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Plasmid mediated metal and antibiotic resistance in marine *Pseudomonas*

D. B. Rajini Rani & A. Mahadevan

Centre for Advanced Studies in Botany, University of Madras, Madras, India

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***Pseudomonas* sp isolated from the Bay of Bengal (Madras coast) contained a single large plasmid (pMR1) of 146 kb. Plasmid curing was not successful with mitomycin C, sodium dodecyl sulfate, acridine orange, nalidixic acid or heat. Transfer of mercury resistance from marine *Pseudomonas* to *Escherichia coli* occurred during mixed culture incubation in liquid broth at 10^{-4} to 10^{-5} ml^{-1}. However, transconjugants lacked the plasmid pMR1 and lost their ability to resist mercury. Transformation of pMR1 into *E. coli* competent cells was successful; however, the efficiency of transformation (1.49×10^2 Hg^r transformants μg^{-1} pMR1 DNA) was low. *E. coli* transformants containing the plasmid pMR1 conferred inducible resistance to mercury, arsenic and cadmium compounds similar to the parental strain, but with increased expression. The mercury resistant transformants exhibited mercury volatilization activity. A correlation existed between metal and antibiotic resistance in the plasmid pMR1.**

Keywords: mercury, arsenic, cadmium, plasmid, restriction analysis, curing, conjugation, transformation

Introduction

In recent years research on the occurrence of metal resistance bacteria in an aquatic environment has received impressive attention due to the environmental hazards that they pose (Rajini Rani & Mahadevan 1989, Summers & Barkay 1989). Much of the research has dealt with detoxification of mercury compounds by bacteria (Barkay *et al.* 1989, 1990, Jobling *et al.* 1988, Rochelle *et al.* 1989) occurring in the aquatic environment. So far, Mahler *et al.* (1986) have isolated cadmium and mercury resistant *Bacillus* strains from a salt marsh and in Boston Harbor, USA.

Resistance of bacteria to mercury is due to enzymatic detoxification (Silver *et al.* 1986), whereas resistance to cadmium, zinc, cobalt, arsenic, chromium and nickel is an energy-dependent metal efflux system (Summers & Barkay 1989). A few metals, such as copper, lead, cadmium and silver, are accumulated intracellularly (Belliveau *et al.* 1987).

The metal resistance genes are located on plasmids and transposons (Foster 1983; Silver & Misra 1988; Rajini Rani & Mahadevan 1989), with a few exceptions on chromosomes (Witte *et al.* 1986, Wang *et al.* 1987). In a few cases, the metal resistance genes are associated with other traits such as multiple drug resistance (Novick & Roth 1968, Kondo *et al.* 1974, Allen *et al.* 1977), degradation of phenols (Chakrabarthy 1976; Pickup *et al.* 1983) and pesticides (Don & Pemberton 1981).

The coastal waters of India, especially adjacent to major cities, contain high concentrations of heavy metals, because of the presence of metal processing industries. The heavy metals pose environmental hazards to bathers—animals and human beings—in addition to a reduction in primary productivity. We isolated a *Pseudomonas* from the Madras coast of the Bay of Bengal (India) and found that it harboured a megaplasmid of 146 kb. Plasmid curing, conjugation and transformation studies presented in this paper indicate that the plasmid pMR1 is stable, non-conjugative, and contains genes resistant to mercury, arsenic and cadmium, and to antibiotics such as ampicillin, kanamycin and tetracycline.

Address for correspondence: Professor A. Mahadevan, Director, Centre for Advanced Studies in Botany, University of Madras, Madras 600025, India.

Materials and methods

Bacterial strains and plasmids

Pseudomonas sp. was isolated from the coastal waters of the Bay of Bengal, Madras. However, the species was not determined. The Hewlett-Peckard 5898A Microbial Identification System (Institute of Plant Protection, Wageningen, The Netherlands) revealed that it had a very low match with *P. aureofaciens*. *Escherichia coli* HB101 was received from the Institute of Genetics, Biological Research Centre, Hungarian Academy of Sciences, Szeged, Hungary. *E. coli* (pMR1) was obtained by direct transformation with plasmid pMR1 from the marine *Pseudomonas* into *E. coli* HB101 (this study).

