***In vivo* complementation and site-specific mutagenesis of the tellurite resistance determinant *kilAtelAB* from IncP α plasmid RK2Te^r**

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The IncP α plasmid RK2 carries a cryptic tellurite resistance (Te^r) determinant. This determinant from RK2Te^r has been previously cloned into a pUC8 plasmid (pDT1558). The Te^r determinant identified as the *kilA* locus comprises an operon of three genes: *kilA*, *telA* and *telB* [also referred to as *klaA*, *klaB* and *klaC* on RK2(Te^s)]. Each of the genes was subcloned into the expression vector pJF118EH behind an inducible *tac*-promotor using PCR. The PCR primers were used to engineer an efficient ribosome-binding site and adjacent sequence to improve protein expression. Expression plasmids were modified by inclusion of different resistance markers for selection during complementation. The tellurite-resistance phenotype was studied with the overexpressing plasmids. The study provides further evidence that all three genes within the *kilAtelAB* operon are required for cells to show resistance to potassium tellurite. Additionally, site-directed mutagenesis was carried out on the two cysteine residues in *TelB*. Changing either Cys¹²⁵ or Cys¹³² to a Ser or Ala residue decreased the resistance mediated by the operon. These mutants demonstrate the requirement of cysteine residues within *TelB* for expression of tellurite resistance.

Keywords: plasmid RK2, *kilA*, tellurite resistance, subunit complementation, Cys mutagenesis

INTRODUCTION

RK2 is a 56.4 kb plasmid of incompatibility group P α which is able to replicate and be stably maintained in a broad range of Gram-negative bacteria (Thomas & Smith, 1987). RK2 appears to encode an unusual network of co-regulated genes known as the *kil-kor* regulon (Figurski *et al.*, 1982). Within this regulon are four *kil* loci (*kilA*, *kilB*, *kilC* and *kilE*) originally detected by their ability to kill *Escherichia coli* cells when unregulated. The deleterious effects of these loci are negatively regulated by different combinations of the determinants *korA*, *korB*, *korC*, *korE* and *korF* (Bechhofer *et al.*, 1986; Figurski *et al.*, 1982; Goncharoff *et al.*, 1991; Jagura-Burdzy *et al.*, 1991; Schreiner *et al.*, 1985; Thomas *et al.*, 1988, 1990; Young *et al.*, 1985, 1987).

Within the *kil-kor* regulon is a tellurite-resistance (Te^r) determinant which is normally cryptic or expressed at very low levels in wild-type RK2 (Bradley, 1985). The resistance determinant was found to map to the *kilA*

locus (Taylor & Bradley, 1987). The *kilA* locus of a tellurite resistance variant of RK2 [RK2(Te^r)] has been cloned into a pUC8 plasmid (pDT1558) (Walter & Taylor, 1989). The Te^r determinant comprises an operon of three genes: *kilA*, *telA* and *telB* (also referred to as *klaA*, *klaB* and *klaC* on RK2(Te^s); Goncharoff *et al.*, 1991; Walter *et al.*, 1991). The operon encodes three polypeptides of 28 kDa (*KilA*), 42 kDa (*TelA*) and 32 kDa (*TelB*) (Goncharoff *et al.*, 1991; Walter *et al.*, 1991).

Insertion mutagenesis of the *kilAtelAB* operon using Tn1000 and mini Mu has been performed (Taylor & Bradley, 1987; Walter *et al.*, 1991). The data showed that all genes within the *kilA* operon are required for tellurite resistance. However, this type of gene interruption experiment has a polar effect within the operon. Therefore, one cannot determine precisely if the second or third genes in the operon can give rise to the phenotype by themselves. The nucleotide sequences for the *kilA* operon from RK2(Te^s) (Goncharoff *et al.*, 1991) and RK2(Te^r) (Walter *et al.*, 1991) were completed at about the same

time. It was found that the only difference between the nucleotide sequence of the Te^r and Te^s determinant is a single base change of T for an A in the *telB* gene. This change gives a cysteine rather than a serine at position 125 in the TelB protein, and might imply that the *telB* gene could alone mediate tellurite resistance. To determine if this is so, in the present study each of the genes of the *kilAtelAB* operon was isolated separately and used for complementation experiments *in vivo* to identify the genetic requirements for tellurite resistance.

