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Plasmid-encoded resistance to arsenic compounds in Gram-negative bacteria isolated from a hospital environment in Venezuela

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Abstract

Resistance to arsenic compounds was examined among amikacin resistant Gram-negative bacteria isolate from a hospital environment. Arsenite resistance (Ars^r) was found in a high proportion of isolates ($> 60\%$) being frequently associated with resistance to tellurite (40%), and to other antimicrobial agents. Ars determinants (27%) were found to be transferable to *E. coli* K12 strains from which large plasmid DNA molecules were isolated and characterized by agarose gel electrophoresis. Plasmids were identified by both classical incompatibility tests, and by replicon typing using DNA specific probes. Most of the amikacin–arsenite (Ak–Ars) conjugative plasmids belong to the H incompatibility group. These results suggest that Ak–Ars resistance linked to IncH plasmids is wide spread in Gram-negative bacteria. © 1997 Elsevier Science B.V.

Keywords: Plasmids; Amikacin; Arsenite; R factors; Incompatibility; Rep probes

1. Introduction

Bacterial R plasmids confer resistance not only to antibiotics but also to heavy-metal ions. Resistance to arsenic compounds was described for the first time in *Staphylococcus aureus* isolates harboring penicillinase plasmids. These plasmids, named pI258, pI147 and pI524, confer inducible resistance to arsenate, arsenite and to other toxic metal ions [1]. Resistance to arsenite has also been reported in *Escherichia coli* transconjugants harboring the mercuric-mercurial plasmids from Datta's collection [2]. Among the plasmids so far identified and classified into incompatibility groups are R476b, R477, R478, R826 and R828 (IncH), isolated from *Serratia marcescens* [3], pR773 (IncF) from *E. coli* [4] and pR46 (IncN) from *Salmonella marcescens* [5]. All these plasmids encode resistance for other antimicrobial agents. Another screening carried on a wide range of enterobacteria revealed that more than 80% of the *E. coli*, *Shigella*, *Proteus* and *Klebsiella pneumoniae* strains were resistant to arsenite, whereas in the *Salmonella* serotypes only a low proportion ($> 7\%$) were resistant [6]. A high proportion (70–90%) of the arsenite encoded resistance plasmids (Ars) were transferred from *Salmonella* serotypes, and *K. pneumoniae* to *E. coli* K12, but only 10% of the plasmids identified in *E. coli* were autotransferable, and no transmissibility from *Proteus* and *Shigella* was reported [6]. None of these plasmids were classified by incompatibility tests, and most of them do not encode resistance to other antimicrobial agents. Therefore, it was of interest to know whether resistance to arsenite could be mediated by plasmids encoding resistance to amikacin and to determine the spreading of both determinants among Gram-negative bacteria in a hospital environment.

In the present work a survey was conducted to characterize the distribution of plasmids encoding for linkage of Amikacin–Arsenite resistance among Gram-negative bacteria isolated from a Venezuelan hospital.

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Classical incompatibility testing was used for plasmid classification. This property is expressed by plasmids sharing one or more elements of replication or partitioning systems [7].

Most of the *E. coli* transconjugants constructed carried plasmids identified as members of the H incompatibility complex. Replicon typing, a new method for the identification of plasmids by hybridization with specific probes (rep probes) developed by Couturier et al. [8] was used to corroborate identification of plasmids belonging to the H complex as well as to discriminate subgroups into this super-group. Transferable plasmids by *E. coli* mating experiments were isolated and characterized by agarose gel electrophoresis and H rep probes. All those identified as belonging to the HI2 sub-group simultaneously exhibit resistance to potassium tellurite and the PacB character, e.g. protection against channel-forming colicins.

2. Materials and methods

2.1. Bacterial strains and plasmids

Amikacin resistant strains were isolated, and identified at the Hospital Universitario de Caracas, and in our laboratory by using the ATBplus Biomerux Identification System. One hundred Amikacin resistant bacteria were randomly selected from 1550 Gram-negative isolates collected during a 6 month period (March–September 1992) from in-patients. All strains are preserved at the Venezuelan Center for Culture Collections (Centro Venezolano de Colecciones de Microorganismos, CVCM). *Escherichia coli* K12 strains used in mating experiments and in other tests are described in Table 1. Plasmids used for incompatibility tests are also described in Table 1.

