Silver-Resistant Mutants of *Escherichia coli* Display Active Efflux of Ag⁺ and Are Deficient in Porins

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Silver-resistant mutants were selected by stepwise exposure of silver-susceptible clinical strains of *Escherichia coli*, two of which did not contain any plasmids, to either silver nitrate or silver sulfadiazine. These mutants showed complete cross-resistance to both compounds. They showed low-level cross-resistance to cephalosporins and HgCl₂ but not to other heavy metals. The Ag-resistant mutants had decreased outer membrane (OM) permeability to cephalosporins, and all five resistant mutants tested were deficient in major porins, either OmpF or OmpF plus OmpC. However, the well-studied OmpF- and/or OmpC-deficient mutants of laboratory strains K-12 and B/r were not resistant to either silver compound. Resistant strains accumulated up to fourfold less 110AgNO₃ than the parental strains. The treatment of cells with carbonyl cyanide m-chlorophenylhydrazone increased Ag accumulation in Ag-susceptible and -resistant strains, suggesting that even the wild-type Ag-susceptible strains had an endogenous Ag efflux activity, which occurred at higher levels in Ag-resistant mutants. The addition of glucose as an energy source to starved cells activated the efflux of Ag. The results suggest that active efflux, presumably coded by a chromosomal gene(s), may play a major role in silver resistance, which is likely to be enhanced synergistically by decreases in OM permeability.

Silver (Ag) is a biologically nonessential metal which is used widely in photographic emulsions. The toxicity of Ag has also led to clinical applications, including the topical treatment of bacterial infections with silver nitrate (AgNO₃) and silver sulfadiazine (AgSD). The action of AgNO₃ and AgSD is assumed to be dependent on the Ag⁺ ions, which strongly inhibit bacterial growth through poisoning of respiratory enzymes and electron transport components and through interference with DNA functions (3, 18).

Ag-resistant bacterial strains have been isolated from both clinical and environmental sources. Examples include strains of *Acinetobacter baumannii* (4), *Escherichia coli* (7), *Enterobacter cloacae* (7), *Klebsiella pneumoniae* (7), *Pseudomonas aeruginosa* (19), *Pseudomonas stutzeri* (5), and *Salmonella typhimurium* (15). In some cases the resistance was shown to be encoded by plasmids (4, 5, 15, 27, 31).

Mechanisms of resistance to other metals such as arsenic, cadmium, copper, and mercury have been elucidated at the molecular level (for reviews, see references 3, 27, and 28). Resistance is sometimes due to enzymatic detoxification as in the case of mercury and organomercurials (28). But in a majority of cases energy-dependent ion efflux seems to be responsible, as exemplified by bacterial resistance to many oxyanions (such as arsenite, antimonite, and chromate) as well as to the cations zinc, cobalt, cadmium, and nickel (3, 27, 28). Solioz and Odermatt (30) have shown recently that a P-type copper efflux ATPase from a gram-positive bacterium, *Enterococcus hirae*, can also pump out, perhaps fortuitously, Ag⁺. Little is known, however, about the mechanism of Ag resistance in gram-negative bacteria. When they contain silver-resistance plasmids, silver-resistant bacteria have sometimes been noted to accumulate less Ag than susceptible strains (4, 31, 32). These data have not been interpreted in terms of active efflux, and the mechanism of non-plasmid-encoded Ag resistance remains unclear.

In order to exert their action on bacterial cells, antimicrobial agents, including heavy metals, must reach their targets inside the cells. In gram-negative bacteria, there are at least two general mechanisms for effectively blocking drug access, i.e., the outer membrane (OM) permeability barrier and active efflux systems (22, 27). Pugsley and Schnaitman (26) reported that mutants of *E. coli* lacking OM porins were more resistant to Ag, and Lutkenhaus (12) obtained porin-deficient mutants of *E. coli* by selecting for resistance to copper. Our preliminary studies also revealed that Ag-resistant strains of gram-negative bacteria (such as *Citrobacter freundii*, *E. coli*, and *K. pneumoniae*) selected on Ag-containing agar plates often lacked major OM proteins. We therefore examined the role of porin deficiency more systematically. In addition, the possible role of active efflux in Ag resistance was examined, as such systems are recognized to play a major role in heavy metal resistance.