Deletion mutagenesis of the *kilA* locus was performed by Goncharoff *et al.* (1991), using a clone from RK2(Te^s). Their study on *klaABC* found that all three of the genes were required in the cell for resistance to low levels of potassium tellurite ($< 5 \mu\text{g ml}^{-1}$). These workers also found that if all three genes were present it was possible to observe spontaneous mutations for resistance to tellurite at concentrations $> 5 \mu\text{g ml}^{-1}$. Their study did not shed any light on whether *telB* alone, from RK2(Te^r), would give rise to tellurite resistance. In addition to the tellurite-resistance phenotype, this locus has also been assigned a phenotype of host lethality (Figurski *et al.*, 1982; Goncharoff *et al.*, 1991).

The present study investigated the ability of the individual genes within the *kilAtelAB* operon, as well as different combinations of the genes from RK2(Te^r), to confer the tellurite-resistance phenotype. It also investigated the importance of two cysteine residues within TelB. A preliminary report has been presented elsewhere (Turner *et al.*, 1992).

METHODS

Bacterial strains, plasmids, and growth conditions.

The *E. coli* strains used were HB101 (Boyer & Roulland-Dussoix, 1969) and JM109 (Yanisch-Perron *et al.*, 1985). The phagemid vectors used for cloning of PCR products and the production of single-stranded DNA template were pTZ18R and pTZ19R (Mead *et al.*, 1986). The expression vector pJF118EH (Furste *et al.*, 1986) was a gift from Dr B. Lemire (University of Alberta, Edmonton); pMS119HE (Strack *et al.*, 1992) was kindly provided by Dr E. Lanka (Max-Planck-Institut für Molekulare Genetik, Berlin, Germany). The plasmid pOG2, which contains the *korA* gene, was provided by Dr C. M. Thomas (University of Birmingham, UK). The vectors pJF118EH and pMS119HE are both pBR322-based plasmids which possess a *tac* promoter under expression control by the *lacI^q* gene, which can be induced with isopropyl β -D-thiogalactopyranoside (IPTG).

All bacterial overnight cultures were grown in Luria-Bertani (LB) broth medium (Sambrook *et al.*, 1989).

For minimum inhibitory concentration (MIC) determination, overnight cultures were diluted 10^4 in Penassay broth and 50 μl inoculated onto LB or Mueller-Hinton plates containing serial dilutions of potassium tellurite (Sigma). For induction, IPTG was spread on the plate 2 h before addition of cells.

Manipulation and analysis of DNA. Plasmid DNA was

prepared on a large scale using a modification of the method of Birnboim & Doly (1979), which included CsCl/ethidium bromide equilibrium density gradient centrifugation. Small-scale preparations were screened as described by Morelle (1989). Single-stranded DNA template for sequencing was prepared by superinfection of JM109/pTZ18R-based recombinants with the helper phage M13K07. DNA cloning manipulations were carried out by standard techniques (Sambrook *et al.*, 1989). DNA sequencing was performed by the TaqDyeDeoxy Terminal Cycle Sequencing method of Applied Biosystems using a model 373A DNA sequencer.

Site-directed mutagenesis. The *kilAtelAB* operon from the clone pDT1558 was subcloned into the phagemid pTZ19R, generating pDT2149. This phagemid was used in order to make single-stranded template for mutagenesis. Oligonucleotide-directed *in vitro* mutagenesis was carried out using the Amersham kit (V2.1). Protocols were carried out as suggested by the manufacturer. Mutants were confirmed by single-stranded DNA sequencing. Confirmed mutant DNA was then transformed into HB101 for further characterization.

PCR mutagenesis. Polymerase chain reactions were performed using a Techne PHC-2 thermocycler. One hundred nanograms of template DNA (pDT1558) and 100 pmol of each of the two primers were subjected to 25 cycles (94 °C, 1 min; 52 °C, 2 min; 72 °C, 2 min) in a buffer containing 1.25 mM dNTP, 10 mM Tris/HCl pH 8.3, 50 mM KCl, 1.5 mM MgCl_2 and 0.01 % gelatin. The same reaction conditions worked well for all three genes. The primers for the 5' side of each gene are given in Fig. 1. These primers generate a *Bam*HI site upstream from a consensus Shine-Dalgarno sequence as well as an efficient adjacent sequence (DeBoer & Hui, 1991). The nucleotides after the start codon were mutagenized to generate a *Bcl*I site in all three genes. This site was incorporated in order to clone the genes in-frame with glutathione-S-transferase to generate a fusion protein using the inducible expression vector pGEX-3X (Smith & Johnson, 1988). The complementary primers for the 3' ends of the genes are shown below. These primers were designed to generate *Eco*RI and *Sal*I sites for the *kilA* (RTUPCR2) and *telA* (RTUPCR4) genes, and *Bam*HI and *Sal*I sites for *telB* (RTUPCR6). The restriction sites are underlined. The stop codon from each gene is in bold type.

