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Plasmid mediated silver resistance in *Acinetobacter baumannii*

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***Acinetobacter baumannii* BL88, an environmental isolate, was resistant to 13 metals and 10 antibiotics. Plumbagin cured resistance to silver, cadmium, antimony, streptomycin and ampicillin at varying frequencies. However, only silver resistance transferred (1×10^{-6} recepient^{-1}) to *Escherichia coli* K12 during conjugation. Correspondingly there was transfer of a 54 kb plasmid (pUPI199) from *A. baumannii* BL88. The plasmid transformed *E. coli* DH5α cells at a frequency of 1×10^{-8} recepient^{-1}. The growth rate of *E. coli* DH5α (pUPI199) was slower as compared with *E. coli* DH5α. Plasmid pUPI199 was 76 and 9.6% stable in the host *A. baumannii* BL88 in the presence and absence of selection pressure, respectively. *A. baumannii* BL88 was found to accumulate and retain silver whereas *E. coli* DH5α (pUPI199) effluxed 63% of the accumulated silver ions.**

Keywords: *Acinetobacter*, conjugation, curing, plasmid, silver uptake, silver resistance, transformation

Introduction

Silver is toxic to bacteria as it interferes with respiration (Bragg & Rainnie 1974, Charley & Bull 1979) and other cell surface associated functions. It forms complexes with membranes, enzymes and nucleic acids (Slawson *et al.* 1992a). Bacteria showing high levels of resistance to multiple metal ions have been isolated from environments contaminated with toxic metals (Summers & Jacoby 1978). Bacterial strains resistant to silver have been isolated from silver mines (Haefeli 1974, Pumpel *et al.* 1986), photographic film waste effluent (Belly 1984) and hospital burn units (Rozenkranz & Carr 1972, McHugh *et al.* 1975). Silver compounds are important chemotherapeutic agents. For example, $AgNO_3$ is used to treat burns (Russell & Chopra 1990) and to prevent eye infections (Slawson *et al.* 1992a). Silver sulfadiazine has been used topically for preventing infections in severe burns (Wysor 1982). Widespread topical use of silver compounds in hospitals has doubtless served as an effective means of selection of silver resistance bacteria (McHugh *et al.* 1975).

Acinetobacter is a ubiquitous, Gram-negative, oxidase-negative bacterium (Juni 1978, Vivian *et al.* 1981). It is an important nosocomial pathogen and is frequently resistant to multiple antibiotics (Larson 1984, Chopade & Towner 1986) and metals (Deshpande *et al.* 1993). *Acinetobacter* is a resident of human skin (Haustein 1989, Chopade, unpublished data) and often causes nosocomial burn wound superinfections (Zaer & Deodhar 1989, Xu 1990, Patwardhan 1990) and eye infections (Zabel *et al.* 1989). Being ubiquitous, it is readily isolated from environments like waste water, and is generally resistant to metals and antibiotics irrespective of the source of isolation (Deshpande *et al.* 1993). Metal and antibiotic resistance genes are frequently found on the same plasmids (Novick *et al.* 1979, Dhakephalkar *et al.* 1992).

Plasmid mediated silver resistance is a rare phenomenon and has only been reported from *Escherichia coli* (Annear *et al.* 1976) *Enterobacter cloacae* (Starodub & Trevors 1990), *Citrobacter freundii* (Goddrad & Bull 1989) and *Pseudomonas stutzeri* (Haefell *et al.* 1984, Slawson *et al.* 1992b). However, to date, there is no report on silver resistance in *Acinetobacter*. It had been proposed that *Acinetobacter* is a reservoir of naturally occuring antibiotic and metal resistance plasmids (Chopade *et al.* 1993a). In the search for antibiotic and metal resistance plasmids, we found a silver resistance plasmid (pUPI199) in *A. baumannii*, which is described in this paper.

