Silver resistance In *Acinetobacter baumannii* BL54 occurs through binding to a Ag-Binding Protein

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Abstract

The mechanism of plasmid mediated silver (Ag) resistance was investigated in Acinetobacter baumannii BL54. The intracellular accumulation of Ag in both original strain BL54 and Escherichia coli K12 transconjugant containing plasmid pUPI276 began immediately and reached a maximum within 60 minutes. This initial accumulation was followed by net loss of Ag which reached a maximum within 180 min. Pre-treatment of cells with 0.5 mM 2,4 dinitrophenol (DNP); 20 mM N, N-dicyclohexylcarbodiimide (DCCD); 3% toluene; 25 mg/ml cefotaxime and polymyxin-B resulted in considerable decrease in the accumulation process. Ag^S plasmid less cured derivative (BL54.1) also accumulated silver but only one-fourth the amount compared to the resistant strain BL54. The intracellular accumulated silver is detoxified by binding to a cysteine rich metal binding protein. The purified Ag-binding protein exhibited maximum absorption at 280/215 nm. From the above data it could be concluded that the intracellular detoxification of silver in A. baumannii BL54 is achieved through binding to a cysteine rich metalloprotein.

Keywords: *Acinetobacter, Silver resistance, Silver accumulation, Metalloprotein.*

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INTRODUCTION

Microorganisms exhibit versatile mechanism of resistance to metal ions (Silver, 1992; William and Silver, 1984). However, there is paucity of information regarding the mechanism of plasmid-mediated silver resistance in bacteria (Haefli *et al*. 1984). A study of silver resistance in *Pseudomonas stutzeri* AG259 (Slawson *et al*. 1994) using energy dispersive X-ray revealed large accumulation of silver deposited on the cell surface in the form of Ag sulfide complexes. Similarly, the mechanism of plasmid encoded Ag^r in *E.coli* R1 was studied (Stradoub *et al*. 1990). An Ag^S derivative accumulated higher amounts of silver as compared to Ag^r strain. Ghandour *et al*. (1988) demonstrated that accumulation of Ag by nongrowing *E.coli* cells was due to surface binding as well as intracellular uptake. Godard and Bull (1989) reported accumulation of Ag in actively growing and nongrowing population of *Citrobacter intermedius*, which was mainly on the cell envelope in the form of a dense deposit. Lie *et al*. (1997) studied silver resistance in *E.coli* mutants, which display active efflux of Ag^+ and were deficient in porins. Treatment of the cells with carbonyl cyanide, m-chlorophenyl hydrazone increased Ag accumulation in Ag susceptible and resistance strains suggesting that even Ag^S strain had an endogenous Ag efflux activity that occurs at higher level in Ag^r mutants. Plasmid encoded both silver and antibiotic resistance genes have been reported in *Salmonella typhimurium* (Mchugh *et al*. 1975) and *E. coli*

(Stradoub and Trevors 1990) but not such report exist in case of *Acinetobacter*. Metal binding proteins play an important role in metal detoxification in eukaryotes (Weser *et al*. 1973). The existences of metalloprotein have been reported in few prokaryotes (Higham *et al*. 1984; Jae-Soon and Cooksey, 1993; Olafson *et al*. 1979; Remacle and Verchval, 1991), however, no such report exists in case of *Acinetobacter*.

The present investigation deals with the mechanism of silver resistance in *A. baumannii* BL54. The role of metalloprotein in detoxification of silver was also studied.

MATERIALS AND METHODS

Bacterial source: *A. baumannii* BL54 was isolated from a clinical specimen from Armed Forced Medical College laboratory in Pune, India. The strain harbored two conjugative plasmids namely pUPI275 (Sm^r, Sd^r) and pUPI276 [(Ag^r, Ap^r, Cb^r, Tc^r, Cm^r (r= resistance)]. These plasmids were transferred by conjugation to *E. coli* K12 and cured by treatment with plumbagin (shakibaie *et al*. 1998, 1999).