RTUPCR2 5' ATGTCGAC GAATTC**TT**ATTGGTTGATGCCCTTC'
RTUPCR4 5' ATGTCGAC GAATTCATACGGACTCCTGTTG'
RTUPCR6 5' ATGTCGAC GGATC**CTT**ATGGCTCTGCACCCG'

Since the PCR was performed using *Taq* DNA polymerase, there was a concern that nucleotide misincorporations may have occurred in the product. Therefore, the genes were sequenced using single-stranded DNA prepared from the pTZ18R-based clones described below. The three clones were sequenced and no mutations were found.

Plasmid construction. The PCR products were digested with *Bam*HI and *Sal*I and cloned into pTZ18R/*Bam*HI, *Sal*I, generating pTZkilA, pTZtelA and pTZtelB. These plasmids were used to sequence the cloned PCR products. For complementation experiments, different antibiotic-resistance determinants were required. A gene for kanamycin resistance was acquired from a *Sma*I digestion of pKIXX (Pharmacia), giving a 1.2 kb Km^r cartridge. A chloramphenicol-resistance gene was obtained from a *Spl*I, *Sma*I digestion of pUOA23 (Wang & Taylor, 1990), giving a 1.7 kb Cm^r gene cassette. Most of the β -lactamase gene in the pJF118EH vector was excised using a *Dra*I digestion (Fig. 2). The blunt-ended resistance cartridges were ligated into the Ap^s pJF118EH/*Dra*I vector giving the plasmids pJFKANEH and pJFCATEH. Once the three clones

kilA CGCGAGGGTGTATCGAATGGGAAGACAAAGCGTGAACATGGCG...

RTUPCR1 ATGGATCCAAGGAGAAATAATATGATCAATCAAGCGTGAACATGGCG
BamHI BclI

telA ACGGCGGGAGAACCCCTATGAACGCACTGAAAACGACGCACG...

RTUPCR3 ATGGATCCAAGGAGAAATAATATGATCAGCACTGAAAACGACGCACG
BamHI BclI

telB CCCAACAGGAGTCCGTATGAATTGCACAAACACCGATGTTTTC...

RTUPCR5 ATGGATCCAAGGAGAAATAATATGATCACCACAAACACCGATGTTTTC
BamHI BclI

Fig. 1. Oligonucleotide primers used for PCR mutagenesis of the Shine–Dalgarno sequences and adjacent sequences of the translation initiation sites for each of the genes in the *kilAtelAB* operon. The PCR primers of the 5' end, labelled RTUPCR1, RTUPCR2 and RTUPCR3, are shown for each of the three genes. The sequences above each of the PCR primers are from the published sequence of the *kilAtelAB* operon within pDT1558 (Walter *et al.*, 1991). The underlined regions show the Shine–Dalgarno sequences. The start codons are in bold. The upstream region from the Shine–Dalgarno sequence on the PCR primers contains a *Bam*HI site. The nucleotides after the start codons were also mutagenized to generate a *Bcl*I site in all three genes.

had been confirmed by DNA sequencing, they were subcloned using *Bam*HI and *Sa*II into pJF118EH and its antibiotic-resistance derivatives. This gave rise to the recombinant plasmids pJFCkilA, pJFKtelA and pJFtelB (Fig. 2), which were used for the complementation experiments. The result of the above mutagenesis and cloning procedures was that all three of the genes were behind the same promoter (*tac*) and contained the same Shine–Dalgarno and adjacent sequences to provide optimal expression.

As a control for the experiments, the *kilAtelAB* operon from the clone pDT1558 was excised using *Hind*III and *Bam*HI. This fragment was ligated into pMS119HE/*Hind*III, *Bam*HI, generating pMS1558. This plasmid contains the *kilAtelAB* operon behind both the *tac* promoter and the *kilA* promoter and is referred to in the text as *tackilAtelAB*.

