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High levels of multiple metal resistance and its correlation to antibiotic resistance in environmental isolates of *Acinetobacter*

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Forty strains of *Acinetobacter* were isolated from different environmental sources. All the strains were classified into four genospecies, i.e. *A. baumannii* (33 isolates), *A. calcoaceticus* (three isolates), *A. junii* (three isolates) and *A.* genospecies3 (one isolate). Susceptibility of these 40 strains to salts of 20 heavy metals and 18 antibiotics was tested by the agar dilution method. All environmental isolates of *Acinetobacter* were resistant to multiple metal ions (minimum 13 metal ions) while all but one of the strains were resistant to multiple antibiotics (minimum four antibiotics). The maximum number of strains were found to be sensitive to mercury (60% strains) while all strains were resistant to copper, lead, boron and tungsten even at 10 mM concentration. Salts of these four metal ions may be added to the growth medium to facilitate selective isolation of *Acinetobacter*. Rifampicin and nalidixic acid were the most toxic antibiotics, inhibiting 94.5 and 89.5% of the acinetobacters, respectively. *A.* genospecies3 was found to be the most resistant species, tolerating high concentrations of all the 20 metal ions and also to a greater number of antibiotics than any other species of *Acinetobacter* tested. An inhibitory concentration (10 mM) of Ni^{2+} and Zn^{2+} was observed to inhibit the growth of all of the clinical isolates but allowed the growth of the environmental isolates, facilitating the differentiation between pathogenic and non-pathogenic acinetobacters.

Keywords: Acinetobacter, antibiotic resistance, genospecies, metal resistance

Introduction

The universal concern caused by the diffusion of toxic metals into our living environment by industrial emissions and leaching from hazardous wastes has motivated surveys of microorganisms potentially useful for biological monitoring (Krishna Murti & Viswanathan 1991). *Acinetobacter* is a Gram-negative, non-motile, non-endospore-forming encapsulated coccobacillus belonging to family Neisseriaceae. It is ubiquitous in nature (Baumann 1968). Association of *Acinetobacter* has been increasingly established with nosocomial infections in recent years (Glew *et al.* 1977, Larsan 1984, Patwardhan 1990). It has also gained importance in recent years as a reservoir of antibiotic resistance genes (Chopade *et al.* 1985). Even though a link between antibiotic and metal resistance has been established in many bacteria (Foster 1983, Witte *et al.* 1986, Wang *et al.* 1987, Silver & Misra 1988), surprisingly, epidemiological surveys carried out on *Acinetobacter* do not comment on metal ion resistance. This may be because metal ion resistances are of less clinical concern than antibiotic resistances. However, such association is significant since knowledge of metal resistance may provide useful information on mechanism(s) of antibiotic resistance (Trevors *et al.* 1985). Microbial metal resistance is also interesting for both basic and applied research, particularly with respect to plasmid genetics, and the physiology and ecology of microorganisms in polluted environments/ecosystems (Trevors *et al.* 1985). Metal resistance may also be used as a genetic marker.

We have already reported resistance of clinical isolates of *Acinetobacter* to heavy metals (Deshpande *et al.* 1993). The purpose of this investigation was to determine natural susceptibility levels of a number of environmental isolates of *Acinetobacter* to metals and antibiotics, and to check if the

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differences between clinical and environmental isolates of *Acinetobacter* are reflected in their response to heavy metals.

Here we report, for the first time, resistance of different genospecies of *Acinetobacter* to different metal ions and its correlation with antibiotic resistance.

Materials and methods

Isolation of Acinetobacter

A total of 40 strains of *Acinetobacter* was isolated from environmental sources such as soil, river water, domestic sewage, treated industrial effluent and laboratory effluent by enrichment culture techniques (Baumann 1968).

Chromosomal transformation assay

The genus *Acinetobacter* of all the isolates was confirmed by the chromosomal transformation assay (Juni 1972). Preparative DNA samples were prepared by the method proposed by Juni (1972). This DNA was then used to transform auxotrophic *A. calcoaceticus* BD413 *trpE* 27. Conversion of tryptophan auxotrophic BD413 to the prototrophic one was taken as a positive transformation assay which confirmed the genus *Acinetobacter* of the strain from which the DNA was isolated.

Biochemical characterization and biotyping

The strains were classified into genospecies according to the scheme proposed by Bouvett & Grimont (1986).

