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Effects of Halides on Plasmid-Mediated Silver Resistance in *Escherichia coli*

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Silver resistance of sensitive *Escherichia coli* J53 and resistance plasmid-containing J53(pMG101) was affected by halides in the growth medium. The effects of halides on Ag^+ resistance were measured with $AgNO_3$ and silver sulfadiazine, both on agar and in liquid. Low concentrations of chloride made the differences in MICs between sensitive and resistant strains larger. High concentrations of halides increased the sensitivities of both strains to Ag^+.

Silver salts and compounds are used widely as environmental biocides and as clinical antimicrobial agents (2, 7, 10, 13, 17). Ag^+-resistant bacteria have been isolated (2, 8, 18), and resistance has sometimes been plasmid linked (5, 12, 19). However, most reports have been preliminary (e.g., references 5, 8, and 16) and the existence of silver-resistant bacteria in clinics has been questioned (10, 17), largely because of the compounding effects of environmental factors on toxicity and resistance (2, 3). Silver-binding components such as microbial biomass, proteins, lignin, and chloride are such factors. Among silver salts, $AgNO_3$ is water soluble, Ag_2SO_4 and Ag-acetate are sparingly soluble, and AgCl, AgBr, AgI, and Ag_2S are basically insoluble (4).

The purpose of this report is to set out easy-to-use conditions for measuring silver sensitivity and resistance in familiar and widely used media, Luria-Bertani (LB) agar and broth (14), so as to facilitate wider identification of silver resistance in nature.

Metal resistances including silver resistance are found in environments exposed to compounds that might provide for selection (3, 7, 17). Environmental toxicity of heavy metals to microorganisms is affected by both biotic and abiotic factors (3, 13, 16). These factors, for example, alter the bioavailability of metal ions (1, 3, 15). For Hg^{2+}, and as we will show for Ag^+ below, addition of Cl^- increases toxicity to bacteria (6), presumedly by increasing membrane permeability (11). The amount of bioavailable Hg^{2+} decreased when Cl^- levels exceeded 1 mM (1). This reduction was suggested to be due to an increase in the proportion of negatively charged mercury or Hg(II) complexes ($HgCl_3^-$/$HgCl_4^{2-}$ [1]). Similarly, in early and preliminary studies, the difference between Ag^+-sensitive and -resistant cells was most clear in the presence of Cl^-, while in the absence of Cl^-, cells with or without the resistance plasmid were both sensitive to Ag^+ (16).

The genes for Ag^+ resistance have been identified recently (9). The cluster of seven genes (and two open reading frames of unknown function) is organized into three divergently transcribed units. The gene products are tentatively identified by homologies with other available sequences, but frequently these are also for proteins that have not been directly isolated. SilE is a periplasmic metal-binding protein that has been purified and measured for Ag^+-binding properties (9). SilS and SilR are a presumed two-component membrane sensor and transcriptional responder, and SilCBA and SilP are a presumed Ag^+ efflux chemiosmotic cation-proton antiporter and a P-type ATPase, respectively.

The effects of halide ions on Ag^+ resistance of *Escherichia coli* K-12 strains J53 (Ag^+ sensitive) and J53(pMG101) (Ag^+ resistant) (9, 12) were measured on LB agar (14) streaked with log-phase cultures at a density of 200 Klett turbidity units (which corresponds to 2 g [wet weight] of cells per liter) (see Fig. 1 and 2). Since Ag^+ is bactericidal rather than bacteriostatic, as are some other toxic heavy metals, the numbers of cells streaked and their growth phase have great influence on growth appearing on the agar plate (reference 16 and data not shown). NaCl alone, without Ag^+, was toxic at high concentrations: at 40 g of NaCl per liter, the resistant J53(pMG101) cells showed reduced growth whereas the sensitive strain J53 reproducibly showed unimpaired growth (data not shown). A similar difference in NaCl sensitivity was found in liquid media (see below). We do not know whether this enhanced sensitivity to NaCl is associated with the silver resistance determinant or another region on the 180-kb (9) plasmid. In LB agar with added Ag^+ and no added NaCl, *E. coli* J53 grew with up to 100 μM Ag^+ and J53(pMG101) grew at 600 μM Ag^+ (Fig. 1A). *E. coli* J53 grew with up to 50 μM Ag^+ with 10 g of NaCl per liter (171 mM [Fig. 1B]), but there was no growth at 50 μM Ag^+ with higher NaCl levels (Fig. 1C and D). Growth of *E. coli* J53(pMG101) was barely reduced at 600 μM Ag^+ and 10 g of NaCl per liter (Fig. 1B), but in 20 g of NaCl per liter there was no growth at 200 μM Ag^+ and a marked reduction in growth at 100 μM Ag^+ (Fig. 1C). In 30 g of NaCl per liter, slight growth of *E. coli* J53(pMG101) was observed only at 50 μM Ag^+ (Fig. 1D). These results on agar media (Fig. 1) were quantitatively reproducible from experiment to experiment and were consistent with those obtained in liquid media (see below). It seemed likely that the effects of NaCl on silver toxicity result from the low solubility of AgCl (solubility prod-