2.2. Culture media

Bacteria were grown either in Luria Bertani broth (LB) or minimal medium (MM). Media were solidified with 1.6% agar (Difco). MM was supplemented to 0.2% glucose, and when necessary, amino acids (20 g/ml) were added.

2.3. Antimicrobial susceptibility and resistance

Antibiotic resistance phenotypes of natural isolates and *E. coli* transconjugants were determined by the disk diffusion method using antibiotics from Difco and Muller-Hinton agar [9]. Sensitivity to aminoglycosides was defined according to the recommendations of the NCCLS [10]. The break-point value for acceptance was a MIC to amikacin higher than 16 mg/l. Resistance to heavy metals and anions was detected by using sodium arsenite ($NaAsO_2$) at 500 μg/ml, and potassium tellurite ($K2TeO_3$) at 50 μg/ml.

2.4. Transfer of arsenite resistant plasmids

Arsenite resistant bacteria/*E. coli* mating were performed under the conditions described previously by Rodríguez Lemoine et al. [11]. Ars^r donor strains grown at 24°C for several generations were separately filtered and gently washed. Recipient cells (grown at 37°C) were poured upon donor organisms (1:10 donor/recipient), filtered and incubated for 2 h at 24°C. Filtered-mating mixtures were suspended in fresh medium and 0.5 ml aliquots (without dilution) were spread on LB plates containing sodium arsenite (500 g/ml) and nalidixic acid (40 g/ml). Plates were incubated at 37°C for 24 h and well-developed colonies were selected for further studies.

The conditions described by Rodríguez Lemoine et al. [11,12] was used to establish whether the Ars plasmids encode a temperature sensitive transfer system. The transfer frequency was calculated as the number of transconjugants per number of donor cells after 1 h mating at 24°C.

2.5. Entry exclusion and incompatibility

Entry exclusion and incompatibility of the plasmids transferred to *E. coli* K12 were measured using the procedure described earlier [13,14], in which 21 transconjugants clones were selected and tested for stable coexistence of the plasmid under investigation with representative plasmids of the H complex.

Table 1
E. coli K-12 strains and plasmids used in this study

Designation	Relevant characteristics	Ref.
E. coli strains		
711	F-pro his trp lac	[3]
J53-1	F-pro met nal	[3]
BZB2101	pColA-CA31, pColB-K260	[10]
Plasmids		
R477	SmSpSuTcArsHgTePhi PacB IncHI2	[3]
R478	CmKmTcArsHgTePhi PacB IncHI2	[3]
Mip235	CmSmSuTePhiPacB IncHI2	[20]
R27	Tc IncHI1	[20]
pULB2433	Ap Tc, repHI2	[8]
pULB2436	Ap, repHI1	[8]

Resistance to Sm, streptomycin; Tp, trimethoprim; Ars, sodium arsenite; Ap, ampicillin; Tc, tetracycline; Hg, mercuric chloride; PacB, colicin protection; Phi, phage inhibition; Te, resistance to potassium tellurite; Su, sucrose utilization; IncHI1 and IncHI2, incompatibility subgroup plasmids. Resistances were determined as indicated in Section 2.

2.6. Detection of the PacB character

Sensitivity of *E. coli* derivatives to the lethal action of channel-forming colicins (PacB character) was determined as described previously [13]. Samples from exponentially growing cultures of the *E. coli* strains carrying colicin encoding plasmids were inoculated, previously induced by UV light into LB and incubated at 37°C overnight. After sterilizing the plates with chloroform vapour, 2.5 ml of melted soft agar containing 2×10^8 cells/ml of the *E. coli* transconjugants under test, was poured on the agar surface. A transconjugant strain carrying plasmid R478 (IncHI2), and a derivative cured from it, were applied on each plate as controls. After incubation at 37°C overnight, the plates were inspected for reduction of the inhibitory effect of colicins on the transconjugants.

2.7. Sensitivity to phage pilHα

To detect presence of plasmids encoding conjugative H pili (supergroup PilH), propagation of phage *pil*Hα was followed under the condition described previously [15].

2.8. Plasmid DNA preparation

Plasmid DNA isolation was performed by the alkaline lysis procedure [16].

