JOURNAL OF BACTERIOLOGY, Apr. 1989, p. 2160–2165
0021-9193/89/042160-06$02.00/0

Vol. 171, No. 4

Comparison of Tellurite Resistance Determinants from the IncPα Plasmid RP4Te^r and the IncHII Plasmid pHH1508a

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Received 17 May 1988/Accepted 7 December 1988

The tellurite resistance (Te^r) determinants of the IncHII plasmid pHH1508a and the broad host range IncPα plasmid RP4Te^r were cloned into pUC8, creating plasmids pDT1364 and pDT1558, respectively. The Te^r region of pDT1364 was localized to a 1.25-kilobase region by using Tn*1000* insertion mutagenesis. Insertions of Tn*1000* into pDT1558 which resulted in tellurite sensitivity spanned 1.75 kilobases of DNA. No similarity between the restriction maps of these two plasmids was observed, and no homology could be detected by DNA-DNA hybridization. Expression in an in vitro transcription-translation system showed that pDT1364 encoded two polypeptides with molecular masses of 23 and 12 kilodaltons (kDa) which were not expressed by pUC8. Some of the Tn*1000* insertion mutants did not express the 23-kDa protein. pDT1558 encoded a 40-kDa polypeptide which was not expressed by pUC8. Both Te^r determinants were expressed constitutively. Our findings suggest that the mechanisms of Te^r encoded by these two plasmids are different.

Tellurium compounds are used in the film and rubber industries and in the manufacture of batteries and are found in fairly large amounts in the human body (21). Potassium tellurite (K_2TeO_3) is toxic to most microorganisms, particularly gram-negative bacteria, possibly because of its strong oxidizing ability (23). Some gram-positive bacteria, including *Corynebacterium diphtheriae* (10), *Streptococcus faecalis* (22), and most strains of *Staphylococcus aureus* (13), are naturally resistant to potassium tellurite. For this reason, tellurite medium has long been used to identify *C. diphtheriae*. Bacteria grown on media containing tellurite form black colonies. Electron microscopy has shown that these bacteria contain black intracellular crystals of metallic tellurium which are often located just inside the inner membrane (27, 29).

Escherichia coli appears to take up tellurite by one of the phosphate transport systems, and non-plasmid-mediated resistance may be due to a defect in phosphate transport (28). In the *Enterobacteriaceae*, tellurite resistance (Te^r) is often mediated by plasmids of the H and P incompatibility groups (5, 6, 23, 26). The mechanism of plasmid-mediated Te^r is unknown. The 56.4-kilobase (kb) broad host range IncPα plasmid RP4 (also called RK2 or RP1), originally isolated from *Pseudomonas aeruginosa*, encodes resistance to ampicillin, kanamycin, and tetracycline. Variants selected on medium containing tellurite express Te^r (RP4Te^r; 5, 24). Tn*7* insertion mutagenesis has shown that the Te^r determinant on this plasmid is located between the *kilA* and *korA* genes, which are involved in plasmid replication control (24). It is on a transposon, Tn*521*, which is about 4.5 kb in size (7).

The H plasmids are large conjugative plasmids that encode multiple drug resistances (1). Te^r bacteria carrying H plasmids are often isolated from film reprocessing sludge, as well as city and hospital sewages (26). The H plasmids are divided into two incompatibility groups, IncHI and IncHII. The IncHI plasmids are further divided into three subgroups, HI-1, HI-2, and HI-3, based on DNA homology (20). IncHI-2 plasmid pMER610 from *Alcaligenes* sp. encodes Te^r and mercury resistance (14). The Te^r determinant on this plasmid specifies four polypeptides and is inducible by prior exposure to subtoxic levels of potassium tellurite (14).

The 208-kb IncHII plasmid pHH1508a, originally isolated from *Klebsiella aerogenes*, determines resistance to streptomycin, trimethoprim, and potassium tellurite (6). Recently, a 96-kb deletion mutant of this plasmid was constructed (pDT1178) which also expressed Te^r although it was nonconjugative (32).

In this study, the Te^r determinants of RP4Te^r and pHH1508a were cloned into pUC8 and expressed in an in vitro transcription-translation system for genetic and physical analyses. (Some of these results were presented at the European Molecular Biology Organization Workshop on Promiscuous Plasmids of Gram-Negative Bacteria held in Birmingham, England from 20 to 24 July 1987.)