RESULTS

PCR mutagenesis and gene segregation

The individual genes within the *kilAtelAB* operon were cloned behind an inducible *tac* promoter using the approach of PCR mutagenesis. This method permitted the generation of modified gene termini containing restriction enzyme sites for cloning and 5' ends with efficient ribosome-binding sites (Fig. 1). The ribosome-binding sequence was designed according to the criteria of Gold & Stormo (1987). The adjacent sequence between the ribosome-binding site and the initiating ATG was also modified for increased efficiency (DeBoer & Hui, 1991). This methodology has been shown to work well to increase the expression of other proteins in our laboratory when the product is cloned behind the IPTG-inducible

tac-promotor of the expression vector pJF118EH (Bishop & Weiner, 1993). Upon harvesting of cells 2 h after induction with IPTG, the gene products expressed from the plasmids pJFCkilA, pJFKtelA and pJFtelB could be visualized by SDS-PAGE after cell fractionation (unpublished results).

It has been suggested previously that *kilA* should not be cloned behind an inducible promoter (Saltman *et al.*, 1991), because of leakiness which may be associated with such promoters. This is based on the idea that expression of *KilA* is lethal and allows no cell growth. However, we did not find this to be a problem. Cultures grew well in the absence of inducer, and demonstrated the lethality phenotype upon induction (unpublished results).

The three pJF118EH-based expression vectors containing the individual genes of the *kilAtelAB* operon were transformed into *E. coli* HB101. There was no observable difference between the transformation efficiencies of the clones and the control vector. This suggests that the clones obtained were not mutants in which the lethal phenotype (Goncharoff *et al.*, 1991) had been eliminated. The presence of the plasmid combinations within the HB101 host was confirmed by dot-blot hybridization with gene probes excised from the original pTZ18R-based clones and detected by hybridization using the DIG DNA labelling and detection kit (Boehringer Mannheim). The plasmids were present even 6 h after induction with IPTG (results not shown).

The *kilA* locus was cloned from RK2(*Te*^r) by using the enzyme *Hinc*II, generating the clone pDT1558, which did not require the *kor* genes for cell viability (Walter *et al.*, 1991). Overnight cultures of cells harbouring pDT1558 are indistinguishable in cell density from a control culture harbouring the cloning vector. Using *Hinc*II to clone the *kilA* locus altered the –35 region of the operon. The change, a T to C, would be expected to give a weaker promoter based on the studies of Hawley & McClure (1983). Cells harbouring pDT1558 grow after an extended lag phase of 5–6 h. Cells harbouring pDT1558 can survive since the weakened promoter would give rise to decreased levels of expression of the *kilAtelAB* operon, yet still express enough protein to mediate tellurite resistance.

MIC of potassium tellurite for the complementated operon

The MICs of potassium tellurite (K_2TeO_3) for *E. coli* HB101 transformed with different genes and gene combinations of the *kilAtelAB* operon are given in Table 1. For *E. coli* cells harbouring the cloning or expression vectors, the MIC was $2 \mu g K_2TeO_3 ml^{-1}$, whereas pDT1558 conferred an MIC of $256 \mu g ml^{-1}$. Cells harbouring RK2(*Te*^r) have a resistance level of $512 \mu g K_2TeO_3 ml^{-1}$ (Walter & Taylor, 1989). HB101 cells harbouring one or two of the genes from the *kilAtelAB* operon were not tellurite resistant, the MIC values being of the same order as those obtained with the control vectors. With the complete operon behind the *tac* promoter (pMS1558) the MIC was decreased and was

Fig. 2. Maps of plasmids used for complementation. The *kilAtelAB* operon was previously cloned from RK2(Te^r) into the cloning vector pUC8 giving the plasmid pDT1558. The individual genes were extracted by PCR mutagenesis and cloned into the vector pTZ18R. The genes were then subcloned into the expression vectors pJFCATEH, pJFKANEH and pJF118EH. This gave rise to the three genes within the *kilAtelAB* operon being separately cloned into the three plasmids pJFckilA, pJFKtelA and pJFtelB shown in this figure.

found to vary from 8 to 32 $\mu\text{g K}_2\text{TeO}_3 \text{ ml}^{-1}$, depending on inducer concentration. The same effect was observed when the genes within the operon were expressed *in trans* by the different plasmids (*kilA/telA/telB*), with the resistance levels being reduced further to a range of 2 to 16 $\mu\text{g K}_2\text{TeO}_3 \text{ ml}^{-1}$ depending on the IPTG concentration.