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Materials and methods

Bacterial strains

A. baumannii BL88 is an environmental isolate. It was confirmed to the genus *Acinetobacter* by the chromosomal transformation assay (Juni 1972). Species identification was performed by the method of Bouvet & Grimont (1986). Other strains used in the study are listed in Table 1. All the strains were maintained on Trypticase Soya agar slants.

Determination of the degree of resistance to metals and antibiotics

The degree of resistance of *A. baumannii* BL88 to metals and antibiotics, in terms of minimum inhibitory concentration (MIC), was determined by the agar dilution method of Reeves *et al.* (1978) and modified as previously described (Deshpande *et al.* 1993). DST agar plates containing antibiotics and GC agar plates (Riley & Taylor 1989) containing metal salts were spot inoculated with 10^4–10^5 c.f.u. ml^{-1} of an overnight culture. Results were read after 18 h incubation at 37°C.

Curing of antibiotic and metal resistance

Curing was done as described by Chopade *et al.* (1993b) using plumbagin (5-hydroxy, 2-methyl, 1,4 napthoquinone). Plumbagin was dissolved in dimethylsulfoxide. An overnight culture of *A. baumannii* BL88 adjusted to 10^5 c.f.u. ml^{-1} was subjected to a range of plumbagin concentrations from 3 to 400 $\mu g\,ml^{-1}$. The growth in a sub-inhibitory concentration (SIC) tube was appropriately diluted in sterile saline and spread plated on Luria agar to obtain isolated clones. A master plate of 50 clones was prepared on Luria agar and replicated on Luria agar plates containing appropriate concentrations of antibiotics and metals. Plates were incubated at 37°C for 24 h. Absence of growth on selection plates indicated curing.

Conjugation

Intergeneric as well as intrageneric matings were performed by the membrane filter mating technique (Chopade 1986). Overnight cultures of the donor (2 ml, 1:10 diluted) and recepient (2 ml) were mixed and filtered on to a Millipore (0.45 μM) membrane filter and incubated on Luria agar plates at 37°C for 24 h. Growth was resuspended in 2 ml of 0.85% saline and dilutions spread plated on DST agar plates containing antibiotics and metal salts. The plates were observed for the appearance of transconjugants for up to 72 h incubation.

Plasmid isolation and gel electrophoresis

Plasmid isolation from *A. baumannii* BL88 as well as *E. coli* K12 transconjugants was performed by a modified alkali lysis method (Sihavy *et al.* 1984). Plasmid separation was done on horizontnal slab gel (Biotech, Bangalore, India) using 0.7% (w/v) agarose, in Tris–acetate–EDTA buffer (Tris–acetate 40 mM, EDTA 10 mM, pH 8.0). Molecular weights of the plasmids were determined using the standard plasmid molecular weight ladder from *Escherichia coli* V517 (Macrini *et al.* 1978).

Transformation

E. coli DH5α cells were rendered competent by $CaCl_2$ treatment (Maniatis *et al.* 1982). Between 0.5 and 0.1 μg of pUPI199 DNA was mixed with 200 μl of competent cells. Cells without added DNA served as control. The transformants were selected on Luria agar plates containing 50 $\mu g\,ml^{-1}$ $AgNO_3$. Transformants were scored after 24 and 48 h incubation at 37°C.

Plasmid stability

A. baumannii BL88 was grown overnight in Luria broth on a shaker (200 r.p.m.) at 37°C in the presence (50 $\mu g\,ml^{-1}$) or absence of $AgNO_3$. Each culture was

