Diversity of silver resistance genes in IncH incompatibility group plasmids

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Silver compounds are used as antimicrobial agents in medicine and bacteria that develop resistance to silver cations (Ag⁺) pose problems similar to those of antibiotic-resistant bacteria. The first set of Ag⁺ resistance genes (*sil*) was from plasmid pMG101, now assigned to the IncHI incompatibility group. Questions of whether *sil* genes are unique to pMG101 or are more widely found, and whether they are associated with a specific incompatibility group or occur in many plasmid groups and on bacterial chromosomes were addressed. *sil* genes were identified in five IncH plasmids, but not in plasmids of the IncP incompatibility group. Three *sil* genes (*silP*, *silR* and *silE*) from these plasmids were PCR-amplified, cloned, sequenced and compared to those of pMG101. Differences of 0–50 nt per kb of sequence were found. Predicted gene products were 0–6% different in amino acid sequence, but the differences did not alter residues thought to be involved in protein function (see supplementary data at <http://mic.sgmjournals.org> or <http://www.uic.edu/depts/mcml/individual/gupta/index.htm>). For representative IncH plasmid R476b and pMG101 the effects of Ag⁺ exposure on resistance levels were measured by growth. The inducibility of *silC*, *silR* and *silE* gene expression after Ag⁺ exposure was studied by reverse transcriptase (RT)-PCR. Silver resistance increased after Ag⁺ exposure for strains carrying plasmid R476b. *silC* and *silE* expression from R476b was inducible after Ag⁺ exposure and was constitutive and high from pMG101. The mRNA levels for the regulatory gene *silR* was constitutive for both pMG101 and R476b. Close homologues for *silABC(ORF96)RS* from pMG101 are clustered on the chromosomes of *Escherichia coli* strains K-12 and O157:H7, without contiguous *silP* and *silE* homologues. Insertion deletions of the *E. coli* K-12 chromosomal homologues for *silA* and *silP* gave Ag⁺ hypersensitivity for growth. The *silA* homologue knockout was complemented back to wild-type resistance by the same gene cloned on a plasmid. Homologues of *sil* genes have also been identified on other enterobacterial genomes.

Keywords: IncH plasmids, *E. coli* chromosome, plasmid incompatibility group

INTRODUCTION

Some metal cations (Zn²⁺, Cu²⁺, Co²⁺ and Ni²⁺) are essential trace nutrients for living cells and function in enzymic reactions. Heavy metal cations (Ag⁺, Cd²⁺, Pb²⁺ and Hg²⁺) are non-essential and toxic. Metal salts

and compounds are often used as antimicrobial agents in medicine and as biocides in hospitals and other human settings.

Silver compounds and products are increasingly common as microbicidal agents in hygiene, agriculture and industry, in addition to clinical uses (reviewed by Gupta & Silver, 1998; Silver *et al.*, 1999a, b). In hospitals, silver sulfadiazine is used in burn dressings (George *et al.*, 1997) to prevent nosocomial infections. The most familiar human exposure to silver is with dental amalgams that contain 35% Ag(0) and 50% Hg(0)

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Fig. 1. (a) Genes and protein products of the plasmid *sil* determinant. Open boxes indicate the nine genes and ORFs. Arrows at the top mark the mRNA transcripts and their orientations. (b) Comparison of the pMG101 *sil* determinant and the *E. coli* K-12 and O157:H7 chromosomal *agr* homologues. The intergenic gaps are given in bp and the sizes of gene products are given in aa. Amino acid identities (%) between gene products are shown.

(Dunne *et al.*, 1997). The slow release of Hg(0) from amalgams is known to select for mercury-resistant bacteria in the gut (Lorscheider *et al.*, 1995). Ag(0) is also released (Lygre *et al.*, 1999), but whether it has any antimicrobial activity that selects for resistance (perhaps in the mouth) has never been tested.

Ag⁺ ions are highly toxic to all micro-organisms, perhaps due to poisoning of the respiratory electron transport chains and components of DNA replication (Modak & Fox, 1973; Russell & Hugo, 1994). Although bacterial Ag⁺ resistance has been periodically reported, the basis was not studied before our recent efforts (Gupta & Silver, 1998; Gupta *et al.*, 1999; Silver *et al.*, 1999a, b). Human exposure to silver compounds has no serious adverse effect (Russell & Hugo, 1994). Prolonged silver use occasionally results in 'argyria', a condition with an irreversible grey to blue-black colouring of the skin and mucous membranes due to Ag(0) or Ag₂S deposits. Argyria, however, is mostly of cosmetic concern (Russell & Hugo, 1994).