The lowered MICs are probably a result of overexpression of the lethal gene combinations. Moving the *kilAtelAB* operon from its weakened promoter in pDT1558 to the stronger *tac* promoter of pMS119HE markedly reduces the MIC conferred by the operon. Therefore, the values from *kilA/telA/telB* suggest that the three genes are all required for tellurite resistance and reduced MICs are due to the expression of the gene products to their lethal levels. This is reflected in the increase in the MIC for both

kilA/telA/telB and *tackilAtelAB* with lower concentration of the inducer IPTG.

MICs of the *kilAtelAB* operon under different copy control

While performing the experiments described above, we observed that the regulation of the operon gives rise to differing degrees of host cell growth inhibition (unpublished results). It is expected that this operon is effectively turned off in the plasmid RK2(Te^r) by the *kor* genes. However, in RK2(Te^r) the *kilAtelAB* operon confers high levels of tellurite resistance (512 $\mu\text{g ml}^{-1}$) (Walter & Taylor, 1989). This implies that very little gene product is required for tellurite resistance, and too much gene product can cause host cell growth inhibition. Results relating to this hypothesis are shown in Table 2.

Table 1. MICs of potassium tellurite

Plasmid(s) present	Gene combination present	MIC ($\mu\text{g ml}^{-1}$)*			
		IPTG concn (mM):			
		0	1.0	0.1	0.01
pUC8	—	2	ND	ND	ND
pJF118EH	—	2	ND	2	2
pMS119HE	—	2	2	2	2
pJFKANEH	—	2	ND	2	ND
pJFCATEH	—	2	ND	2	ND
pJF118EH, pJFKANEH, pJFCATEH	—	1	ND	ND	ND
pJFCkilA	<i>kilA</i>	1	1	1	1
pJFKtelA	<i>telA</i>	2	2	4	4
pJFtelB	<i>telB</i>	2	2	2	2
pJFCkilA, pJFKtelA	<i>kilA/telA</i>	2	2	2	2
pJFKtelA, pJFtelB	<i>telA/telB</i>	2	2	4	4
pJFCkilA, pJFtelB	<i>kilA/telB</i>	2	2	2	2
pJFCkilA, pJFKtelA, pJFtelB	<i>kilA/telA/telB</i>	8	2	4	16
pMS1558	<i>tackilAtelAB</i>	256	8	16	32
pDT1558	<i>kilAtelAB</i>	256	128	256	256
RK2(Te ^r)	<i>kilAtelAB</i>	512	ND	ND	ND
RK2(Te ^s)	<i>klaABC</i>	2	ND	ND	ND

* MICs were determined with plasmids harboured by HB101; ND, not determined.

When the operon is on a plasmid of lower copy number or under the control of the *kor* genes, then the resistance to tellurite is high. In contrast, when the operon is over-expressed, the resistance is much lower and can be lethal (no growth on plates without tellurite). These observations help to explain the lowered MICs of the complemented operon when expressed behind the *tac*-promotor *in trans*. The data suggest that very little gene product is required to give rise to a high level of resistance.

Mutagenesis of cysteines 125 and 132 of TelB

Site-directed mutagenesis was performed on Cys¹²⁵ as well as the neighbouring Cys¹³² in TelB. These two cysteine residues are both required for tellurite resistance in RK2(Te^r). Changing either or both Cys¹²⁵ or Cys¹³² to Ala or Ser decreased the tellurite resistance mediated by the *kilAtelAB* operon (Table 3). Attempts to delete the cysteine-containing region (residues 125–132) by loop-out mutagenesis were unsuccessful. However, a small duplication mutation (pRx6B-11) was obtained. This mutant also has a residue deleted at position 132, with the rest of the gene still in-frame, resulting in the sequence GGEFCGFGGEFMM for residues 121–133.

These mutations resulted in a marked reduction in the level of resistance to potassium tellurite in both broth and solid media, with the MIC dropping from 256 $\mu\text{g ml}^{-1}$ down to 8–16 $\mu\text{g ml}^{-1}$ on LB plates and 1–2 $\mu\text{g ml}^{-1}$ on Mueller–Hinton plates. The MICs were tested several times with identical results after introduction of the mutant plasmids into both JM109 and HB101. The

significance of the resistance levels being slightly higher for the Ser/Ser, Ala/Cys and Cys/repeat/deletion combinations is unclear. In contrast to the Ala and Ser mutants, changing Cys¹³² to Ile or Arg gave rise to intermediate levels of resistance (64–128 $\mu\text{g ml}^{-1}$), although these mutants sometimes showed resistance levels in the range 4–8 $\mu\text{g ml}^{-1}$. These results suggest that this residue is more likely to have a structural role in the protein than to be involved directly at the active site.