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The plasmids used in this study are listed in Table 1. All plasmids were maintained in derivatives of *Escherichia coli* K-12. Unless otherwise stated, the concentrations of antibiotics were as follows (in micrograms per milliliter): ampicillin, 48; carbenicillin, 500; chloramphenicol, 16; nalidixic acid, 24; potassium tellurite (Difco Laboratories), 50; tetracycline, 16; trimethoprim, 10. All antibiotic plates were made from Luria broth agar (GIBCO Laboratories) or MacConkey agar (Difco), except for tellurite plates, which were made from brain heart infusion agar (Difco), and trimethoprim plates, which were made from diagnostic sensitivity testing agar (Oxoid Ltd.).

Expression of Te^r by RP4Te^r and pHH1508a. Overnight cultures of *E. coli* carrying RP4 or pHH1508a were inoculated into fresh Luria broth, and induction was performed by adding potassium tellurite to some of the flasks to give a final concentration of 0.1 or 0.26 μg/ml. After 100 min, the cultures were challenged with a high concentration of potassium tellurite (19.5 μg/ml) and growth rates at 37°C were monitored by measuring the optical density at 600 nm in a Coleman Junior IIA spectrophotometer.

Plasmid DNA isolation. Plasmid DNA was isolated by the Sarkosyl lysate method (31) or by a scaled-up version of the

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TABLE 1. MIC of tellurite for *E. coli* harboring various plasmids

Plasmid(s)	Resistance marker(s)[a]	MIC[b] K_2TeO_3 (μg/ml)	Source	Reference
pHH1508a	Sm Su Tp Te	512[c]	*Klebsiella aerogenes*	6
pDT1178	Tp Te	ND	Deletion mutant of pHH1508a	32
pDT1364	Ap Te	128[c]	Te^r pUC8 clone derived from pHH1508a	This study
pDT1364-1 to pDT1364-20[d]	Ap	0.25–1.0	Tn*1000* insertion mutants of pDT1364	This study
RP4Te^r	Ap Km Tc Te	512[c]	*Pseudomonas aeruginosa*	24
pRK2102	Ap Te	ND	Laboratory derived from RK2 (RP4)	11
pDT1366	Ap Te	256[c]	Te^r pUC8 clone derived from RP4Te^r	This study
pDT1558	Ap Te	256	Te^r pUC8 clone derived from pDT1366	This study
pDT1558-1 to pDT1558-3	Ap	0.125	Tn*1000* insertion mutants of pDT1558	This study
pDT1558-4	Ap	8.0	Tn*1000* insertion mutant of pDT1558	This study

[a] Ap, Ampicillin; Km, kanamycin; Sm, streptomycin; Su, sulfonamides; Tc, tetracycline; Te, potassium tellurite; Tp, trimethoprim.
[b] The MICs of potassium tellurite were determined as described in Materials and Methods and Results. ND, Not determined.
[c] Previously reported (27).
[d] This represents a series of 20 derivatives of pDT1364, each of which contains a Tn*1000* insertion which results in a loss of tellurite resistance (Fig. 2b).

method of Birnboim and Doly (3), followed by cesium chloride-ethidium bromide density gradient centrifugation.

Restriction enzyme digestion and analysis. Restriction enzymes (*Acc*I, *Bam*HI, *Bgl*I, *Eco*RI, *Hin*cII, *Hin*dIII, *Pst*I, *Sal*I, *Sma*I, *Sst*I, *Sst*II, *Xba*I, and *Xho*I) were obtained from Boehringer Mannheim Biochemicals, Montreal, Quebec, Canada, or Bethesda Research Laboratories Canada Ltd., Mississauga, Ontario, Canada. *Bss*HII was obtained from New England BioLabs, Beverly, Mass. Samples of restriction endonuclease digests were subjected to electrophoresis in horizontal agarose gels in Tris-borate-EDTA buffer (19). The sizes of restriction fragments were determined with reference to bacteriophage lambda DNA digested with *Hin*dIII.

Construction of clones. Te^r clones were constructed by ligation of restriction enzyme-digested DNA into the multiple cloning site of pUC8 (30) by using T4 DNA ligase (Bethesda Research Laboratories) (17). Recombinant DNA was transformed into *E. coli* JM83 (Δ*lacZ*) by using calcium chloride (9), and transformants were selected on agar plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, isopropyl-β-D-thiogalactopyranoside (Boehringer Mannheim), and 75 μg of ampicillin per ml (30). Lactose-negative colonies were then tested for Te^r. Further subclones were constructed by cloning Te^r regions into restriction enzyme sites in plasmid pACYC184 (8), followed by recloning into pUC8.