Media and growth conditions

Marine *Pseudomonas* was initially grown in ZoBell's 2216E marine agar medium (Aaronson 1970) and later in a modified medium containing 2% NaCl, 0.15% $MgSO_4 \cdot 7H_20$, 0.16% KCl, 0.1% yeast extract and 0.3% peptone. Mercuric chloride, phenyl mercury acetate (PMA), sodium arsenate, sodium arsenite and cadmium chloride were filter sterilized (0.2 μm) and aseptically added to the medium. *E. coli* and its transformants were grown in Luria broth at 37 °C in an orbitol incubator.

Resistance to antibiotics was determined by microsensitivity discs (Span diagnostics, Surat, India; Bauer *et al.* 1966).

All experiments were repeated three times.

Plasmid isolation

Various plasmid isolation protocols such as those proposed by EMBO (Dharmalingam 1985), Kado & Liu (1982) and Birnboim & Doly (1979) yielded good separation of plasmid DNA. The plasmid DNA was further purified by cesium chloride centrifugation (Maniatis *et al.* 1982).

Restriction analysis of pMR1

Restriction endonucleases were purchased from Promega (Leiden, The Netherlands) and used as specified by the manufacturer. Fragments were separated on 1% (w/v) agarose gel (electrophoresed at 50 V for 5–6 h to detect very small fragments). Using a λ*Hin*dIII standard DNA obtained from Promega, the molecular weight of the fragments was calculated with the aid of a computer programme (Ulaganathan & Mahadevan 1989).

Plasmid curing

Several curing agents were tested. These included 0.2, 0.5 and 1.0 $\mu g\ ml^{-1}$ of mitomycin C; 20, 50 and 100 $\mu g\ ml^{-1}$ of acridine orange; 20, 50 and 75 $\mu g\ ml^{-1}$ of sodium dodecyl sulfate (SDS); 2, 5 and 10 $\mu g\ ml^{-1}$ of nalidixic acid, and temperature treatments of 37 and 39 °C for 24 and 48 h. Filter sterilized curing agents were added separately to marine nutrient medium. The overnight grown culture (0.1 ml) was used to inoculate the medium containing the curing agents. The flasks were incubated for 24 h at 30 °C on a shaker at 120 rpm. Flasks without any curing agents served as control. Samples from the treated cultures that had moderate growth after 24 h were diluted serially and spread on marine agar medium, which served as master plate.

Temperature treatments that did not include curing agents were carried out similarly. From the master plate, colonies were transformed to selective agar plates containing mercuric chloride (10 $\mu g\ ml^{-1}$). Ten colonies that developed poorly were randomly picked and screened for plasmid.

Conjugation

In vitro transfer of mercury resistance from marine *Pseudomonas* (donor) to *E. coli* (recipient) was studied in 20 ml of medium (marine broth) at 30 °C by the technique of Curtiss (1981). Donor and recipient cultures (10^7–10^8 cells ml^{-1} in the logarithemic phase of growth) were mixed in proportions up to 1:100, incubated at 30 °C, and putative plasmid transconjugants were scored after 8, 12 and 24 h on mercury (10 $\mu g\ ml^{-1}$) containing Luria agar plates.

Transformation

E. coli HB101 was rendered competent for uptake of plasmid DNA (Maniatis *et al.* 1982). To 100 μl of the competent cells, 0.5–1 μg of plasmid DNA was added. Cells without any added DNA served as control. Metal resistant transformants were selected on Luria agar containing 10 $\mu g\ ml^{-1}$ $HgCl_2$.

Bacterial volatilization of mercuric chloride

A qualitative method for the detection of mercury volatilization activity without using ^{203}Hg proposed by Nakamura & Nakahara (1988) was adopted. *E. coli* transformants were grown in Luria broth containing 50 $\mu g\ ml^{-1}$ $HgCl_2$ overnight. Cultures were streaked on Luria agar plates containing 50 $\mu g\ ml^{-1}$ $HgCl_2$ and incubated at 30 and 37 °C overnight. Cell masses were collected individually by sterile tooth picks and resuspended in reaction mixture in one well of a microdilution plate (Shiratori *et al.* 1989). X-ray film (Kodak X-OMAT AR) and an acrylic plate were sequentially mounted over the plate in a dark room and both the ends were fixed with clips. The plate was kept in a black box, incubated for 60 min at 37 °C and the films developed.