Bacterial resistances to different toxic metal ions are encoded by genes located mostly on plasmids, but sometimes on bacterial chromosomes (reviewed by Silver, 1998; Silver & Phung, 1996; Silver *et al.*, 2000). These resistances are selected frequently when metal salts are used as antiseptics. Ag⁺-resistant bacteria have been reported periodically from sources such as hospitals and burn wounds where silver toxicity might be expected to select for resistance (Annear *et al.*, 1976; Bridges *et al.*, 1979; Hendry & Stewart, 1979; McHugh *et al.*, 1975; Pruitt *et al.*, 1998). The physiological, biochemical and molecular basis of bacterial Ag⁺ resistance were not described prior to our studies (Gupta *et al.*, 1999). The first report on the genetic and molecular basis for Ag⁺ resistance concerned a *Salmonella typhimurium* isolate, from the Massachusetts General Hospital, that killed several patients and required the closing of the burn ward in 1975 (Gupta *et al.*, 1999; McHugh *et al.*, 1975). Plasmid pMG101 from this strain confers resistances to Ag⁺, Hg²⁺ and tellurite, as well as to several antibiotics, for example ampicillin,

chloramphenicol, tetracycline and streptomycin (Gupta *et al.*, 1999; McHugh *et al.*, 1975).

The Ag⁺ resistance determinant from pMG101 contains nine ORFs (Fig. 1a) in 12.5 kb of sequence (GenBank accession no. AF067954; Gupta *et al.*, 1999). The ORFs are arranged in three transcriptional units (Fig. 1a; Gupta *et al.*, 1999). Functions for the seven named genes were assigned on the basis of homologies to known proteins for other metal resistances; the two unnamed ORFs lack such homologues. The first gene identified, *silE*, encodes a 123 aa periplasmic metal-binding protein (Gupta *et al.*, 1999). Upstream from *silE* and in the same orientation is a presumed two-component gene pair, *silRS*, encoding a transcriptional regulatory responder protein and a membrane sensor kinase, homologous to other members of the two-component family (Hoch & Silhavy, 1995).

The remaining six ORFs in the Ag⁺ resistance system are transcribed divergently from *silRSE* (Fig. 1a; Gupta *et al.*, 1999). The *silCBA* genes immediately upstream of *silRS* encode a presumed three-polypeptide chemiosmotic cation/proton antiporter that is a member of the resistance, nodulation and cell division (RND) family of transporters (Saier *et al.*, 1994). This protein complex consists of an inner-membrane proton/cation antiporter (SilA), a membrane fusion protein that spans the inner and outer membranes of Gram-negative bacteria (SilB) and an outer-membrane protein (SilC). Between *silC* and *silB*, a 96 codon ORF of unassigned function was identified (Gupta *et al.*, 1999).

The product of the last gene of the Ag⁺ resistance determinant, SilP, is predicted to be a P-type ATPase, a member of the family of heavy-metal resistance ATPases (Rensing *et al.*, 1999; Silver & Phung, 1996). The Ag⁺ resistance determinant is unique to date in encoding both a metal-binding protein and two biochemically different efflux mechanisms.

With the characterization of the first Ag⁺ resistance determinant, it is important to understand how widely such systems are to be found in clinical isolates (exposed or not exposed to silver) and in those from silver-stressed non-clinical environments. To begin, this study focused on the occurrence of *sil* genes on different groups of plasmids, rather than on the molecular mechanism of Ag⁺ resistance. Different laboratory stock plasmids of the IncH and IncP incompatibility groups known to carry multiple antibiotic resistance markers were tested for the presence of *sil* genes. These plasmids were originally isolated from varying geographic locations (see supplementary data at <http://mic.sgmjournals.org> or <http://www.uic.edu/depts/mcmmi/individual/gupta/index.htm>). The identification and diversity of non-plasmid, chromosomally located *sil* homologous determinants in genome sequences from different bacteria is also discussed. The data presented are starting points for molecular epidemiological studies with clinical and non-clinical bacterial isolates.

A wide distribution of *sil* homologous determinants, localized on plasmids or on the bacterial chromosomes

might pose a threat toward effective use of silver compounds as antiseptics, analogous to the development of antibiotic-resistant bacteria when antibiotic usage increases (Liu, 1999; Salyers & Amabile-Cuevas, 1997).

METHODS

Media, chemicals and culture methods. Luria–Bertani (LB) broth was used (Ausubel *et al.*, 2001). When indicated, the medium was supplemented with ampicillin (100 µg ml⁻¹). Recombinant clones were selected on LB agar supplemented with 0.15 mM IPTG and 69 µM X-Gal. Unless otherwise indicated, all chemicals and reagents were purchased from Difco or Sigma. Ag⁺ resistance of the different *E. coli* strains was assayed on LB agar plates without added NaCl (Gupta *et al.*, 1998). Exponential-phase cultures were streaked on plates and incubated at 37 °C for 16 h before recording growth.