Chemicals

The heavy metals were purchased from Merck (Darmstadt, Germany) as sodium meta arsenite, sodium arsenate, silver nitrate, cadmium chloride, cobalt nitrate, potassium chromate, copper chloride, mercury chloride, sodium molybdate, nickel chloride, lead acetate, potassium antimony tartarate, sodium tungstate, aluminum chloride, bismuth sulfate, lithium chloride, selenium dioxide, stanus chloride, boric acid and zinc chloride. Stock solutions were prepared in distilled water and were sterilized by filtration through 0.22 μm membrane filters (Sartorius). Freshly prepared solutions were used on the same day. All the antibiotics were purchased from Hindustan Antibiotics (Pune, India) or Ranbaxy (New Delhi, India). Stock solutions of antibiotics were prepared in appropriate sterile solvents as described by Rippere & Johnson (1986).

Susceptibility testing

For determination of minimum inhibitory concentrations (MICs), an agar dilution method was used (Riley & Taylor 1989). Tubes containing 20 ml melted Diagnostic Sensitivity Test agar (Hi Media, Bombay, India) and different concentrations of metals or antibiotics were poured onto plates on the day of experiment. The range of concentrations for all heavy metals tested was 0.001–20 mM and that for antibiotics was 2–1024 $\mu g\,ml^{-1}$. The agar plates were dried at 37 °C for 1 h and inoculated with 22 spots, each with 10^4–10^5 microorganisms from exponentially growing cultures. DST agar plates without metals or antibiotics inoculated with test organisms were kept as controls. The plates were read after incubation of 24 h at 30 °C. The highest concentration that allowed the growth of microorganisms was termed as the subinhibitory concentration (SIC) while the lowest concentration that inhibited the growth of microorganisms was termed the MIC. The strains not inhibited by 25 $\mu g\,ml^{-1}$ of antibiotics were termed resistant strains. On the other hand, unlike antibiotic resistance, there is no acceptable metal concentration to specify metal resistance (Trevors *et al.* 1985). Therefore, strains which were not inhibited by 10 mM arsenic, copper, lead, zinc, molybdenum, boron, tin and nickel; 1 mM cadmium, sodium, tungsten, lithium, aluminum, colbalt, chromium, bismuth, silver and antimony; and 0.1 mM mercury were regarded as resistant. *A. calcoaceticus* ATCC 33305, ATCC 15150, EBF65/65 C426 and C4169 were used as reference strains.

Results

Isolation and characterization of Acinetobacter

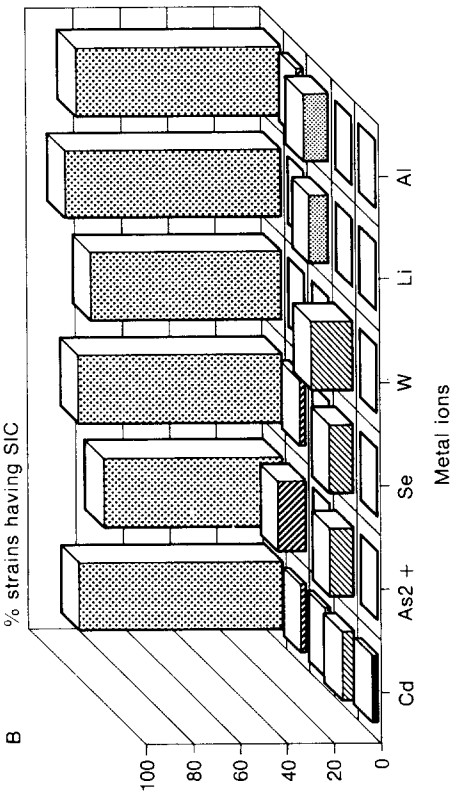
Forty environmental isolates were confirmed to be genuine acinetobacters by performing the transformation assay. These strains were classified into four different genospecies according to the scheme proposed by Bouvet & Grimont (1986). The distribution of the strains into four genospecies and the sources from which the strains were isolated are described in Table 1.