Results

Isolation of plasmid DNA

A single plasmid with low mobility was detected without any chromosomal contamination and was designated as pMR1 (Figure 1). The procedure of

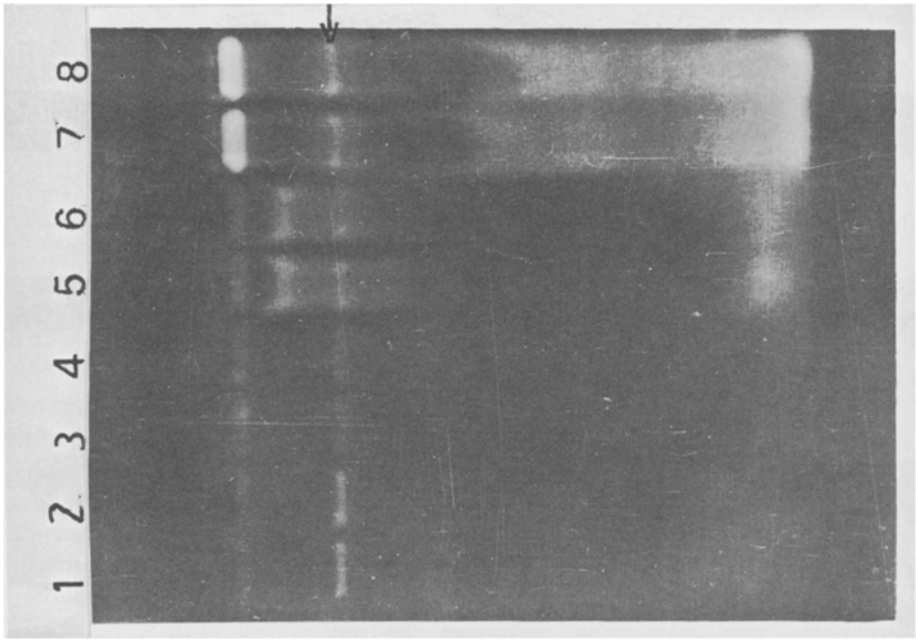

Figure 1. Gel electrophoresis of plasmid pMR1 separated by using different protocols: lanes 1 & 2, Birboim & Doly (1979); lanes 3 & 4, EMBO (Dharmalingam, 1985); lanes 5–8, Kado & Liu (1981). Arrow indicates plasmid DNA.

Birnboim & Doly (1979) facilitated a good yield of plasmid DNA. The size of the pMR1 supercoiled form was about 146 kb as confirmed by restriction enzyme analysis.

Restriction analysis of pMR1

Fragment patterns of different restriction endonucleases were determined with plasmid pMR1 DNA. The results were reproducible. The enzymes *Pst*I and *Eco*RI had 27 and 20 recognition sites, whereas *Sa*II, *Bam*HI and *Hin*dIII had 18, 15 and 13 recognition sites, respectively. *Xho*I did not produce discrete bands except for a smear. The molecular weights of the fragments calculated with the aid of the UMA-DNA computer programme are presented in Table 1.

Plasmid curing

The curing experiment, set up using mitomycin C, acridine orange, SDS, nalidixic acid, and heat at 37 and 39 °C, revealed that the treatments did not eliminate the plasmid DNA but delayed the growth of cells on marine agar plates amended with mercury (data not presented). The cells resumed normal growth, similar to the control without any metal, after 48–56 h of incubation.