Bacterial strains. In this study, a series of *E. coli* strains carrying IncH plasmids from the University of Alberta collection were used (see supplementary data): J53(R476b), J53-2(R826), J53-2(R826-1), J53-2(R828), RG486(MIP233), RG486(pWR23), RG486(MIP235), RG192(TP116), RG1763(R478), RG192(pAS-251-2), RG192(pJT1), J53-1(R1022), J53(R27); J53-1(pHH1532b-1), JE2571(pHH1457), J62-1(pHH1457-1), J53(MG223), J53(MG224) and J53(MG225). *E. coli* J53 without a plasmid and J53(pMG101) (Gupta *et al.*, 1999; McHugh *et al.*, 1975) were used as Ag⁺-sensitive and -resistant controls, respectively.

Identification and characterization of *sil* gene homologues

Dot-blot hybridization. An aliquot (50 µl) of each overnight culture was placed on a nylon hybridization membrane using a dot-blot apparatus (Schleicher & Schuell). The bacterial cells were lysed. The liberated DNA was denatured, neutralized and fixed to the membrane (Ausubel *et al.*, 2001). A ³²P-labelled *silA* probe was hybridized to the filter-immobilized DNA. The DNA for the *silA* probe was generated by PCR amplification using *silA* gene-specific oligonucleotide primers and radiolabelled with [³²P]dCTP by using the MegaPrime DNA labelling kit (Amersham Life Science). Hybridization signals were visualized by exposure to X-OMAT AR film (Eastman Kodak).

PCR. The homologues of pMG101 *sil* genes were amplified from the boil-lysis supernatants (Ausubel *et al.*, 2001) of overnight culture aliquots, using *sil* gene-specific primers and PlatiTaq DNA polymerase (Life Technologies). The amplification products were separated on a 0.7% agarose gel and visualized under UV after staining with ethidium bromide.

Cloning and DNA sequence analysis. PCR products of the *sil* genes were cloned into the pGEM-Teasy vector (Promega) and transformed into *E. coli* DH5α by electroporation of competent cells (Ausubel *et al.*, 2001; Shigekawa & Dower, 1988). Plasmid DNA was isolated (Ausubel *et al.*, 2001) and cloned DNA was sequenced by using M13 universal forward and reverse sequencing primers by the dideoxy chain termination method using an ABI automated sequencer at the University of Illinois Sequencing Facility. DNA sequences were compared and analysed using CLUSTAL X version 1.64b and DNA Sequencer version 3.1.1 software.

Transcript analysis. Total RNA was isolated from *E. coli* strain J53(R476b) and J53(pMG101) cells exposed to 0 or 25 µM Ag⁺ for 2 h at 37 °C during growth in LB broth. RNA was isolated using the RNeasy total RNA preparation kit (Qiagen) and treated with RNase-free DNase (Life Technologies). For reverse transcriptase (RT)-PCR, 1 µg RNA was

used for cDNA synthesis at 42 °C using Superscript II RT according to the manufacturer's protocol (Life Technologies). Subsequent PCR was performed using PlatiTaq DNA polymerase (Life Technologies). For quantitation of fluorescence intensities of the RT-PCR products, the ethidium-bromide-stained agarose gel was recorded using a Sony CCD (charge-coupled device) camera attached to the Nucleotech gel documentation system. The fluorescence intensities were measured using GelExpert 97 version 2.0 software.

Construction of *agrA* deletion mutant and growth measurements. Chromosomal *agrA* was deleted by insertion of an antibiotic (kanamycin) resistance marker. The kanamycin cassette DNA contained flanking sequences homologous to *agrA* and was incorporated into the chromosome using homologous recombination as described by Datsenko & Wanner (2000). DH5 α *ΔagrA*(pAG59) carries a pUC57T (MBI Fermentas) derivative containing a PCR DNA fragment for the *agrABC* genes. Growth was measured for *E. coli* strains K-12 DH5 α , DH5 α *ΔagrA* and DH5 α *ΔagrA*(pAG59) by inoculation of LB broth (–NaCl) containing different concentrations of AgNO₃ with an exponential-phase culture at 0.2 Klett turbidity units. The cultures were incubated with shaking for 16 h at 37 °C prior to measurements with a Klett turbidity meter.