Table 1. Bacterial strains

Strain	Relevant information[a]	Reference
1. *A.baumanni* BL88	Ap^r, Cb^r, Cx^r, Ce^r, Cl^r, Cz^r, Tb^r, Tp^r, Sm^r, Nf^r, Cu^r, Ag^r, Zn^r, Pb^r, Bi^r As^r, Cd^r, Al^r, Li^r, W^r	this study
2. *A. baumannii* BL88.1	cured by plumbagin (Ag^s, Cd^s Sb^s, Ap^s, Sm^s)	this study
3. *E. coli* K12 J53.2	F^-, pro^-, met^-, Rp^r	Coetzee *et al.* (1972)
4. *E. coli* K12 J53.2 (pUPI199)	transconjugant pUPI199 (Ag^r)	this study
5. *E. coli* K12 KT1090	F^-, lac^-, thi, ser, azi, hsd^r, hsd^m, Sm^r, Rp^r	Chopade *et al.* (1985)
6. *E. coli* KT1090 (pUPI199)	transconjugant pUPI199 (Ag^r)	this study
7. *E. coli* K12 DH5α	*Sup* E44, *lac* U169(80 *lac* ZM15), hsd R^{17}, *rec* A1 end A1, *gyr* A96, thi 1, relA1, Nal^r	Raleigh *et al.* (1989)
8. *E. coli* DH5α (pUPI199)	transformant pUPI199 (Ag^r)	this study
9. *E. coli* V517	pVA517(A–H)	Macrina *et al.* (1978)

[a]Abbreviations: Ap, ampicillin; Cb, carbenicillin; Cx, cloxacillin; Ce, cephaloridine; Cl, cefalexin; Cz, cefazolin; Tb, tobramycin; Tp, trimethoprim; Sm, streptomycin; Nf, nitrofurantoin; Cu, copper; Ag, silver; Zn, zinc; Pb, lead; Bi, bismuth; As, arsenate; Cd, cadmium; Al, aluminum; Li, lithium; W, tungstun.

serially diluted and 0.1 ml of each dilution was spread plated on Luria agar plates containing (50 $\mu g\,ml^{-1}$) and lacking $AgNO_3$. Plates were incubated at 37°C for 24 h. Stability in the presence and absence of selection was determined as

$$\% \text{ stability} = \frac{\text{TVC on selective plate}}{\text{TVC on DST plate}} \times 100.$$

Growth curves

Fresh overnight cultures of *E. coli* DH5α and *E. coli* DH5α (pUPI199) grown in Luria broth and Luria broth plus $AgNO_3$ (50 $\mu g\,ml^{-1}$), respectively, were used to inoculate fresh broth (0.1% inoculum) in Erlenmeyer side armed flasks. The flasks were incubated at 37°C on a rotary shaker (200 r.p.m.). Culture absorbance was measured periodically at 660 nm. Growth curves were plotted as time of incubation versus log OD_{660}.

Silver uptake studies

A. baumannii BL88 and *E. coli* DH5α (pUPI199) pregrown in the presence of silver (20 $\mu g\,ml^{-1}$) were grown to mid to late log phase in the absence of selection. $AgNO_3$ solution was added to these cultures at a final concentration of 75 $\mu g\,ml^{-1}$. Then 1 ml was removed after every 20 min starting from 0 min up to 2 h and then after 18 h. The samples were centrifuged and cell pellets washed three times with Luria broth. To the cells as well as the pooled broth 1 ml of concentrated HNO_3 was added. The samples were kept at room temperature overnight, appropriately diluted and silver concentration was determined by atomic absorption spectrophotometry (Chemita, India). Uninoculated controls were run simultaneously. *E. coli* DH5α served as a negative control for the transformant.

Results

Metal and antiobiotic resistance

A. baumannii showed resistance to 13 metals and 10 antibiotics (Table 2). Interestingly, the degree of resistance of ampicillin, cefalexin, tobramycin and nitrofurantoin was more than 1024 $mg\,l^{-1}$, and the isolate was a β-lactamase producer. This strain was also resistant to many metals important from medical and environment points of view (Table 2). *A. baumannii* BL88 was thus undertaken for further investigations.

Curing

Plumbagin at SIC (50 $\mu g\,ml^{-1}$) cured silver resistance at a frequency of 69%. Along with Ag^r other markers were also cured, i.e. Cd^r, Sb^r, Ap^r and Sm^r, at varying frequencies (Table 3). This was taken as *prima facie* evidence of involvement of the plasmid in the resistance to silver and other cured markers.