Isolation of Te^s Tn*1000* insertion mutants. Te^r clones were transformed into *E. coli* W1485 containing the F plasmid, which carries transposon Tn*1000* (γδ). The resulting strains, selected on ampicillin plates, were mated with *E. coli* JE2571-1 (Nal^r) overnight in Penassay broth (Difco) at 37°C. The mating mixture was diluted in phosphate buffer, and transconjugants were selected on medium containing both nalidixic acid and carbenicillin. *E. coli* JE2571-1 strains into which the Te^r clones had been transferred during conjugative transfer to the F factor were tested for loss of Te^r (due to insertion of Tn*1000*).

Analysis of Tn*1000* insertion sites and orientation. Tn*1000* (γδ) is 5.7 kb long and has an *Sst*I site located close to the δ end (12). The positions and orientations of the Tn*1000* insertions into the Te^r clones were determined by comparing double digestions which included *Sst*I with the restriction maps of Tn*1000* and each clone. The γδ orientation is defined as having the γ end closer to the left side than is the δ end in the restriction endonuclease maps shown in the figures.

MIC determination. The MIC of potassium tellurite was determined by using the agar dilution method as described previously (27).

DNA-DNA hybridization. DNA fragments isolated from low-melting-point agarose (Bethesda Research Laboratories) by phenol-butanol-chloroform extraction were labeled with [^{32}P]dATP by nick translation (17). Dot blots were prepared as described previously (15). DNA-DNA hybridization was performed under conditions of low stringency (50% formamide; 37°C).

Analysis of plasmid-determined proteins. Plasmid proteins were analyzed by using an *E. coli* in vitro transcription-translation system (Amersham Canada, Oakville, Ontario, Canada). Polypeptides synthesized in this system were labeled with L-[^{35}S]methionine (New England Nuclear Corp., Boston, Mass.). Polypeptides were separated on 12% polyacrylamide gels containing 0.1% sodium dodecyl sulfate and 5% glycerol (16). The gel was prepared for fluorography (4), dried under vacuum, and then exposed to Kodak X-Omat AR film for various times at −70°C.

RESULTS

Expression of Te^r by RP4 and pHH1508a. Early-log-phase bacterial cultures of *E. coli*(RP4Te^r) and *E. coli*(pHH1508a) were challenged with a high level of potassium tellurite. No difference was seen in the growth rates of those cultures which had been previously exposed to subtoxic concentrations of tellurite compared with those of cultures not previously exposed. This indicates that the Te^r determinants of RP4Te^r and pHH1508a are expressed constitutively in *E. coli*, in contrast to the Te^r determinant of the IncHI-2 plasmid pMER610 (14).

Cloning of Te^r determinants. Plasmid pRK2102 carries a 12-kb fragment corresponding to the region between 50.4 and 8.5 kb on the map of RP4 (11). By using the restriction enzyme *Hin*cII, the Te^r region from pRK2102 was cloned into the *Hin*cII site of pUC8, creating the Te^r Ap^r plasmid pDT1366 (Fig. 1a). pDT1366 carries two pUC8 sequences separated by two *Hin*cII fragments of RP4. Because *Hin*cII creates blunt ends, these sites were lost during religation and no *Hin*cII sites are present in this plasmid. Since the *Hin*cII site about 2.2 kb away from the *Eco*RI site in RP4 is in the promoter of the *kilA* gene, which is transcribed in the leftward direction (33), it is possible that the *kilA* gene in pDT1366 is expressed from a hybrid promoter formed during ligation of the fragments or from the β-galactosidase promoter of pUC8. The *korA* and *korB* genes are located within

