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Mobilization of *Escherichia coli* R1 silver-resistance plasmid pJT1 by Tn5-Mob into *Escherichia coli* C600

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Summary. *Escherichia coli* R1 is an Ag^+-resistant strain that, as we have shown recently, harbours at least two large plasmids, pJT1 (83 kb) and pJT2 (77 kb). Tn5-Mob was introduced into the *E. coli* R1 host replicon via conjugation on membrane filters. The transfer functions of plasmid RP4-4 were provided in this process and Tn5-Mob clones mated with *E. coli* C600 yielded Ag^+-resistant transconjugants. This mobilization procedure allowed transfer and expression of pJT1 Ag^+ resistance in *E. coli* C600. Prior to use of Tn5-Mob mobilization, it was not possible to transfer Ag^+-resistant determinant(s) into *E. coli* by conjugation or transformation including high-voltage electroporation. *E. coli* C600 containing PJT1 and PJT2 displayed decreased accumulation of Ag^+ similar to *E. coli* R1. *E. coli* C600 could not tolerate 0.1 and 0.5 mM Ag^+, rapidly accumulated Ag^+ and became non-viable. Tn5-Mob mobilization may be useful in the study of metal resistance in bacteria, especially in strains not studied for resistance mechanisms.

Key words: Silver-resistance-accumulation – *Escherichia coli* – Mobilization – Tn5-Mob – Plasmid

Introduction

Silver resistance in selected bacterial strains has been studied by Belly and Kydd (1982), Haefeli et al. (1984), Hendry and Stewart (1979), Kaur and Vadehra (1986), Starodub and Trevors (1989, 1990) and reviewed by Trevors (1987). Numerous researchers (Charley and Bull 1979; Ghandour et al. 1988; Goddard and Bull 1989a, b; Pumpel and Schinner 1986) have investigated Ag^+ accumulation; however the mechanism of Ag^+ resistance remains uncertain. Goddard and Bull (1989a, b) reported that silver accumulation by *Citrobacter intermedius* B6 was associated with the cell envelope, surface absorption to cells being discounted as the accumulation process. Ghandour et al. (1988) observed that batch culture growth of *E. coli* K12 was completely inhibited at $AgNO_3$ concentrations above 2.5 μM in a chloride-free medium.

Starodub and Trevors (1989, 1990) reported that an Ag^+-resistant *Escherichia coli* R1 strain contained an 83-kb and a 77-kb plasmid. Curing the 83-kb plasmid produced an Ag^+-sensitive strain that accumulated higher concentrations of Ag^+ than the resistant wild type. Previous conjugation and transformation experiments were unsuccessful in introducing the pJT1 plasmid into bacterial strains. Furthermore, high-voltage electroporation of *E. coli* recipients with pJT1 did not yield Ag^+-resistant transformants (Starodub and Trevors 1989, 1990). Transfer of the suspected Ag^+-resistance plasmid, pJT1, into an Ag^+-sensitive recipient and expression of Ag^+ resistance would provide evidence that pJT1 carried the genetic determinant for Ag^+ resistance.

Simon (1984) constructed a Tn5-Mob transposon useful in the mobilization of Gram-negative bacterial replicons. Tn5-Mob can be inserted into the host DNA of Gram-negative bacterial replicons. A host replicon carrying Tn5-Mob can be mobilized into Gram-negative species using plasmid RP4-4 (Simon 1984). In the present study, Tn5-Mob was used to introduce plasmid pJT1 into *E. coli* C600. An Ag^+-resistant transconjugant of *E. coli* C600 and *E. coli* R1 was also studied for differences in their Ag^+-accumulating ability.

Materials and methods

Bacterial strains and plasmids. Bacterial strains and plasmids are listed in Table 1. *E. coli* strains were grown for 16 h in 100 ml Luria broth (LB) pH 7.2 (Acumedia, Baltimore, USA) at 37° C with shaking at 120 rpm. Antibiotics and $AgNO_3$ were used at the following concentrations: 100 μg/ml nalidixic acid (Nx), 5 μg/ml tetracycline (Tc), 40 μg/ml neomycin (Nm), 20 μg/ml kanamycin (Km), 200 μg/ml ampicillin (Ap) and 0.5 mM $AgNO_3$.

