were used as antibiotic-susceptible recipients of transduced determinants of resistance.

Transduction experiments. The transduction procedure, selection of transductants on mono-antibiotic plates and analysis of transductant colonies for the presence of transduced determinants of antibiotic resistance were described in detail previously (Olsen and Thomas, 1973; Holloway, 1975; Seginková *et al.*, 1986; Blahová *et al.*, 1997). The multiplicity of infection was 0.1 PFU per cell. One ml of suspension of recipient strain contained approximately 1 x 10^9 CFU/ml. The time period for phenotypic expression of transduced determinants was set, after preliminary experiments, to 60 mins after addition of a phage lysate to a suspension of recipient strain. Transductants were selected on Nutrient Agar (Difco) plates containing the following concentrations of single antibiotics: 100 mg/l for STR, KAN and CAR, and 30 mg/l for CTX, CAZ and ATM. The plates were incubated at 35°C and the number of transductants was recorded after 36 hrs of incubation.

Analysis of transductants. An indirect selection procedure was used for demonstration of the spectrum of transduced resistance determinants after each transduction experiment A representative number of transductant colonies were picked up into 0.5 ml of Nutrient Broth (Difco) in minitubes and statically incubated for 8 hrs, when their titre reached approximately 10^5 CFU/ml. Then, 0.05 ml aliquots of each suspension were applied by a calibrated multiloop applicator on the surface of a series of single-antibiotic agar plates containing identical concentration of each antibiotic to which the donor strains were resistant. The appearence of macro-colonies on each antibiotic plate was then recorded after an overnight incubation of plates at 35°C, and the spectrum of multiple antibiotic resistance of each transductant macro-colony was deduced.

Results

HFT by phage isolate AP-343

As it can be seen from Table 1, determinants of resistance to CTX and/or CAZ were transduced by the phage isolate AP-343 to all four recipient strains with an unusually high frequency (3.2 x 10^{-6} – 1.8 x 10^{-6}) with exception of CAZ-selected transductants of *P. aeruginosa* PAO-1670 strain, where the frequency of transduction was lower , i.e. 9.4 x 10^{-7}. The frequency of appearance of STR-selected transductants was 1.0 – 1.5 x 10^{-7} and that of KAN- or CAR-selected transductants was in the range of 10^{-6} to 10^{-7}, depending on the recipient strain. No transduction of resistance to IMP, meropenem or flurochinolones was recorded.

A multiple antibiotic resistance was transduced by the phage isolate AP-343 to all recipient strains (more than 600 transductants were investigated). There were no spontaneous mutants as confirmed by negative controls run in each transduction experiment.

HFT by recipient-restrictive phage isolate AP-103

When the lytic potential (i.e. the titre) of the phage isolate AP-103 was tested by plating of each dilution of the phage lysate on a series of plates inoculated with a suspension of each of the recipient strains, the lytic reaction appeared only with the PAO-1670 recipient strain. There was no lytic reaction with any of the three strains of ML series, i.e. ML-1008, ML-1292 and ML-M88. We performed transduction experiments to demonstrate that a phage which is apparently not lytic for certain strains, could be, despite the unexpected absence of lytic reaction, a satisfactorily transducing phage for them.

Table 1. Frequency and multi-antibiotic resistance spectrum of transductants obtained in individual recipient strains of *Pseudomonas aeruginosa* with phage isolate AP-343

Selection on	Frequency and spectrum of antibiotic resistance transduced by recipient strains[a]			
	PAO-1670	ML-1008	ML-1292	M-88
Streptomycin (STR)	1×10^{-7} STR/KAN/CAR/CTX/CAZ	No transduction	1.5×10^{-7} STR/KAN/CAR/CTX/CAZ	Not tested[b]
Kanamycin (KAN)	3×10^{-7} STR/KAN/CAR/CTX/CAZ	3×10^{-6} STR/KAN/CAR	8.3×10^{-6} STR/KAN/CAR/CTX/CAZ	3×10^{-7} KAN/CAR/CTX/CAZ
Cefotaxime (CTX)	2.8×10^{-6} STR/KAN/CAR/CTX/CAZ	2.5×10^{-6} STR/KAN/CAR/CTX/CAZ	2.2×10^{-6} STR/KAN/CAR/CTX/CAZ	2.9×10^{-6} KAN/CAR/CTX/CAZ
Ceftazidime (CAZ)	9.4×10^{-7} STR/KAN/CAR/CTX/CAZ	2.1×10^{-6} STR/KAN/CAR/CTX/CAZ	1.8×10^{-6} STR/KAN/CAR/CTX/CAZ	3.2×10^{-6} KAN/CAR/CTX/CAZ
Carbenicillin (CAR)	1.6×10^{-7} STR/KAN/CAR/CTX/CAZ	No transduction	No transduction	3.8×10^{-7} KAN/CAR/CTX/CAZ

[a]Demonstrated by indirect selection procedure.
[b]Not tested for selection since *P. aeruginosa* M-88 strain was highly resistant to STR by chromosomal mutation.