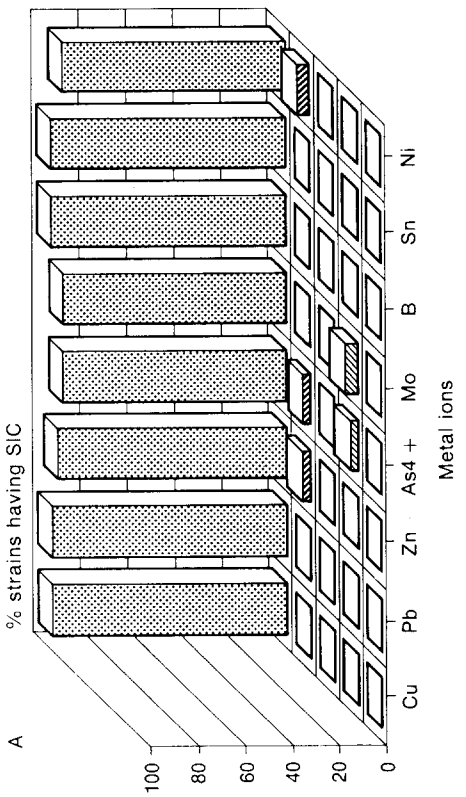
Resistance to metals

Figure 1 shows the response of all the isolates to 20 different metal salts. These metal ions were divided

Table 1. Isolation of *Acinetobacter* strains from environmental sources

Genospecies	Source	Number of strains
A. baumannii	soil	8
	river water	5
	domestic waste	14
	treated effluent	4
	laboratory waste	2
A. calcoaceticus	river water	2
	laboratory waste	1
A. junii	river water	2
	industrial waste	1
A. genospecies3	soil	1





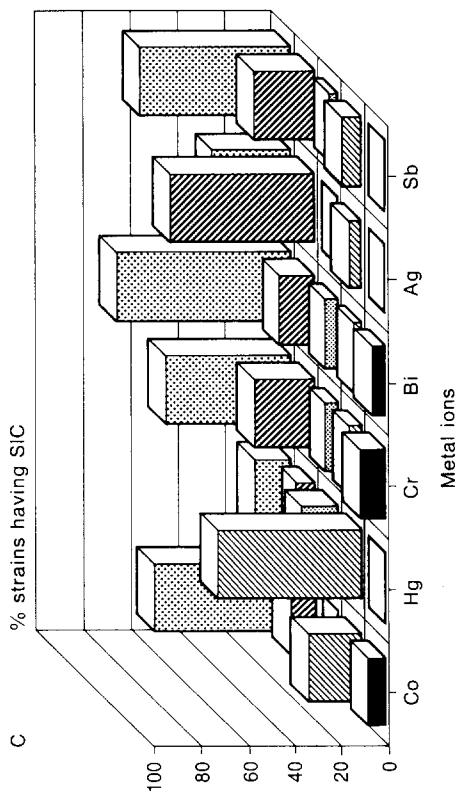


Figure 1. Response of environmental isolates of *Acinetobacter* to: (A) group I metals, (B) group II metals and (C) group III metals. ■, 0.001 mM; ▨, 0.01 mM; ▩, 0.1 mM; ▧, 1 mM; ▦, 10 mM.

into three groups based upon the response of environmental acinetobacters. Group I (Figure 1A) includes the metal ions against which more than 95% of the isolates showed high level of resistance (SIC = 10 mM). All the isolates were resistant to copper, lead, boron and tin even at 10 mM concentration. Growth of only two strains was inhibited by 10 mM arsenate, molybdenum and nickel, while only one strain was inhibited by the same concentration of zinc. The overall response of all the isolates was homogenous against all the metals belonging to group I since MIC values of these metals were the same for more than 95% of the environmental isolates of *Acinetobacter*.

The metals against which moderate numbers of isolates (65–95%) displayed high resistance levels were grouped in group II (Figure 1B). Only 7.7 and 23.1% of the strains were sensitive to lithium and arsenic, respectively, at 10 mM concentration. Thus arsenic was the most toxic and lithium was the least toxic of the group II metals.

The most toxic metals were included in group III (Figure 1C). There was great heterogeneity in the response of environmental isolates since the four minimum MIC values were obtained for metals belonging to this group. This fact suggests that the individual levels of the susceptibility of the acinetobacters against group III metals may occur within a relatively wide range of metal concentrations, in contrast to the response to other metals such as metals belonging to group I.