(A preliminary report of some of this work was presented at the 36th Interscience Conference on Antimicrobial Agents and Chemotherapy [10].)

**MATERIALS AND METHODS**

**Heavy metals, antibiotics, and other agents.** AgNO₃ and AgSD were from BDH Chemicals Ltd. (Toronto, Canada) and Polysciences Inc. (Warrington, Pa.), respectively. Radioactive silver nitrate (110AgNO₃ in 0.1 M nitric acid, 2.1 mg of Ag ml⁻¹, and 25.5 MBq ml⁻¹) was obtained from Amersham Life Science (Oakville, Canada) and was diluted 200-fold in water to produce a working solution. Other heavy metal salts (analytical grade) were from commercial sources. The sources of antibiotics were as previously described (8, 9). Carbonyl cyanide m-chlorophenylhydrazone (CCCP), disodium ATP, and sodium sulfadiazine were purchased from Sigma Chemical Co. (St. Louis, Mo.). HEPES was obtained from BDH Chemicals.

**Bacterial strains and their cultivation.** The strains 116, 496, and B₁ of *E. coli* were Ag-susceptible clinical isolates and were identified by using the Vitek AutoMicrobe System (bioMerieux, Hazelwood, Mo.) and maintained in the Department of Microbiology of the University of Saskatchewan, Canada. Ag-resistant mutants were selected by stepwise selection on tryptic soy broth (Difco, Detroit, Mich.) agar plates containing increasing concentrations of either
AgNO₃ or AgSD, without the use of mutants. In this article, AgNO₃R and AgSDR following the names of strains are used to represent these laboratory-selected Ag-resistant mutants. Some OmpF- and/or OmpC-deficient mutants and their isoapgenic parental strains of E. coli K-12 (JF568, JF701, JF703, JM101, and HB119) or Br (CM6 and CM7) (2, 24, 33) were used as control strains, as well as strain Ag⁺, an E. coli strain containing a silver resistance plasmid (7, 31). Bacteria were routinely grown in Luria-Bertani (LB) broth (1% Difco tryptone, 0.5% Difco yeast extract, 0.5% NaCl) at 37°C. Where indicated, LB broth without NaCl and M63 minimal medium with glucose (17) were used.

### Antibiotic and heavy metal susceptibilities.

The susceptibilities of Ag-resistant mutants of *E. coli* were examined in the Discussion section. The differences in doubling times when the strains were grown in LB broth with good aeration. The antibiotic and heavy metal susceptibilities. All five Ag-resistant mutants of *E. coli* were resistant to both AgNO₃ and AgSD (Table 1). The ratios of MICs in LB broth between the mutants and the parent strains were greater than or equal to 128 for AgNO₃ or 64 for AgSD. However, the ratios of MICs between the resistant and susceptible strains were much lower in a medium without NaCl (Table 1). A similar observation was made with Ag-resistant clinical isolates of gram-negative bacteria including *E. coli* (data not shown). This halide effect is examined in the Discussion section.

### Comparison of the susceptibilities of Ag-resistant mutants and parental strains to antibiotics and non-Ag heavy metals.

Comparison of the susceptibilities of Ag-resistant mutants and parental strains to antibiotics and non-Ag heavy metals showed that Ag-resistant mutants tended to have decreased susceptibilities to cephalosporins (fourfold increase in MICs) and possibly tetacycline and chloramphenicol (Table 1) but had sensitivities to carbencillin, benzylpenicillin, kanamycin, and ciprofloxacins that were similar to those of the parental strains (data not shown). The B1 strains were less susceptible to β-lactams than were the 116 and 496 strains, because B1