Growth medium: To understand the mechanism of plasmid mediated Ag resistance in this strain, *A. baumanii* BL54 was primarily grown in PYL (Peptone yeast extract and lactose) medium containing peptone 2 gr; yeast extract 1 gr; NaH_2PO_4 0.22 gr; K_2HPO_4 0.36 gr; Lactose 2 gr; $MgSO_4$ $7H_2O$ 0.1 gr; 500 ml D/W pH 7.2 and subsequently transferred to *Acinetobacter* minimal medium (AMM) with the following composition, sodium succinate 5 gr; sodium acetate 1gr; $(NH4)_2SO_4$ 1 gr; L-glutamic acid 1 g; lactose 1 gr; Na_2HPO_4 0.1 gr; KH_2PO_4 0.01 gr in 1000 D/W supplemented with 25 mg tryptophan and histidine. For growth of the organism a loopful of the culture was inoculated into 200 ml above medium in 500 ml Erlenmeyer flask on rotary shaker [200 rpm] (Certomate -R Germany) and incubated at 30°C for 48 hours.

Silver accumulation: The accumulation of silver in strain BL54, plasmid-less derivative BL54.1 and *E.coli* K12 transconjugants (pUPI276) was studied by atomic absorption spectrophotometry (Slawson *et al*. 1994) and by Dithiazone (diphenylthiocarbazone) method (Haefli *et al*. 1984). A loopful of the organisms (10^8 CFU/ml) inoculated into 20 ml minimal medium in 100 ml flask containing 200 µg/ml $AgNO_3$ and incubated with shaking at 30°C for 24 hours. One set was kept as control without adding silver. Culture (1 ml) from each set was withdrawn immediately and centrifuged in micro-centrifuge tube at 10,000 xg for 5 min. Pellets were washed once with 0.01 M phosphate buffer, pH 8.0 and then with 2 mM piperazine N, N bis [2-ethane sulfonic acid] (PIPES) buffer, pH 6.7 to remove the growth medium. Similarly, samples were withdrawn at 5, 10, 20, 30 min. and intervals up to 18 hours then treated as above. The cell pellets were digested with 1 ml concentrated HNO_3 and incubated at room temperature overnight. The digests were diluted to 10 ml with sterile deionized D/W and heated at 80°C for 30 min. using a reflux condenser. The silver content of each sample was measure with atomic absorption spectrophotometer.

Effect of inhibitors on silver uptake: Similarly, the effect of 2, 4-DNP (2,4 dinitrophenol), DCCD (N, N-dicyclohexylcarbodiimide), toluene, cefotaxime and polymyxin-B on accumulation of silver was studied. Aliquots of the culture treated with these compounds individually was removed at various intervals and examined for Ag accumulation (Slawson *et al*. 1994) To study the silver uptake in nonviable cells, *A. baumannii* BL54 ($CFU10^7$) was exposed to 25 µg/ml cefotaxime and polymyxin-B for 2h before $AgNO_3$ addition. Metabolite inhibitor DCCD (Sigma grade) was dissolved in ethanol to give stock solution of 100 mM concentration and kept at 4°C. Diluted solution was prepared fresh and the amount of ethanol (2%) was not inhibitory to the cells. EDTA (10 mM) was used to remove surface bound silver (Stradoub *et al*. 1990). The viable count of the organisms in presence of 2,4-DNP was determined prior to addition of silver.

H2S production: To understand whether the accumulation of silver in BL54 is through silver–sulfide (Ag-S) formation, the production of H_2S gas was

tested by lead acetate strip test as well as gas chromatography (GC) (Godre, 1989). In case of GC, analysis was done with gas chromatograph (Model-8150, Shimadzu, Toshniwal, India) equipped with thermal conductivity detector. Data computation was done on integrator spectra (SPG 27) USA. Two sets were prepared, one set containing different concentrations of $AgNO_3$ (0.01-0.1 mM) in 50 ml L/B (Luria Bertani) broth. The H_2S was then determined in these samples and compared with a control set containing no silver.