Table 2. Frequency and multi-antibiotic resistance spectrum of transductants obtained in individual recipient strains of *Pseudomonas aeruginosa* with phage isolate AP-103

Selection on	Frequency and spectrum of antibiotic resistance transduced by recipient strains[a]			
	PAO-1670	ML-1008	ML-1292	M-88
Kanamycin (KAN)	8.2×10^{-5} KAN/CAR/CTX/CAZ/ATM	1.0×10^{-6} KAN/CAR/CTX/CAZ/ATM	$3..3 \times 10^{-6}$ KAN/CAR/CTX/CAZ/ATM	3.0×10^{-5} KAN/CAR/CTX/CAZ/ATM
Carbenicillin (CAR)	6.0×10^{-6} KAN/CAR/CTX/CAZ/ATM	9.0×10^{-6} KAN/CAR/CTX/CAZ/ATM	3.8×10^{-6} KAN/CAR/CTX/CAZ/ATM	5.5×10^{-5} KAN/CAR/CTX/CAZ/ATM
Cefotaxime (CTX)	5.5×10^{-6} KAN/CAR/CTX/CAZ/ATM (60%) KAN/CAR/CTX/(30%) KAN/CAR/CTX/CAZ/(10%)	2.0×10^{-6} KAN/CAR/CTX/CAZ/ATM (100%)	2.5×10^{-6} KAN/CAR/CTX/CAZ/ATM (100%)	2.0×10^{-5} KAN/CAR/CTX/CAZ/ATM (100%)
Ceftazidime (CAZ)	2.0×10^{-5} KAN/CAR/CTX/CAZ/ATM (50%) KAN/CAR (30%) CTX/CAZ/ATM (20%)	5.0×10^{-5} KAN/CAR/CTX/CAZ/ATM (90%) CTX/CAZ/ATM/(10%)	9.0×10^{-6} KAN/CAR/CTX/CAZ/ATM (90%) CTX/CAZ/ATM (10%)	3.5×10^{-5} KAN/CAR/CTX/CAZ/ATM (100%)
Aztreonam (ATM)	3.0×10^{-6} KAN/CAR/CTX/CAZ/ATM (100%)	1.0×10^{-5} KAN/CAR/CTX/CAZ/ATM (50%) CAZ/ATM (40%) ATM (10%)	5.0×10^{-5} KAN/CAR/CTX/CAZ/ATM (80%) CTX/CAZ/ATM (10%) CAZ /TM (10%)	4.0×10^{-5} KAN/CAR/CTX/CAZ/ATM (100%)

[a]Demonstrated by indirect selection procedure.

The phage isolate AP-103/98 transduced with a high frequency (10^{5} – 10^{-6}) a more or less complete spectrum of antibiotic resistance determinants (Table 2), in dependence on the properties of recipient strains and the antibiotic used for selection (Fig. 1). E.g., in transductants of the PAO-1670 strain selected on media with CTX or CAZ, there was observed not only a complete spectrum of five resistance determinants, but, in 30% of transductants, the presence of only three determinants of resistance, i.e. KAN, CAR and CTX, and in 10% of transductants, the presence of four determinants of resistance including CAZ but not ATM. Two types of transductants were recorded also in the combinations of ML-1008 and CAZ, and of ML-1292 and CAZ (Fig. 2). Interestingly enough, in transductants of the ML-1008 recipient strain selected on ATM, transductants that accepted a single antibiotic resistance determinant, i.e. ATM (10%), or two determinants, i.e. ATM plus CAZ (40%), could be selected indicating a relatively stable association of CAZ

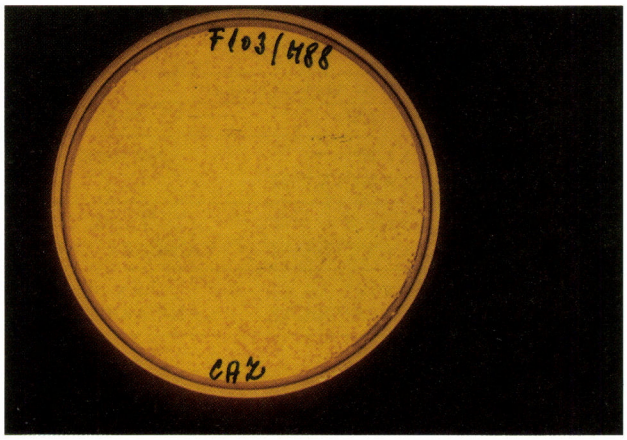

Fig. 1
Transduction of CAZ resistance determinant by phage isolate AP-103 from *P. aeruginosa* No. 103 to *P. aeruginosa* ML-M88 recipient strain
Frequency of transduction was 3.5 x 10^{-5}.