Table 2. Resistance of *A. baumannii* BL88 to selected antibiotics and metals

Antibiotic	MIC ($mg\,l^{-1}$)[b]	Metal salt	MIC (mM)[b]
Ampicillin	>1024	$Co(NO_3)_2$	1
Carbenicillin	64	$BiSO_4$	>10
Cloxacillin	>1024	As_2O_4	>10
Cephaloridine	128	$CuSO_4$	>10
Cefazolin	128	K–Sb–tartarate	1
Cefalexin	1024	$AgNO_3$	1
Streptomycin	128	$ZnSO_4$	10
Tobramycin	>1024	$(CH_3COO)_2{\cdot}Pb$	10
Trimethoprim	64	$CdSO_4$	1
Nitrofurantoin	>1024	$NiCl_2$	> 1
		$AlCl_3$	>10
		$LiCl_2$	>10
		Na_2MoO_4	>10

[a]Serial 2-fold dilutions of antibiotics in the range 2–1024 $\mu g\,ml^{-1}$ were made in DST agar.
[b]Serial 10-fold dilutions of metal salts in the range 0.001–10 mM were made in GC agar (Riley *et al.* 1989) supplemented with 0.5% yeast extract.

Table 3. Curing of resistance markers from *A. baumannii* BL88 using plumagin[a]

Marker tested	Number of clones		Curing (%)
	tested	cured	
Ag^r	74	51	69
Cd^r	68	64	94
Sb^r	67	32	45
Ap^r	72	27	38
Sm^r	60	43	88

[a]The MIC of *A. baumannii* BL88 for plumbagin was determined by broth dilution. Isolated clones from the SIC tube were used to test for loss of resistance markers.

Transfer of resistance genes by conjugation

During conjugative transfer only Ag^r transferred to *E. coli* K12 (Table 4). Co-transfer of the other cured markers was not detected. It was observed that further intrageneric transfer of silver resistance within *E. coli* also occured at a frequency similar to the intergeneric mating (Table 5).

Isolation of plasmid DNA

The plasmid profile of *A. baumannii* BL88 showed the presence of two plasmids. The molecular sizes of

Table 4. Conjugative transfer of resistance markers, from *A. baumannii* BL88 to *E. coli* K12

Recepient	Frequency of transfer marker selected				
	Ap^r	Sm^r	Ag^r	Sb^r	Cd^r
E. coli K12 J53.2	NT[a]	NT	2×10^{-6}	NT	NT
E. coli KT1090	NT	NT	1.5×10^{-6}	NT	NT

[a]NT = not transferred.

Table 5. Conjugative transfer of pUPI199 (Ag^r) within *E. coli* K12

Donor	Recepient	Frequency of transfer[a]
E. coli DH5α (pUPI199)	*E. coli* K12 J53.2	1.4×10^{-6}

The transconjugants appeared after 48–72 h incubation in all the experiments. Each experiment was repeated at least twice.

[a]Frequency of conjugation =

$$\frac{\text{number of transconjugants appeared on selection medium} \times \text{dilution factor}}{\text{TVC of the recepient}}$$

these were estimated to be about 54 and 4.0 kb by comparison with the standard plasmid ladder obtained from *E. coli* V517. The plasmid profile of *E. coli* K12 transconjugant showed the presence of a 54 kb band. This plasmid was designated pUPI199 (Figure 1).

Plasmid transformation

The plasmid pUPI199 coded Ag^r trait was further confirmed by transforming *E. coli* DH5α with the plasmid preparation of pUPI199. Silver resistant transformants were obtained at a frequency of 10^{-8} per recepient cell. Transformants was confirmed by growth on the same selective medium and by plasmid isolation (Figure 1).