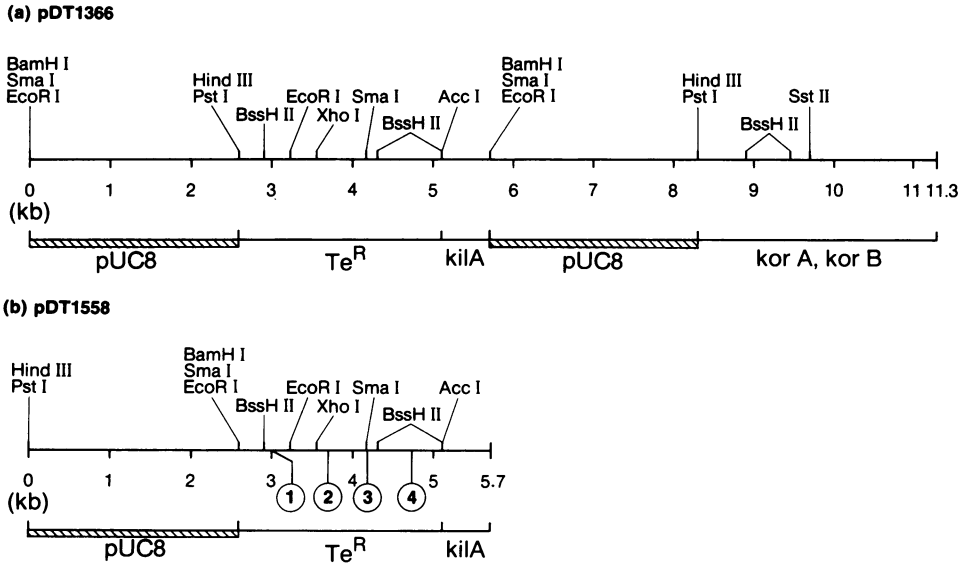

FIG. 1. Restriction endonuclease maps of pDT1366 (a) and pDT1558 (b). The circled numbers below pDT1558 indicate the positions of some Tn*1000* insertions which resulted in Te^s.

the *Hin*cII fragment between 52.5 and 55.6 kb on the map of RP4 (2). pDT1366 appears to contain this *Hin*cII fragment and therefore probably encodes both *korA* and *korB*. This would prevent the *kilA* gene from killing the host cell. Plasmid pDT1558 (Fig. 1b) was constructed by cloning the *Bam*HI-*Hin*dIII fragment of pDT1366 containing the Te^r region and the *kilA* region of RP4 into the corresponding sites in pACYC184 (8), followed by recloning into the *Hin*cII site of pUC8 by using the Klenow fragment of DNA polymerase to create blunt ends on the insert. Since the pUC8 sequence in pDT1558 is in the orientation opposite to that of those in pDT1366, *kilA* may not be expressed in this plasmid.

The Te^r region of pHH1508a (32) was cloned into the *Sal*I site of pUC8, creating the Te^r Ap^r plasmid pDT1364 (Fig. 2a). This plasmid carries a 7-kb *Sal*I fragment of pHH1508a. The restriction endonuclease map of pDT1364 shows no similarity to that of pDT1558 or to the Te^r determinant of pMER610 (14).

Tn*1000* insertion mutants of Te^r clones pDT1364 and pDT1558. Twenty Te^s mutants of pDT1364 were obtained by Tn*1000* insertion mutagenesis. Restriction mapping of these mutants showed that insertions of Tn*1000* spanned a 1.25-kb region of pDT1364 (Fig. 2b). Fifteen of these insertions were in the δγ orientation, and five were in the γδ orientation. This indicates that a minimum of about 1.25 kb of the 7-kb insert on this plasmid is involved in Te^r. The sites of insertion of Tn*1000* in four Te^s derivatives of pDT1558 are shown in Fig. 1b. pDT1558-3 and pDT1558-1 have Tn*1000* inserted in the γδ orientation, whereas pDT1558-2 and pDT1558-4 have Tn*1000* inserted in the δγ orientation. These mutants span a