DNA isolation and electrophoresis. Plasmid DNA was isolated using alkaline (pH 12.4) lysis with sodium dodecyl sulfate and puri-

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Table 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics[a]	Source or reference
Bacterial strains		
E. coli C600	Nx^r	Pasteur Institute
E. coli R1	Ag^r	Hendry and Stewart (1979) Starodub and Trevors (1989; this study)
E. coli S17-1	294 recA, chromosomally integrated RP4 derivative	Simon (1984)
E. coli C600 (PJT1, PJT2, RP4-4)	Ag^r, Ap^r, Tc^r, Km^s	this study
Plasmids		
RP4-4	Ap^r, Tc^r, Km^s	Simon (1984)
pSUP5011	pBR325-Mob; Ap^r, Cm^r	Simon (1984)
pJT1	Ag^r	Starodub and Trevors (1989; this study)

[a] *r*, resistant; *s*, sensitive

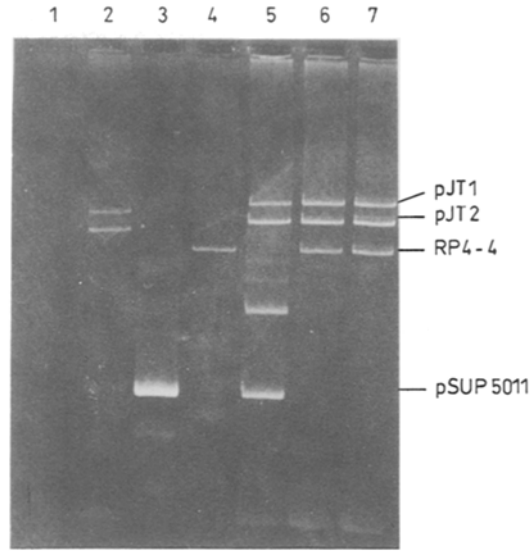

Fig. 1. Agarose (0.7% mass/vol.) gel electrophoresis. Lane 1, *E. coli* C600 plasmidless recipient; lane 2, *E. coli* R1 containing pJT1 and pJT2; lane 3, *E. coli* Tn5-Mob (pSUP5011); lane 4, *E. coli* (RP4-4); lane 5, *E. coli* R1 containing pJT1, pJT2, and pSUP5011; lane 6, *E. coli* R1 containing pJT1, pJT2 and RP4-4; lane 7, *E. coli* C600 containing pJT1, pJT2 and RP4-4. pJT2 is known not to carry any Ag^+-resistant determinants (Starodub and Trevors 1989). *E. coli* R1 containing pJT1, pJT2, RP4-4 and pSUP5011 is not shown

fied by horizontal agarose (0.7% mass/vol.) gel electrophoresis (Starodub and Trevors 1989, 1990). Gels were stained in an aqueous solution of 0.5 μg/ml ethidium bromide, destained in distilled water and visualized under 302-nm transillumination.

Conjugation crosses. Donor and recipient cells were grown to mid-exponential phase in 20 ml PenAssay broth (Difco, Detroit, USA) supplemented with appropriate antibiotic(s) or $AgNO_3$, mixed in a 1:1 cell ratio in sterile 1.5-ml centrifuge tubes, and centrifuged at $12000 \times g$ for 30 s. The mating mixtures were resuspended in 50 μl PenAssay broth and spread on sterile 0.45-μm cellulose acetate filters (25 mm diameter) on LB agar plates (Luria broth plus 1.5% agar; Simon 1984). Matings were conducted for 16 h at 37° C, at which time cells were resuspended and serially diluted in 0.85% (mass/vol.) sterile NaCl; 200-μl aliquots of undiluted and 100-fold diluted solutions were spread on the surface of LB agar supplemented with appropriate antibiotic(s) or $AgNO_3$. *E. coli* S17-1 containing Tn5-Mob (pSUP5011) was mated with *E. coli* R1 to obtain *E. coli* R1 (pJT1, PJT2) Tn5-Mob strains. Transconjugants were selected on LB agar supplemented with 0.5 mM $AgNO_3$ and 40 μg/ml Nm. In the second mating, 30 Nm-resistant clones were filter-mated individually with *E. coli* (RP4-4). Transconjugants were selected on LB agar supplemented with 0.5 mM $AgNO_3$, 40 μg/ml Nm, 200 μg/ml Ap and 5 μg/ml Tc. One transconjugant clone from each mating (total of 30) was subjected to plasmid isolation and horizontal agarose gel electrophoresis; 30 clones containing pJT1, pJT2, RP4-4 and Tn5-Mob (pSUP5011) were individually mated with *E. coli* C600 (resistant to 100 μg/ml Nx). About 50% of donor strains transferred Ag^+ resistance to *E. coli* C600 at a frequency of about 10^{-6}/recipient cell. The *E. coli* C600 strains were resistant to 100 μg/ml Nx and 0.5 mM $AgNO_3$ and contained pJT1, pJT2 and RP4-4 plasmids.

