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Chromate Resistance Plasmid in *Pseudomonas fluorescens*

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Chromate resistance of *Pseudomonas fluorescens* LB300, isolated from chromium-contaminated sediment in the upper Hudson River, was found to be plasmid specified. Loss of the plasmid (pLHB1) by spontaneous segregation or mitomycin C curing resulted in a simultaneous loss of chromate resistance. Subsequent transformation of such strains with purified pLHB1 plasmid DNA resulted in a simultaneous reacquisition of the chromate resistance phenotype and the plasmid. When pLHB1 was transferred by conjugation to *Escherichia coli*, the plasmid still conferred chromate resistance.

Plasmids confer resistance to many toxic metal and metalloid ions, including those of antimony, arsenic, bismuth, boron, cadmium, cobalt, lead, mercury, nickel, silver, tellurium, and zinc (6, 9, 12, 14, 16, 17). Plasmid-specified chromate resistance has been reported for both *Streptococcus lactis* (7) and *Pseudomonas aeruginosa* (18). In each case, the plasmid-bearing strain is approximately 10-fold more resistant to chromate than is the plasmidless strain.

Analyses of sediments from the upper Hudson River in New York State have indicated that very high chromium concentrations may exist in some locations (unpublished data). Substantial bacterial populations reside in these sediments even though the amount of chromium present as chromate might be expected to inhibit bacterial growth (10, 13, 15). Chromate-resistant (Cr^r) strains of bacteria were isolated from these chromium-contaminated river sediments, and the basis of the chromate resistance of the strains was investigated.

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MATERIALS AND METHODS

Bacterial strains and cultivation conditions. The strains used in this study, their relevant characteristics, and their origins are listed in Table 1.

Vogel-Bonner (VB) broth was prepared by aseptically mixing 10 ml of VB concentrate, 10 ml of a 25% (wt/vol) D-glucose solution, and 480 ml of distilled water (all previously sterilized). For agar medium, 7.5 g of Bacto-Agar (Difco Laboratories) was added to the distilled water before autoclaving. VB concentrate contained the following (in grams per liter of distilled water): K_2HPO_4, 500; $Na(NH_4)HPO_4 \cdot 4H_2O$, 175; citric acid, 100; and $MgSO_4 \cdot 7H_2O$, 10.

Luria (L) broth contained the following (in grams per liter of distilled water): tryptone (Difco), 5; yeast extract (Difco), 2.5; NaCl, 2.5; and D-glucose, 0.5. For agar medium, 15 g of Bacto-Agar was added.

Nutrient (N) agar was prepared from dehydrated nutrient agar (Difco).

Isolations of chromate-resistant strains were made from anaerobic enrichments which had been prepared in VB broth containing 0, 50, 100, or 200 μg of K_2CrO_4 per ml. Incubation was at 30°C in tightly closed screw-cap tubes which had been filled to capacity after inoculation. Portions of enrichments were concentrated by centrifugation, suspended in small volumes of L broth, and plated on N agar containing 100 μg of K_2CrO_4 per ml. After 36 h of aerobic incubation, clones representing different colony types were transferred to separate tubes of L broth and incubated overnight at 30°C. (Anaerobic enrichment with chromate followed by aerobic selection for chromate resistance was designed to increase the chance of isolating strains which were chromate resistant and capable of anaerobic growth with chromate as the electron acceptor. LB300 is such a strain. Chromate reduction by this organism will be discussed in a separate paper.) After plating, single colonies were transferred into L broth and incubated. From these broth cultures, stock cultures were prepared. They were maintained in 50% (vol/vol) glycerol at −20°C. Purity was determined microscopically and by examination of colony morphology on the plates. Strain LB300 was identified as belonging to the species *Pseudomonas fluorescens* by Bender Hygienic Laboratory, Albany, N.Y.