2.9. Probes and hybridization

Plasmids pULB2436 and pULB2433 (repHI1 and repHI2 respectively) were derived and used following the replicon typing technique described by Couturier et al. [8]. They were labeled by random primer using Genius non-radioactive DNA labeling Kit (Boehringer). Southern and slot blots hybridization was performed under high stringency conditions. Positive signals were detected using Lumiphos Kit (Boehringer).

3. Results and discussion

Studies on the epidemiology, genetics and biochemistry of resistance to metals and other antimicrobial agents suggest that the selection of resistant organisms and the subsequent spread of these phenotypes could result from exposure of bacteria to a specific metal or antibiotic in a particular environment. Alternatively, a correlation between resistance to metals and the exposure of bacteria to an environment highly selective for antibiotic resistance, but not necessarily to metals (i.e. a hospital), has also been proposed [17,18].

In a previous study we reported that more than 10% of *S. marcescens* isolates from hospital sources carry plasmids encoding resistance to tellurium compounds, and that a high proportion of these plasmids also encode for resistance to arsenite [19]. Most of the plasmids were identified as members of the incompatibility complex H [20]. Resistance to these compounds was found to be mediated by the same plasmids that determine resistance to antibiotics.

Gram-negative isolates were collected to survey the distribution of Arsenite–amikacin resistance determinants among plasmids transferable to *E. coli* strains. Attention was given to establish their relationship with other transmissible characters. Amikacin is a semisynthetic derivate of kanamycin, it has in vitro efficacy against a broad spectrum of Gram-negative bacilli, being used as a first line drug for infection control. An increase of Amikacin resistance has been observed in many countries [21–23]. A survey conducted in twelve Venezuelan hospitals between 1988 and 1995 showed an increase of resistance from 14.8 to 23%. [24].

Results from the present survey show that the Ak^r isolates exhibited complex patterns of resistance to a wide range of antimicrobial agents, including ampicillin, tetracycline, kanamycin, chloramphenicol, streptomycin, tobramycin, gentamicin, nalidixic acid and arsenite (results not shown). Resistance to arsenite was found in a high proportion (60%) of the 100 amikacin resistant isolates selected for study. Resistance to both antimicrobial agents is widely distributed being found among the following genera: *Acinetobacter lowfii* (2/6), *Acinetobacter anitratus* (7/7), *Acinetobacter calcoaceticus* (7/13), *Citrobacter freundii* (5/6), *Enterobacter aerogenes* (2/3), *Enterobacter agglomerans* (8/12), *E. cloacae* (4/5), *Escherichia coli* (5/8), *K. pneumoniae* (11/17), *Proteus* (1/1), *Pseudomonas* (7/15), *Serratia marcescens* (1/7). In the remaining genera resistance to arsenite was not detected, e.g. *Acinetobacter lowfii*, *A. baumanii*, *Enterobacter* sp., *Flavobacterium* sp., *S. liquefaciens* (results not shown). By mating experiments using *E. coli* K-12 as recipient (selecting by amikacin) all transconjugants were arsenite resistant and those selected by arsenite were resistant to amikacin. Linked transfer of Ak–Ars from *E. coli* to *E. coli* was demonstrated in 40% of the natural amikacin–arsenite isolates. Table 2 shows the distribution of the resistance determinants transferred from natural isolates to *E. coli* K12 strains. This study reveals that arsenite resistance was transferred concomitantly with other antimicrobials agents including tellurium compounds. The ability of arsenite resistant isolates to grow on tellurite could occur by two well differentiated mechanisms, reduction of tellurium compounds to metallic tellurium (black brilliant colonies on tellurite solid media) and by efflux of these toxic oxianions mediated by the arsenite pump (pale-gray colonies on tellurite solid media). The former resistance mechanism is frequently encoded on plasmids belonging to the H complex: incompatibility groups HI

