The mercury resistance operon of the IncJ plasmid pMERPH exhibits structural and regulatory divergence from other Gram-negative *mer* operons

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The bacterial mercury resistance determinant carried on the IncJ plasmid pMERPH has been characterized further by DNA sequence analysis. From the sequence of a 4097 bp *Bgl*II fragment which confers mercury resistance, it is predicted that the determinant consists of the genes *merT*, *merP*, *merC* and *merA*. The level of DNA sequence similarity between these genes and those of the *mer* determinant of Tn21 was between 56.4 and 62.4%. A neighbour-joining phylogenetic tree of *merA* gene sequences was constructed which suggested that pMERPH bears the most divergent Gram-negative *mer* determinant characterized to date. Although the determinant from pMERPH has been shown to be inducible, no regulatory genes have been found within the *Bgl*II fragment and it is suggested that a regulatory gene may be located elsewhere on the plasmid. The cloned determinant has been shown to express mercury resistance constitutively. Analysis of the pMERPH *mer* operator/promoter (O/P) region *in vivo* has shown constitutive expression from the *mer* P_{TCPA} promoter, which could be partially repressed by the presence of a *trans*-acting MerR protein from a Tn21-like *mer* determinant. This incomplete repression of *mer* P_{TCPA} promoter activity may be due to the presence of an extra base between the –35 and –10 sequences of the promoter and/or to variation in the MerR binding sites in the O/P region. Expression from the partially repressed *mer* P_{TCPA} promoter could be restored by the addition of inducing levels of Hg²⁺ ions. Using the polymerase chain reaction with primers designed to amplify regions in the *merP* and *merA* genes, 1.37 kb pMERPH-like sequences have been amplified from the IncJ plasmid R391, the environmental isolate SE2 and from DNA isolated directly from non-cultivated bacteria in River Mersey sediment. This suggests that pMERPH-like sequences, although rare, are nevertheless persistent in natural environments.

Keywords: pMERPH, *mer* operon, mercury resistance in bacteria

INTRODUCTION

Bacterial resistance to mercury (Hg^R) is widespread in clinical and environmental isolates, in both Gram-negative and Gram-positive bacteria, and has a global dis-

tribution (Summers, 1986; Foster, 1987). Resistance, often borne on plasmids and/or transposons, is encoded by the genes of the *mer* operon, of which the best studied are those borne on the transposons Tn501 and Tn21. Resistance to inorganic mercury (narrow spectrum resistance) is mediated by a NADPH-dependent reduction of inorganic Hg²⁺ ions to the less toxic Hg⁰ which volatilizes from the cell surface. This reaction is catalysed by mercuric reductase, the product of the *merA* gene. In addition to *merA*, the *mer* operon consists of two or three genes encoding transport proteins (*merT*, *merP* and *merC*) and two regulatory genes (*merR* and *merD*). Broad

Abbreviations: CAT, chloramphenicol acetyltransferase; O/P, operator/promoter.

The EMBL accession numbers for the nucleotide sequence of the pMERPH operon and of the pHG106 *merA* region reported in this paper are Z49146 and Z49200, respectively.

Table 1. Plasmids and constructs

Plasmid	Relevant phenotype*	Construct	Source
pBluescript KS ⁺	Ap ^R	—	Stratagene
pACYC184	Cm ^R Tc ^R	—	Chang & Cohen (1978)
pKK232.8	Ap ^R	—	Brosius (1984)
pHG106	Ap ^R Tc ^R Hg ^R	4.2 kb <i>Hind</i> III broad spectrum <i>mer</i> from pDU1358 into pBR322	Griffin <i>et al.</i> (1987)
pMERPH	Hg ^R	—	Peters <i>et al.</i> (1991)
R391	Km ^R Hg ^R	—	Coetzee <i>et al.</i> (1972)
pSP100	Cm ^R Hg ^R	4.1 kb <i>Bgl</i> II pMERPH <i>mer</i> into pLV59	Peters <i>et al.</i> (1991)
pSE20R1	Ap ^R Hg ^S	0.52 kb <i>merR</i> O/P PCR product from SE20 into KS ⁺	Osborn <i>et al.</i> (1995)
pPH101	Ap ^R Hg ^S	<i>Bgl</i> II 4.1 kb from pSP100 into KS ⁺	This study
pPH102		<i>Hinc</i> II-A 0.77 kb from pPH101 into KS ⁺	
pPH103		<i>Hinc</i> II-D 0.63 kb from pPH101 into KS ⁺	
pPH104		<i>Hinc</i> II-B 0.38 kb from pPH101 into KS ⁺	
pPH105		<i>Hinc</i> II-C 1.0 kb from pPH101 into KS ⁺	
pPH106		<i>Hinc</i> II-E 1.32 kb from pPH101 into KS ⁺	
pPH107		<i>Sst</i> I/ <i>Hind</i> III 0.27 kb from pPH102 into KS ⁺	
pPH108		<i>Hind</i> III/ <i>Hinc</i> II 0.33 kb from pPH102 into KS ⁺	
pPH109		<i>Hind</i> III 0.18 kb from pPH102 into KS ⁺	
pPH110		<i>Kpn</i> I/ <i>Cla</i> I 0.5 kb from pPH105 into KS ⁺	
pPH111		<i>Cla</i> I 0.5 kb from pPH105 into KS ⁺	
pPH112		Blunted <i>Hinc</i> II/ <i>Sst</i> I from pPH102 into <i>Sma</i> I pKK232.8	
pPH113		As pPH112 but in opposite orientation	
pACSE20R	Tc ^R Cm ^S Hg ^S	<i>Pvu</i> II 1 kb from pSE20R1 into pACYC184	

* Ap, ampicillin; Cm, chloramphenicol; Hg, mercuric ion; Km, kanamycin; Tc, tetracycline; R, resistant; S, sensitive.

spectrum resistance to organomercurials in addition to Hg²⁺ is conferred by the product of the *merB* gene which cleaves the organo group from the mercuric ion, prior to reduction of Hg²⁺ by MerA (for a review, see Misra, 1992). Expression of the *mer* operon is inducible and is controlled by MerR, which binds to the *mer* operator/promoter (O/P). In the absence of Hg²⁺ ions MerR represses expression. On addition of Hg²⁺ ions, the MerR-*mer* O/P complex undergoes a conformational change which allows RNA polymerase to transcribe the structural genes (for a review, see Summers, 1992).