Table 1. Size of restriction enzyme fragments in plasmid pMR1

Restriction enzymes	Number of fragments	Size (kb)[a]
*Pst*I	27	48, 17.5, 11.49, 9.98, 9.96, 7.6, 6.95, 6.8, 6.65, 5.86, 4.91, 4.43, 3.94, 3.74, 2.86, 2.59, 2.51, 2.39, 2.27, 2.11, 2.06, 2.0, 1.9, 1.84, 1.76, 1.66, 1.65.
*Eco*Rl	20	20.13, 14.63, 12.30, 10.76, 10.20, 9.42, 8.40, 7.48, 6.8, 6.56, 5.8, 5.54, 4.86, 4.39, 4.13, 3.69, 3.15, 2.85, 2.22, 1.56
*Sal*I	18	27.61, 21.44, 17.92, 12.82, 10.38, 8.29, 7.49, 6.9, 6.39, 4.86, 4.39, 4.39, 3.44, 3.41, 2.72, 2.51, 2.4, 2.03, 1.52
*Bam*HI	15	28.77, 18.97, 16.54, 15.35, 11.83, 10.20, 9.08, 7.28, 6.81, 5.80, 4.96, 3.77, 2.72, 2.4, 1.69
*Hin*dIII	13	22.92, 18.97, 17.92, 13.98, 12.30, 10.57, 10.56, 9.22, 9.22, 7.49, 5.18, 4.76, 2.81

[a]Kilobase pairs.

Plasmid conjugation

The frequency of plasmid pMR1 transfer from marine *Pseudomonas* to *E. coli* strain at 30 °C for 24 h in Luria broth culture was 6.9×10^{-4} transconjugants per donor cell. *E. coli* transconjugants obtained on Luria agar plates supplemented with mercury were small, flat, circular and moderate sized colonies. Plasmid analysis by agarose gel electrophoresis revealed that Hg^r transconjugants had not acquired plasmid DNA from marine *Pseudomonas*. However, mercury resistance in *E. coli* transconjugants was unstable and was lost immediately after subculturing.

Plasmid transformation

Cesium chloride purified plasmid DNA of marine *Pseudomonas* was used to transform competent cells of *E. coli* HB101. Mercury chloride resistant colonies developed after 48 h incubation on Luria agar plates supplemented with 10 $\mu g\ ml^{-1}$ $HgCl_2$. The efficiency of transformation was $1.49 \times 10^2\ \mu g^{-1}$ pMR1 DNA. The control plates without any added DNA did not produce any colonies. Twenty five presumptive Hg^r transformants were screened for plasmid DNA by agarose gel electrophoresis of clear lysates. All *E. coli* transformants randomly screened contained the plasmid with the same mobility. This result indicated the presence of *mer* genes on plasmid pMR1.

Resistance to mercury compounds

One of the *E. coli* transformants (pMR1) grew well in Luria broth containing more than 100 $\mu g\ ml^{-1}$ $HgCl_2$ (Figure 2) and 30 $\mu g\ ml^{-1}$ PMA (Figure 3), within 24–42 h of incubation at 37 °C, with shaking. *E. coli* (pMR1) possessed more than a 40-fold increase in *mer* expression than the wild-type strain, thus conferring broad spectrum resistance in plasmid pMR1 of marine *Pseudomonas*. It also efficiently volatilized $HgCl_2$ to Hg^0 (not shown). The fogged areas on the X-ray film were the result of reduction of the Ag^+ emulsion by mercury vapor (Nakamura & Nakahara 1988).

Multimetal and antibiotic resistance in plasmid pMR1

A correlation existed between metal and antibiotic resistance in *E. coli* having the plasmid pMR1. The plasmid pMR1 was not only resistant to mercury compounds but also resisted very high concentrations of arsenic (sodium arsenate, 5 $mg\ ml^{-1}$) (Figure 4) and cadmium (cadmium chloride, 50 $\mu g\ ml^{-1}$) (Figure 5) within 24–42 h of incubation with shaking at 37 °C. When tested for antibiotic resistance, it produced no zone of inhibition for 10 $\mu g\ ml^{-1}$ ampicillin, 30 $\mu g\ ml^{-1}$ tetracycline and 30 $\mu g\ ml^{-1}$ kanamycin (Table 2), indicating it to be plasmid encoded.

Discussion

The *Pseudomonas* isolated from the Bay of Bengal is resistant to heavy metals such as mercury, PMA, arsenic, cadmium, copper, lead and zinc and antibiotics such as ampicillin, kanamycin, tetracycline, penicillin and carbenicillin. The mechanism of mercury resistance in this bacterium is direct enzymatic detoxification by the inducible enzyme mercuric reductase, similar to other mercury resistant bacteria (Weiss *et al.* 1977, Schottel *et al.* 1978; Belliveau & Trevors 1990).