Accumulation of Ag^+ by whole cells. Silver accumulation was measured using the method described by Gadd et al. (1989) and Starodub and Trevors (1990). *E. coli* R1, *E. coli* C600 (RP4-4) and *E. coli* C600 (pJT1, pJT2, RP4-4) were grown in 100 ml Luria broth at 37° C for 4 h at 120 rpm, both in the absence and presence of various concentrations of $AgNO_3$. When *E. coli* C600 was grown in the presence of $AgNO_3$ the concentration used was 0.05 mM as this organism is sensitive to Ag^+. Cells were harvested by centrifugation at $8000 \times g$ for 15 min at 4° C, washed twice in sterile Luria broth, suspended in 18 ml Luria broth in a sterile 125-ml Nalgene flask and incubated in a water bath at 37° C with shaking at 120 rpm. Silver nitrate was then added at the desired concentration. At intervals, 1.0-ml aliquots were removed and centrifuged at $12000 \times g$ for 20 s. The cells were washed once in Type I ultrapure water (17.6 MΩ/cm) and repelleted at $12000 \times g$ for 20 s. Cell pellets were digested in 1 ml 6 M ultrapure HNO_3 at 90° C for 1 h and Ag^+ concentrations determined with a Buck 200A atomic absorption spectrophotometer (Buck Scientific, East Norwalk, USA) using operating conditions recommended by the manufacturer. A standard curve of Ag^+ prepared from an Ag^+ atomic absorption standard (Fisher Scientific, Toronto, Canada) was linear over the concentration range 0–100 μM. Ag^+ accumulation data were expressed relative to dry mass. Dry masses were obtained by drying cell pellets taken at the start of each experiment, at 80° C for 18 h, and weighing on a balance accurate to 0.1 mg. Viable cell counts were conducted after termination of Ag^+ accumulation experiments by spreading 100-μl aliquots of serial dilutions (prepared in 0.85% sterile NaCl) on LB agar plates. Colony forming units were enumerated after incubation for 48 h at 37° C. Absorbance of cell cultures at 600 nm was also determined after completion of Ag^+ accumulation assays to ensure culture growth had not occurred.

Results

Tn5-Mob successfully mobilized pJT1 into *E. coli* C600 (Fig. 1): lane 5 shows an agarose gel of *E. coli* R1 containing pJT1, pJT2, and Tn5-Mob plasmid (pSUP5011). When 30 clones of this strain were mated with *E. coli* C600 (lane 1) (Nx^r, plasmidless recipient), *E. coli* C600 (pJT1) Ag^+-resistant transconjugants were recovered that contained pJT1 plasmid, pJT2 and RP4-4.

The *E. coli* R1 wild type and *E. coli* C600 (pJT1, pJT2, RP4-4) transconjugant previously not exposed to