Table 2
Arsenite autotransferable plasmids: resistance pattern in *E. coli*

Strain of origin	Plasmid denomination	*E. coli* resistance pattern
Acinetobacter annitratus	pAa56	AkTcKm-CmGmSmApArs
	pAa58, pAa120, pAa121	AkTcKmCmSmApArs
Acinetobacter calcoaceticus	pAc36, pAc40	AkTcKm-CmSmApTeArs
	pAc28	AkKmCmSmApArs
Citrobacter freundii	pCf13, pCf119	AkKm-CmSmApTeArs
	pCf52, pCf67	AkKmCmSmApArs
Enterobacter aerogenes	pEa47, pEa113	AkCmSmT-mGmApTeArs
	pEa10, pEa78	AkKm-CmSmApTeArs
Enterobacter agglomerans	pEag35	AkTcKm-CmSmApTeArs
	pEag94, pEag61	AkTcKmCmSmApArs
Enterobacter cloacae	pEcl122,pEcl127,pEcl131	AkTeArs
Escherichia coli	pEc69	AkKmCmSmT-mGmTeArs
	pEc51,pEc60	AkTcKm-CmSmApTeArs
Klebsiella pneumoniae	pKp5,pKp85,pKp112, pKp86	AkKmGmT-mGmApTeArs
	pKp1,pKp2,pKp72	AkTcKmCmSmT-mGmApTeArs
	pKp33,pKp59	AkTcKm-CmSmApTeArs
	pKp4,pKp124	AkTcKmCmSmApArs
	pKp106	AkKmCmSmApArs
	pKp7	AkKmCmApArs
Proteus mirabilis	pPm12	AkTcKm-CmSmApTeArs

Ak, amikacin; Tc, tetracycline; Km, kanamycin; Cm, chloramphenicol; Sm, streptomycin; Tm, tobramycin; Gm, gentamycin; Ap, ampicillin; Te, tellurite; Ars, arsenite. The underlined plasmids (plasmid denomination column) belong to the HI incompatibility group. Those belonging to the HI2 sub-group also exhibit the PacB character, a temperature sensitive transfer system and sensitivity to phage pilH.

(subgroups HI2 and IncHI3 but not to IncHI1) and HII plasmids [25], as well as by the IncP(α) plasmid RP4 [26]. The arsenite pump is also plasmid-encoded (Ars operon) and present in plasmids of other groups [27].

Among the Ars–Te transconjugants characterized, the tellurite resistant phenotype observed was typically expressed by H plasmids, e.g. reduction of tellurium compounds to metallic tellurium. Another property also linked to the IncH plasmids is the ability to reduce natural sensibility of *E. coli* K12 strains to the lethal action of channel-forming colicins (PacB character) [12,13]. Although the mechanism of protection against these colicins remains unknown, this property has successfully been used as a criterion to facilitate identification of H complex plasmids from other incompatibility groups [19], and to differentiate IncHI2 and IncHI3 subgroups from those belonging to IncHI1 subgroup [13]. Table 2 shows that 21 of the Ak–Ars *E. coli* transconjugants constructed, simultaneously acquired the PacB character, exhibit a transfer system that is temperature sensitive and encode for a pili which allows adsorption of the RNA specific-phage pilHα (underlined in Table 2), which are specific characteristics of plasmids belonging to H complex. These properties were determined as described in Section 2.

By classical incompatibility tests using representatives plasmids of the H complex, we found that all transconjugants exhibiting simultaneously the characters above described, carried plasmids that were incompatible with those of the HI incompatibility group. Nevertheless, analysis of extrachromosomal DNA content from *E. coli* transconjugants using gel electrophoresis revealed a complex pattern of DNA molecules (Fig. 1A). In some of them, both original isolates and *E. coli* transconjugants, up to eight well separate DNA bands were identified with molecular size ranging from 2×10^3 to more than 80×10^3 bp. It appears, therefore, that transfer (and/or cotransfer) of plasmids other than H plasmids might occur simultaneously at 24°C. As previously postulated, dispersion of antibiotic and heavy metal resistance appears to occur among different genera of bacteria even in the absence of a specific selective pressure. Our results on mating experiments support this interpretation. That is, the presence of no selected plasmids were detected in some transconjugants. By Southern hybridization using specific probes for different H replicon control systems (rep probes) we were able to discriminate plasmids of the H complex from other replicons carried by the *E. coli* transconjugants constructed under selective pressure for amikacin and/or arsenite. Hybridization allowed identification of the largest molecules (80 kbp) as members of the IncHI replicon group (Fig. 1B). By using slot blots and rep probes (only for IncHI, subgroups 1 and 2, since no rep probes for subgroup 3 and the IncHII group are available yet) 16% of the H plasmids were unequivocally identified as members of HI2 subgroup. Surprisingly 27% were also positive for HI1 and 30% for both HI1 and HI2 (Fig. 2). As previously reported, plasmids identified as members of the IncHI1 subgroup do not encode for resistance to Te-PacB [12]. This apparent inconsistency could be explained assuming presence of several replicons