In Gram-negative bacteria a distinct subset of closely

related *mer* determinants (represented by Tn501, Tn21, pKLH2 and pMER419) has been identified and is commonly found in bacteria from natural environments and in clinical isolates from around the globe (Osborn *et al.*, 1995). Peters *et al.* (1991) identified novel mercury resistance determinants from the conjugative IncJ plasmids pMERPH and R391, which were believed to be integrated into the chromosome. Both determinants confer narrow spectrum mercury resistance and detoxify HgCl₂ by reduction and subsequent volatilization. Maxicell polypeptide analysis identified four polypeptides of which three were of similar size to the MerP, MerC and MerA polypeptides of Tn21. However, the pMERPH and

R391 *mer* determinants failed to hybridize to the Gram-negative *mer* determinants of Tn501 and Tn21 at a stringency which detected DNA homologies of greater than 70%. In addition, these *mer* determinants, although inducible, did not *trans*-complement MerR mutants of Tn21 (Foster & Ginnity, 1985). Initial studies into the frequency of pMERPH-like sequences in natural environments suggests they are relatively rare when compared with those of the Tn501/Tn21/pKLH2/pMER419 subgroup (Peters *et al.*, 1991).

The aims of the present study were threefold: firstly, to determine the DNA sequence of the *mer* determinant of pMERPH to allow comparison with the other *mer* determinants sequenced to date; secondly, to investigate the regulation of the pMERPH *mer* operon; and finally, to attempt to identify using PCR other pMERPH-like sequences in the natural environment.

METHODS

Bacterial strains, plasmids and oligonucleotides. *Escherichia coli* strains used were AB1157 (Sm^R F⁻ *thi-1 thr-1 leu-6 proA2 argE3 his-4 rpsL31 supE44 galK2 lacY1 ara-14 xyl-5 mtl-1 tsx-33*) and XL-1-Blue (*supE44 hsdR17 recA1 endA1 gyrA96 thi-1 relA1 lac* {F'*[proAB⁺ lacI^q lacZΔM15 Tn10 (tet^R)]*}). XL-1-Blue was used as the host strain for all DNA manipulations, except for the construction of pACSE20R. AB1157 was used as the host strain in the promoter probe experiments and as the host for the conjugative plasmids pMERPH and R391. The environmental strain SE2 (Hg^R) was isolated from a sediment sample taken from Fiddlers Ferry, River Mersey (Osborn *et al.*, 1993). Plasmids (Table 1) were propagated in *E. coli*. Oligonucleotides used in this study are listed in Table 2.

Cloning and sequencing. DNA fragments for sequencing were subcloned into pBluescript KS⁺ using standard protocols (Sambrook *et al.*, 1989). Template DNA was prepared by alkaline lysis (Birnboim & Doly, 1979) and further purified by caesium chloride density gradient centrifugation. pMERPH subclones were sequenced on both strands using Sequenase 2.0 (USB) with the M13 -20 and Reverse (RP) primers together with a number of custom-synthesized primers (Table 2). The pHG106 *merA* region was sequenced on both strands using an ABI 373A DNA automated sequencer with the primers 1358E3 and 1358E5.

The promoter probe clones pPH112 and pPH113 were constructed by ligation of a 0.7 kb blunt-ended *HincII/SstI* fragment from pPH102 bearing the pMERPH *mer* O/P region into the promoter probe vector pKK232.8 in both orientations. The *merR* and *mer* O/P region of the Tn21-like Hg^R determinant from SE20 was subcloned from pSE20R1 (Osborn *et al.*, 1995) into the *PvuII* site of pACYC184 and insert-bearing clones identified by the insertional inactivation of the chloramphenicol resistance gene.

Sequence and numerical analysis. The pMERPH *mer* sequence was analysed using the UWGCG suite of sequence analysis programs (Devereux *et al.*, 1984). Evolutionary relationships between *merA* gene sequences were constructed using the methods described by Osborn *et al.* (1995).

Chloramphenicol acetyltransferase (CAT) assays. Crude protein extracts were made by Brij lysis (Forster, 1984) prior to assaying for CAT activity by direct measurement of the production of [¹⁴C]acetyl chloramphenicol (Shaw, 1975). Briefly, 0.5 ml of an overnight culture was added to 50 ml Luria Broth (LB) and shaken at 37 °C at 200 r.p.m. for 30 min. HgCl₂ was added to a final concentration of 2 µM for induction experiments and then the cultures were grown for a further 30 min. Cells from 10 ml of culture were harvested by centri-