### TABLE 1. MICs of heavy metal compounds and antibiotics for Ag-susceptible and Ag-resistant strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>AgNO₃ (µg/ml)</th>
<th>AgSD (µg/ml)</th>
<th>HgCl₂ (µg/ml)</th>
<th>Cephalexin (µg/ml)</th>
<th>Cephalexin (µg/ml)</th>
<th>Cefepine (µg/ml)</th>
<th>Cefpirom (µg/ml)</th>
<th>Tetracycline (µg/ml)</th>
<th>Chloramphenicol (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>116</td>
<td>16</td>
<td>16</td>
<td>4</td>
<td>4</td>
<td>0.03</td>
<td>0.06</td>
<td>0.8</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>116AgNO₃R</td>
<td>&gt;1,024 (64)</td>
<td>&gt;1,024</td>
<td>6.4</td>
<td>16</td>
<td>32</td>
<td>0.13</td>
<td>0.25</td>
<td>0.8</td>
<td>6</td>
</tr>
<tr>
<td>496</td>
<td>16</td>
<td>16</td>
<td>4</td>
<td>4</td>
<td>0.06</td>
<td>0.06</td>
<td>1.0</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>496AgNO₃R</td>
<td>&gt;1,024 (64)</td>
<td>1,024</td>
<td>6.4</td>
<td>8</td>
<td>32</td>
<td>0.13</td>
<td>0.13</td>
<td>1.3</td>
<td>5</td>
</tr>
<tr>
<td>496AgSDR</td>
<td>&gt;1,024 (64)</td>
<td>&gt;1,024</td>
<td>12.8</td>
<td>16</td>
<td>32</td>
<td>0.13</td>
<td>0.13</td>
<td>1.0</td>
<td>5</td>
</tr>
<tr>
<td>B1</td>
<td>16</td>
<td>16</td>
<td>16</td>
<td>16</td>
<td>32</td>
<td>0.13</td>
<td>0.13</td>
<td>0.6</td>
<td>3</td>
</tr>
<tr>
<td>B1AgNO₃R</td>
<td>&gt;1,024 (64)</td>
<td>&gt;1,024</td>
<td>2.8</td>
<td>128</td>
<td>64</td>
<td>1.00</td>
<td>1.00</td>
<td>1.0</td>
<td>5</td>
</tr>
<tr>
<td>B1AgSD</td>
<td>&gt;1,024 (64)</td>
<td>&gt;1,024</td>
<td>1.4</td>
<td>256</td>
<td>64</td>
<td>0.50</td>
<td>1.00</td>
<td>1.0</td>
<td>2</td>
</tr>
</tbody>
</table>

* MICs of CoSO₄ (440 µg/ml), CrCl₃ (1.35 µg/ml), and CuSO₄ (1.25 µg/ml) were identical for all strains, except for B1AgNO₃R, which showed a twofold higher value. MICs of MnSO₄ (1.60 µg/ml) and ZnCl₂ (170 µg/ml) were also identical for all strains, except that of MnSO₄ for B1AgSDR (211 µg/ml) and that of ZnCl₂ for 496 (340 µg/ml). The MIC of Na₂HAsO₄ was identical (500 µg/ml) for the two strains tested, 116 and 116AgNO₃R.

**a** The values in parentheses are the MICs determined in LB broth without NaCl.

**b** MICs of this compound were unchanged, in every case, in LB broth without NaCl.
produced a higher level of \( \beta \)-lactamase, encoded by a plasmid in this strain (data not shown). Ag-resistant mutants were also somewhat resistant to \( \text{HgCl}_2 \) (up to a four- to eightfold increase in MICs) (Table 1). They usually had an unaltered susceptibility to \( \text{CuSO}_4 \), \( \text{Na}_2\text{HAsO}_4 \), \( \text{CoSO}_4 \), \( \text{CrCl}_3 \), \( \text{MnSO}_4 \), \( \text{HgCl}_2 \), and \( \text{ZnCl}_2 \) (Table 1). All strains, independent of their Ag susceptibility, were highly resistant to sodium sulfadiazine (MIC >2,048 \( \mu\text{g/ml} \)).

**Membrane proteins.** Since cross-resistance to some cephalosporins and Ag compounds was observed, we analyzed the membrane (OM and CM) proteins of the Ag-resistant mutants by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. As shown in Fig. 1, all five Ag-resistant mutants were essentially deficient in either OmpF porin (mutants of 116 and 496) or both OmpF and OmpC porins (mutant of B1). All strains had OmpA. There were no detectable differences in the CM proteins either among the mutant strains or between the mutants and the parental strains (data not shown).

