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The role of TerW protein in the tellurite resistance of uropathogenic *Escherichia coli*

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Abstract: The *ter* operon, which is found on a large conjugative plasmid, pTE53, from the uropathogenic strain *Escherichia coli* KL53, mediates heavy metal ion resistance to tellurium compounds. Here we present the function of the *terW* gene, which is transcribed from its own promoter in the opposite direction to the *terZABCDE* loci and encodes a protein of 155 amino acids. A TerW protein containing an N-terminal His-tag was overexpressed, purified and analysed for the *in vitro* DNA-binding. Green fluorescent protein fusions, a fluorimetric assay, nonradioactive gel retardation and capillary electrophoresis footprinting were used to examine the interaction between the bioinformatically-predicted DNA-binding region and TerW. Here we show that TerW binds specifically to the potential promoter region of the *terZABCDE* genes, which are responsible for the tellurite resistance of the *Escherichia coli* host cells. Our results suggest that the transcriptional regulation of *ter* genes is operon-like.

Key words: tellurite resistance; *ter* genes; TerW biological function

Abbreviations: CE, capillary electrophoresis; EMSA, electromobility shift assay; GFPuv, green fluorescent protein; IPTG, isopropyl-β-D-1–thiogalactopyranoside; MIC, minimal inhibitory concentration; PPRR, potential promoter rich region; qRT, quantitative real time; Te^R, tellurite resistance.

Introduction

Tellurium is a rare element, belonging to the group known as chalcogens, that shares chemical properties with biologically important elements such as oxygen, sulphur and selenium (Fischer 2001). Both organic and inorganic tellurite soluble salts have been found to be toxic for most forms of life at very low concentrations (Chasteen et al. 2009). Determinants of tellurite resistance (Te^R) have been described as a part of plasmids (IncHI, IncHII and IncP) and/or chromosomes, representing typical pathogenicity-associated islands (Perna et al. 1998; Taylor 1999; Tarr et al. 2000).

Plasmids of the P incompatibility group have the ability to transfer between, replicate in and remain stable in the full variety of Gram-negative bacteria (Thomas & Smith 1987). The RK2, RP4, RP1, R18 and R68 plasmids were originally isolated from Gram-negative bacteria in Birmingham (UK) in 1969 (Holloway & Richmond 1973; Ingram et al. 1973). The RK2 Te^R determinant has been located between the *kilA* and *korA* genes, which are involved in the control of plasmid replication (Taylor & Bradley 1987). This 3 kb region contains an operon encompassing the three genes (*kilA*, *telA* and *telB*), which are necessary for the expression of high-level resistance to tellurite, and no DNA or amino acid sequence homology to the IncHI or IncHII Te^R determinants has been detected (Goncharoff et al. 1991; Walter et al. 1991). The *kilA* gene was previously identified by Figurski et al. (1982), and a lethal effect was observed when it was cloned separately from the *korA* (kill-override) gene, which negatively regulates transcription from the *kilA* promoter (Young et al. 1987). Further work by this group suggested that each of three genes (*kilA*, *telA* and *telB*) can express a host-lethal phenotype (Goncharoff et al. 1991).

The determinant of *ter* gene family was first found on a pMER610 (IncHI2) plasmid of *Alcaligenes* sp. (Jobling & Ritchie 1987); it has also been found on the plasmid pHH1508a (IncHII) from *Klebsiella aerogenes* (Walter & Taylor 1989), the large conjugative plasmid pR478 (IncHI2) from *Serratia marcescens* (Whelan et al. 1997) and the chromosomes of *Proteus mirabilis* (Toptchieva et al. 2003) and *Escherichia coli* O157:H7 (Perna et al. 2001). Several enteric pathogens share a similar locus of *ter* genes, which provides the high level resistance that is currently used for the isolation and identification of many pathogens, including the emergent enterohemorrhagic *E. coli* O157:H7 strain (Zadik et al. 1993). This human pathogen probably acquired these genes by horizontal gene transfer, as a part of a large tellurium-resistance and adhesin-conferring island that also conferred urease, colicin and phage protection proteins (Tarr et al. 2000).

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Table 1. Strains and plasmids used in this study.