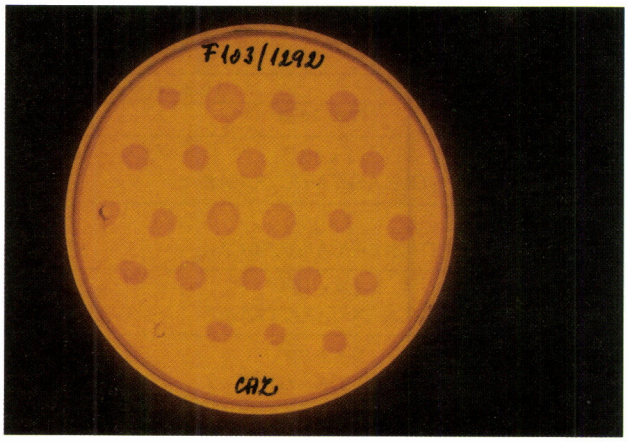

Fig. 2
Analysis of transductants obtained with phage isolate AP-103 in *P. aeruginosa* ML-1292 recipient strain on the medium with CAZ
All clones were resistant to CAZ and grew as macro-colonies on this medium.

and ATM determinants in the genetic system of multiple antibiotic resistance of the original *P. aeruginosa* 103/98 strain , from which the phage isolate AP-103 was obtained.

This observation differs from that recorded in transduction experiments with the phage isolate AP-343 originating from *P. aeruginosa* from a different patient hospitalised in a different country. As it can be seen from Table 1, a total spectrum of resistance to five antibiotics is usually transduced with exception of the ML-1008 recipient strain, which did not accept genes coding the resistance against "advanced" cephalosporins, i.e. CTX and CAZ. In addition, no transductants appeared with this bacteriophage in recipient strains ML-1008 and ML-1292 on media with CAR and STR.

Discussion

Acquisition of determinants of antibiotic resistance by nosocomial strains of *P. aeruginosa* might further aggravate the natural insensitivity of this species to various groups of antibiotics and chemoterapeutics. The resistance can be transferred from resistant to susceptible strains of *P. aeruginosa* by bacterial conjugation including the mobilisation (Lešická-Hupková *et al.*, 1996) of non-conjugative plasmids carrying genes for antibiotic resistance (Mugnier *et al.*, 1996; Bissonette and Roy, 1992). Alternatively, various genes of resistance including that against IMP may be transposed among pseudomonads via integrons (Bissonette and Roy, 1992; Yobe *et al.*, 1996). Phages isolated from wild-type hospital strains of multiple-antibiotic-resistant strains of *P. aeruginosa* (Blahová *et al.*, 1997) as well as generalised transducing phages (Masuda and Ohya, 1992), all transducing antibiotic resistance determinants, have been reported. The species-specific phage transduction can thus significantly contribute to dissemination of new determinants of antibiotic resistance among hospital strains of *P. aeruginosa*.

In earlier communications (Blahová *et al.*, 1997; Masuda and Ohya, 1992), however, the frequency of transduction of antibiotic resistance genes ranged between 10^{-8} and 10^{-9}, and only exceptionaly between 10^{-7} and 10^{-8}, i.e. in the range of frequency of transduction of chromosomal genes of *P. aeruginosa* (Haas and Holloway, 1977; Rella and Haas, 1982).

Quite recently, however, two phages were isolated from nosocomial multiple-antibiotic-resistant strains of *P. aeruginosa* from patients in hospitals in two countries, which transduced antibiotic resistance determinants with frequency in the range of 10^{-6} (phage isolate AP-343) to 10^{-5} (phage isolate AP-103) in dependence on the recipient antibiotic-susceptible strain of *P. aeruginosa* (PAO and/or ML series) and the antibiotic resistance determinant(s) used for selection of transductants. Existence of such HFT phages might reflect an increased efficiency of transduction of antibiotic resistance among *P. aeruginosa* strains, and thus an increased risk of spread of antibiotic resistance even to new anti-pseudomonad antibiotics among pseudomonads with unfavourable and unwanted epidemiological consequences in hospital conditions.

The two HFT phage isolates characterised here, AP-103 and AP-343, have demonstrated a difference in their transducing capacity. Apart from the higher frequency of transduction by AP-103 in comparison with AP-343, the

former demonstrated a peculiar restriction in its lytic but not transducing properties, being lytic only for the PAO but not ML recipient strains. In addition, AP-103 showed, in contrast to AP-343, a capacity to transduce also some of antibiotic resistance determinants present in the lysogenic wild-type *P. aeruginosa* 103/98 strain from which it was isolated. Various spectra of resistance in individual transductants (e.g. selected by CTX, CAZ or ATM in PAO and some ML recipient strains) pointed again to the capacity of this phage to disseminate various combinations of antibiotic resistance determinants to different susceptible strains.

In conclusion, it seems that phages isolated from some lysogenic, wild-type, nosocomial, multiple-antibiotic-resistant strains of *P. aeruginosa* demonstrate an increased transduction potency expressed in an increased frequency of transduction to individual recipient antibiotic-susceptible strains. Thus, they might present an increased negative epidemiological significance in their potential to spread antibiotic resistance determinants from resistant to susceptible bacteria.

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