Resistance tests. Resistance to chromate was determined by placing 1.0% (vol/vol) inocula from overnight cultures into tubes of VB broth containing 0 to 2,500 μg of K_2CrO_4 per ml and then incubating the broth at 30°C with shaking. Growth was scored at 24 and 48 h. Resistances to other metal ions as well as to chromate were determined by inoculating freshly prepared N-agar plates containing from 0.1 to 100 mM

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TABLE 1. List of bacterial strains and their characteristics

Strain	Relevant characteristics	Source
E. coli		
AC80	*thr leu met hsdM hsdR*	W. Arber
AC156	*thr leu met hsdM hsdR* Ap^r Km^r Tc^r(RP4)	Transformation of AC80
LB317	*thr leu met hsdM hsdR* Cr^r(pLHB1)	By conjugation: LB308 by AC80
LB318	*thr leu met hsdM hsdR* Ap^r Km^r Tc^r Hg^r Cr^r(RP4::Tn*1861*, pLHB1)	By conjugation: AC1020 by LB317
P. fluorescens		
LB300	Cr^r(pLHB1)	New isolate
LB303	Cr^s	Spontaneous segregant of LB300
LB308	*trp* Cr^r(pLHB1)	Mutagenesis of LB300
LB309	*ade* Cr^r(pLHB1)	Mutagenesis of LB300
LB310	*his* Cr^r(pLHB1)	Mutagenesis of LB300
LB311	*lys* Cr^r(pLHB1)	Mutagenesis of LB300
P. putida		
AC151	*ilv* Ap^r Km^r Tc^r(RP4)	This laboratory
AC1020	*trp leu* Ap^r Km^r Tc^r Hg^r(RP4::Tn*1861*)	This laboratory

metal salts with about 3×10^7 cells from overnight cultures. Plates were examined for visible growth after incubation for 48 h at 30°C. Antibiotic resistances were determined with antibiotic-impregnated disks (BBL Microbiology Systems) on N-agar plates which had been seeded with about 3×10^7 cells from overnight broth cultures. Zones of inhibition were measured after incubation at 30°C for 48 h.

Isolation and characterization of plasmid DNA. Plasmid DNA was isolated and purified by the method of Currier and Nester (5). Ethidium bromide was removed from the DNA by extraction with cold isopropanol saturated with cesium chloride. Plasmid DNA was then dialyzed at 4°C against TES buffer (50 mM Tris, 50 mM NaCl, 0.5 mM EDTA; pH 8.0) containing Dowex 50W-X8 cationic exchange resin (to trap ethidium bromide). The dialyzed DNA was stored at 4°C until used.

DNA was prepared for electron microscopy by the Kleinschmidt (11) aqueous spreading technique, stained with uranyl acetate, and rotary shadowed with platinum. Circular λ phage DNA (15.7 μm) was used as an internal size standard. Contour lengths were determined from electron micrographs with a Numonics Corp. electronic graphics calculator.

Electrophoresis of plasmid DNA was performed in horizontal 0.7% (wt/vol) agarose slab gels (13 by 12 by 0.3 cm), using E buffer (40 mM Tris-hydrochloride, 50 mM sodium acetate, 1 mM EDTA; pH 8.0). Gels were run for about 3 h at 60 V (measured across the resolving gel), stained for 30 min with ethidium bromide (5 μg/ml), and photographed while illuminated from below with a 260-nm UV light source.

Mutagenesis and selection of auxotrophs. Mutagenesis was with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine under conditions such that 95 to 99% of the exposed cells were killed. Single colonies of the surviving populations were grown on N agar with K_2CrO_4 and then replica plated onto VB agar plates containing K_2CrO_4. All colonies which grew on N agar with chromate but not on VB agar with chromate were screened for mutation identification, using amino acid-vitamin-nucleotide crisscross pools.

Conjugation, transformation, and mobilization. Solid-surface matings were done in a soft (0.6% [wt/vol] agar) L-agar overlay on basal (1.5% [wt/vol] agar) L-agar plates. Donor and recipient cells were grown as previously described (1). Equal volumes of each strain were suspended in molten (45°C) soft agar and poured onto a basal L-agar plate. Incubation was at 30°C for 6 h. After incubation, part of the soft agar (containing the cells) was transferred with a sterile spatula to a minimal volume of sterile 0.15 M NaCl and mixed by vortexing. The resultant suspension was plated on appropriate selective medium and incubated at a temperature optimal for growth of the recipient strain.

Transformation of recipient strains with purified plasmid DNA was essentially as described by Chakrabarty et al. (3).