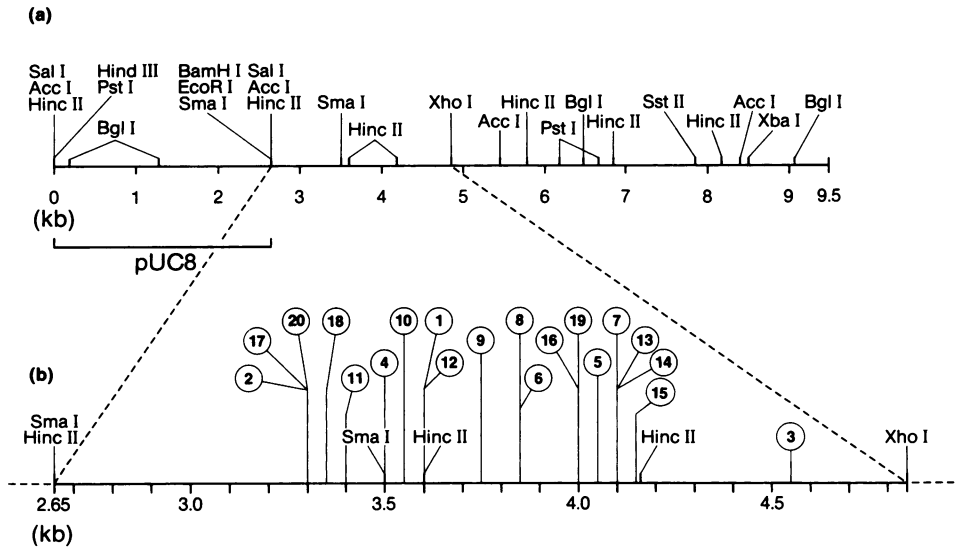

FIG. 2. Restriction endonuclease maps of pDT1364 (a) and the Te^r region of pDT1364 (b), showing the sites in which transposon Tn*1000* was inserted to form Te^s mutants.

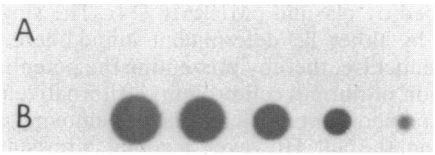

FIG. 3. DNA-DNA hybridization between Te^r regions of pDT1558 and pDT1364. Fourfold dilutions of DNA fragments carrying the IncPα (A) or IncHII (B) Te^r region were hybridized with the ^{32}P-labeled Te^r region from pDT1364.

1.75-kb region which is close to the 1.9-kb size of this Te^r determinant estimated by Taylor and Bradley (24).

The MICs of potassium tellurite for *E. coli* strains carrying the insertion mutants of pDT1364 were all between 0.25 and 1 μg/ml (Table 1), which is about the same as the MIC for the plasmid-free strains of *E. coli* and is much lower than that of *E. coli*(pDT1364), which was about 128 μg/ml (27).

The MIC for most of the Tn*1000* insertion mutants of pDT1558 was 0.125 μg/ml (Table 1), which is slightly lower than that for plasmid-free *E. coli*. An exception is insertion mutant pDT1558-4, which is located near the rightmost end of the region believed to be involved in Te^r. This mutant had an MIC of 8 μg/ml, which is intermediate between the MIC of *E. coli*(pDT1558) (256 μg/ml) and that of plasmid-free *E. coli*. In comparison, the MIC of *E. coli* strains carrying either of the original plasmids pHH1508a and RP4Te^r was 512 μg/ml (27).

Lack of DNA homology between Te^r determinants. Equal amounts of DNA fragments specifying Te^r from pDT1558 and pDT1364 were denatured, applied to nitrocellulose, and hybridized with the Te^r determinant from pDT1364 (Fig. 3). No homology between the Te^r determinants from the IncPα and IncHII plasmids could be detected, suggesting that the two determinants are not closely related.

Plasmid-determined proteins. Both pDT1558 (Fig. 4A) and pDT1366 (data not shown) were found to encode a single 40-kilodalton (kDa) polypeptide which was not expressed by pUC8. Coincidentally, transposon Tn*1000* also produces a protein of about 40 kDa, as well as several other proteins (25). Because of this transposon-encoded protein, it was not possible to determine whether insertion mutant pDT1558-4, which expressed an intermediate level of Te^r, still expressed the 40-kDa Te^r protein. No unique polypeptides were expressed by either pDT1558-4 or pDT1558-1 in comparison with other insertion mutants (data not shown). However, Te^s Tn*1000* insertion mutants pDT1558-2 and pDT1558-3 also produced proteins of 37 and 32 kDa, respectively (Fig. 4A). These polypeptides may be truncated forms of the 40-kDa protein formed by insertion of a stop codon coded by Tn*1000* into the reading frame of the Te^r protein.

pDT1364 encoded two polypeptides of 23 and 12 kDa which were not expressed by pUC8 (Fig. 4B). All of the Te^s Tn*1000* insertion mutants of pDT1364 were examined by this system, and the proteins expressed by three of these are shown in Fig. 4(B). Many of the Tn*1000* insertion mutants did not express the 23-kDa polypeptide. One Tn*1000* insertion mutant, pDT1364-1, did not express the 23-kDa polypeptide but instead expressed a 9.5-kDa polypeptide. This polypeptide may be a truncated mutant of the 23-kDa protein. No unique polypeptides could be detected in the other insertion mutants. None of the mutants showed any change in the 12-kDa polypeptide, suggesting that this protein is not involved in Te^r.