**OM permeability.** Plasmid pBR322 was transformed into Ag-susceptible and -resistant strains of 116 and 496 so that the strains produced sufficient levels of \( \beta \)-lactamase for the OM permeability assay. This did not change the susceptibility of the strains to \( \text{AgNO}_3 \) and AgSD (data not shown). The OM permeability was determined by using cephaloridine and cephalothin as the substrates as described in Materials and Methods. The quantitative results indicated that the permeability of the laboratory-selected Ag-resistant mutants was at least five times lower than that of the parental strains (data not shown).

**Heavy metal susceptibility of the known OmpF- and/or OmpC-deficient strains.** Our Ag-resistant mutants were selected by a multistep process and could have contained other changes in addition to the loss of porins. We therefore examined, as controls, various well-studied, isogenic porin-containing or porin-deficient \( E. \text{coli} \) strains. The results showed that the strains with or without OmpF and/or OmpC had an essentially unaltered susceptibility to Ag compounds and \( \text{Cu}^{2+} \) (Table 2) and to many other metals such as \( \text{HAsO}_4^{2-} \), \( \text{Co}^{2+} \), \( \text{Cr}^{3+} \), \( \text{Mn}^{2+} \), and \( \text{Zn}^{2+} \) (data not shown) but the porin-deficient strains were somewhat more resistant to \( \text{HgCl}_2 \) (Table 2).

**Ag accumulation by Ag-susceptible and -resistant strains.** Ag accumulation studies were carried out mainly using one pair of strains, \( E. \text{coli} \) 116 and 116\( \text{AgNO}_3 \)-R. \( E. \text{coli} \) 116\( \text{AgNO}_3 \)-R accumulated less Ag (up to fourfold) than its parental Ag-sensitive strain when the external concentrations of Ag\( \text{NO}_3 \) were 20 \( \mu\text{M} \) or lower (Fig. 2A and B). It usually took about 5 to 10 min for the accumulation to reach a steady state. Interestingly, the accumulation difference was dependent upon the substrate concentration used. When 100 or 500 \( \mu\text{M} \text{AgNO}_3 \) was used, the difference between the susceptible and the resistant strains disappeared (Fig. 2C and D). Assuming that proteins make up about 50% of the cell (dry weight) and that the cell volume is around 3 \( \mu\text{l/mg} \) (dry weight) and that we can convert the accumulation data (such as those shown in Fig. 2) into nominal intracellular Ag concentrations (micromolar) (Table 3), although most of the Ag might be bound to cellular macromolecules. The estimated intracellular concentrations were much lower than the external concentrations when 5 or 20 \( \mu\text{M} \text{AgNO}_3 \) was used. At higher external concentrations (100 or 500 \( \mu\text{M} \)), the intracellular concentrations were close to the external ones (Table 3). Another Ag-resistant strain of \( E. \text{coli} \), 496\( \text{AgNO}_3 \)-R, also accumulated about 40% less Ag than

![FIG. 1. OM proteins of Ag-susceptible strains of \( E. \text{coli} \) and their laboratory-selected Ag-resistant mutants. Lanes: 2, 496; 3, 496\( \text{AgNO}_3 \)-R; 4, 496\( \text{AgSD} \); 5, 496\( \text{AgSD} \); 6, 116\( \text{AgNO}_3 \)-R; 7, 116\( \text{AgSD} \); 8, 116; 9, 116\( \text{AgNO}_3 \)-R; 10, 496\( \text{control, OmpF}^- \text{OmpC}^- \); 11, Ag; 1 and 12, molecular mass standards. OmpF and -C and OmpA are indicated by arrows; OmpF and OmpC usually run very close in this system, but in strain B1 (lane 5) OmpF (marked by an open triangle) and OmpC (the lane below) are well separated.](http://jb.asm.org/)

![FIG. 2. Accumulation of \( ^{115}\text{AgNO}_3 \) by intact cells of \( E. \text{coli} \) 116 (C) and its Ag-resistant mutant 116\( \text{AgNO}_3 \)-R (C). Cells were grown in LB broth, harvested, and resuspended in HEPES buffer as described in Materials and Methods. After 5 min at 37°C, \( ^{115}\text{AgNO}_3 \) was added to final concentrations of 5 \( \mu\text{M} \) (A), 20 \( \mu\text{M} \) (B), 100 \( \mu\text{M} \) (C), and 500 \( \mu\text{M} \) (D). The entry of Ag into the cells was assayed by centrifugation through silicone oil, as described in the text. The results shown are the averages of two to four separate experiments.](http://jb.asm.org/)
its parent Ag-sensitive strain, at the external concentration of 20 μM (data not shown).