Mobilization of the chromate resistance plasmid was attempted using the broad-host-range, antibiotic resistance plasmid RP4 and RP4::Tn*1861*, a derivative of RP4 containing a mercury resistance transposon (8). These plasmids were placed within chromate-resistant strains by either conjugation or transformation, as described above. Mobilization experiments involved solid-surface mating of strains with appropriate recipients, followed by selection for simultaneous transfer of either antibiotic and chromate resistance or antibiotic, chromate, and mercury resistances.

Curing. Chromate-resistant strains were cured either by treatment with mitomycin C (1) or by growth for several generations without selection for chromate resistance. In the latter case, a 1% (vol/vol) inoculum of cells grown with chromate was placed in L broth and grown overnight at 30°C with shaking. After 24 h the culture had reached a density of about 10^{10} cells per ml. A 1% (vol/vol) inoculum from this culture was then used to inoculate fresh L broth, and the culture was incubated as described above. This inoculation and overnight growth cycle was repeated five times without chromate. The resulting cells were then diluted and plated on N agar at a density such that 50 to 100 colonies per plate arose. Single colonies were spotted onto VB agar containing 250 μg of K_2CrO_4 per ml, VB agar without chromate, and N agar without chromate.

Plates were incubated and then screened for chromate-sensitive (Cr^s) colonies. All Cr^s colonies were purified and retained for further investigation.

RESULTS

Loss of the chromate resistance phenotype. Chromate-resistant *P. fluorescens* LB300, which is capable of growth in VB broth containing more than 1.5 mg of K_2CrO_4 per ml, lost the Cr^r phenotype at a frequency of ca. 1% when grown in the absence of chromate for five overnight culture cycles. Chromate-sensitive strains derived in this way were unable to grow in VB broth containing as little as 10 μg of K_2CrO_4 per ml. Furthermore, reversion of such strains to chromate resistance could not be detected. Treatment with mitomycin C at a concentration of 5 μg/ml resulted in the loss of the Cr^r phenotype in 99% of the colonies of strain LB300 tested. Several Cr^s single colonies derived by mitomycin C treatment were checked for reversion to the Cr^r phenotype, but the frequency in every case was below the limits of detection.

Physical characterization of plasmids. The plasmid DNA isolation and purification procedure described above was used on chromate-resistant *P. fluorescens* LB300 and on its chromate-sensitive derivative, LB303. An intense plasmid band was observed in the cesium chloride-ethidium bromide buoyant density gradient for the Cr^r strain, but only one band was seen in the gradient for the Cr^s strain. All bands were collected, and the DNA was analyzed by agarose gel electrophoresis. Several plasmid bands were present in the presumptive plasmid fraction from the chromate-resistant strain. However, repeated analysis of the DNA extracted from the chromate-sensitive variant, LB303, revealed no plasmids.

Electron micrographs of plasmid DNA isolated from chromate-resistant strain LB300 showed 10.8-, 21.7-, and 27.9-μm circles (Table 2).

Genetic properties of plasmid associated with chromate resistance. Attempts were made to transform the chromate-sensitive, plasmidless segregant (LB303) derived from Cr^r strain LB300, as well as strains of *Pseudomonas putida* and *Escherichia coli* (which had proven to be excellent recipients in other studies), using plasmid DNA isolated from Cr^r strain LB300. RP4 plasmid DNA isolated from *P. putida* AC151 was used as a positive control. The results indicated that *E. coli*, *P. putida*, and *P. fluorescens* strains could be transformed with *P. putida*-derived RP4 plasmid DNA at frequencies ranging from 1.8×10^{-3} to 3.2×10^{-6} transformants per recipient. However, only the chromate-sensitive segregant, LB303, could be transformed with pLHB1 plasmid DNA. In that case the frequency was 1.7×10^{-6} transformants per recipient.

Several auxotrophic mutants were derived from Cr^r strain LB300 by mutagenesis for use as donor strains in conjugation experiments. *Klebsiella pneumoniae*, *E. coli*, *P. putida*, and *P. fluorescens* strains were among the recipients used. The results of these experiments (Table 3) indicated that the Cr^r phenotype could readily be transferred by conjugation to both LB303 (the Cr^s segregant of Cr^r strain LB300) and *E. coli* AC80, but not to any of the other strains tested.