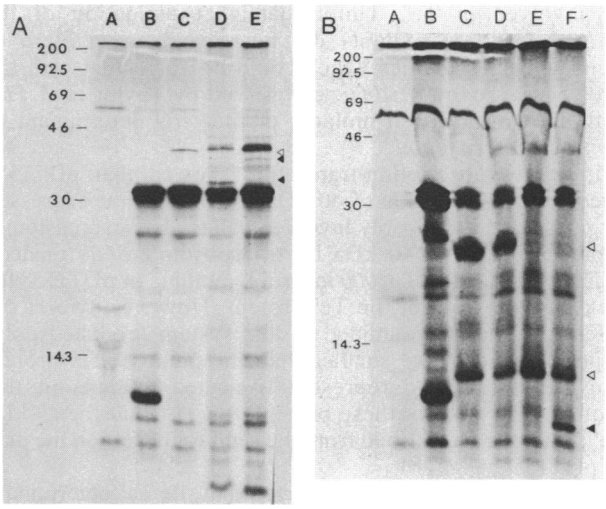

FIG. 4. Proteins expressed by Te^r clones and Te^s derivatives in an in vitro transcription-translation system. (A) Proteins of the RP4 Te^r determinant. Lanes: A, no DNA added; B, pUC8; C, pDT1558; D, pDT1558-3; E, pDT1558-2. The open triangle indicates the 40-kDa polypeptide expressed by pDT1558 but not by pUC8. The closed triangles indicate the positions of the altered proteins expressed by the two Tn*1000* insertion mutants. (B) Proteins encoded by the pHH1508a Te^r determinant. Lanes: A, no DNA added; B, pUC8; C, pDT1364; D, pDT1364-17; E, pDT1364-6; F, pDT1364-1. Open triangles indicate the 23- and 12-kDa polypeptides expressed by pDT1364 but not by pUC8. The closed triangle indicates the altered protein expressed by Tn*1000* mutant pDT1364-1. The molecular masses and positions of the standard proteins are indicated to the left (myosin, 200 kDa; phosphorylase *b*, 92.5 kDa; bovine serum albumin, 69 kDa; ovalbumin, 46 kDa; carbonic anhydrase, 30 kDa; lysozyme, 14.3 kDa).

DISCUSSION

This study reports the cloning and comparison of Te^r determinants from IncHII plasmid pHH1508a and IncPα plasmid RP4. In contrast to the Te^r determinant of pMER610 (14), the expression of which is induced by prior exposure to low levels of potassium tellurite, the Te^r determinants of pHH1508a and RP4Te^r were expressed constitutively.

The restriction endonuclease maps of the Te^r clones pDT1364 and pDT1558, derived from pHH1508a and RP4Te^r, respectively, showed no similarity to each other or to that of the Te^r determinant of pMER610 (14). In addition, no homology was detected between the Te^r determinants of pDT1364 and pDT1558 by DNA-DNA hybridization under conditions of low stringency, indicating that the two determinants are not closely related.

By using Tn*1000* insertion mutagenesis, the Te^r determinant on pDT1364 was localized to a minimal 1.25-kb region. The Te^r determinant of RP4Te^r spans a minimum of 1.75 kb of DNA, and the Te^r determinant of pMER610 spans a minimum of 3.55 kb (14). These different size estimates for the Te^r determinants on these three plasmids suggested differences in the sizes of the proteins that could be produced. This was confirmed by in vitro transcription-translation. pDT1364 expressed two polypeptides of 23 and 12 kDa which were not encoded by pUC8. The absence of the 23-kDa polypeptide in some of the Te^s Tn*1000* insertion mutants of pDT1364 suggests that this protein is involved in Te^r. Since none of the Tn*1000* insertion mutants showed any change in the 12-kDa polypeptide, this polypeptide may not

be involved in Te^r. Unfortunately, examination of the Tn*1000* insertion mutants did not reveal any pattern of truncated polypeptides which could be correlated with the site of insertion of Tn*1000* as observed by Taylor et al. (25) with Tn*1000* insertion mutants of the *tetO* determinant of *Campylobacter jejuni*.