Ag accumulation was also examined with the OmpF-deficient K-12 strain JF703 and its parent JF568 at 20 μM AgNO₃. There appeared to be a small difference in the initial rates of accumulation at early time points, the accumulation in JF703 being about 20% lower than that in JF568 at 1 or 2 min. However, there was no difference in the steady-state level of accumulation (essentially reached at 5 to 10 min) between these two strains (data not shown). These data are consistent with the similar MICs observed for these strains (Table 2).

**CCCP increases the accumulation levels of Ag.** Since differences in Ag accumulation were found between Ag-susceptible and -resistant strains, the effect of energy inhibitors on Ag accumulation was determined. When the cells were treated with the proton ionophore CCCP at 100 μM for 5 min at 37°C before initiating the Ag accumulation assay, the steady-state level of Ag accumulation was increased, and there was no longer a difference between the susceptible and resistant strains (Fig. 3). The steady-state levels of accumulation in CCCP-poisoned cells of both strains were very close to the external Ag concentration (Table 3). In contrast, other energy inhibitors such as arsenate at 1 mM and vanadate at 10 mM had no effect on Ag accumulation in both Ag-susceptible and -resistant strains (data not shown).

**Efflux induced by the addition of energy source.** Cells of strain 116AgNO₃R were washed three times in buffer and starved for 1 h at 37°C to delete the endogenous energy source, as described in Materials and Methods. When 110mAgNO₃ (5 μM) was added to these cells, Ag⁺ accumulation was maximal, probably because the efflux pump could not function owing to the lack of energy. After 15 min, the suspension was divided, and glucose was added to half of the suspension. Under these conditions, rapid, active efflux of 110mAgNO₃ out of the cells was observed (Fig. 4), probably being the result of an energy-coupled efflux process.

**Ag uptake by everted membrane vesicles.** Everted membrane vesicles provide a means of characterizing energy-dependent efflux systems in vitro. Such vesicles use energy to concentrate substances which intact cells expel. Vesicles prepared from Ag-susceptible and -resistant derivatives of strain 116 were assayed as described in Materials and Methods. When 20 mM sodium lactate or 0.5 mM disodium ATP was used, the vesicles accumulated about two-fold more Ag compared to the vesicles without an added energy source (data not shown). The higher concentration of ATP appeared, however, to interfere with the assay since the vesicle suspension became slightly more turbid after the addition of ATP. Therefore, the experimental parameters need to be examined more carefully before firm conclusions can be drawn.

**DISCUSSION**

Absence of major porins in Ag-resistant mutants. The laboratory-selected Ag-resistant mutants of *E. coli* were deficient in their major porins (Fig. 1). This finding was supported by the lowered OM permeability in the mutants (see Results). It is likely that the absence of porins contributed substantially to the low-level cross-resistance between Ag and several structur-
ally unrelated antibiotics (Table 1) (see, for example, reference 6). Porin-deficient mutants of *E. coli* grow at normal rates when high concentrations of nutrients are available, but their growth rate decreases when the nutrient concentrations become low (2,12). Our laboratory-selected Ag-resistant mutants grew at normal rates in well-aerated LB broth, but the growth became slower compared to that of their parent strains on solid medium, presumably because at the interior of colonies the cells were starved for nutrients.

But the absence of major porins alone cannot explain the Ag resistance, because various well-characterized OmpF- and/or OmpC-deficient mutants showed almost identical susceptibilities to Ag⁺ (Table 2). As described below, we believe that Ag⁺ is actively pumped out by resistant (and even susceptible) cells of *E. coli* and that lowered OM permeability acts synergistically with this efflux mechanism to raise the level of resistance (23), as has been shown for the endogenous tetracycline efflux system of *E. coli* (33).