Strain/Plasmid	Relevant genotype	Reference
Strain *E. coli*		
DH5α	F', *end*A1, *hsd*R17, (r_k^- m_k^+), *sup*E44, *thi*-1, *rec*A1, *gyr*A (Nal^R), *rel*A1, Δ(*lacIZYA-arg*F) U169, *deo*R, (Φ80*dlac*Δ(*lacZ*) M15)	Woodcock et al. (1989)
BL21(DE3)	F-, *omp*T, *hsd*S_β (r_β-m_β-), *dcm*, *gal*, (DE3) tonA	Novagen
KL53	Cm^R, Tc^R, Te^R, clinical isolate	Burian et al. (1998)
KL53-W1	KL53 containing the expression plasmid pETW1.1, λDE3, Cm^R, Tc^R, Km^R, Te^R	This work
KL53-W1b	KL53 containing the expression plasmid pRSFW1b, λDE3, Cm^R, Tc^R, Km^R, Te^R	This work
Plasmid		
pJET 1.2/blunt	A/T cloning vector, Ap^R	Fermentas
pET28a(+)	ColE1 replicon, Km^R	Novagen
pRSFDuet-1	RSF1030 replicon, Km^R	Novagen
pACYC184	p15A replicon, Cm^R, Tc^R	Chang & Cohen (1978)
pBAD-GFPuv	ColE1 replicon, Ap^R	Crameri et al. (1996)
pPMZ-JET	PPRR of *ter* operon in pJET1.2/blunt, Ap^R	This work
pMBAD1	PPRR of *ter* operon fused with *gfp*UV in pBAD-GFPuv, Ap^R	This work
pMAC1	PPRR of *ter* operon fused with *gfp*UV in pACYC184, Cm^R	This work
pETW1.1	*terW* in pET28a(+), Km^R, His-TerW $\sim$ 20.25 kDa	This work
pRSFW1b	*terW* in pRSFDuet-1, Km^R, His-TerW $\sim$ 18.42 kDa	This work

The *ter* operon (*terXYW* and *terZABCDEF*) has been found in our laboratory on a large conjugative plasmid pTE53, isolated from the uropathogenic strain *E. coli* KL53 (Burian et al. 1998). The minimal part of the operon, which is essential for the tellurite resistance phenotype, contains the *terBCDE* genes (Kormutakova et al. 2000). The whole determinant was cloned using *in vivo* methods based on the miniMu derivative cloning system, resulting in the pNT3B plasmid (Kormutakova et al. 2000; Tu et al. 2001; Vavrova et al. 2006). The presence of the *ter* operon improved the fitness of bacterial strains by enabling them to escape from macrophage attack (Valkova et al. 2007). In the present study, we present the biological function of the TerW protein.

Material and methods

Strains and cultivation media

Escherichia coli DH5α cells were used for the cloning and amplification of all recombinant plasmids. *E. coli* BL21(DE3) cells were used for the expression and overproduction of His-tagged fusion proteins. The *E. coli* KL53 strain is the original uropathogenic clinical isolate from the Department of Urology, Faculty of Medicine, Comenius University in Bratislava (Burian et al. 1998). LB medium with appropriate antibiotics was used for the maintenance and growth of the *E. coli* strains. Antibacterial agent concentrations were used as follows: 0.5–10 mM K_2TeO_3 (Biomark Laboratories), 100 μg/mL ampicillin, 25 μg/mL kanamycin, 34 μg/mL chloramphenicol and 25 μg/mL tetracycline.