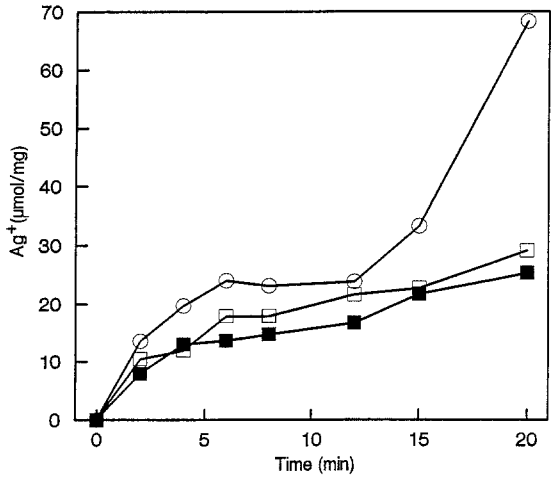

Fig. 2. Ag^+ accumulation from an external Ag^+ concentration of 0.1 mM in Luria broth at 37° C. All strains were previously grown in the absence of Ag^+ prior to the experiment. (○) *E. coli* C600; (□) *E. coli* C600 (pJT1, pJT2, RP4-4); (■) *E. coli* R1 wild type. Data points in Figs 2–4 are means of duplicate experiments

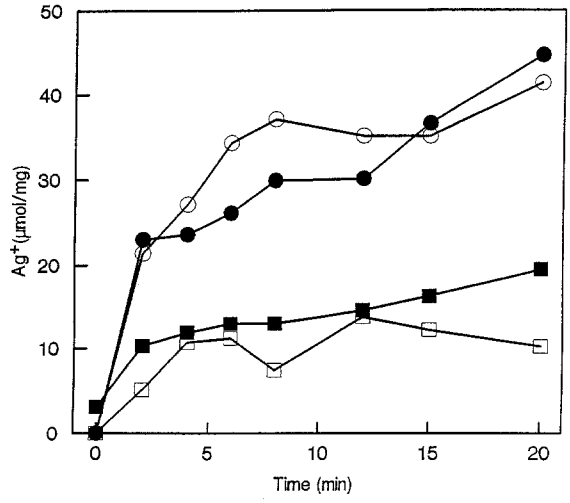

Fig. 4. Ag^+ accumulation from an external Ag^+ concentration of 0.5 mM in Luria broth at 37° C. Strains previously not grown in the presence of Ag^+: (○) *E. coli* C600 (pJT1, pJT2, RP4-4); (●) *E. coli* R1 wild type. Strains previously grown in presence of 0.05 mM Ag^+ prior to experiment: (□) *E. coli* C600 (pJT1, pJT2, RP4-4); (■) *E. coli* R1 wild type

Ag^+ accumulated low concentrations (about 25–29 μmol/mg) of Ag^+ after a 20-min incubation in the presence of 0.1 mM Ag^+. *E. coli* C600 (recipient organism) was sensitive to Ag^+ and accumulated 68 μmol Ag^+/mg dry mass during the 20-min assay (Fig. 2). Plating of cell suspensions at termination of the accumulation experiments revealed the Ag^+-sensitive *E. coli* C600 strain was essentially non-viable as demonstrated by a 10^7 decrease in colony forming units. The *E. coli* R1 wild type and transconjugant remained viable during the accumulation assay (data not shown). These findings suggested that pJT1 plasmid was responsible for decreased Ag^+ accumulation and maintenance of viability in the presence of Ag^+.

The *E. coli* strains used in the first Ag^+-accumulation experiments were previously grown in Luria broth not supplemented with $AgNO_3$ (Fig. 2). In another experiment these strains were grown in Luria broth supplemented with 0.05 mM Ag^+, a concentration not inhibitory to the Ag^+-sensitive *E. coli* C600 strain. These cultures were harvested, washed, and used in Ag^+-accumulation assays (Fig. 3). The *E. coli* C600 plasmidless recipient accumulated the highest concentration of Ag^+ during a 20-min period. The shape of the accumulation curve may represent a biphasic accumulation process in this Ag^+-sensitive strain. Fluctuations in accumulation by *E. coli* C600 may indicate alternating Ag^+ uptake and release or, alternatively, cell death and lysis. Accumulation was about sevenfold less than the concentration accumulated by the same strain previously not grown in the presence of Ag^+ (Fig. 2). This indicated that previous exposure to Ag^+ saturated binding sites for this metal, and little subsequent accumulation was possible (Fig. 3). The *E. coli* strains resistant to Ag^+ accumulated 5–6-fold less Ag^+ (Fig. 3) than the same strains previously grown in the absence of Ag^+ (Fig. 2).