The only mechanism so far reported for arsenic resistance has been the energy-dependent arsenate efflux system (Mobley & Rosen 1982, Silver & Keach 1982). This system was responsible for arsenic resistance in this bacterium (unpublished results). The synthesis of ATP in glucose by endogenous cells of *Pseudomonas* is in accordance to Mobley & Rosen (1982), where rapid arsenate efflux occurred in *E. coli* only in the presence of glucose but not in the presence of succinate. This result was further confirmed by atomic absorption spectrophotometer analysis, that detected 98% of total arsenate in the culture filtrate of 24 h grown arsenate induced cells. Interestingly, 50% of the cadmium was detected in the cells of *Pseudomonas* and the remaining in the surrounding medium (unpublished results), clearly a dual mechanism of detoxification exists: intracellular accumulation and metal extrution; however, it should be further confirmed by performing cadmium transport assays using labelled cadmium compounds. A similar dual mechanism of Cd^{2+} influx and Cd^{2+} efflux was reported in a *Bacillus subtilis* (Laddaga *et al.* 1985).

Agarose gel electrophoresis revealed the presence of a single plasmid DNA with low mobility. The plasmid, designated as pMR1, produced a large number (13–27) of fragments when digested with restriction enzymes. The total size of the restriction fragments produced by the individual enzymes was the same and therefore the molecular weight of pMR1 was calculated to be about 146 kb. Indeed, much of the large proportion of *mer* plasmids in natural waters are large; more than 100 kb in size (Bale *et al.* 1988, Jobling 1988; Rochelle *et al.* 1989).

The metal resistance patterns for the treated cells

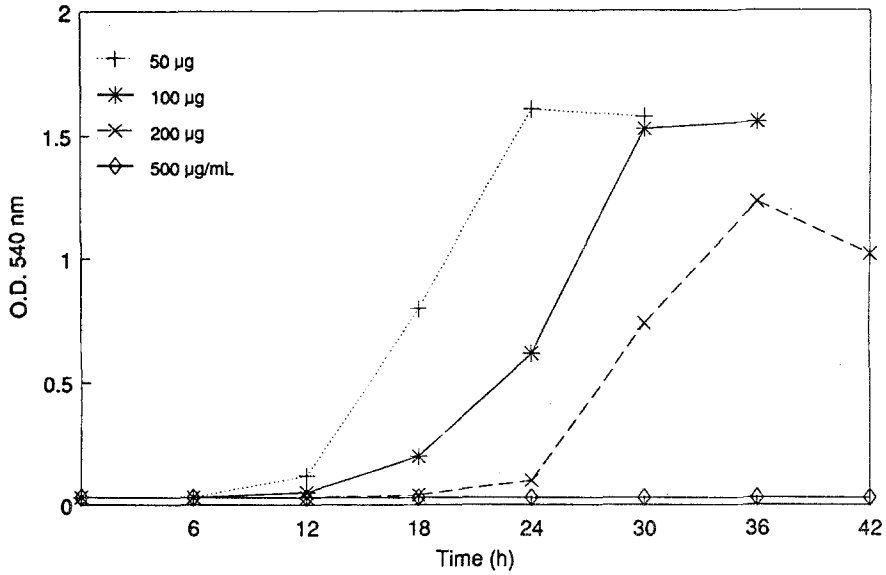

Figure 2. Growth of *E. coli* (pMR1) on mercuric chloride.