Table 2. Oligonucleotides

Oligonucleotide	Sequence
M13 reverse primer	5'-AAC AGC TAT GAC CAT G-3'
M13 -20 primer	5'-GTA AAA CGA CGG CCA GT-3'
102A	5'-CAA TAG CGG CAA TAA CAC-3'
103A	5'-TAC CCG AGC AAG GGA TCT-3'
103B	5'-CTG CTC GGT TCC ATC AT-3'
103C	5'-TCC ACG CCA ACC ATT CCT-3'
103D	5'-GTT GTG CTG GGT AAC GA-3'
104A	5'-GCC TGA ACG CTT TAG CT-3'
105A	5'-GTT TGG TTG CTT TCA GTG-3'
105B	5'-TGT TTG CCT GGA TTG CCT-3'
106A	5'-ACC GCC CGT GCA CTT TGA-3'
106B	5'-AAG AGC AAG CCA ACG CA-3'
106C	5'-CTT CTT AGG CTT AAG CG-3'
106D	5'-GTT GTG CTG GGT AAC GA-3'
106E	5'-CTC CAC CAT AGT CAG GT-3'
106F	5'-GTG GTG ATG GTT ATA AG-3'
110A	5'-GAC TGC TGT GAC AAC GA-3'
111A	5'-CGT TGA GTA CCA AGG CA-3'
1358E3	5'-AGC GTG CGA CTG TCG GTT-3'
1358E5	5'-TGC CGA AGG GAT CAA GGT-3'

fugation at 10000 r.p.m. for 3 min, and the resultant pellet was resuspended in 0.4 ml 25% sucrose/0.01 M Tris, pH 8.0. EDTA (30 µl, 0.1 M, pH 7.8) and lysozyme (40 µl, 1 mg ml⁻¹ in 0.01 M Tris, pH 8.0) were added, the vessel contents mixed by inversion and left on ice for 2 min. MgSO₄ was added to a final concentration of 50 mM together with 100 µl 5% Brij-58, the vessel contents mixed by inversion and left on ice for 1 h. Cell debris was pelleted by microcentrifugation at 10000 r.p.m. for 15 min. CAT activity was assayed after incubation of 20 µl crude protein extract with 40 µl 0.25 M Tris, pH 8.0, 20 µl 8 mM chloramphenicol and 20 µl acetyl-CoA mixture (18 µl 0.5 mM acetyl-CoA and 2 µl [¹⁴C]acetyl-CoA (3.7 kBq) at 37 °C for 1 h. Ethyl acetate (0.8 ml) was added, and the mixture briefly vortexed prior to microcentrifugation at 13000 r.p.m. for 15 s. A portion (0.7 ml) of the upper organic layer was then added to 4 ml Econofluor scintillation fluid (NEN) and assayed for the presence of ¹⁴C-labelled chloramphenicol 3-acetate in an LKB Rackbeta scintillation counter, compared against reagent blanks. CAT activity was calculated as the mean value from at least three experiments.

PCR amplification and hybridization analysis of *mer* regions.

Template DNA from Hg^R bacteria was prepared as described previously (Osborn *et al.*, 1993). DNA isolated directly from soil was prepared by the method of Bruce *et al.* (1992). PCR products were amplified using *Taq* DNA polymerase (2.5 U) with the primers 104A and 103B. Reactions were performed for either 30 cycles (94 °C for 1 min, 48 °C for 1 min, 72 °C for 2 min) for crude bacterial extract or 35 cycles for purified soil DNA, followed by 1 cycle of 72 °C for 10 min. Primer, dNTP (dATP, dCTP, dGTP and dTTP) and MgCl₂ concentrations were 30 pmol, 50 µM and 1.5 mM, respectively. PCR products were visualized following electrophoresis on 0.7% agarose TBE gels containing ethidium bromide (1 µg ml⁻¹). Following Southern transfer, hybridization with the 4.1 kb *Bgl*II *mer* fragment from pSP100 was performed as described previously (Osborn *et al.*, 1993) at a stringency to detect DNA homologies greater than 70%. Membranes were exposed to Fuji RX film for 20–60 min at –70 °C.

RESULTS

Sequence and evolutionary analysis of the structural *mer* genes of pMERPH

Analysis of the DNA sequence of the 4097 bp *Bgl*II fragment from pMERPH identified four putative *mer* genes (*merT*, *merP*, *merC* and *merA*) on the basis of homology to published *mer* sequences (Fig. 1). The predicted proteins encoded by these genes were of similar size to those of the Tn21 *mer* determinant. However the predicted protein sequences differed significantly from those of Tn21, for example 57.8% identity and 71.5% similarity for MerA, and 49.1% identity and 67.5% similarity for MerT. Analysis of the predicted sequence of the MerA protein of pMERPH identified an additional eight cysteine residues when compared against those of Tn21 and Tn501 (Fig. 1), whilst the other eight cysteine residues were conserved with those of Tn21. No such increase was found in the number of cysteine residues in the predicted sequences of MerT, MerP or MerC of pMERPH. Furthermore the positions of the cysteine residues in these proteins was conserved between Tn21 and pMERPH. The MerA protein of pMERPH was most closely related to that from the *Thiobacillus ferrooxidans*

Hg^R determinant (Inoue *et al.*, 1989) (59.1% identity, 74.6% similarity), whilst the MerA proteins of the Gram-positive Hg^R determinants were the most distantly related to that of pMERPH, for example p1258 showed 41.6% identity and 62.9% similarity (Laddaga *et al.*, 1987). A neighbour-joining (Saitou & Nei, 1987) phylogenetic tree of *merA* gene sequences was constructed (Fig. 2). The previously incomplete sequence of the *merA* gene of pDU1358 (Griffin *et al.*, 1987; Nucifora *et al.*, 1989) was completed by sequencing a 300 bp region, to enable the pDU1358 *merA* gene to be included in the phylogenetic analysis. The robustness of the tree was confirmed by bootstrap analysis and in addition the tree topology was consistent with that generated using the method of Fitch & Margoliash (1967) (data not shown). This *merA* dendrogram suggests that the pMERPH *merA* gene is approximately equally related to that of the *T. ferrooxidans* Hg^R determinant (Inoue *et al.*, 1989) and to the subgroup of closely related Gram-negative *mer* determinants Tn21 (Barrineau *et al.*, 1984), Tn501 (Brown *et al.*, 1983), pDU1358 (Griffin *et al.*, 1987), pKLH2 (Kholodii *et al.*, 1993) and pMER419 (Hobman *et al.*, 1994).