**NaCl and Ag resistance.** Ag⁺ interacts with Cl⁻ to form sparingly soluble AgCl. Based on the solubility of AgCl (14 μM [1]), the presence of excess NaCl (85 mM in LB broth) is expected to lower the concentration of free Ag⁺ to about 2 nM, regardless of the nominal concentrations of AgNO₃ added. It was surprising, then, that the differences in the AgNO₃ MICs for resistant and susceptible strains in LB broth were greater than 100-fold (Table 1). Silver et al. (29) explained this phenomenon by assuming that susceptible cells bind Ag⁺ so tightly that they extract it from AgCl, whereas resistant cells do not. In view of our observation on the efflux-based mechanism of resistance, we propose a modified hypothesis. Both susceptible and resistant cells interact with particles of AgCl, and molecules on the cell surface extract or leach out Ag⁺ from AgCl. Subsequently, resistant cells are able to pump out more of the Ag⁺ ions that enter the cytoplasm. A similar hypothesis can explain the large differences in MICs seen with AgSD (Table 1), which is less soluble than AgCl (3 μM [21]).

**Evidence for the efflux of Ag⁺.** Active efflux is well-known as a major mechanism of both antibiotic and heavy metal resistance (22, 27). Ag-resistant strains accumulated much lower steady-state levels of Ag than the susceptible parent strains. This difference cannot be explained by differences in permeability alone and requires the assumption that Ag is extruded out of the resistant cells (see Fig. 2 of reference 23).

We obtained additional data that support the existence of a contribution by an active efflux process. (i) The deenergization of cells by pretreatment with proton conductor CCCP increased the levels of Ag accumulation in the resistant strain (Fig. 3). (ii) The addition of an energy source (glucose) to starved cells activated the efflux process for Ag (Fig. 4). The CCCP experiment also suggested that the wild-type, Ag-sensitive strains have Ag efflux activity.

Interestingly, the differences in accumulation levels in susceptible and resistant strains became smaller and smaller as the external concentrations of Ag were increased (Fig. 2). These data suggest that the Ag efflux system may become saturated at high concentrations and that the influx of Ag may overwhelm efflux. Under these conditions, the estimated intracellular Ag concentrations became close to the external concentrations, as expected (Table 3).

It appears very likely that our system is encoded by chromosomal genes because the parent strains we used were fully Ag susceptible and plasmid DNA could not be detected in either 116 or 496. Although a β-lactamase-encoding plasmid was present in strain B1, the Ag resistance phenotype could not be transferred into *E. coli* DH15a by transformation of the plasmid. We are not aware of previous reports of lowered accumulation of Ag⁺ in resistant strains not containing plasmids. However, such chromosomally encoded Ag⁺ efflux processes may be widespread in the bacterial world. For example, *P. aeruginosa* PA04068 (a derivative of PA01 [8]) was shown to accumulate four- to fivefold more Ag, from 20 μM external ¹¹⁰mAgNO₃ when pretreated with 100 μM CCCP (11). In view of these results, it seems exceedingly likely that the level-of-accumulation of Ag⁺ reported for plasmid-containing silver-resistant strains (4, 31) is also due to the active efflux of this ion. Indeed, Matsui and coworkers (14) have sequenced genes from the silver resistance plasmid pMG101 (15) and found that both an RND-type proton-coupled efflux transporter and a P-type ATPase are encoded by these genes.

Although our data demonstrate that an active efflux process exists for Ag⁺, the identity of this putative system remains to be established. In the gram-positive species *E. faecalis*, a chromosomally coded, P-type ATPase, CopB, has recently been shown to pump out Ag⁺, a process interpreted as “fortuitous” by the authors (30). We believe that our pump is likely to be quite different, as the endogenous *E. coli* pump appears to use proton-motive force as the energy source. Recently, Nakajima et al. (20) showed that overexpression of RobA in *E. coli* produced multiple resistance to antibiotics and heavy metals, including Ag. Since RobA activates the transcription of *acrAB* efflux pump genes (13), it may also increase the expression of a similar efflux system that is capable of pumping out heavy metal ions.

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