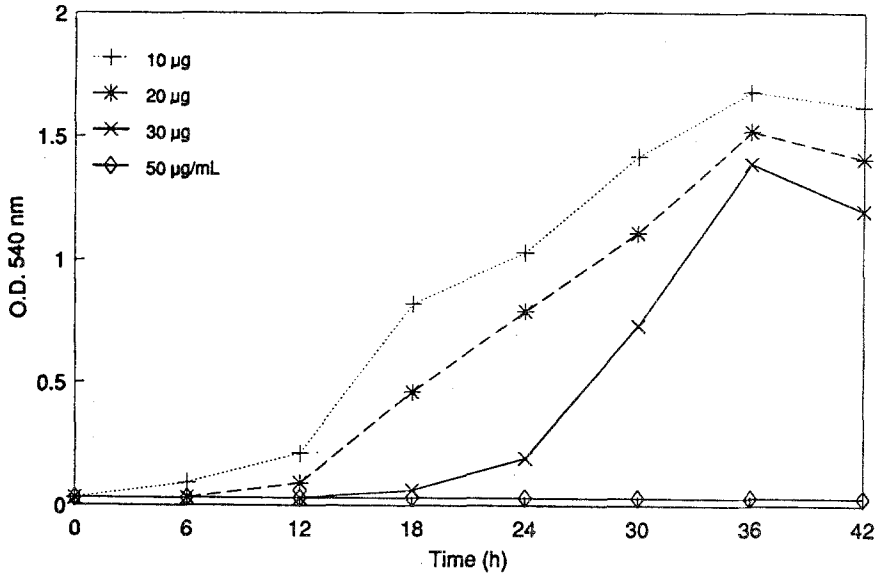

Figure 3. Growth of *E. coli* (pMR1) on PMA.

and agarose gel profiles showing the presence of the plasmid in the cells suggested that plasmid pMR1 was stable and carried genes resistant to mercury. Gauthier *et al.* (1985) observed no curing effect in a mercury resistant marine Pseudomonad treated with ethidium bromide.

Agarose gel electrophoresis of Hg^r *E. coli* transconjugants indicated that plasmid pMR1 was non-conjugative and the region coding for mercury resistance may have inserted into the chromosome of *E. coli* recipients. However, the *E. coli* Hg^r transconjugants were non-fertile since they lost their mercury resistance trait. A similar observation was made by Bale *et al.* (1989) and Belliveau & Trevors (1990) in mercury resistant *Pseudomonas* and *Bacillus* sp. occurring in water.

E. coli cells were successfully transformed with pMR1 DNA of marine *Pseudomonas*; however, the frequency of plasmid pMR1 transformation was low and it could be obviously due to its large size. Belliveau & Trevors (1990) transformed *Bacillus* strains with the large *mer* plasmid pGB130 of *B. cereus* by high voltage electroporation. The transformation efficiency ranged from 10^3 to 10^4 Hg^r transformants μg^{-1} pGB130 DNA. Transformants expressed inducible mercury resistance and

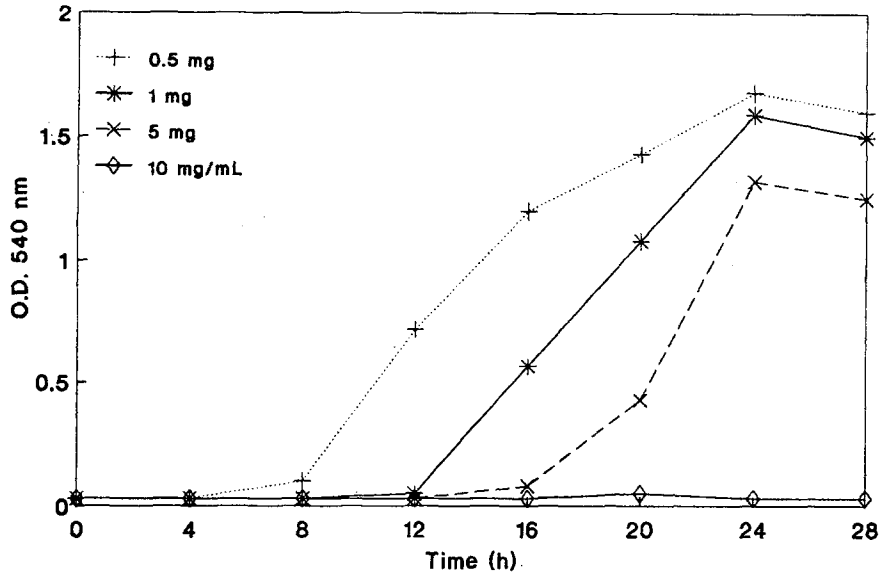

Figure 4. Growth of *E. coli* (pMR1) on sodium arsenate.