Regulation of the pMERPH *mer* operon

No putative regulatory genes (*merR* or *merD*) were identified on analysis of the 4097 bp *Bgl*II fragment from pMERPH, and an inducible response to sub-toxic levels of HgCl₂ is not shown by the cloned determinant (data not shown). Sequence analysis of the O/P region of pMERPH identified a number of differences from the *mer* regions of other Gram-negative *mer* determinants. –35 and –10 sequences for the *mer* P_{TCPA} promoter were found (Fig. 1), but there were no such sequences for the divergently transcribed regulatory gene (*merR*) which is found in all other Gram-negative plasmid-borne *mer* operons. Moreover the spacer region between the –35 and –10 sequences for the P_{TCPA} promoter was 1 bp longer than that found in other Gram-negative *mer* determinants such as Tn21, and only a 5 bp dyad symmetry (CCGTA–6 bp–TACGG) was found between the –35 and –10 sequences of the P_{TCPA} promoter (Fig. 1) as opposed to a 6 or 7 bp sequence in other determinants. Variation was also found in the MerR binding region (GTAG–4 bp–GTAC in pMERPH as opposed to GTAC–4 bp–G/CTAC in all other *mer* determinants). Promoter activity in both directions from the pMERPH *mer* O/P region was investigated using the promoter probe constructs pPH112 and pPH113 (Fig. 3). Analysis of promoter activity by CAT assays showed that CAT activity increased significantly only when the pMERPH *mer* O/P region was cloned such that the CAT gene was expressed from the P_{TCPA} promoter (pPH113). Negligible expression was observed when this region was cloned in the opposite orientation (pPH112) (data not shown). Furthermore, addition of inducing levels of HgCl₂ had no effect on CAT expression in strains containing either construct. Regulation of the pMERPH *mer* O/P region was further investigated by the introduction of a trans-acting MerR protein, produced by the plasmid pACSE20R, into *E. coli* AB1157(pPH113). The presence

1 GATCTGCTGTTGGAAGCGCGGACATTAAACACAGAAGATTGCCGGGATTAAGAAGCTTGCGCCACACGGTGCGCAAGT

81 GTTGGTGCTCAACTTTGGAAGCGGTTGCTCGACGACCAACATCATCAAGAAGAATCAACACAGGATAAAAAGCGTTAACCGG

161 AAAGCGGTTACAGAGATTTGGGTTCACTTTGGAAGAATCTCCAAATTACTACGCCCTTAATGACACTCCCTGTATAGAAG

241 TGCAGAAATGACTTTACACAGCTTGCAGTGTGGAAGCGCAAAATAGCTAGCTTTTCCGCCGTAGAAAGCGGTTCTACAA

321 GAATTATGTAATGAGTGTATCTTAATACTAACCAATCCCACTGCCATTATGATTCTCCACTCTCTCGAAGATTAAATA

401 ATGAGTCAAAATTTTATCTTTACTCTCCGTATGGGTACGCGGTTTAAAGCTGTTTACTTAAAGTACGATAAGTAGAGTAG

481 TTATGTCAAAAAGTAATCCCAACTTTGCAATCATCGCGGTGTTATTGCGCGTATTGCGCGTGCTTTGCTGCGCTGCTGT

Tn21
.....catatcgcttgatcgctacatgtagc-ggaagtaaggttatcgctatccaatccaattcaaaaggcgca
.....gatatagcgaactgaggcatgtactcatc-ccttcattcccaatgcgataggttaggtttaagtttttcccggt
←Tn21 merL -10 merR -35 merR

pMERPH merT-
M S K S N P N F P I I G G V I A A I G A G L C C A G
481 TTATGTCAAAAAGTAATCCCAACTTTGCAATCATCGCGGTGTTATTGCGCGTATTGCGCGTGCTTTGCTGCGCTGCTGT

Tn21 merT-
M
agttagt.....
tcaatc.....

F V L L L L L L G V S G S W I G N I L T L L E P Y R P I F
561 CCTTCTGCTTGTGCTGCTAGGCGTGAGTGCTCTGGAATTGGTAATTTAACTCTGTTAGAACCTTATGCTGCCAATTTT
I L L V L L L L G F G A G W K V I R P V E D C E P G F S
641 TATCTCGTGCTGTTTAACTGCTTTATGCTGCTCCAGCGTGGAAAGCTTATGCGCCCTCGAGGACTGTGAGCCAGCGACT
C A V P Q V R K R R Q V I F W L T A L T A L V L V T
721 CCTGTGACGTCTCCAAGTCCGAAACCTCGGAGGTGATCTTTTGGTATGACGCACTGACTGCGTGGTTTTAACTACCC
S N Y I W I V F A *
801 AGTAATTTAGGATTGCTGGTTCGCTGATGACGATTCTCAACTCTTTATTTGATTAAATCTTATAGCTTGCTCTTG