Stability of pUPI199

pUPI199 was 76% stable in *A. baumannii* BL88 pregrown in 50 $\mu g\,ml^{-1}$ $AgNO_3$. However when *A. baumannii* BL88 was grown for 48 h without $AgNO_3$ selection, the stability of the plasmid dropped to less than 10%. Instability of the Ag^r trait was further evidence of its plasmid coded nature (Table 6).

Growth curve studies

Growth curve studies on *E. coli* DH5α and *E. coli* DH5α (pUPI199) revealed that the overall rate of growth was low in the case of *E. coli* DH5α (pUPI199) as compared with its plasmid-less derivative (Figure 2).

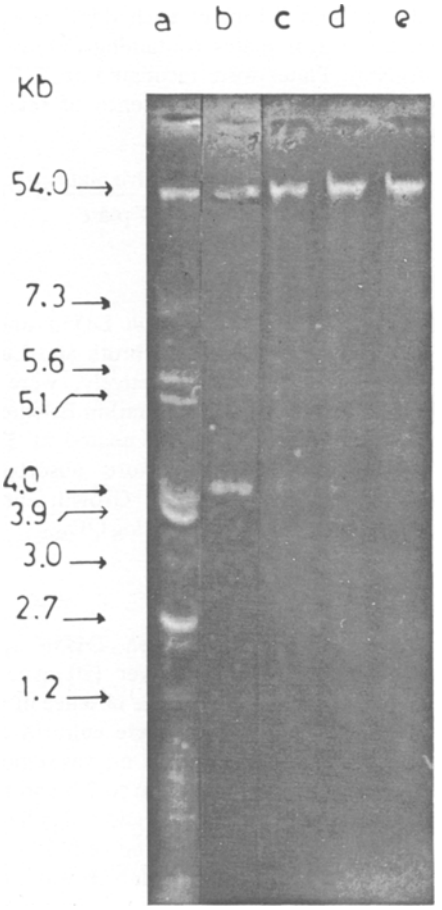

Figure 1. Plasmid profiles of *A. baumannii* BL88 and silver resistance transconjugants. Lane a, plasmid ladder from *E. coli* V517; lane b, *A. baumannii* BL88; lane c, *E. coli* K12 J53.2 (pUPI199); lane d, *E. coli* K12 DH5α (pUPI199); lane e, *E. coli* K12 KT1090 (pUPI199).

Table 6. Stability of plasmid pUPI199 in *A. baumannii* BL88

Selection	TVC (c.f.u. ml^{-1})		Stability (%)
	DST	(DST + $AgNO_3$)	
LB + $AgNO_3$ (20 μml^{-1})	8×10^9	6.1×10^9	76
LB	7.3×10^9	7.8×10^8	9.4

Silver uptake studies

Silver uptake by the cells was observed in *Acinetobacter* as well as *E. coli* almost immediately after

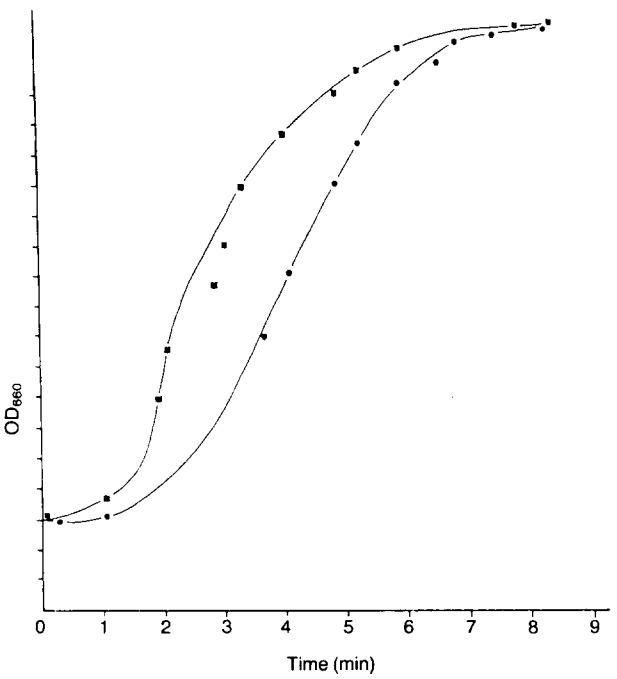

Figure 2. Growth curves of *E. coli* DH5α (pUPI199) (●) and *E. coli* DH5α (■).