DISCUSSION

It has been previously reported that unregulated expression of any one of the three genes within the *kilA* operon of RK2(Te^r), *kilA*, *telA* and *telB*, will cause the death of *E. coli* host cells (Goncharoff *et al.*, 1991). Saltman and coworkers have investigated the phenotypes associated with the expression of *klaA* from the *kilA* locus cloned from RK2(Te^s) (Saltman *et al.*, 1991, 1992). These experiments indicate that a vector containing *kilA* cannot be present in a cell without the repressors *korA* or *korB* (Saltman *et al.*, 1991). Therefore, cloning behind a strong promoter should be lethal to host cells. In our expression system, the regulation of the *tac* promoter by *lacI^q* decreases the expression to levels too low to give a lethal phenotype in the same manner as the *kor* genes regulate the *kilA* promoter. Modulation of the –35 region of the *kilA* promoter can reduce expression of the genes to levels too low to give a lethal phenotype (Young *et al.*, 1985). An apparent balance exists between the level of expression of the *kilA* operon and the degree of growth

Table 2. MICs of potassium tellurite conferred by the *kilAtelAB* operon in different plasmids of differing copy number and promoter control

Plasmid	Copy number	Relevant promoters/genes present	MIC ($\mu\text{g ml}^{-1}$)	
			No IPTG	+0.1 mM IPTG
pUC8	500–700	<i>lac</i>	2	
pDT1558	500–700	<i>kilA</i>	256	
pMS1558	20–30	<i>tac-kilA</i>	256	2–16
pDTK1558*	500–700	<i>kilA</i>	256	
pMSK1558*	20–30	<i>tac-kilA</i>	256	2–8
pDT1555†	10–12	<i>kilA</i>	512–1024	
RK2Te ^s	2–5	<i>klaA/korAB</i>	2	
RK2Te ^r	2–5	<i>kilA/korAB</i>	512	
RK2Te ^s + pMS1558	2–5/20–30	<i>klaA/korAB + tac-kilA</i>	1024	0
RK2Te ^s + pDT1558	2–5/500–700	<i>klaA/korAB + kilA</i>	1024	
RK2Te ^s + pMSK1558	2–5/20–30	<i>klaA/korAB + tac-kilA</i>	1024	0
RK2Te ^s + pDTK1558	2–5/500–700	<i>klaA/korAB + kilA</i>	1024	
pOG2	20–30	<i>korA</i>	2	
pOG2 + pMSK1558	20–30/20–30	<i>korA + tac-kilA</i>	1024	0–4
pOG2 + pDTK1558	20–30/500–700	<i>korA + kilA</i>	1024	

* pDTK1558 and pMSK1558 are pDT1558 and pMS1558 with their ampicillin resistance gene replaced with a kanamycin resistance gene.

† pDT1555 is a pACYC184 vector with the *kilAtelAB* operon from pDT1558.

Table 3. MICs of potassium tellurite for TelB Cys¹²⁵/Cys¹³² site-directed mutants

Plasmid	Sequence*	MIC ($\mu\text{g ml}^{-1}$)	
		Mueller-Hinton	LB
pTZ19R	Control	2	2
pDT2149	EF C GFVRSV C M	256	256
pDT2168	EF s GFVRSV C M	2	8
pDT2182	EF C GFVRSV s M	1	8
pDT2183	EF s GFVRSV s M	1	16
pRx5A-3	EF a GFVRSV C M	1	16
pRx5C-25	EF C GFVRSV a M	1	8
pRx5C-32	EF a GFVRSV a M	1	8
pRx3B-4	EF C GFVRSV i M	64	128
pRx3B-5	EF C GFVRSV r M	8	64
pRx6B-11	GGE F CG F g g ef_MM	2	16

* Mutant residues are in lower-case type.

inhibition. This is reflected in the tellurite MICs shown in Tables 1 and 2.