RESULTS

Plasmid *sil* determinants

Plasmid pMG101 (harbouring the *sil* system) has now been assigned to the IncHI2 incompatibility group (data not shown; incompatibility and surface exclusion

measurements). The IncH incompatibility group of plasmids consists of two compatible groups, IncHI and IncHII. The IncHI group contains three self-incompatible subgroups, IncHI1, IncHI2 and IncHI3. Nineteen plasmids belonging to different IncH incompatibility divisions were analysed by dot blot DNA–DNA hybridization and homologues for the pMG101 *silA* gene were identified in five IncHI (three IncHI2 and two IncHI3) plasmid-carrying strains (Fig. 2). Neither of the two IncHII plasmids tested showed positive hybridization (Fig. 2, spots 18 and 19). In addition, six IncP group plasmids were tested and did not hybridize (data not shown). The positively hybridizing strains were *E. coli* strains J53(R476b), RG486(MIP233), RG486(pWR23), RG486(MIP235) and RG1763(R478). The probe hybridization was at 65 °C. To pick up all cross-hybridization, low-stringency wash conditions at 25 °C were used. These conditions gave low-level hybridization to DNA from other *E. coli* strains on the filter (including the plasmidless control strain *E. coli* J53; Fig. 2, spot 20), which probably reflects the homology between the plasmid *silA* gene and the homologous *E. coli* K-12 chromosomal *agrA* gene (see below).

Next, homologues for three additional *sil* genes, *silP*, *silS* and *silE* (each representing a separate transcript for the pMG101 system; Gupta *et al.*, 1999), were identified by PCR (data not shown) in the strains that hybridized positively to ³²P-labelled *silA* DNA probe (Fig. 2). All the PCR primers were identical to the DNA sequence for the corresponding *sil* genes of pMG101. PCR amplification products of the predicted sizes, 2659, 1549 and 469 bp, were obtained for the IncHI *silP*, *silS* and *silE* genes, respectively (data not shown).

Sequences of plasmid-located *sil* genes

The *silP*, *silS* and *silE* genes amplified by PCR from *E. coli* strains J53(R476b), RG486(MIP233), RG486(pWR23), RG486(MIP235) and RG1763(R478) were cloned and the DNA for each new *sil* gene was sequenced. The sequences were compared with pMG101 sequences (Table 1). The DNA and protein alignments from which the detailed data in Table 1 were calculated are given in supplementary data. The *silE* genes from plasmids in strains J53(R476b), RG486(MIP233) and RG486(MIP235) were identical in DNA sequence (Table 1) to pMG101 *silE*; *silE* from strains RG1763(R478) and RG486(pWR23) differed from *silE* of pMG101 at 19 or 1 positions, respectively (Table 1). The 10 predicted histidine residues of SilE that are involved in Ag⁺–protein binding (Gupta *et al.*, 1999; Silver *et al.*, 1999a) are conserved in the SilE products from the additional IncHI plasmids (see supplementary data).

None of the new *silP* and *silS* genes were identical to those of pMG101. Four of the five new plasmid *silP* genes were more similar in sequence to *silP* of pMG101 than was *silP* of R476b (Table 1). The *silS* variants each differed from one another in similar numbers of positions (Table 1; see supplementary data). The

Fig. 2. Dot blot DNA–DNA hybridization with the *silA* probe of DNA released from cultures of *E. coli* strains. Spots: 1, J53-2(R828); 2, J53(R476b); 3, RG192(TP116); 4, RG486(MIP235); 5, J53-1(R1022); 6, J53-2(R826); 7, RG486(pWR23); 8, RG192(pJT1); 9, J53(MG225); 10, RG1763(R478); 11, J53(MG224); 12, RG192(pAS-251-2); 13, J53(R27); 14, J53-1(pHH1532b-1); 15, J53-2(R826-1); 16, J53(MG223); 17, RG486(MIP233); 18, J62-1(pHH1457-1); 19, JE2571(pHH1457); 20, negative control strain J53.

Table 1. DNA and amino acid comparison of three *sil* gene homologues from IncHI plasmids