In vitro transcription-translation showed that pDT1558 encoded a polypeptide of 40 kDa which was not expressed by pUC8 and is probably involved in Te^r. The expression of a protein of about 40 kDa by transposon Tn*1000* made it difficult to examine Tn*1000* insertion mutants of pDT1558 for lack of expression of the Te^r protein. However, two of the insertion mutants examined in this system produced polypeptides which were smaller than the putative Te^r protein and therefore could represent truncated mutants of this protein. The sizes of these proteins suggest that the 40-kDa Te^r protein is transcribed from the right to the left on the map of pDT1558 (Fig. 2b).

Jobling and Ritchie (14) reported that the Te^r determinant of the IncHI-2 plasmid encodes four polypeptides of 15.5, 22, 23, and 41 kDa. Tn*1000* insertions into the Te^r region of pMER610 prepared by Jobling and Ritchie (14) could be divided into two groups: those that caused complete loss of Te^r, probably because of a structural mutation, and those which resulted in only partial loss of Te^r, possibly because of a mutation in the regulatory region. In this study, Tn*1000* insertions into the Te^r region of pDT1364 all resulted in a lowering of the MIC of potassium tellurite down to the level of plasmid-free *E. coli*. This is consistent with the presence of a single structural gene and no regulatory regions. In contrast, while most Tn*1000* insertions into pDT1558 resulted in a slightly lower level of resistance than that of plasmid-free *E. coli*, one mutant had an intermediate level of resistance. This may indicate that Tn*1000* was inserted into the promoter region of the Te^r gene, resulting in a lower level of gene expression and therefore a lower level of resistance. Alternatively, Tn*1000* could have been inserted at the end of the gene, creating a truncated protein with partial activity. Expression of this insertion mutant in the in vitro transcription-translation system was not able to distinguish between these possibilities. With either determinant, we were unable to find regions in which Tn*1000* insertions caused significant hypersensitivity to tellurite. This might be expected if a tellurite transport system was encoded.

The levels of Te^r encoded by clones pDT1364 and pDT1558 were lower than those of the parent plasmids. This may have been due to a toxic effect of expression of the Te^r determinants on a high-copy-number plasmid, as found with a tetracycline resistance determinant (18).

The initial isolation of pDT1366 suggested that the *korA* and *korB* genes of RP4 play a role in the expression of Te^r, but the absence of the *korA* and *korB* genes on pDT1558 indicates that these genes are not required for expression of this Te^r determinant. The relationship of Te^r to these plasmid replication control genes and the reason why they all appear to be encoded together on a transposon (7) are unclear.

Earlier studies on Te^r specified by plasmids of the H incompatibility group found an association between resistance to tellurium compounds and the ability to inhibit the development of coliphages, such as bacteriophage λ (26). The presence of Te^r clone pDT1364 did not result in phage inhibition (Phi) (unpublished data), which suggests that the Te^r and Phi phenotypes are not functionally associated.

Our findings suggest that the mechanisms of Te^r specified by plasmids RP4Te^r and pHH1508a are much simpler than that encoded by plasmid pMER610 (14). The single protein expressed by either Te^r determinant studied here may function as a reductase, thereby preventing the potentially harmful oxidation of normal cell proteins. Alternatively, it could act as a transport protein capable of removing potassium tellurite from the cell. However, a complex resistance mechanism resembling plasmid-mediated mercury resistance would require a larger number of proteins, such as those found in the pMER610 Te^r system.

The existence of at least three different types of plasmid-encoded Te^r determinants suggests that this resistance is important to gram-negative bacteria in the environment or possibly in the colonization of the human body, in which tellurium compounds appear to be relatively abundant (21). It is well known that gram-positive bacteria, such as *Corynebacterium diphtheriae*, *Streptococcus faecalis*, and *Staphylococcus aureus* are commonly resistant to tellurium compounds, but it is not known whether this resistance is chromosome encoded or plasmid encoded or whether the mechanism of resistance of these bacteria is related to those found in gram-negative bacteria.

ACKNOWLEDGMENTS

This work was supported by a grant from the Medical Research Council of Canada. E.G.W. is the recipient of a Medical Research Council studentship. D.E.T. is a Heritage Medical Scholar.

We thank D. H. Figurski for pRK2102, W. Yan for pDT1178, and B. Walter and R. Sherburne for photographic assistance.

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