contact between the cells and Ag^+. The cell bound concentration of Ag^+ in *Acinetobacter* increased in the first 40 min of incubation and was then maintained at about the same value up to 18 h (Figure 3). *E. coli* DH5α (pUPI199) accumulated much higher amounts (13 p.p.m.) steadily up to 2 h. The concentration decreased to 4.8 p.p.m. in 18 h. *E. coli* DH5α also showed maximum accumulation (9.7 p.p.m.) at 120 min with an initial plateau. The plasmid-containing *E. coli* DH5α accumulated and retained higher levels than the plasmid-less containing strain. Indirect evidence for silver accumulation was obtained from growth of Ag^r cells on Luria agar plus $AgNO_3$. *A. baumannii* BLK 88 as well as the *E. coli* K12 J53.2 transconjugant showed blackening and a metallic sheen to the colonies when grown on $AgNO_3$ containing medium indicating that the cells accumulate silver.

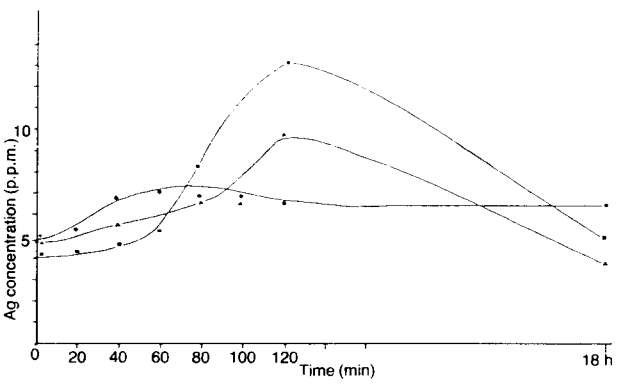

Figure 3. Silver uptake by *A. baumannii* BL88 and silver resistant transformant. (●) *A. baumannii* BL88, (■) *E. coli* DH5α (pUPI199), (▲) *E. coli* DH5α (control).

Discussion

A. baumannii BL88 was resistant to $AgNO_3$ at a concentration of 128 $mg\,l^{-1}$ (0.75 mM). Neito *et al.* (1989) has reported that all the 24 murine *Acinetobacter* species tested were intolerant to 1 mM $AgNO_3$ concentration. However, 22 of the 39 *Acinetobacter* isolates in our collection showed growth at concentrations of 1 mM $AgNO_3$ or above (Deshpande *et al.* 1993).

Five resistance markers were cured at varying frequencies from BL88 by plumbagin, indicating the involvement of a plasmid. High instability of silver resistance is also indicative of its plasmid coded nature (Annear *et al.* 1976). However, during conjugation only silver resistance was transferable. Transferable silver resistance is a rare phenomenon (Russell & Chopra 1990). Summers (1984) screened hundreds of Ag^r strains, out of which only one carried a plasmid. The Ag^r plasmid pKK1 was transferred from *Pseudomonas stutzeri* to *Psuedomonas putida* by high voltage electrotransformation (Trevors & Starodub 1990) and not by conjugation. However, Haefeli (1984) has reported intrageneric transfer of silver resistance in *Pseudomonas*. Also, plasmid transfer from *Acinetobacter* to *E. coli* is a rare phenomenon. (Chopade *et al.* 19856). In *A. baumannii* BL88, Ag^r transferred intergenerically although at a low frequency. The later *E. coli* to *E. coli* intrageneric transfer also occured at a similar frequency. This behavior of pUPI199 is unusual because intrageneric transfer of *Acinetobacter* plasmids appears at a higher frequency than intergeneric transfer (Chinchalkar *et al.* 1992).