Gene and plasmid	Nucleotide differences						Coding differences						Identity to pMG101 (%)	
	pMG101	R476b	MIP233	pWR23	MIP235	R478	pMG101	R476b	MIP233	pWR23	MIP235	R478	DNA	Protein
<i>silE</i> (424 bp sequenced out of 429 bp)														
pMG101	—	0	0	19	0	1	—	0	0	9	0	1	—	—
R476b	0	—	0	19	0	1	0	—	0	9	0	1	100	100
MIP233	0	0	—	19	0	1	0	0	—	9	0	1	100	100
pWR23	19	19	19	—	19	20	9	9	9	—	9	10	95.5	92.9
MIP235	0	0	0	19	—	1	0	0	0	9	—	1	100	100
R478	1	1	1	20	1	—	1	1	1	10	1	—	99.7	99.3
<i>silP</i> (1256 bp sequenced out of 2475 bp)														
pMG101	—	53	8	7	8	6	—	29	6	5	6	6	—	—
R476b	53	—	47	48	48	49	29	—	23	24	25	25	95.8	91.9
MIP233	8	47	—	3	4	4	6	23	—	1	2	2	99.4	98.3
pWR23	7	48	3	—	1	1	5	24	1	—	1	1	99.4	98.6
MIP235	8	48	4	1	—	2	6	25	2	1	—	2	99.4	98.3
R478	6	49	4	1	2	—	6	25	2	1	2	—	99.5	98.3
<i>silS</i> (1064 bp sequenced out of 1494 bp)														
pMG101	—	46	39	48	34	34	—	13	13	18	12	12	—	—
R476b	46	—	15	22	14	14	13	—	2	7	3	3	95.7	96.1
MIP233	39	15	—	25	9	9	13	2	—	7	1	1	96.3	96.1
pWR23	48	22	25	—	18	18	18	7	7	—	8	8	95.5	94.7
MIP235	34	14	9	18	—	0	12	3	1	8	—	0	96.8	96.4
R478	34	14	9	18	0	—	12	3	1	8	0	—	96.8	96.4

sequences from all six *sil*-positive IncHI plasmids (including pMG101) were 0, 0.3 or 4% different at the nucleotide level for *silE*, 0.5–4% for *silP* and 3–4% for *silS* (Table 1; see supplementary data). The corresponding protein products differed in slightly higher percentages of positions (Table 1), since many of the nucleotide changes resulted in coding changes (see supplementary data). In the *silP* gene products, the novel *SilP* N-terminal sequence, His₅AspHis₂, implicated as a determinant of cation specificity or for modulation of transport, was conserved. The high level of sequence identity between *sil* gene PCR products shows that the amplified products correspond to plasmid-encoded sequences and are not from the *E. coli* K-12 chromosome (see below).

The order of *sil* genes is the same on different plasmids

In pMG101, the genes *silP*(ORF105)*AB*(ORF96)*C* are oriented divergently from *silRSE*. The *sil* gene order and orientation on the five new IncHI plasmids were determined by PCR amplification using one primer located in the 5' region of one gene and a second primer located toward the 3' end of a downstream gene. With primers in the 5' region of *silR* and the 3' end of *silS*, a PCR product of approximately 2184 bp was obtained from the six *sil*-positive plasmids (Fig. 3a), showing that *silR* and *silS* are contiguous and similarly oriented. With primers in the 5' region of *silR* and the 3' end of *silE* (see

Fig. 1a), a PCR product of approximately 2812 bp was obtained from all six plasmids (Fig. 3b), indicating that *silRS* was contiguous with and in the same orientation as *silE*. PCR products of the expected size (6424 bp) were obtained with primers in the 5' region of *silA* and the 3' end of *silP* (Fig. 3c). These results show that *silA* and *silP* are contiguous and similarly oriented in all six plasmids. The organization of the *sil* genes on the IncHI plasmids from *E. coli* strains J53(R476b), RG486(MIP233), RG486(pWR23), RG486(MIP235) and RG1763(R478) is therefore considered to be the same as the *sil* genes on pMG101.

Silver resistance and transcription from the *sil* genes

Growth. Silver resistance levels of *E. coli* strains J53(R476b) (Fig. 4), RG486(MIP233), RG486(pWR23), RG486(MIP235) and RG1763(R478) (additional data not shown) were measured. *E. coli* strains J53 and J53(R476b) grew on plates supplemented with up to 100 µM Ag⁺ (Fig. 4a, lanes 1 and 2), whereas strain J53(pMG101) grew above 600 µM Ag⁺ (Fig. 4a, lane 3; only data up to 200 µM Ag⁺ shown). These data are representative of three independent experimental results. The growth of *E. coli* strains RG486(MIP233), RG486(pWR23), RG486(MIP235) and RG1763(R478) was similar to that for J53(R476b) (data not shown). The absence of a high pMG101-like level of Ag⁺ resistance with *E. coli* strains carrying the five new IncHI plasmids, although all of the *sil* genes are present, might

Fig. 3. PCR products spanning different genes were obtained for (a) *silR silS*, (b) *silR silS silE* and (c) *silA silP* from different IncHI plasmids and visualized on ethidium bromide-stained agarose gels. The PCR products were from *E. coli* strains. J53(pMG101) (lane 1), J53(R476b) (2), RG486(MIP233) (3), RG486(pWR23) (4), RG486(MIP235) (5) and RG1763(R478) (6). The sizes of pMG101 products are indicated and the primers used for PCR are given below each panel.