The reference strains exhibited uniform response to all the metal ions tested except Cr^{2+}, Ag^{+} and As^{3+}. Two of the reference strains were resistant to silver (ATCC 15150 and ATCC 33305) and arsenite (ATCC 15150 and EBF65/65 C426) while only ATCC 33305 was resistant to chromium. All reference strains were sensitive to cadmium and mercury, and resistant to the remaining metal ions tested.

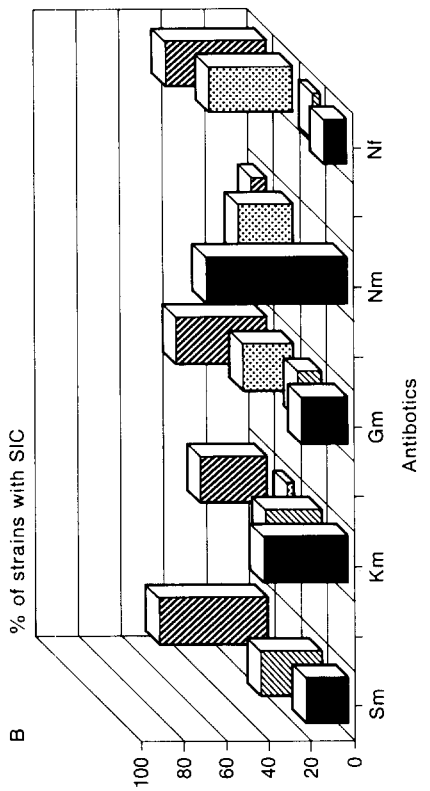
All environmental isolates of *Acinetobacter* tested were resistant to multiple metal ions: two isolates were resistant to all 20 metal ions (5.2%), 16 were resistant to 19 (40.8%), 13 were resistant to 18 (33.2%), one was resistant to 17 (2.6%), two were resistant to 16 (5.2%), one each was resistant to 15, 14 and 12 metal ions (2.6%), and two were resistant to 13 metal ions (5.6%). The distribution of frequencies of resistance for all strains for each metal ion as per genospecies is shown in Table 2.

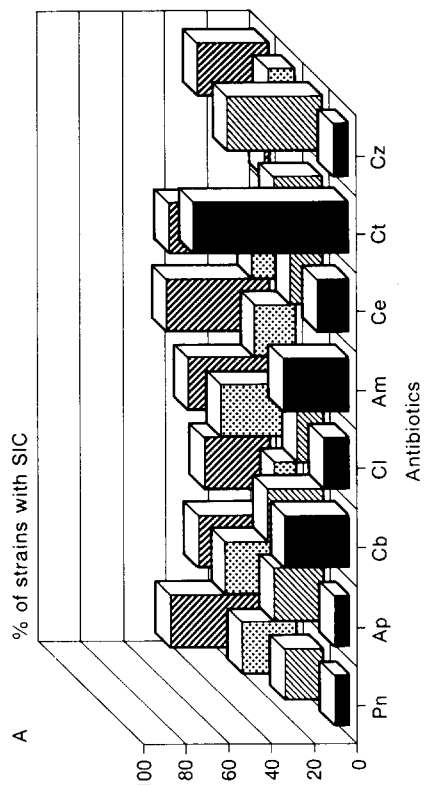
Resistance to antibiotics

Figure 2 shows the response of the environmental isolates of *Acinetobacter* to different antibiotics. Most of the isolates showed high levels of resistance to multiple antibiotics. More than 71% of the strains showed resistance to all beta-lactam group antibiotics except carbenicillin and cephotaxime, to

Table 2. Response of *Acinetobacter* to metal ions

Metal ions	Resistant strains (%)				
	A. baumannii	*A. calcoaceticus*	*A. junii*	*A.* genospecies3	reference strains
Co	81.25	0	0	100	100
Cu	100	100	100	100	100
Hg	28.125	66.66	100	100	0
Pb	100	100	100	100	100
Zn	100	100	100	100	100
Cd	90.625	100	66.66	100	0
Cr	75	100	100	100	25
Bi	87.5	100	100	100	100
Ag	93.75	100	100	100	50
As^{4+}	93.75	100	100	100	100
As^{3+}	71.88	100	100	100	50
Sb	90.625	66.66	100	100	100
Mo	93.75	100	100	100	100
B	100	100	100	100	100
Se	87.5	100	100	100	100
W	78.125	100	100	100	100
Sn	100	100	100	100	100
Ni	100	66.66	100	100	100
Li	90.625	100	100	100	100
Al	87.5	100	100	100	100