Mobilization of chromate resistance by RP4, which is known to mobilize some nontransmissi-

TABLE 2. Size of plasmids isolated from chromate-resistant *P. fluorescens* LB300 and *E. coli* LB317

Strain	Size (μm)	10^6 Mol wt	No. of plasmids measured	% of total
LB300	10.8 ± 1.0	22.2 ± 2.1	104	71
LB300	21.7 ± 1.8	44.7 ± 3.7	32	22
LB300	27.9 ± 1.7	57.5 ± 3.5	10	7
LB317	10.7 ± 1.1	22.0 ± 2.3	100	100

TABLE 3. Conjugal transfer of chromate resistance plasmid

Donor	Recipient[a]	Transfer of Cr^r (frequency)[b]
P. fluorescens	*E. coli*	
LB303	AC80	$<10^{-8}$
LB308	AC80	5.0×10^{-6}
LB309	AC80	1.0×10^{-6}
LB310	AC80	3.0×10^{-8}
LB311	AC80	3.0×10^{-8}
P. fluorescens	*P. fluorescens*	
LB303	LB303	$<10^{-8}$
LB308	LB303	4.0×10^{-8}
LB309	LB303	1.5×10^{-6}
LB310	LB303	1.0×10^{-4}
LB311	LB303	3.0×10^{-8}
E. coli	*P. fluorescens*	
AC156	LB303	$<10^{-8}$[c]
LB318	LB303	3.5×10^{-8}[c]

[a] Five other *E. coli*, three *P. putida*, and two *K. pneumoniae* strains were used as recipients in crosses with *P. fluorescens* donors bearing the Cr^r phenotype and pLHB1 plasmid. No transfer occurred in these cases. Recipients were checked for growth on selective plates. In no case did Cr^r colonies arise when 10^8 cells were spread on a control plate.

[b] Selection was on VB agar containing 250 μg of K_2CrO_4 per ml and supplemented as necessary to support the growth of the recipient strains.

[c] Selection was on VB agar containing 250 μg of K_2CrO_4 per ml and 25 μg of $HgCl_2$ per ml and either ampicillin, kanamycin, or tetracycline.

ble plasmids (2), was attempted. RP4$^+$-, pLHB1$^+$-carrying strains of *P. fluorescens* (LB313 and LB314) derived from LB300 were used as donors in conjugation experiments with several recipient strains (other than AC80 and LB303). Selection was for simultaneous transfer of chromate resistance and any one of the antibiotic resistance markers (ampicillin, kanamycin, or tetracycline) on RP4. All such attempts proved to be unsuccessful (data not shown).

Transposon-mediated transfer of the Cr^r plasmid was attempted with LB318, an *E. coli* strain containing both RP4::Tn*1861* (Table 1) and pLHB1, as the donor and a plasmidless Cr^s segregant of *P. fluorescens*, LB303, as the recipient. Transconjugants were recovered from this mating which had simultaneously acquired antibiotic, mercury, and chromate resistances, but which had no amino acid requirements (Table 3).

Screening for additional plasmid-specified characteristics. The plasmid-bearing, chromate-resistant *P. fluorescens* strain, LB300, and its plasmidless, chromate-sensitive variant, LB303, were screened in parallel for differences in their resistance to the following metal and metalloid compounds and antibiotics: $Na_2B_4O_7$, $NaBO_3$, $NaBO_2$, K_2CrO_4, $K_2Cr_2O_7$, $CoCl_2$, $NiSO_4$, $CuSO_4$, $ZnSO_4$, $NaAsO_2$, Na_2HAsO_4, Na_2MoO_4, $AgNO_3$, $CdSO_4$, $SnCl_2$, Na_2TeO_3, $BaCl_2$, $HgCl_2$, and $Pb(NO_3)_2$ and amikacin, carbenicillin, chloramphenicol, cloxacillin, colistin, gentamicin, kanamycin, rifampin, streptomycin, sulfadiazine, tetracycline, and tobramycin. In addition, both strains were screened for their ability to use *m*-toluate, salicylate, and xylenes as the sole sources of carbon and energy. The plasmid-bearing strain was found to be much more resistant to chromate and slightly more resistant to dichromate than was the plasmidless segregant. Further experiments showed that these differences were approximately 200- and 2-fold, respectively. No other resistance markers were identified. Neither strain was capable of growth with *m*-toluate, salicylate, or xylenes as the sole source of carbon and energy.