When the external Ag^+ concentration was increased from 0.1 mM to 0.5 mM in accumulation assays (Fig. 4), *E. coli* R1 and the Ag^+-resistant *E. coli* C600 (pJT1, pJT2, RP4-4) transconjugant exhibited decreased accumulation of Ag^+ at corresponding times after prior exposure to 0.05 mM $AgNO_3$. The same strains accumulated 3–4-fold more Ag^+ when cells previously not exposed to Ag^+ were used in accumulation assays. This demonstrated that Ag^+-resistant strains are influenced by previous exposure to Ag^+. A majority of the Ag^+ was accumulated during the first 5 min of the assay. However, during the remaining 15 min, fluctuations in accumulation were observed. Ag^+-accumulation studies were not possible with the *E. coli* C600 Ag^+-sensitive strain at the higher Ag^+ concentration.

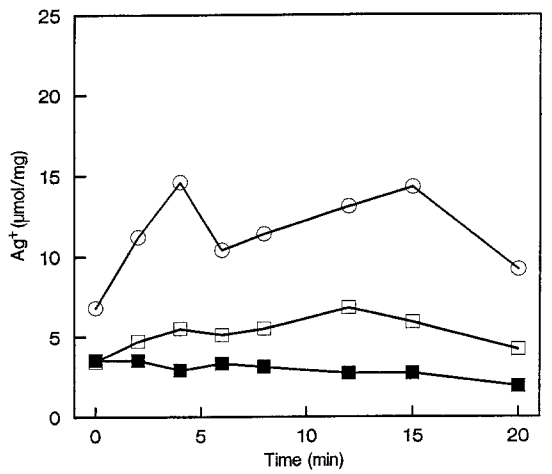

Fig. 3. Ag^+ accumulation from an external Ag^+ concentration of 0.1 mM in Luria broth at 37° C. All strains were previously grown in the presence of 0.05 mM Ag^+ prior to the experiment. Legends and symbols as in Fig. 2

Discussion

Tn5-Mob mobilization of pJT1 provided evidence that pJT1 was responsible for Ag^+ resistance as postulated by Starodub and Trevors (1989, 1990), who observed that *E. coli* R1 cured of pJT1 was sensitive to Ag^+. Plasmid curing provides preliminary evidence that a determinant is carried on an extrachromosomal plasmid (Trevors 1986). The Tn5-Mob system was previously used to mobilize and transfer a cryptic plasmid from *Azospirillum lipoferum* into a plasmid-free *A. tumefaciens* strain (Bally and Givaudan 1988). However, the Tn5-Mob system has not been extensively used with non-conjugative plasmids suspected of carrying metal-resistance determinant(s). In addition, Tn5-Mob can also label chromosomal DNA which may in turn be mobilized. We believe the acquired Ag^+ resistance in the transconjugant *E. coli* C600 (pJT1, pJT2, RP4-4) could not have been due to mobilization of a chromosomal element from *E. coli* R1 by Tn5-Mob because curing *E. coli* R1 of pTJ1 rendered that strain sensitive to Ag^+ (Starodub and Trevors 1989, 1990). Previously, Haefeli et al. (1984) mobilized the non-conjugative Ag^+-resistance plasmid, pKK1 from *Pseudomonas stutzeri* AG259 into *P. putida* using plasmid R68.45. The *P. putida* transconjugant expressed Ag^+ resistance.