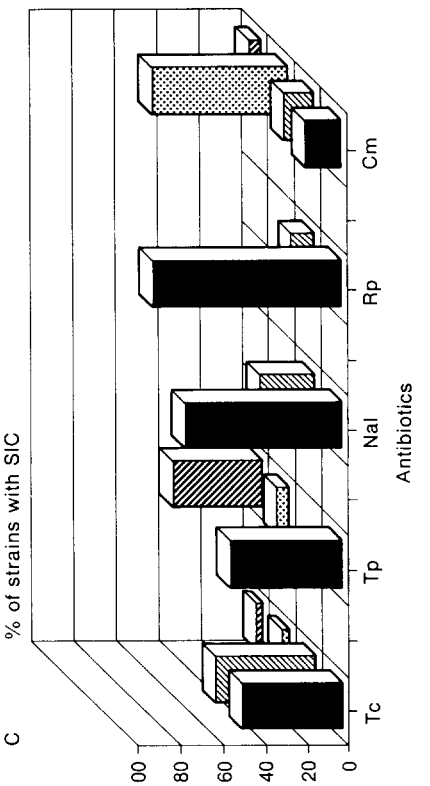


Figure 2. Response of environmental isolates of *Acinetobacter* to: (A) beta-lactam antibiotics, (B) aminoglycoside antibiotics and (C) other antibiotics. ■, 0–32 μg ml^{-1}; ▨, 32–128 μg ml^{-1}; ▩, 128–512 μg ml^{-1}; ▨, 512–1024 μg ml^{-1}.

which 55 and 21% of the strains were found to be, respectively, resistant. Similarly more than 78% of the strains were resistant to most of the aminoglycoside antibiotics, except neomycin to which only 34.65% of the strains were resistant. The majority of the environmental isolates were sensitive to all of the other antibiotics tested except to chloramphenicol (84.16% strains resistant). Rifampicin and nalidixic acid were the most toxic antibiotics, inhibiting growth of more than 90% of the strains even at very low concentration.

The reference strains were sensitive to all of the the tested antibiotics, except penicillin and rifampicin. All were resistant to penicillin, while only EBF65/65 C426 and C4169 were resistant to rifampicin.

A. calcoaceticus was found to be the most resistant genospecies since all the *A. calcoaceticus* isolates were resistant to 12 of the 18 antibiotics tested (Table 3). The high level of multiple resistance displayed by the strains of *A. calcoaceticus* was followed by the strains of *A.* genospecies3, *A. junii* and *A. baumannii* in that order.

Discussion

A wide range of concentrations of metals was tested because of the paucity of information about the susceptibility of *Acinetobacter* to heavy metals. A similar range was also used in the previous investigation with clinical isolates of *Acinetobacter* (Deshpande *et al.* 1993). It was hoped that an inhibitory concentration would be obtained that could differentiate between clinical and environmental isolates. It was observed that more than 95% of the environmental isolates were resistant to 10 mM Ni^{2+} and 10 mM Zn^{2+}. In the previous investigation all the clinical isolates were sensitive to both Ni^{2+} and Zn^{2+} at this concentration. Thus 10 mM concentration of Ni^{2+} and Zn^{2+} could serve as a guide for differentiating pathogenic strains of *Acinetobacter* from nonpathogenic strains.

The overall response exhibited by the strains belonging to genospecies *A. calcoaceticus* or *A. junii* or *A.* genospecies3 was very homogenous since strains belonging to the same genospecies showed similar MIC values against individual metals. However, *A. baumannii* strains showed a heterogenous response to the metal ions tested. This was evident from the fact that as many as four MIC values were obtained for these strains against individual metal ions, such as Co^{2+}, Hg^{2+}, As^{3+} and W^{2+}. This emphasizes the need for further differentiation of the strains belonging to *A. baumannii* into subgroups. Metal ion resistance may provide a potential basis for further classification of such strains into subgroups. Such a resistogram typing