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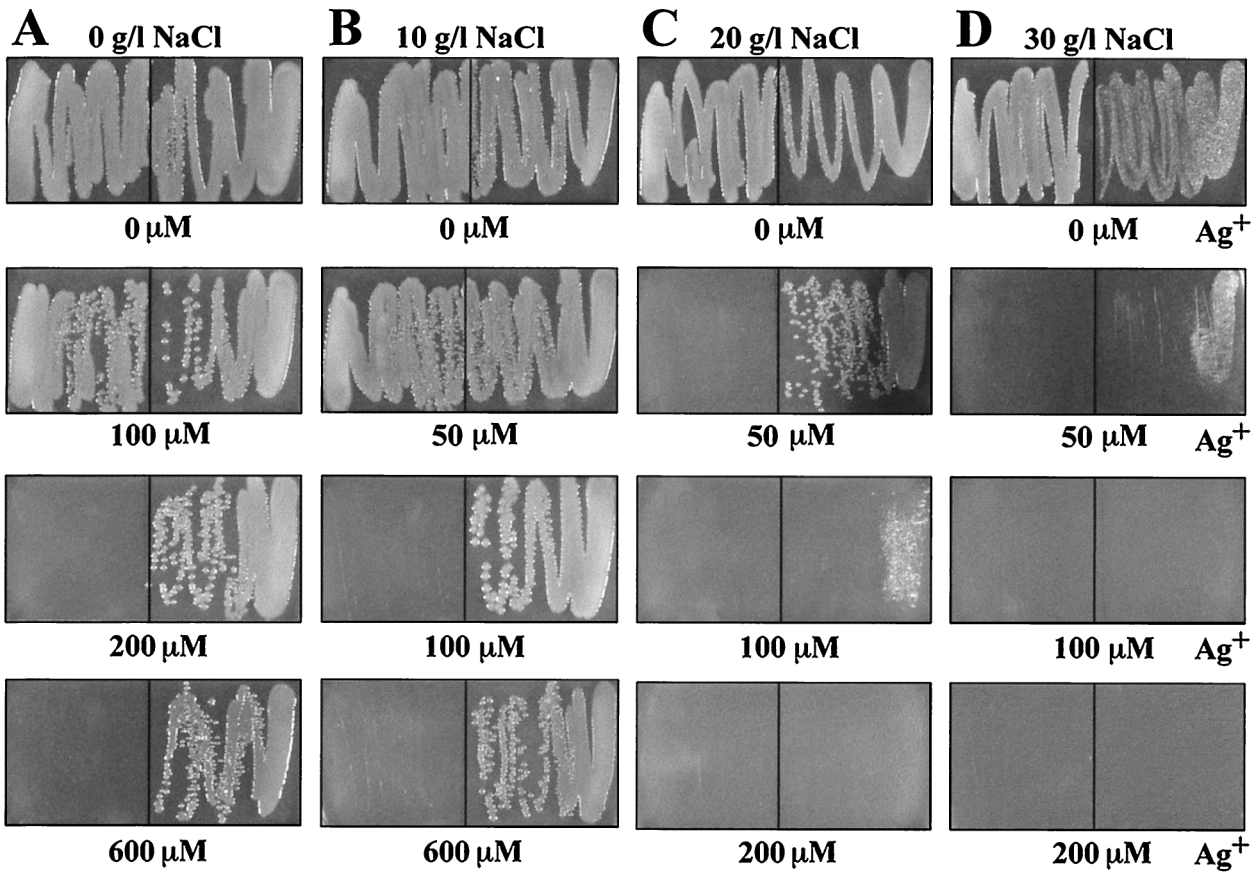