M K T L A L M S L F V L T S L N A L A A A A A A G T
881 GAGAAATTTAGAAACACTGCCCTTAATGTCTGCTTGTGCTAGCAGCACTGAACGCTTTAGCTGCCCGCCAAKACAGT
T L E V P T M N C V T C P F T V E K A L Q K V D G V S
961 TACTTTGGAAGTCCCAACCTGAATCTGACTTGCTGCTTCCCTTACCGTGAAAGAACCTTACAAAAAGTGAAGCGGTCA
K A E V T F K T K L A V I V T F D E K S T V K A L T
1041 GTAAAGCGAAGTGACCTTTAAGACCAAACTGGCGGTGTCACCTTCGACGACGAAAAATCCACGCTTAAAGCACTGACC

merC→
E A T T N A G Y P S T L K E * M L I R L L L T R F
1121 GAAGCCACCACTAACCGGGGTTATGCTCAAGCGCTCAAGGACAAAGCATAGAAATGCTAATAGCTGATGACCGCGGTT
G D K I S S L G A L V S A M G C V M C F P A I A S L G
1201 CGGGATAGATTAGCTCATTTGGCGCGCTGGTTTCTGCTAGGATGCGCTATGTTTTCGCCGCCATTGCGACGTGCG
A A V G L G L S T Q W E G L F V S T S L L P L F W A I
1281 CGGCTCGCTAGGACTGGGTTTCTACGCAATGGGAAGCTGTGTTTATGACAGCACTTATAGCCCTTGTTCGGCTGATT
L L L N L N G L G W F S H R Q W H R S A L G V A G I P I
1361 GCCTTGCTACTCAACGACTTGGCTGTTTACGCAACGGCAATGGCAAGCTAGTGAAGCTGGGTGTGGCGCGCCCAATTT
L L L S L Y P F F F Q Y G W S S Y V T Y S A L G L M V A
1441 ATTGCTGTATGCTCTATCGGTTTTCAGTAGCGGCTGAGGACAGCTATGTACCTTATACGACTAGGCTGTAGTGCTG
V S L W D I F S P A N K R C D D D S C A V *
1521 CCGTATGCTTTGGGATATTTTTCACCGCGCAATAAAGCGCTGTGATGATGACAGCTGTGCTGTTTACACAGCGCAATG

merA→
M K L S I E I G T T P S S V A H V K
1601 CCTGACTACTTTTGAGGAAACGAATGATTTACTATGCTAGTAGAGGATGACTTCTCAAGTTCGCTGCCACCGTTA
E A L D A I E G V N K V E I S Y E N A R A T I T T N
1681 AAGAAAGCACTCGATCGGATGAGCGGTTTAATAGAGTGAAATATCTTATGAAACCGGACGACAACTGCTACCGAAG
G V S V T V L I G A I E A L G I A E S T G T A K
1761 GTGGGGTACGCGTAACGCTTCTATTGAGCAATAGAGCTCTCGGTTATATCGAAAGAAATCCATGGCACTGCAAA
E I T S P N D C C D N E N A S N T E S N C T Q H V A I
1841 AGAAATTTCTACCAAGCACTGCTGTGACCAAGAAATAGCAAGCACTGAAGCAACAACTCAACATGTGGCA
I G T S G A F A C A I K A A E G C A K V T L I E G
1921 TTATGCTAGCGGTTTGGCGGCTTTTCCGTGCGCTATTAAGACCGCTGAAGTGCTGCAAGCTCACTTTATTAGGGA
A D V I G C G G V N V G G V P S K I L I R A Q L A Q
2001 CGCGATGCTATGCGCGCTTCTGCTGGAATGCGGCTGCTGACCCCTAAAAATTTAATCGCGCGGCTCAATGTGGCTCA
Q Q R N N P F T G A G G A G G L Q S R A L L T Q Q Q
2081 CGACGAAACGATAATCTCCTTTACCGAGCTGGAATAATCGGCCCAATTTGAGCTGAGCCTTTGTGACTAGCAGAGA
A R V E E L A A A K Y O N I L E T N P A L L L K G
2161 CGCGCTCGGTAAGAGCTGCGCGCGGCAAAATGACAGAAATTTCTGAGACAAATCTTCGCGCTTAGTTTACTTAAAGGT
W A Q F K N A L L T V R A K N D G T E A G Q V A H D K I
2241 TGCGCGCAATTAAAAAGCAACACTCTGATGTAGCAAAAATGTGAACCAAGCAGGAGCACTACGCGGATAAAAT
L I A T G S T P T I F P I D G L T E T F Y W T S T E A
2321 CCTGATGCTACCGGCTCAAGCGCAACATCTCTCTTATGATGTTTACTGAAAGCACTTATTTGGACCTTACGGAAG
L F A Q G G T Q P H L V I V G S S V A A E I A Q A Y
2401 CCGTGTTTGCTCAGAAATTCGCCGAGCACTGGTGTGATGTGTTGTGCGGTTTAGCGCTGGAATATGCGGAGCTTAT
R R L G S E V T I L A R K T L L Y R D E D P L L G E K A L
2481 CGCGCTTAGGACGTGAAGTACCAATATAGCAAGACTATCTGTTTCTGCGGAAGACTCCCTTCTGTGGAACCACT
T G C F E K E G I R V L N S T Q A T C T V T H D G S Q F
2561 CACCGACTGTTTGAAGAAGAAGCACTTCAAGTATTAACAGTACGCAAGCTACCAAGGTACCCACAGGTGAAGCGCAAT