Protein purification: Isolation of Ag-binding metallo-protein followed by its purification, the Sephadex G-100 gel filtration and DEAE-Cellulose ion exchange chromatography were used as described below. For purification purpose, first the organism was cultured in 500 ml Erlenmeyer flasks containing 200 ml Tris-base minimal medium at 30°C with shaking at 200 rpm. Two sets were run one of which contained and $AgNO_3$ (200 µg/ml) was added to one set. Cells were harvested after 48h of incubation by centrifugation at 8,000Xg for 5 min then washed in Tris-buffer (0.01 mM, pH 8.0) and re-suspended in one ml of the same buffer. The cell suspension was sonicated using a temperature controlled ultrasonicator (Degersheim, Schweiz, Germany) and cell lysis was monitored microscopically. The lysate was then centrifuged at 12,000 xg for 5 min at room temperature and supernatant was passed through a column (1.6x9 cm) packed with Sephadex G-100 equilibrated with the same buffer. Similarly, DEAE-Cellulose column was prepared in 0.01 M Tris-HCl was prepared and loaded with fraction 19 obtained from Sephadex column (Ghandour *et al.* 1988) and eluted with gradient salt (0.01 to 0.6 mM) according to Weser *et al*. (1973). The fractions collected and protein in each fraction was monitored at 280/215 nm. (U.V./Vis Elico, India). The concentration of Ag was measured using atomic absorption spectrophotometry. The cysteine content of purified metalloprotein was estimated by 5,5-dithiobis [2-nitrobenzoic acid] using Ellman`s reagent (1959). The crude lysate as well as purified metalloprotein were subjected to sodium dodecylsulfate (SDS) polyacrylamide gel electrophoresis.

Reverse phase HPLC (R-HPLC): The amino acid composition of the purified Ag-binding protein was determined by R-HPLC. Purified protein was lyophilized and hydrolyzed under HCl vapor at 116?C for 24h. The whole solution derivatized with phenylisothiocyanate. Quantification of the amino acids was carried out using R-HPLC (Shimadzu, model 221-25412) with a follow rate of 1 ml/min. Cysteine content of the protein was estimated separately (Godard and Bull, 1989).

RESULTS

These results show that the organism is unable to produce H2S regardless the Ag content of the media. These findings were confirmed by gas chromatography (data not shown). Silver was taken up almost immediately and reached to a maximum within 60 min. (3.6 mg/gr biomass) as shown in Fig. 1. The initial accumulation was followed by loss of Ag (Fig. 1). In contrast the plasmidless cured derivative (BL54.1) accumulated little amounts of Ag (0.5 mg/g biomass) and no silver was lost from the cell pellet. Estimation of silver by atomic absorption spectrophotometry and dithiazone method revealed highest accumulation of Ag in the original isolate BL54 (2.85 mg/gr biomass) and lowest in cured derivative BL54.1 (0.18 mg/gr biomass) (Fig. 1). The results were compared with sensitive culture of *E.coli* K12 J53.2 where only 0.205 mg Ag/gr biomass was accumulated in the cell.

Washing the cells with 10 mM EDTA did not reduce accumulated silver significantly and only a small quantity (300-400 µg/gr biomass) of surface bound Ag was lost in the original strain BL54 (Fig. 1). Treatment of the cells with cefotaxime and polymyxin-B antibiotics resulted in considerable reduction in Ag accumulation in nonviable cells, the results are shown in Figure 1. In presence of 2,4 DNP and DCCD, Ag was taken up for 60 min. (2.7 mg/gr biomass for DNP and 2.2 mg/gr biomass for DCCD) as in untreated cells but the excess of the accumulated Ag was not lost from the cells (Fig. 1).