FIG. 1. Growth of *E. coli* J53 (sensitive, left) and J53(pMG101) (resistant, right) on LB agar with NaCl and $AgNO_3$ after 20 h at 37°C. LB was supplemented with $AgNO_3$ and no added NaCl (A), $AgNO_3$ and 10 g of NaCl per liter (B), $AgNO_3$ and 20 g of NaCl per liter (C), and $AgNO_3$ and 30 g of NaCl per liter (D). The numbers below the panels represent the $AgNO_3$ concentration added to each petri dish.

uct, 1.56×10^{-10} mol/liter [20]) and the formation of water-soluble complex anions, $AgCl_2^-$ and $AgCl_3^{2-}$ (4), at higher chloride levels. Similar chemistry occurs with the other halides, bromide and iodide (solubility products of AgBr and AgI, 7.7×10^{-13} and 1.5×10^{-16} mol/liter, respectively [20]), and formation of anionic complexes (AgX_2^- and AgX_3^{2-}, where X is Cl, Br, or I) with relative stabilities of $I^- > Br^- > Cl^-$ (4). In addition to the anionic silver complexes, cationic species (Ag_2X^+ and Ag_3X^{2+}) are formed in aqueous solutions (4). The water-soluble ionic Ag-halide complexes might have increased access to the cell membrane, resulting in increased bioavailability of Ag^+. At low halide levels, silver is excluded by precipitating as AgX, but at higher concentrations there is an increase in the proportion of accessible anionic complexes, resulting in decreased Ag^+ resistance.

Growth experiments were conducted with NaBr- and NaI-supplemented LB agar and $AgNO_3$ (Fig. 2). The Ag^+ sensitivity of *E. coli* J53 increased with the addition of low levels (2.5 g/liter) of NaBr (Fig. 2A), although NaBr alone was not toxic up to 30 g/liter (data not shown). A high level (30 g/liter) of NaBr reduced the Ag^+ resistance of J53(pMG101) substantially (Fig. 2A), as was found with 30 g of NaCl per liter (Fig. 1D). The addition of 5 g of NaI per liter (35 mM) reduced the Ag^+ sensitivity of *E. coli* J53 (Fig. 2B), allowing growth in 0.6 mM Ag^+, presumably by removing the Ag^+ as precipitated AgI. Higher I^- levels (10 or 20 g/liter [Fig. 2B]) increased the sensitivity of the resistant strain, presumably by solubilizing Ag^+ in a more "bioavailable" anionic complex.

Since Ag-sulfadiazine is the primary silver compound in clinical use (7, 13), it is appropriate to measure differences between $AgNO_3$ and Ag-sulfadiazine. Sulfadiazine in this compound is thought to function not as an antibiotic, but rather to complex silver in a water-insoluble form that is "slow released" for bioactivity (2, 13). The blackening on the skin from Ag^+ reduction with $AgNO_3$ is not seen with Ag-sulfadiazine. In the absence of added NaCl, Ag-sulfadiazine was slightly more toxic than $AgNO_3$ (compare Fig. 3 with Fig. 1A). Growth of sensitive *E. coli* J53 was reduced at 100 μM Ag-sulfadiazine, and resistant J53(pMG101) grew at up to 600 μM Ag-sulfadiazine. The toxicity of Ag-sulfadiazine to sensitive and resistant cells increased with the addition of NaCl (Fig. 3), as had been the case with $AgNO_3$ (Fig. 1). Comparing the toxicity of $AgNO_3$ with that of Ag-sulfadiazine, the latter was less (roughly twofold) toxic after the addition of NaCl (compare Fig. 3 with Fig. 1). For example, growth of resistant J53(pMG101) was reduced at 200 μM Ag-sulfadiazine, compared to 100 μM $AgNO_3$ (with 20 g of NaCl per liter), and growth of resistant J53(pMG101) occurred at 100 μM Ag-sulfadiazine, compared to 50 μM $AgNO_3$ (with 30 g of NaCl per liter [Fig. 3]).

Although growth on agar plates is the more ready means of distinguishing Ag^+-sensitive from Ag^+-resistant *E. coli* (Fig. 1 to 3), liquid growth experiments showed basically similar re-