Some expression of the *kilA* operon occurs in RK2-containing cells under normal growth conditions (Goncharoff *et al.*, 1991). Although the operon is effectively repressed by the *kor* genes, enough gene product is made to mediate tellurite resistance in RK2(Te^r) (Bradley *et al.*, 1988). The bactericidal effects of the *kilA* operon were

suggested to be a result of the level of expression (Goncharoff *et al.*, 1991), and this was confirmed by a similar study by our group (unpublished results). The reduced MICs observed from the genes of the *kilAtelAB* operon expressed either *in cis* or *in trans* behind the *tac* promoter are most probably due to the effect of the growth inhibition phenotype. There would be a delicate balance within the cell to produce enough gene product to mediate tellurite resistance but yet not enough to inhibit cell growth or cause cell death. This is seen with cells harbouring either pMS1558 (*tackilAtelAB*) or pJFC-kilA/pJFKtelA/pJFtelB (*kilA/telA/telB*) (Table 1). When the IPTG is titrated from 1.0 mM to 0.01 mM the MIC increases from 2 to 16 $\mu\text{g ml}^{-1}$ for *kilA/telA/telB*, and from 8 to 32 $\mu\text{g ml}^{-1}$ for *tackilAtelAB*. The higher MICs for cells containing pMS1558 were probably obtained because this operon still has poor adjacent sequences between the ribosome-binding sites and the start codons for the three genes. Therefore, the level of expression of the lethal genes from *tackilAtelAB* may be lower for a given amount of IPTG than for the PCR-mutagenized genes. The results in this study (Table 1) clearly demonstrate that it is not TelB alone which mediates the tellurite resistance, but that the products of the three genes in the operon are working in concert.

Site-directed mutagenesis on the cysteine residues in TelB was prompted by the sequence difference between the *kilA* operon between RK2(Te^s) and RK2(Te^r). There are only two cysteine residues in all of the three proteins coded for by the operon; both are found in TelB. TelB is

predicted to be an integral membrane protein, with Cys¹²⁵ expected to be found in a loop and Cys¹³² to be within a transmembrane segment close to the membrane interface. In RK2(Te^s), Cys¹²⁵ is a Ser residue. The results of the mutagenesis (Table 3) suggest that both Cys residues are required for resistance and may play a role in the interaction with the TeO₃²⁻ oxyanion. However, this hypothesis is not consistent with the results seen after Arg and Ile replacements to position 132. Cys¹³² is modelled at the membrane interface. By changing this residue to an isoleucine the transmembrane helix would be positioned deeper within the membrane, thereby stabilizing the structure. In the case of the Arg mutant, the positive charge of the side chain could interact with the polar lipid head groups and again stabilize the transmembrane helix and the structure (Nilsson & von Heijne, 1990). In both the Arg¹³² and Ile¹³² mutations the shift of the third transmembrane helix deeper into the membrane could result in an increased exposure of Cys¹²⁵. Another possibility is that these mutations do not interfere with subunit assembly to the same degree as the Ala and Ser mutations at the 132 position.

It remains unclear why the resistance does not drop to 2 µg ml⁻¹ for the Cys¹²⁵/Ser mutation. However, in light of the information that different levels of expression and/or promoter type give rise to marked changes in the resistance level, the vector differences may be responsible for this anomaly. The results from this mutagenesis study confirm the importance of Cys¹²⁵ and suggest that the residue at position 132 is important in maintaining the integrity and/or function of Kila-Tela-TelB.

The results of the present study demonstrate that the biochemical activities responsible for the tellurite-resistance phenotype can be complemented *in vivo* by expressing the individual genes of the *kilA* locus *in trans*. Although we have little experimental evidence at this time, it may be inferred from the above results that the three proteins Kila, Tela and TelB probably work together as a complex. Our study adds to the list of other *E. coli* proteins and protein complexes which have been functionally complemented. These include lactose permease (Bibi & Kaback, 1992; Wrubel *et al.*, 1990), cytochrome *d* terminal oxidase complex (Newton & Gennis, 1991), nitrate reductases (Blasco *et al.*, 1992a, b) and fumarate reductase (Condon & Weiner, 1988). In all cases the complemented activities were lower than those obtained when the proteins were expressed from their wild-type polycistronic message. The explanation for this difference may reside in the loss of the translational coupling and regulation which are absent when the genes are separated. These regulatory effects may be contributing to the differences we found for the *kilAtelB* operon phenotypes.

Overall, the data reported herein show that all three genes, *kilA*, *telA* and *telB*, are required for tellurite resistance, and lower levels of expression give higher resistance levels. Additionally, Cys¹²⁵ (and to a lesser extent Cys¹³²) is important for a functional tellurite-resistance phenotype.

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