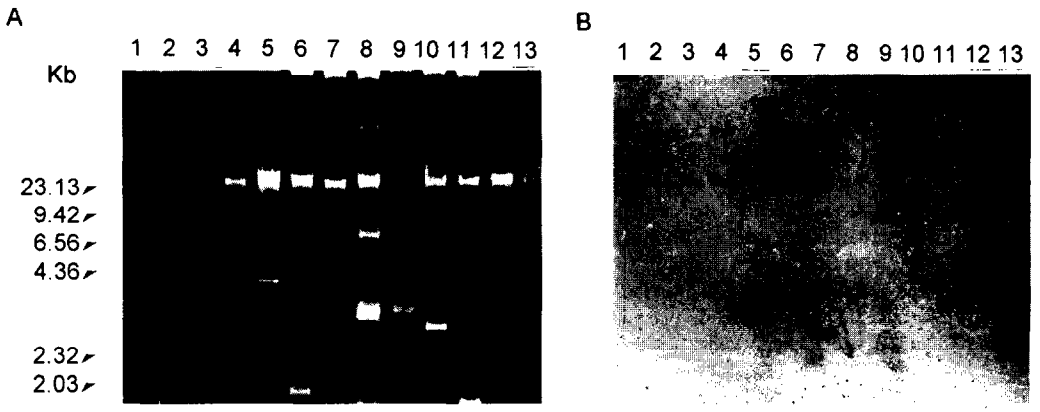

Fig. 1. (A) Ethidium bromide-stained plasmid DNA isolated from transconjugants *E. coli* strains (lanes 2–12); lanes 1 and 13, plasmids R-27 (IncHI1) and R478 (IncHI2), respectively. (B) Autoradiogram of a blot of the gel from A, probed with repHI2.

(cointegrates) among the large size plasmids identified. As an example, plasmid R27 (IncHI1) contains three replicon regions, two from HI1 subgroup and the other bears homology to the RepFIA plasmid replicon [28]. Plasmid molecules that hybridize with the HI1 and HI2 probes could also contain both replicons regions. Those plasmids that hybridize with HI1 probe and show tellurite and PacB characteristics must be cointegrated.

Using rep probes as a complementary test we were able to corroborate that H plasmids are widely distributed among different genera commonly found in a hospital environment; and that this dissemination could occur independently of the selective pressure exhorted on the bacterial population. Studies using all available rep probes, including those being constructed in our laboratory, are currently in progress in order to identify other plasmids present in transconjugant bacteria, and to know their transfer relationship with H plasmids in hospital environments.

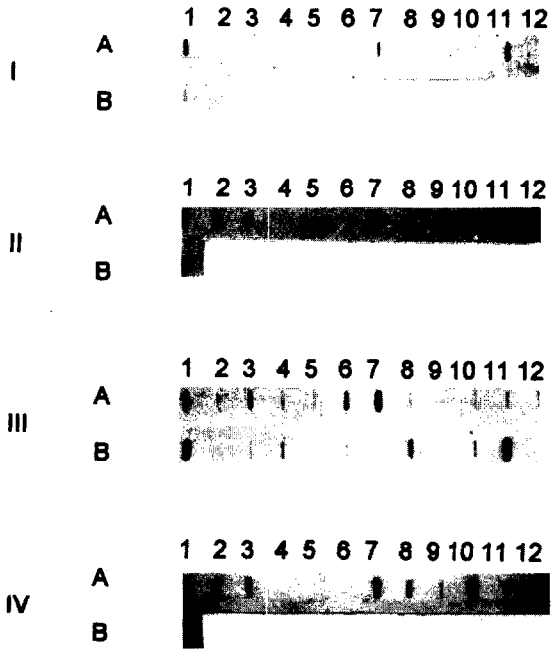

Fig. 2. Slot blot hybridization. Blots II y IV, slots A9, A10, A11, A12 and B1 are controls. The other slots are plasmids from natural and transconjugants strains. I and II, hybridization with repHI1 probe; III and IV, hybridization with repHI2 probe.

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