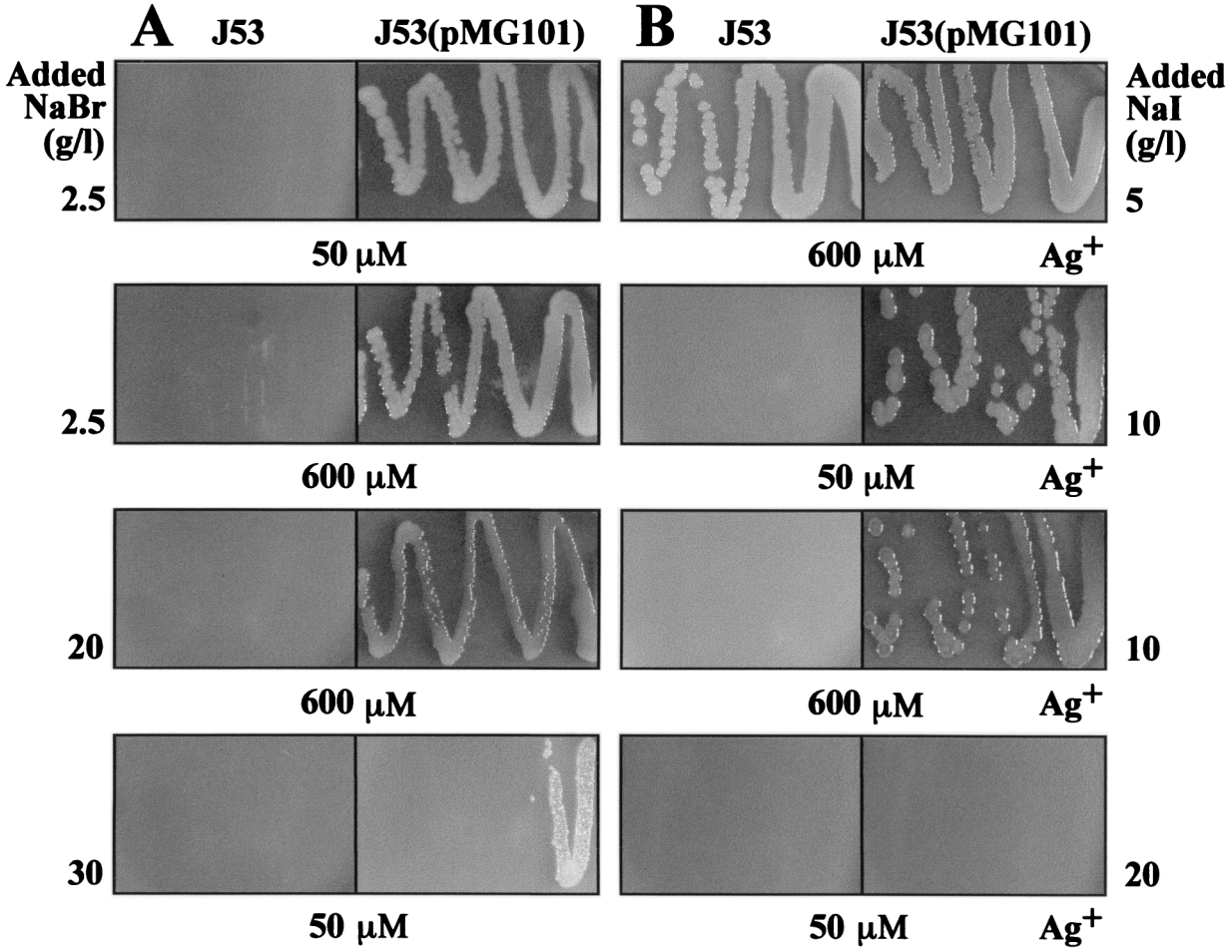

FIG. 2. Growth of *E. coli* J53 and J53(pMG101) on LB agar plates supplemented with NaBr and $AgNO_3$ (A) or NaI and $AgNO_3$ (B). The numbers on the sides of the panels represent the NaBr or NaI concentration, and the numbers below the panels represent the $AgNO_3$ concentration added to each petri dish.

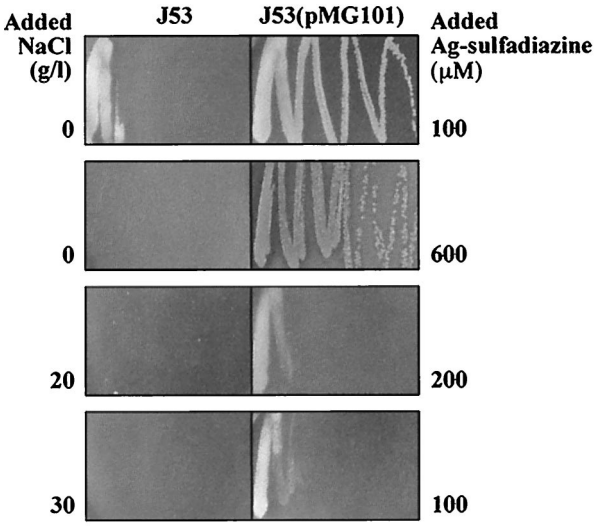

FIG. 3. Growth of *E. coli* J53 and J53(pMG101) on LB agar supplemented with NaCl and Ag-sulfadiazine. The numbers on the sides of the panels represent the NaCl or Ag-sulfadiazine concentration added to each petri dish.