To find out whether intracellular detoxification of Ag was due to binding to a metal binding protein, Sephadex G-100 gel filtration of cell lysate in pres-

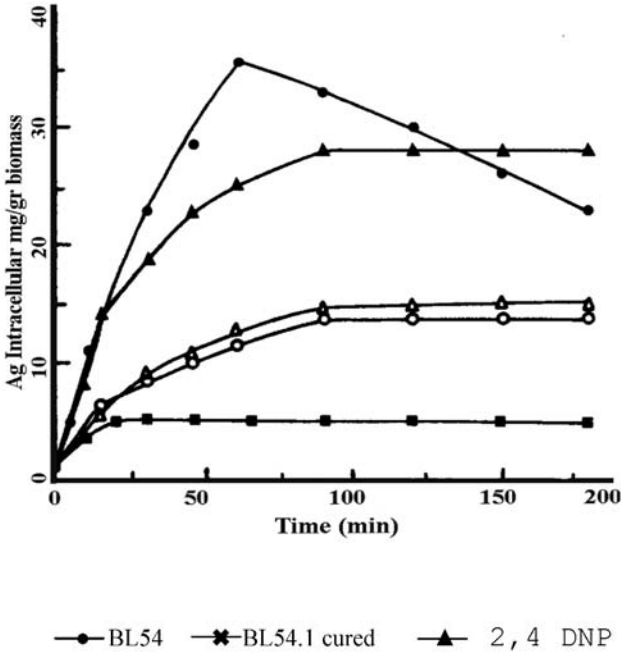

Figure 1. Accumulation of silver in *A. baumannii* BL54 and the plasmid cured derivative. Effect of inhibitors on the accumulation processes. The above results are average of two replicate experiments.

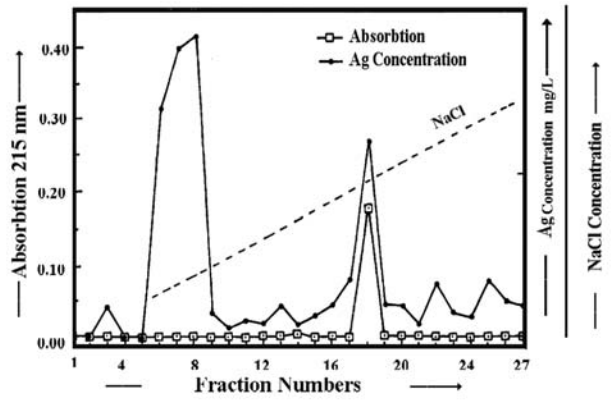

Figure 2. Ion exchange chromatography on DEAE-Cellulose of Ag-binding protein. A column (4 x 25 cm) of partially purified Ag-binding metallo-protein from fraction -19 of sephadex G-100.

ence and absence of 200 µg/ml $AgNO_3$ was carried out (data not shown). Fraction 19 exhibited maximum peak for silver corresponding to maximum absorption at 280/215 nm. However, cells grown in absence of Ag showed no such peak. This protein was further purified on DEAE-Cellulose ion exchange chromaography as shown in Figure 2. Two

Table 1. Amino acid composition (%) of the Ag-binding metalloprotein isolated from *A. baumannii* BL54.

Amino acid	(%)
Lysine	1.2
Histidine	6.9
Argenine	0.5
Threreonin	4.6
Serine	0.8
Glutamic acid	2.4
Proline	0.4
Glycine	4.8
Alanine	2.2
Cysteine	10.6

protein peaks (A280) were observed, Ag was associated with protein in fraction 18. This fraction exhibited maximum absorption at 280/215 nm. Quantitative estimation of amino acid composition (%) of the purified protein by reverse phase HPLC is shown in table 1. Further estimation of cysteine content of the fraction 18 showed presence of 10.6% cysteine (Table 1).

The purified Ag-binding metallo-protein was isolated from fraction 18 and lyophilized, the concentrate (60 ?l) was loaded on 7.5% native as well as reducing SDS-PAGE (Fig. 3). In SDS-PAGE containing 10% urea and 5% mercaptoethanol two bands were observed, while in native gel one band was detected. The purity of the protein further confirmed by dissolving the segment of the gel containing protein in 1 ml of 0.01 M phenol and presence of silver in protein the was etected by atomic absorption spectrophotometry. The results indicated that silver actually is bound to the protein.