T L E T N A G D L R C D R L L V S T G R H A N T E Q
2641 TTACATTTGAAACTAGCGGGGATATAGCTGCAGCTGTTTCTGTGTAGAACCGCGGCGGACCGCAACTATTTGCCAA
L N L G A G G V G T T N N K K E I V V N E R N E M T P
2721 CTCATCTCGGCGGCTGGTGTATGACGACCAAAAGGCGGCAATCTGTGTTAAGCAACGATGGAAGAAGATGTGTC
G I Y A A G C G C N H P Q V V A A A G S R S G I
2801 CGGTATTATACCGCGCGGAGCTGTCGAACATGCCGCAATCTGTCTATGTGCGGCGCGCGGAGTACGCTTTCGCGCA
N M T G G Y A K L D S T M P A V I F T D P Q V A T
2881 TCAACATGACGGGGATCGCCCAACTGATCTATCCACATGCGCAAGATTTTACCAGCTCCCGAGGTGTGCAACT
V G L T E Q A N A Q D I E T S R V L E N V P R
2961 GTTGGGCTACCGGAAGCAAGCCACCAAGCAATGAACACGACAGCTGTGTATGAGATGAGAGACGTGCCCGG
A L A N F E T D G F I K L V T E K A T G R L I G A Q I
3041 CGCACTGGCAATTTGCAAGCCGATGTTTATCAAAATGTGTACGAGCAAAAAGCACTGCGCGCGCTGTACGGGCGCGAGA
L H A E G G E L I Q S A A L A I R N R M T V T E L A
3121 TTCTGGCGCATGAGGTGAGAACATGATCAGAGTGGCGGCTGGCGATCCGACCGCTATGACGGGTGACGAAATAGCC
D Q L F P L T M V E G G L K L C A Q T F N K D V K A L
3201 GACCAAGTTTCTTCCTTACTGCTGATGTGGAGGTTTGAAGCTTGGCGGCAAACTTTTAAACAGATGTGCAAGAAGT

S G S G
3281 GTCTCTGCTGCTGGGTAGCAAACTTGAGTTGATCCGATTTCATTGATGGAATGGCAATTTTTCATTCTGTAGTACGAA

3361 TGCCAACTAATTTTTGAGTATAAGTAAAAAGCACTCAATTTACTACGCTTGTTTTTTAGGGAGGATAGATGAGCG
3461 ACCATCTGCGAATTCGTTAACCCAGTACCATAATTTTTTTCGCTCAGGCTAGTGTCTCAGACGGTACTTTACAGTTGTC
3521 TTTGAATAAATGGATTAGTCGCCGTTAAGCCGTAAGAAGCGTAAACCGACACATAAAATGTCCAACCTGCTTGCCAAATAT
3601 GCGATTAGCTAAAACATAGTGTGATGTTATAGGCGGATAGGACATGCAATCTGTCATGCCCAAGTACGTGAAAAA
3681 AAGCATGCTGACAAATCAGAAATAGCAAGCGCGCTAATCTGCGAGATGCGCTTAATATTTCTGAGGAGAAAAAATGACGCG
3761 CAGCCAAAGTCGCCAAAAAGGCCCAAGTGATATAACCAAGCAGCGCTGCTGTTAAAGATTAAAGTAAATTTATCATCTTA
3841 GTACTCTCAAGGTGCACGGGCGGTGCACAAATCAATGATCAGCATGTTAGTGTCTCAAAAGAGGCTCTCATGTGGTTTG
3921 CTGCGCCATAATCTCAACAGGACATTTAAGCTGAATGCGCTGTTGAGATACGCTCAATACAGACTCGCGCATCGAC
4001 ACCGGTGTAGGAGTGCAGCGTGATCTTAAATCAGCAGTAGAACCAACCTTCATTGATGTGCAGTTTTTCCACAGGGG
4081 ATTCAGCTGGGAAGATC

Fig. 1. Nucleotide sequence of the pMERPH *mer* operon and deduced amino acid sequence of the MerT, MerP, MerC and MerA proteins. The Tn21 *mer* O/P region (Barrineau *et al.*, 1984) (shown in lower case) is aligned with that of pMERPH. Putative -35 and -10 sequences are indicated by $\Delta\Delta\Delta$ or $\Pi\Pi\Pi$, regions of dyad symmetry by horizontal dashed arrows, and the expected site of MerR binding by vertical lines. A single vertical arrow shows the position of the extra base in the spacer region between the -35 and -10 sequences of the *mer* P_{TCPA} promoter. In MerA cysteine residues which are conserved between pMERPH and Tn21 are shown in bold and are double underlined. Other cysteine residues, also in bold, are single underlined.

of a *trans*-acting MerR on the pMERPH *mer* O/P was seen partially to repress P_{TCPA} promoter activity in the absence of Hg²⁺ ions when compared with constitutive CAT expression in the absence of MerR (Fig. 4). Addition of inducing levels of Hg²⁺ ions to AB1157(pPH113 and pACSE20R) resulted in an increase in CAT expression from the P_{TCPA} promoter to a level similar to that of the constitutive system (Fig. 4). Homologous control of the pMERPH *mer* O/P was investigated by introducing pMERPH into *E. coli* AB1157(pPH113). An increase in CAT expression by AB1157(pPH113 and pMERPH) was observed above that of AB1157(pPH113) in both the presence or absence of inducing levels of Hg²⁺, with additional activity seen upon addition of 2 μ M HgCl₂ (Fig. 4).