Silver is a toxic metal with no known function in bacteria (Trevors 1987). It can complex with proteins, amino acids, phosphate and chloride ions (Trevors 1987). Therefore, Ag^+-resistant bacterial strains must prevent Ag^+ from exerting a lethal effect. In the present study, decreased accumulation of Ag^+ was observed by the Ag^+-resistant *E. coli* R1 wild type and the C600 transconjugant into which plasmids pJT1 and pJT2 were mobilized. Silver resistance may be explained by a difference in affinity for Ag^+: sensitive cells bind Ag^+ more tightly than resistant cells. Further studies are in progress to determine if pJT1 and the Ag^+-resistance plasmid pKK1 in *P. stutzeri* AG259 share a common resistance mechanism to Ag^+, such as the diminished presence or lack of specific outer-membrane protein(s) (Starodub and Trevors 1989, 1990). The resistance mechanism(s) for *E. coli* R1, *P. stutzeri* AG259 and numerous other Ag^+-resistant bacterial strains have not been determined (Trevors 1987). However, it is known that the Ag^+-resistant *P. stutzeri* AG259 strain accumulates low concentrations of Ag^+ and that accumulation is dependent on conditions used in accumulation assays (i.e. buffer versus broth) and previous growth conditions such as absence or presence of Cu^{2+} or Ag^+ in the growth medium (Gadd et al. 1989). For example, cells grown in the presence of sub-inhibitory concentrations of Cu^{2+} or Ag^+ accumulated less Ag^+ in subsequent assays (Gadd et al. 1989). Similar findings were observed with *E. coli* grown in the presence of Ag^+ in the present study. In addition, Ag^+ accumulation in *P. stutzeri* AG259 was dependent on the Cl^- concentration. It is possible that Ag^+-sensitive bacteria bind Ag^+ tightly and accumulate it, whereas Ag^+-resistant cells bind the metal ions to a lesser extent and as a result do not accumulate Ag^+. The results of this study suggest this is the mechanism of Ag^+ resistance. Therefore, Ag^+ does not exert a toxic effect until the metal concentration reaches a critical level that even resistant cells cannot tolerate (Trevors 1987). It would appear that such a resistance mechanism, which excludes Ag^+ from accumulating, would be more effective in protecting cells from Ag^+ than the synthesis of metal-binding proteins which may be specific for Ag^+ or possibly non-specific for several metals. Continuing research in this area will bring forth information on resistance mechanisms, as well as the location of resistance determinants.

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References

Bally R, Givaudan A (1988) Mobilization and transfer of *Azospirillum lipoferum* plasmid by the Tn5-Mob transposon into a plasmid-free *Agrobacterium tumefaciens* strain. Can J Microbiol 34:1354–1357

Belly RT, Kydd GC (1982) Silver resistance in microorganisms. Dev Ind Microbiol 23:567–577

Gadd GM, Laurence OS, Briscoe PA, Trevors JT (1989) Silver accumulation in *Pseudomonas stutzeri* AG259. Biol Metals 2:168–173

Charley RC, Bull AT (1979) Bioaccumulation of silver by a multispecies community of bacteria. Arch Microbiol 123:239–244

Ghandour W, Hubbard JA, Deistung J, Hughes MN, Poole RK (1988) The uptake of silver ions by *Escherichia coli* K12: toxic effects and interaction with copper ions. Appl Microbiol Biotechnol 28:559–565

Goddard PA, Bull AT (1989a) The isolation and characterization of bacteria capable of accumulating silver. Appl Microbiol Biotechnol 31:308–313

Goddard PA, Bull AT (1989b) Accumulation of silver by growing and non-growing populations of *Citrobacter intermedius* B6. Appl Microbiol Biotechnol 31:314–319

Haefeli C, Franklin C, Hardy K (1984) Plasmid-determined silver resistance in *Pseudomonas stutzeri* isolated from a silver mine. J Bacteriol 158:389–392

Hendry AT, Stewart JO (1979) Silver-resistant Enterobacteriaceae from hospital patients. Can J Microbiol 25:915–921

Kaur P, Vadehra DV (1986) Mechanisms of resistance to silver ions in *Klebsiella pneumoniae*. Antimicrob Agents Chemother 29:165–167

Pümpel T, Schinner F (1986) Silver tolerance and silver accumulation of microorganisms from soil materials of a silver mine. Appl Microbiol Biotechnol 24:244–247

Simon R (1984) High-frequency mobilization of Gram-negative bacterial replicons by the in-vitro-constructed Tn5-Mob transposon. Mol Gen Genet 196:413–420

Starodub ME, Trevors JT (1989) Silver resistance in *Escherichia coli* R1. J Med Microbiol 29:101–110

Starodub ME, Trevors JT (1990) Silver accumulation and resistance in *Escherichia coli* R1. J Inorg Biochem (in press)

Trevors JT (1986) Plasmid curing in bacteria. FEMS Microbiol Rev 32:149–158

Trevors JT (1987) Silver resistance and accumulation in bacteria. Enzyme Microb Technol 9:331–333