result from low-level gene expression and a history of not having been selected for Ag⁺ resistance in laboratory passage. To test if a higher level of Ag⁺ resistance is obtained after induction by Ag⁺, exponential-phase cultures of *E. coli* strains J53, J53(pMG101) and J53(R476b) were grown with 25 µM Ag⁺ for 2 h and then streaked on LB agar plates containing higher concentrations of Ag⁺ (Fig. 4b). Growth of *E. coli* J53 occurred only up to 100 µM Ag⁺ (Fig. 4b, lane 1), similar to results with uninduced cells (Fig. 4a, lane 1). However, growth of strain J53(R476b) occurred up to 400 µM Ag⁺ (Fig. 4b, lane 2), but not at 600 µM Ag⁺ (data not shown). *E. coli* J53(pMG101) grew at 400 µM Ag⁺ (Fig. 4b, lane 3) and above (data not shown). These data show that induction with Ag⁺ increased the Ag⁺ resistance of *E. coli* strain J53(R476b), suggesting that gene expression increased.

Transcript analysis. To test *sil* gene expression at the transcriptional level, total RNA was isolated from cultures of *E. coli* J53(R476b) and J53(pMG101), grown with and without 25 µM Ag⁺ for 2 h and the RNA analysed by RT-PCR (Gupta, 1999; Gupta *et al.*, 1999). For each RT reaction, equivalent amounts of RNA extracted from cultures grown in the presence and in the absence of Ag⁺ were used. As an internal RNA control, a primer for the 3' region of the *E. coli* 16S rRNA gene was included in each RT reaction, which also included

primers corresponding to the 3' regions of *silC*, *silS* or *silE*, respectively. Thus each RT reaction included its own internal control (shown in Fig. 5d). Next, a pair of PCR primers, corresponding to the 5' and 3' regions of *silC*, *silR*, *silE* or control *E. coli* 16S rRNA genes, were used for amplification of single gene products from the cDNA (Fig. 5). The *E. coli* 16S rRNA gene control (Fig. 5d) demonstrated that equivalent amounts of RNA were taken for the RT reactions and therefore any differences in intensities of the PCR products with *sil* genes represent changes in transcript abundance.

The PCR products for the *silR* genes (Fig. 5a) were obtained from the RT product for which the primer for the 3' region of *silS* was used. This result confirms the contiguity of *silS* and *silR* (Fig. 1; Gupta, 1999; Gupta *et al.*, 1999) for plasmid R476b. The *silR* PCR products generated for the two plasmids and from induced and uninduced cultures were all approximately equal in amount (Fig. 5a). A comparison of the fluorescence intensities of the PCR products showed that the differences (a range of 15 %) were within experimental error (analysis not shown). It appears that there was no induction of transcription for *silRS* with both plasmids after exposure to Ag⁺.

Quantitation of the PCR products for *silC* indicated 1.8-times more *silC* transcript with RNA from induced strain J53(R476b) cells than from uninduced cells (Fig. 5b, lanes 3 and 4; analysis not shown) and equivalent amounts of *silC* RNA with uninduced and Ag⁺-induced cultures of J53(pMG101) (Fig. 5b, lanes 1 and 2). The diffusion of the band in Fig. 5(b) lane 1 is thought to be an artifact of the gel and equivalent total fluorescence was obtained for lanes 1 and 2. Furthermore, the amount of *silC* PCR product from induced strain J53(R476b) cells was similar to that from pMG101. This indicates that *silC* transcription from strain J53(R476b) is low, but can be induced, while in J53(pMG101) *silC* transcription is constitutive and high. These data represent a first step toward explaining the differences in Ag⁺ resistance levels between the two strains.

The PCR products for *silE* show a lower but 1.32-fold inducible level of transcription with strain J53(R476b) (Fig. 5c, lanes 3 and 4; analysis not shown) compared with a higher and constitutive level of transcription with strain J53(pMG101) (Fig. 5c, lanes 1 and 2). The transcript level from induced cells of strain J53(R476b) (Fig. 5c, lane 4) was 73 % of that from J53(pMG101) cells, perhaps consistent with the lower resistance level of J53(R476b) (Fig. 4b).

Chromosomal homologues of the *sil* determinant

The *sil* determinant from pMG101 contains seven named genes (and two ORFs of unassigned function) (Fig. 1a; Gupta *et al.*, 1999). The closest homologues for *silAB*(ORF96)CRS in the GenBank database are from the published *E. coli* K-12 and O157:H7 chromosomes (Blattner *et al.*, 1997; Perna *et al.*, 2001). These ORFs are listed in GenBank as hypothetical genes and are desig-

Fig. 4. Ag^+ resistance. Exponential-phase cultures, grown (a) in the absence of Ag^+ or (b) for 2 h with $25 \mu\text{M}$ Ag^+ , were streaked on LB agar supplemented with Ag^+ . Growth was measured with *E. coli* strains J53 (column 1), J53(R476b) (2) and J53(pMG101) (3). Triangular 'slices' of the agar plates with or without bacterial growth and the concentration of Ag^+ added to each plate are shown.