Amplification of pMERPH-like *mer* genes from cultivated and non-cultivated bacteria

Using the primers 104A and 103B, a 1.37 kb *mer*Δ*PCΔA* region was amplified by the PCR (30 cycles) from *E. coli* AB1157 strains bearing the IncJ plasmids pMERPH and R391 and from the positive control XL-1-Blue(pPH101). PCR products were also amplified from the environmental Hg^R isolate SE2, which had previously been shown to bear a Hg^R determinant that did not hybridize to a *merRTPA* probe from Tn501 at a level of 70 % homology (Osborn *et al.*, 1993). PCR products (1.3 kb and 0.8 kb) were also amplified from DNA extracted directly from bacteria from River Mersey sediment, without cultivation, following 35 cycles of PCR. No PCR products were amplified from the negative controls (sterile distilled water) in either experiment (Fig. 5a). PCR products from all the cultivated *mer* determinants and from the total DNA extracted from River Mersey sediment hybridized to a 4.1 kb *Bgl*II *mer* probe from pMERPH at a level of homology of greater than 70 % (Fig. 5b). The smaller

Fig. 2. Neighbour-joining distance tree of *merA* genes showing the evolutionary position of pMERPH. The source of other *merA* genes are as follows: pI258, Laddaga *et al.* (1987); *Bacillus* RC607, Wang *et al.* (1989); *Streptomyces lividans* 1326, Sedlmeier & Altenbuchner (1992); *Thiobacillus ferrooxidans* E15, Inoue *et al.* (1989); pMER419, Hobman *et al.* (1994); Tn21, Barrineau *et al.* (1984); Tn501, Brown *et al.* (1983); pKLH2, Kholodii *et al.* (1993); and pDU1358, Griffin *et al.* (1987), Nucifora *et al.* (1989) and data from this study. The distance in k_{nu} between any two sequences is the sum of the lengths of the horizontal branches between the two. Bootstrap percentages (100 replicates) are shown to the left of the node being considered.

Fig. 3. Map of plasmid pPH113, derived from the promoter probe vector pKK232.8. The CAT gene is expressed from the P_{TCPA} promoter in the *mer* O/P region. pPH112 (not shown) differs from pPH113 in that the *mer* insert is cloned in the opposite orientation.

PCR products amplified from the cultured bacteria (~ 100 bp and in addition, from SE2, one of ~ 300 bp) did not hybridize to the *Bgl*II *mer* probe.

DISCUSSION

Comparison of the pMERPH *mer* operon with those of previously sequenced *mer* determinants identified the presence of four *mer* genes (*merT*, *merP*, *merC* and *merA*). The predicted molecular masses of the Mer proteins calculated from the pMERPH sequence were 12.5, 9.7, 14.9 and 59 kDa for MerT, MerP, MerC and MerA, respectively. Maxicell analysis of the polypeptides encoded by the 4.1 kb *Bgl*II fragment of pMERPH had identified polypeptides of 59, 22, 14 and 6.5 kDa (Peters *et al.*, 1991). The predicted sizes of the MerA and MerC polypeptides (59 and 14.9 kDa) are similar to the sizes of

Fig. 4. The effects of repression by, and Hg²⁺ induction of, *trans*-acting MerR (transcribed on the plasmids pACSE20R and pMERPH, representing heterologous and homologous control, respectively) on expression from the pMERPH P_{TCPA} promoter as measured by CAT activity of the promoter probe construct pPH113. CAT activity values for *E. coli* AB1157 containing the control plasmids were as follows: pKK232.8, +Hg, 119.5 ± 30.4 , -Hg, 122.0 ± 19.8 ; pACSE20R, +Hg, 56.3 ± 30.6 , -Hg, 66.0 ± 45.4 ; pMERPH, +Hg, 146.0 ± 33.0 , -Hg, 126.3 ± 40.6 .

two of the polypeptides observed in maxicell analysis. In the MerP of Tn21 a 19-amino-acid signal peptide is cleaved from the amino-terminal end to leave a 72-amino-acid processed protein (Summers, 1986). Such processing of the MerP of pMERPH would leave a protein with a predicted molecular mass of 7.7 kDa. The 6.5 kDa polypeptide observed in maxicell analysis (Peters *et al.*, 1991) is probably this processed form of MerP. The identity of the 22 kDa polypeptide observed in maxicell analysis remains unknown, whilst the MerT protein may have run with the larger MerC on the SDS-PAGE gel.

Evolutionary trees derived from MerR (amino acid) and *merR* (nucleotide) sequences (Silver & Walderhaug, 1992; Osborn *et al.*, 1995) have identified a distinct subset of closely related Gram-negative *mer* determinants (Tn501, Tn21, pDU1358, pKLH2 and pMER419) and phylogenetic analysis of the *merA* genes (Fig. 2) is consistent with this observation. The pMERPH *merA* gene is quite distinct from both this Gram-negative subgroup and from that of the *T. ferrooxidans merA* gene and suggests that the pMERPH *mer* determinant is the most divergent Gram-negative *mer* determinant characterized to date. The MerA of pMERPH is distinguished by the presence of a large number of cysteine residues, when compared against the sequences of other Gram-negative *mer* determinants; however the role of these additional cysteine residues is unknown. The cysteine pairs involved in Hg²⁺ reduction (Distefano *et al.*, 1989; Miller *et al.*, 1989) found

Fig. 5. (a) Agarose gel electrophoresis of the PCR products amplified from cultivated and non-cultivated bacteria with the primers 104A and 103B. Lanes: 1 and 7, sterile distilled water after PCR; 2–5, crude extracts of DNA after PCR of XL-1-Blue(pPH101), AB1157(pMERPH), AB1157(R391) and SE2, respectively; 6, kilobase ladder; 8–10, 0.082, 0.0082 and 0.00082 μ g, respectively, total DNA extracted from non-cultivated bacteria from Fiddlers Ferry sediment. Lanes 1–5 are after 30 cycles and lanes 7–10 after 35 cycles of PCR. (b) Southern hybridization analysis of the agarose gel in (a) hybridized to the *Bgl*II *mer* probe from pMERPH.