sults (Fig. 4). The effects of chloride ions on Ag^+ resistance of *E. coli* J53 and J53(pMG101) were measured in LB broth (14) containing NaCl and/or $AgNO_3$, inoculated with 2 Klett turbidity units (20 μg [wet weight] of cells per ml) of log-phase cells. Low NaCl levels enhanced Ag^+ resistance of *E. coli* J53 (pMG101) from 400 μM Ag^+ to more than 600 μM Ag^+ (Fig. 4A, C, and D). Growth of sensitive *E. coli* was unaffected at 50 μM Ag^+, but turbidity was reduced at 100 μM Ag^+. With added higher NaCl concentrations, there was reduced growth of sensitive cells at 50 μM Ag^+ (Fig. 4D to F). Addition of 5 or 10 g of NaCl per liter (85 or 171 mM) to LB allowed growth of *E. coli* J53(pMG101) in 600 μM Ag^+ (Fig. 4C and D), but growth was reduced by Ag^+ at higher levels (20 or 30 g/liter) of NaCl (Fig. 4E and F). In LB liquid cultures, NaCl (50 or 60 g/liter) by itself, without added silver, was more toxic for strain J53(pMG101) than for the plasmidless strain J53 (Fig. 4B).

These results provide a basis for measurement of Ag^+ sensitivity and resistance by bacterial isolates, which is useful since silver compounds are increasingly being used as microbicidal agents (2, 10, 13, 17). Environmental silver-binding components are quite diverse and radically affect the proportion of silver that is bioavailable. In clinical settings, Cl^- and proteins bind Ag^+; in soil and most aqueous environments, Cl^- and organic S and N ligands predominate for Ag(I) binding.

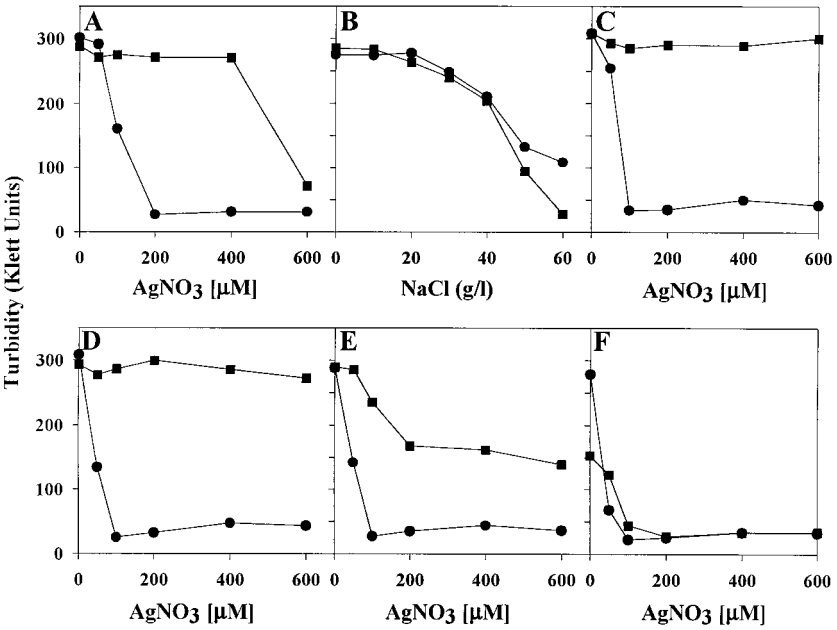

FIG. 4. Growth of *E. coli* J53 (●) and J53(pMG101) (■) in LB liquid broth. LB media were supplemented with $AgNO_3$ (no added NaCl) (A), NaCl (no added $AgNO_3$) (B), $AgNO_3$ and 5 g of NaCl per liter (C), $AgNO_3$ and 10 g of NaCl per liter (D), $AgNO_3$ and 20 g of NaCl per liter (E), and $AgNO_3$ and 30 g of NaCl per liter (F). Turbidity was measured after 20 h of growth at 37°C by a Klett-Summerson colorimeter with a Kodak 56 green filter.

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