Metal resistance genes are located on plasmids conferring antibiotic resistance (Novick *et al.* 1979). McHugh *et al.* (1975) reported transferable Ag^r which was associated with resistance to mercury, ampicillin, chloramphenicol and sulfonamide. However, our observation is that plasmid pUPI199 bears only an Ag^r marker as co-transfer or co-transformation of other markers was not detected. The molecular weight of pUPI200 was estimated to be about 54 kb. The silver resistance plasmids described earlier in *Pseudomonas stutzeri* pKK1 (75 kb) (Haefeli *et al.* 1984) and *E. coli* (83 and

77 kb) (Starodub & Trevors 1989) are thus different from pUPI199.

The low growth rate of the transformant *E. coli* DH5α (pUPI199) as compared with the recipient is evidence of the metabolic burden posed by maintainance of the plasmid in the cell. The cultures showed slower growth rates in presence of Ag^+ (data not shown) indicative of an inducible property. A decrease in the specific growth rate on exposure of resistant bacteria to high metal concentrations has been described previously (Horitsu & Kato 1980, Rajini Rani & Mahadevan 1992). Studies on Ag^r *Enterobacter cloacae* (Annear *et al.* 1976) revealed that Ag^r is expressed after acclimatization of the cells and only when silver ions are present in the medium.

At the present time, the mechanism of plasmid determined silver resistance or silver resistance in general is not clearly understood. In our studies, *A. baumannii* and *E. coli* showed an immediate accumulation of Ag^+ after addition of $AgNO_3$. The same has been observed by Pumpel & Schinner (1986) for silver accumulation in fungi and by Galun *et al.* (1982) for uranium accumulation in *Penicillium* biomass. It was observed that *A. baumannii* accumulated increasing concentrations of Ag^+ and then a plateau occured. It is possible that Ag^+ binding occurs at the surface rather than intracellularly. The *Thiobacillus* sp. are known to accumulate large quantities of silver by mere surface deposition (Pooley 1982). Specific surface receptors on BL88 may be binding silver ions. Once all the surface receptors were saturated, a plateau was observed. This also explains the inducibility and slow growth rate of *A. baumannii* BL88 in the presence of Ag^+. The lag phase may be essential to dilute out the sensitive surface receptors to be replaced by those inhibiting the entry of Ag^+ by chelating them. A similar phenomenon has been explained by Smith (1967) for resistance due to alternation in membrane permeability. In the sensitive *E. coli* DH5α cells, a gradual and then a rapid increase in accumulation occured. In a previous report, sensitive cells of *E. coli* were found to accumulate silver but not the resistant *E. coli* (Starodub & Trevors 1989). *E. coli* DH5α (pUPI199), however, steadily accumulated much higher levels of Ag^+ in the same time interval, possibly due to the plasmid coded specific surface receptors. In a detailed investigation on silver accumulation in *P. stutzeri*, Slawson *et al.* (1992b) observed that both sensitive and resistant cells accumulated silver, although at different final concentrations. Resistant cells accumulate 4-fold higher concentration than their sensitive counterparts (Slawson *et al.* 1992a). Our observation with *E. coli* K12 recepients and transformants is consistent with this. However, their studies suggest active intracellular uptake of Ag^+ which may not be the case with pUPI199 coded resistance. Moreover, it has been suggested that the resistant cells must exclude or efflux the accumulated Ag^+ unless some type of intracellular detoxification mechanism exists (Gadd 1989). Such a mechanism has not been identified so far.

Overall, this study has shown transferable plasmid mediated silver resistance in *A. baumannii* which constitutes the first report in the genus *Acinetobacter*. This is consistent with our hypothesis that *Acinetobacter* serves as a reservoir of naturally occuring metal resistant plasmids. Further studies on host range, incompatibility and the cloning of silver resistance genes are under investigation.

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