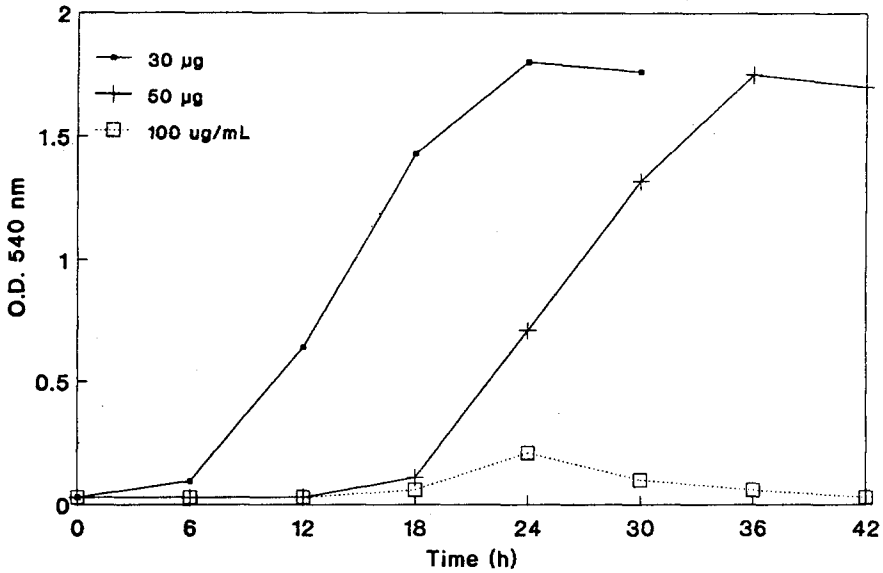

Figure 5. Growth of *E. coli* (pMR1) on cadmium chloride.

Table 2. Antibiotic resistance phenotypes in plasmid pMR1[a]

Strains	Antibiotics				
	Ampicillin	Kanamycin	Tetracyclin	Penicillin G	Streptomycin
Marine *Pseudomonas* SP. (pMR1)	R*	R*	R*	R	S
E. coli HB101	S	S	S	S	R
E. coli HB101 (pMR1)	R	R	R	R	R

[a]R, resistant; S, sensitive; *, plasmid encoded.

rapid volatilization when grown in the presence of either $HgCl_2$ or PMA. Volatilization of mercury in the presence of PMA indicated the presence of the organomercurial detoxifying enzyme organomercurial lyase, encoded by plasmid pMR1. Only bacteria possessing broad spectrum mercury determinants volatilized Hg^{2+} to Hg^0 upon induction by PMA (Jobling *et al.* 1988). Prior induction of *E. coli* transformant cells to a sub-toxic concentration of $HgCl_2$ or PMA exhibited a 40-fold increase in *mer* expression compared with marine *Pseudomonas*. For the first time we have demonstrated the expression of broad spectrum resistance genes of *Pseudomonas* in *E. coli* and the operons for both the metals are present in the plasmid pMR1.

Apart from plasmid bound broad spectrum *mer* resistance, *E. coli* transformants also displayed parallel expression of pMR1 DNA encoding for arsenic and cadmium resistance. The growth pattern in the metals and presence of plasmid in *E. coli* transformants indicated that mercury, arsenic and cadmium resistance genes are plasmid borne.

Cells having plasmid pMR1 also displayed multiple antibiotic resistance (e.g ampicillin, tetracyclin and kanamycin resistance). Plasmid-encoded metal and antibiotic resistance is widespread in *E. coli, Staphylococcus aureus, Pseudomonas* sp. *Bacillus*, enteric bacteria and *Alcaligenes* sp. (Baya *et al.* 1986, Silver & Misra 1988, Rajini Rani & Mahadevan 1989, Summers & Barkay 1989). This is an ecological advantage to the bacterium to survive in a milieu containing both heavy metals and antibiotics, and by virtue of their capability to clean up heavy metals they can be used in polluted waters containing heavy metals.

Acknowledgements

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