Fig. 5. RT-PCR analysis of transcripts. The transcripts for (a) *silR*, (b) *silC*, (c) *silE* and (d) *E. coli* 16S rRNA genes were analysed in total RNA isolated from *E. coli* strains J53(pMG101) (lanes 1 and 2) and J53(R476b) (3 and 4). RNA was isolated from cultures uninduced (UI) or induced (I) with $25 \mu\text{M}$ Ag^+ for 2 h during growth. The primers used for the RT reaction and for subsequent PCR amplifications are given.

nated *ybdE*, *ylcD*, *ylcC*, *ylcB*, *ylcA* and *ybcZ*, respectively (for *E. coli* K-12, GenBank accession no. AE000162, amino acid accession nos 1786788–1786783; for *E. coli* O157:H7: GenBank accession nos AE005236 and AE005237, amino acid accession nos AAG54903:1–AAG54908:1; respectively) (Blattner *et al.*, 1997; Perna *et al.*, 2001). Based on the data presented in this paper, we propose to name these genes *agrA* (*ybdE*), *agrB* (*ylcD*), ORF110 (*ylcC*), *agrC* (*ylcB*), *agrR* (*ylcA*) and *agrS* (*ybcZ*) (*agr* for Ag^+ resistance; Fig. 1b). The mnemonic *agr* is introduced rather than calling the chromosomal genes *sil*, as it has not yet been demon-

strated whether they are paralogous or only orthologous in function. The *E. coli* chromosomal *sil* gene homologues are present on what is being called the 'common *E. coli* backbone' of 2.6 Mb shared between the two strains (Perna *et al.*, 2001). Between the two chromosomal sets, only the *SilR* homologues (*AgrR*) are 100% identical while the other homologue pairs vary between 0.74 and 2.3% at the amino acid level and to similar degrees at the nucleotide level. The chromosomal genes are oriented in the same direction as the plasmid *sil* genes and the spacing between genes in most cases is rather similar (Fig. 1b). The amino acid identities

Fig. 6. Growth of *E. coli* strains DH5 α (■), DH5 α Δ*agrA* (▲) and DH5 α Δ*agrA*(pAG59) (●) in LB broth (-NaCl) containing different concentrations of AgNO₃. The cultures were incubated for 16 h at 37 °C prior to measurements with a Klett turbidity meter.

between translation products of the plasmid *sil* determinants and the putative paralogous *E. coli* chromosomal products are shown in Fig. 1(b) and detailed alignments between the *sil* and the chromosomally encoded products are available in the supplementary data.

The *E. coli* chromosomal genes are proposed to encode a three-polypeptide cation efflux transporter (AgrABC, equivalent to SilABC), plus a two-component kinase/responder transcriptional regulatory pair (AgrRS, equivalent to SilRS). These five genes and their products are related to those of the *czcABC*RS (for Cd²⁺, Zn²⁺ and Co²⁺ resistances) plasmid system of *Ralstonia* (Nies, 1995). Between ORFs *agrC* and *agrB* is ORF110, which is homologous to ORF96 in the *sil* determinant (Fig. 1a and b). An equivalent ORF is not present in the homologous *CzcABC*RS system. A deletion in *agrA* (homologous to *silA*) on the *E. coli* chromosome resulted in Ag⁺ hypersensitivity (Fig. 6), but did not change the sensitivity level to high Cd²⁺ and Cu²⁺. Similar observations have also been made by Franke *et al.* (2001). The introduction of an intact *agrA* gene on a plasmid restored resistance to silver (Fig. 6). These results suggest that the chromosomal genes also function for Ag⁺ resistance.

In addition to the *E. coli* K-12 and O157:H7 chromosomal *agr* genes, *sil* gene homologues are found in the partial genomes of *Salmonella paratyphi*, *S. typhimurium* and *Klebsiella pneumoniae*. The genome sequences are available from the Washington University Genome Sequencing Center (<http://genome.wustl.edu/gsc>). In *K. pneumoniae*, there are homologues for all seven *sil* genes (predicted products with over 90% amino acid identity) and the novel features of specific Sil proteins are conserved, for

example the His₅AspHis₂ N-terminal motif of SilP and the 10 specifically placed histidine residues of SilE. In contrast, the homologues from *Salmonella* are less similar to those of pMG101 (predicted products only 30–40% identical at the amino acid level) and the *Salmonella* P-type ATPase homologue lacks the His₅AspHis₂ motif.