in the active site and at the carboxyl terminus of the Tn21 MerA are conserved in pMERPH (Fig. 1), as is the GGxCVxxGCxP motif at the MerA active site (Fig. 1, encoded by DNA from position 2013–2045). The occurrence in pMERPH of all four of the *mer* structural genes found previously in the *mer* determinants of Tn21 (Barrineau *et al.*, 1984) and pKLH2 (Kholodii *et al.*, 1993) suggests that the pMERPH *mer* determinant has diverged from a Tn21/pKLH2-like ancestor, and that the maintenance of all four structural genes is highly advantageous even in extensively diverged *mer* determinants. No regulatory genes have been identified adjacent to the pMERPH *mer* structural genes and the determinant may have evolved from a Tn21/pKLH2-like ancestor with subsequent movement of the regulatory genes to elsewhere on the pMERPH plasmid outside the region sequenced to date. Separation of the *mer* structural and regulatory genes has previously been seen in the chromo-

somal *mer* determinant of *T. ferrooxidans* E15 (Inoue *et al.*, 1991).

It has been shown that the *mer* determinants of the IncJ plasmids R391 and pMERPH fail to *trans*-complement Tn21 derivatives with transposon insertions in *merR* (Foster & Ginnity, 1985; Peters *et al.*, 1991). In this study it has been shown that the MerR protein of the Tn21-like determinant of SE20 can both partially repress expression from the *mer* P_{TCPA} promoter and, in the presence of inducing levels of Hg²⁺, can induce expression (Fig. 4). This incomplete repression of the P_{TCPA} promoter by the MerR of SE20 may be a result of variation in the length of both the spacer region between the –35 and –10 sequences, and of the dyad symmetry in the pMERPH *mer* O/P region. Parkhill & Brown (1990) have shown that increasing the spacer region from 19 to 20 bp in the *mer* O/P of Tn501 resulted in a reduction in induction of the promoter by MerR in the presence of inducing levels of Hg²⁺, and a complete loss of repression by MerR in the absence of Hg²⁺ ions. Twenty bp spacer regions are found in both the *Bacillus* RC607 and p1258 *mer* O/P regions (Wang *et al.*, 1989; Skinner *et al.*, 1991) and the additional base pair in pMERPH is at the same site as in these determinants. Helmann *et al.* (1989) showed that the MerR protein from Tn501 bound with equal affinity to both the Tn501 and *Bacillus* RC607 *mer* promoters, suggesting that the variation in length of the spacer region may not on its own be the cause of the reduced repression observed in the study of Parkhill & Brown (1990). A reduction in both induction and repression of the P_{TCPA} promoter activity of Tn501 by MerR was observed in the mutant C565A (a substitution of A for C in the left hand GTAC box in the MerR binding region) (Parkhill & Brown, 1990). Consequently, in pMERPH, the replacement of the C in the left hand GTAC box with a G (Fig. 1) may have resulted in the observed reduction in repression of P_{TCPA} promoter activity in the presence of a *trans*-acting MerR (Fig. 4), due to a decrease in binding of the MerR to the *mer* O/P region. Homologous control of the pMERPH *mer* O/P by pMERPH *in trans* resulted in a significant increase in CAT expression when compared against heterologous control by the MerR from SE20 (Fig. 4), suggesting that a regulatory element must be found on the pMERPH plasmid. The homologous system appears to show an Hg²⁺-independent activation of transcription to levels far greater than those of the constitutive promoter, whilst addition of Hg²⁺ results in further activation. Such a phenomenon has previously been seen in the MerR^{AC} double mutant of Tn501 where increased transcriptional activation in the absence of Hg²⁺ was accompanied by distortion of the *mer* O/P region (Parkhill *et al.*, 1993).

pMERPH-like sequences had previously been isolated from the River Mersey, from a clinical isolate of *Proteus rettgeri* in South Africa (Coetzee *et al.*, 1972), and from the River Rhine (Peters, 1988). In this study pMERPH-like sequences were amplified from both cultivated and non-cultivated bacteria (Fig. 5a) from a River Mersey sediment sample collected six years after the isolation of the *Pseudomonas putrefaciens* strain bearing pMERPH,

suggesting at least localized persistence of pMERPH-like sequences. It is further possible that some of the pMERPH-like sequences present may have suffered a deletion as is suggested by the amplification of 0.8 kb products from total DNA which hybridized to the 4.1 kb *Bgl*II probe (Fig. 5b). In our earlier study (Bruce *et al.*, 1992) using *mer* primers derived from the sequences of Tn501 and Tn21, such truncated PCR products were also amplified from soil DNA. Although these products failed to hybridize to a Tn501 *merRTPA* probe, partial sequence analysis suggested that these truncated PCR products were related to Tn501. The presence of pMERPH-like sequences around the globe, in a manner similar to that of Tn501 or pKLH2-like sequences (Osborn *et al.*, 1995), taken together with their evolutionary distance from other Gram-negative *mer* determinants (Fig. 2) suggests that pMERPH-like sequences have an ancient history.

In this study the pMERPH *mer* determinant has been identified as the most divergent of those sequenced to date, and is probably a derivative of an ancestral Tn21/pKLH2-like determinant which has diverged considerably, especially in the *merA* gene and in the regulation of the operon. The determinant, although found less commonly than other Gram-negative *mer* determinants, has been found to persist in natural environments, and despite its apparent rarity has been found across vast geographical distances.

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