DISCUSSION

The chromate-resistant strain of *P. fluorescens* (LB300) isolated from the Hudson River for this study was found to be 40- to 200-fold more resistant to chromate than were either pseudomonads taken from other environments or other bacteria taken from the same environment. Since resistance in bacteria to many toxic metal and metalloid ions (6, 9, 12, 14, 16, 17) and to chromate in particular (7, 18) is known to be conferred by plasmids, we suspected that this strain might harbor a chromate resistance plasmid.

The following results indicate that chromate resistance is probably plasmid associated in strain LB300. First, chromate-sensitive variants arose at a frequency of 10^{-2} after growth without selection for chromate resistance for five overnight growth cycles. Second, curing with mitomycin C resulted in the loss of chromate resistance in more than 99% of the single colonies examined. Finally, plasmid DNA was successfully isolated from strain LB300 but not from its Cr^s variant, LB303. Agarose gel electrophoresis of the plasmid DNA isolated from Cr^r strain LB300 revealed several plasmid bands. Three different size classes of plasmid (10.8, 21.7, and 27.9 μm) were subsequently identified by electron microscopy. The role of these plasmids in chromate resistance was deduced from the observations that (i) repeated attempts to isolate plasmid DNA from a Cr^s variant of Cr^r strain LB300 were unsuccessful (this variant also could not be shown to revert to the Cr^r phenotype) and (ii) when this apparently plasmidless strain was transformed with plasmid DNA from Cr^r *P. fluorescens* LB300, the transformants not only had chromate resistance characteristics identical to those of the donor strain but also had multiple plasmid bands corresponding in size to those of the original Cr^r strain. The acquisition of multiple plasmids during a single transformation suggests that the different plasmids are actually multimeric forms of the same plasmid and that the monomeric unit is 10.8 μm in size.

Further evidence from experiments with *E. coli* supports this interpretation. When *E. coli* AC80, a plasmidless, chromate-sensitive strain, was mated with Cr^r derivatives of *P. fluorescens* LB300, a Cr^r *E. coli* transconjugant, LB317, resulted. This strain was examined for the presence of plasmid DNA. A single 10.7-μm plasmid species was isolated (Table 2). No other plasmid species was identified in strain LB317. This evidence shows that the 10.8-μm plasmid was transferred to *E. coli* by conjugation but suggests that, unlike in *P. fluorescens* LB300, multimers cannot form in this strain. It is interesting that the degree of chromate resistance of Cr^r *E. coli* LB317 is only 40-fold greater than that of the corresponding plasmidless strain (AC80), whereas the *P. fluorescens* plasmid-bearing strain (LB300) is 200-fold more resistant to chromate than is its plasmidless counterpart (LB303). The presence of multimers in LB300 may result in a gene dosage effect and account for the difference in the degree of resistance that the plasmid affords to *P. fluorescens* and *E. coli*.

The experimental results described above indicate that pLHB1 has very restricted transmissibility. Transfer by conjugation was only successful with the Cr^s *P. fluorescens* segregant

(LB303) and Cr^s *E. coli* AC80 as recipients and *P. fluorescens* strains as donors. Matings involving LB317 as the donor (*E. coli* AC80 containing pLHB1) were unsuccessful. Also, matings between RP4$^+$-, pLHB1$^+$-carrying strains of *P. fluorescens* and a variety of recipients showed that RP4 could be transferred at high frequency but that there was no cotransfer of RP4 and pLHB1. The fact that RP4 could be transferred to many of these recipients demonstrated that failure to mobilize pLHB1 was not due to restriction by the recipients of foreign DNA originating in the Cr^r *P. fluorescens* donors. Since transmission of nonconjugative plasmids can be mediated by transposons (4), an attempt was also made to mobilize pLHB1 with a derivative of RP4 containing a transposon. The results of this experiment indicated that transmission of pLHB1 is indeed promoted by RP4::Tn*1861*.

Further investigation is under way to more fully elucidate the nature of this unique chromate resistance factor and its effects on the host.

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