DISCUSSION

pMG101 belongs to the IncHI2 incompatibility group of plasmids (D. E. Taylor, unpublished data), which are large multi-antibiotic resistance plasmids found widely in the *Enterobacteriaceae* and that are transferred by conjugation only at lower temperatures (see references at <http://www.uic.edu/depts/mcmi/individual/gupta/index.htm>). The identification of new *sil* genes on five additional plasmids, all of which are IncHI2 or IncHI3, and homologous genes on the chromosomes of *E. coli* K-12 and O157:H7 and other bacteria raises important concerns about the development of Ag⁺-resistant bacteria. Strains carrying the five IncHI plasmids showed moderate Ag⁺ resistance (Fig. 4 and data not shown). Ag⁺ resistance of representative strain J53(R476b) increased upon induction with Ag⁺. This induction coincided with a measured increase in transcription of *sil* genes and, hence, likely an increase in the cellular proteins (one for Ag⁺ binding and the other for Ag⁺ efflux).

pMG101 conferred strong Ag⁺ resistance and a higher constitutive level of transcription, compared to plasmid R476b. It is unclear and remains to be tested why plasmid R476b shows lesser resistance to Ag⁺ and a lower level of constitutive, but inducible expression. The newly identified *sil* genes may not provide a high level of Ag⁺ resistance in the strains as isolated, but may provide a selective advantage to the bacteria that possess these genes on exposure to Ag⁺. Mutations that allow higher expression from these genes and increased resistance would be selected. Ag⁺-resistant clinical *E. coli* isolates selected by step-wise exposure to higher concentrations of Ag⁺ showed active efflux of Ag⁺ (Li *et al.*, 1997). It is possible that the pMG101 *sil* system has been already selected in this regard by repeated growth on high concentrations of Ag⁺ during the more than 25 years of laboratory culturing since it was isolated. It is also notable that pMG101 was found in a *Salmonella* isolate from a burn ward where silver sulfadiazine was used to prevent nosocomial infections (McHugh *et al.*, 1975).

Contiguous homologues for five of the seven *sil* genes were identified on the *E. coli* K-12 and O157:H7 chromosomes. There are no ORFs on the *E. coli* K-12 or O157:H7 chromosomes homologous to *silE*, suggesting that this gene was added late to the plasmid system. *E. coli* K-12 has six genes for P-type ATPases, and the closest with regard to amino acid product sequence on both the K-12 and O157:H7 chromosomes to that of pMG101 SilP is *ybaR* (GenBank accession U58330;

renamed *copA*; Rensing *et al.*, 2000), which is involved in Cu⁺ efflux. *copA* is located approximately 60 kb away from the *agrABORF110CRS* system. *copA* may also mediate efflux of Ag⁺ since a deletion in *copA* resulted in hypersensitivity to Ag⁺ (A. Gupta, data not shown) as well as to Cu⁺ (Rensing *et al.*, 2000). The Ag⁺ efflux reported in clinical *E. coli* isolates (Li *et al.*, 1997) may involve the homologous *agrAB(ORF110)CRS* chromosomal regions (Fig. 1b). The likely cation substrates for efflux by the chromosomal *agr* system are Ag⁺ and Cu⁺, since a shared Ag⁺/Cu⁺ efflux transporter has been identified in *Enterococcus* (Solioz & Odermatt, 1995) and a deletion in *agrA* renders the cell hypersensitive to Ag⁺ (Fig. 6). The differences between the two *E. coli* K-12 and O157:H7 chromosomal *agr* gene systems, although small (Fig. 1b), are sufficient to suggest that a common ancestor of both strains, that are predicted to have diverged about 4.5 million years ago (Perna *et al.*, 2001), already contained the *agr* gene system. The differences between the two *E. coli* chromosomal systems are similar in percentage to those seen between the *sil* systems from different plasmids of the IncHI group, as reported in this study (Table 1). This suggests that the plasmid *sil* gene determinants have also been present for a sufficiently long time to establish heterogeneity in DNA and protein sequences. With increasing numbers of bacterial genomes being sequenced and homologues of the *sil* system being found in increasing numbers, it is reasonable to designate the chromosomal genes as *agr* to allow more accurate subsequent annotation of new DNA sequences.

The availability of DNA sequences for *sil* genes on six IncHI plasmids and identification of close homologues on bacterial chromosomes are starting points for molecular epidemiological studies of *sil* genes in clinical and environmental isolates of concern that may or may not have been exposed to silver. Such molecular diversity studies of the *sil* genes will be helpful in addressing questions regarding nosocomial infections, toxic metal bioremediation and the effective use of Ag⁺ as a biocide.

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