Table 3. Resistance of *Acinetobacter* to antibiotics

Antibiotics	Resistant strains (%)				
	A. baumannii	*A. calcoaceticus*	*A. junii*	*A.* genospecies3	reference strains
Pn	83.85	100	100	100	100
Ap	80.625	100	100	100	0
Cb	45.15	100	100	100	0
Cx	77.4	100	66.66	100	0
Am	65.15	100	66.66	100	0
Ce	64.5	100	66.66	100	0
Ct	16.125	66.66	33.33	0	0
Cz	90.3	100	100	100	0
Sm	82.75	66.66	66.66	100	0
Km	48.375	100	100	100	0
Gm	77.4	100	66.66	100	0
Nf	90.35	100	100	0	0
Tc	54.825	66.66	33.33	0	0
Tp	41.95	100	66.66	0	0
Na	6.45	33.33	33.33	0	0
Rf	3.225	33.33	0	0	50
Cm	80.625	100	100	100	0

Pn, penicillin; Ap, ampicillin; Cb, carbenicillin; Cx, cloxacillin; Am, amoxicillin; Ce, cephaloridine; Ct, cephotaxime; Cz, cephazolin; Sm, streptomycin; Km, kanamycin; Gm, gentamicin; Nf, nitrofurantoin; Tc, tetracycline; Tp, trimethoprim; Na, nalidixic acid; Rf, rifampicin; Cm, chloramphenicol.

scheme has been proposed for other bacteria (Elek & Higney 1970).

Holton (1983) proposed a selective medium for the isolation of *Acinetobacter*. This medium contained antibiotics such as ampicillin (16 $\mu g\,ml^{-1}$), vancomycin (10 $\mu g\,ml^{-1}$) and cefsulodin (10 $\mu g\,ml^{-1}$) as selective agents. Results obtained in this investigation show that 15.4% of the total isolates as well as four reference strains were inhibited in the presence of 16 $\mu g\,ml^{-1}$ of ampicillin, emphasizing the unsuitability of Holton's selective medium for the isolation of environmental isolates of *Acinetobacter*. One interesting and potentially useful observation from this study showed that all isolates tested were resistant to Cu^{2+}, Pb^{2+}, B^{2+} and Sn^{2+}, even at concentrations as high as 10 mM. Thus salts of these metal ions at such high concentration may be used as selective agents instead of using antibiotics. This possibility is being investigated further.

The overall response of the acinetobacters was quite homogenous since, for more than 90% of them, the MICs of copper, lead, zinc, arsenate, molybdenum, boron, stannum, nickel, cadmium, selenium, tungsten, lithium and aluminium were the same. Interestingly, the only strain belonging to *A.* genospecies3 was resistant to all 20 metal ions tested. All the strains belonging to *A. junii* were resistant to 18 metal ions while all *A. calcoaceticus* and *A. baumannii* strains were resistant to 16 and six metal ions, respectively. The resistance levels exhibited by the environmental acinetobacters against individual metal ions were much higher as compared with those against the clinical isolates (Deshpande *et al.* 1993), halophilic acinetobacters (Nieto *et al.* 1989) or the culture collection strains (this study). The toxicity of the metal ions against the environmental isolates was in the following order: Hg > Co > arsenite > Cr, W > Cd > Bi, Sb, Se, Li > Ag > arsenate, Mo > Al > Cu, Pb, Zn, B, Sn, Ni.

All of the strains tested in the present investigation produce exopolysaccharides. These extracellular polysaccharides may trap the metal ions and prevent them from entering into the cells, and thereby rendering them harmless to the cell. This property may have a potential biotechnological application for the acinetobacters in the removal of metal ions from polluted environments.

In the present investigation all but one of the multiple metal resistant environmental isolates were also resistant to multiple antibiotics. Interestingly, 88.2% of the cadmium resistant strains were also resistant to penicillin and ampicillin. Such association between genes encoding resistance to penicillins and cadmium has been established in *Staphylococcus aureus* (Novick & Roth 1968). Similarly all mercury resistant strains were resistant to ampicillin, carbenicillin and chloramphenicol, while 87.5% of these strains were also resistant to kanamycin and tetracycline, indicating a possible association between these resistances. Such association has been established in *Serratia marcescens, Klebsiella aerogenes, Pseudomonas aeruginosa, Providencia* sp. and *Proteus mirabilis* (Schottel *et al.* 1974). In all these cases genes encoding resistance to metals and antibiotics were located on plasmids. Whether metal and antibiotic resistances reported in the present study are plasmid borne or not requires further investigation.

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