The molecular weight of the protein was estimated by comparing its relative mobility in SDS-PAGE with that of standard M.W. Markers, ovalbumin, trypsine inhibitor, and lysozyme as well as by relativemobilities of the above proteins in Sephadex G-100 column. The molecular weight of Ag-binding metallo-protein was 22 KD.

DISCUSSION

The mechanism of resistance to silver in *A. bauman-*

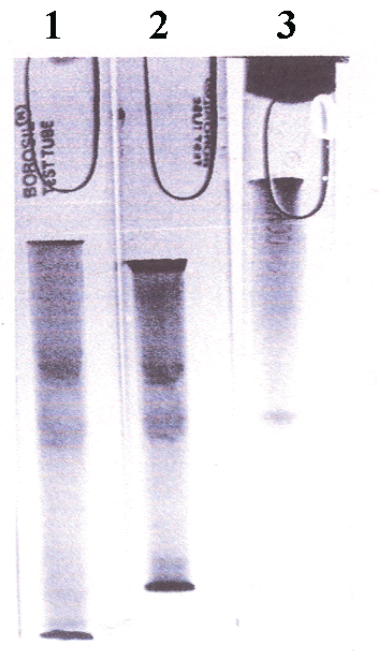

Figure 3. SDS-Polyacrylamide tube gel electrophoresis of purified Ag-binding metallo-protein from fraction 18 of DEAE cellulose column chromatography. The protein was concentrated by lyophilization and 60 ?l of the solution was loaded into the gel. The SDS gel was also containing 10% urea and 5% mercaptoethanol.

nii BL54 seems to be different from pervious reports (Mergeays *et al*. 1978; Silver, 1992; Slawson *et al*. 1994; Stradoub and Trvors,1990). Study of silver accumulation in strain BL54 and plasmidless sensitive derivative (BL54.1) revealed large amounts of silver associated with resistant strain BL54. The accumulation of silver was evident from washing the cells with EDTA that removes surface bound Ag and by treatment with toluene and antibiotics. The results were further confirmed by DCCD and DNP. The above information support the energy dependent Ag accumulation in strain BL54 and activity of this process was inhibited by uncoupler of proton motive force (DNP) and F_1F_0 ATP synthetase in the membrane (DCCD). Accumulation of Ag may occur in two stages:

1) Attachment of Ag to the cell surface which is non-specific and energy independent as occurs in case of sensitive cells.

2) Intracellular accumulation, which is metabolically active and accomplished by tight binding of Ag to the metalloprotein in the form of inert complexes.

The metalloprotein was purified by DEAE-Cellulose ion exchange chromatography and its amino acid composition was determined. The interesting observation was presence of high cysteine content (10.6%) in the protein. From this results and that of SDS-PAGE, it would be suggested that Ag binding metalloprotein consist of two subunits hold together by S-S linkages, these linkages get separated in presence of reducing agents like mercaptoethanol and urea. The intracellular sequestration of Ag by metalloprotein prevents the inhibitory activity of silver against cellular machinery. Indeed, Ag is known to be highly toxic for growth of microorganisms. It binds strongly to proteins, enzymes and DNA (Slawson *et al*. 1994). Silver at 86 µM inhibits oxidation of glucose, glycerol, fumarate and succinate (Williams and silver, 1984). Silver resistance in *E. coli* mutants has been studied. Such resistant mutants displayed active efflux of Ag and were deficient in porins (Lie *et al*. 1997).

The metalloproteins have been isolated from various microorganisms (Higham *et al*. 1984; Lie *et al*. 1997; Van-Noort *et al*. 1991) but there is no such report in case of *Acinetobacter*.

CONCLUSION

From above data it could be concluded that detoxification of intracellular silver in *A. baumannii* BL54 was achieved through a plasmid encoded energy dependent uptake (shakibaie *et al*. 1999). Metabolite inhibitors affected this activity.

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