In silico analysis and cloning of a potential promoter sequence

The potential promoter rich region (PPRR) of the *ter* operon was determined with the help of the Neural Network Promoter Prediction software (http://www.fruitfly.org/seq_tools/promoter.html) and Softberry-BPPROM software (http://www.softberry.com/berry.phtml). DNA manipulations were carried out as described by Sambrook et al. (1989). Plasmid DNA was isolated from overnight culture in LB medium with appropriate antibiotics using QIAprep spin miniprep kits (Qiagen). For all PCR amplifications, total DNA from *E. coli* KL53 was used as the template. PCR products were purified using the Promega Wizard PCR product purification kit. All generated plasmids are listed in Table 1. The construction of the plasmid called pPMZ-JET consisted of the insertion of an 830 bp PCR fragment DV3-ZfuzR (bearing PPRR) into the A/T cloning vector pJET1.2/blunt (Fermentas). Primers DV3 (5'-ATACCGGGCTGTCCGAGAAC-3') and ZfuzR (5'-AAGCTAGCGAGTGATACCGTCTGG-3') were derived from the sequence of the large conjugative plasmid pTE53, isolated from uropathogenic *E. coli* KL53. To simplify the cloning and preparation of an in-frame *gfpUV* fusion, we used a reverse ZfuzR primer with an artificial *Nhe*I restriction site. Amplification was performed using the following thermal program: initial denaturation at 94 °C for 2 min, 29 cycles of 94 °C for 30 s, 55 °C for 45 s and 72 °C for 2 min, with a final extension cycle at 72 °C for 8 min. The PCR amplification product comprised 36 bp from the *terZ* gene and a 794 bp region preceding the *terZ* gene; we assumed that this region was large enough to include the native *ter* operon promoter. The extracted fragment DV3-ZfuzR (*Xba*I/*Nhe*I) from the plasmid pPMZ-JET was subcloned into the vector pBAD-GFPuv (Crameri et al. 1996) as follows: (i) we removed the natural *gfpUV* promoter from the pBAD-GFPuv vector by cutting with restriction endonucleases *Eco*RV and *Nhe*I; and (ii) the PCR fragment of the *ter* PPRR (DV3-ZfuzR) was subcloned into linearised pBAD-GFPuv vector. Using the aforementioned subcloning process, we prepared the plasmid pMBAD1 (pBAD-GFPuv derivative with *Eco*RV and *Xba*I restriction sites removed by Klenow treatment and re-ligation). After the digestion of plasmid pMBAD1 with the restriction enzymes *Cla*I and *Eco*RI, a DNA fragment 1,950 bp in size remained, which included the PPRR with the *gfpUV* fusion. Plasmid pMAC1 contains the fragment described above, recloned into pACYC184 (Chang & Cohen 1978). New plasmid constructs were verified by restriction analysis and sequencing by an ABI 3100-Avant Genetic Analyzer (Applied Biosystems).

Construction of expression plasmids

The entire *terW* coding region (468 bp) was amplified by PCR using the sense primers pETWF (5'-CGGGATCCAT GCAATTAAATACCAGACA-3') and pETWFb (5'-CGGG ATCCAATGCAATTAAATACCAGACA-3'), respectively, and the antisense primer pETWR (5'-CGGAATTCGGAC TTTTTAGCGCTGTATT-3'). The PCR amplification methods were as described above. The PCR products were digested with *Bam*HI and *Eco*RI restriction enzymes and cloned between the same sites of the pET28a(+) and pRSFDuet-1 expression vectors (Novagen), yielding the expression plasmids pETW1.1 and pRSFW1b, respectively. The inserted *terW* genes carried a 6–His tag fused to their N terminus. The identities of the recombinant plasmids were confirmed by PCR amplification and double restriction endonuclease (*Bam*HI and *Eco*RI) digestion.

Expression and purification of proteins

The expression plasmids (pETW1.1 or pRSFW1b) were transformed into *E. coli* BL21(DE3) cells after verifying of the reading frame by sequence analysis. Transformed BL21(DE3) cells were cultivated overnight in LB medium supplemented with kanamycin at 37℃. Bacteria were diluted with fresh LB medium containing the appropriate antibiotic (1:20), and the cells were grown to the exponential phase ($OD_{600} \sim 0.6$–0.8) at 37℃. The expression of recombinant protein was then induced by the addition of isopropyl-β-D-1-thiogalactopyranoside (IPTG) at a final concentration of 1 mM, and the cells were further incubated with shaking at 37℃ for 3 h. The bacteria cells that expressed the TerW protein were collected by centrifugation, resuspended in equilibration buffer (50 mM sodium phosphate pH 7.4, 300 mM NaCl), lysed by sonication at 4℃ (total time of 10 min, with 15 s bursts and 35 s cooling periods) and treated with DNase I (Promega). Cell debris was removed by centrifugation, and the supernatant was applied onto an equilibrated 1 mL Ni^{2+} chelating agarose column (Qiagen). The proteins were washed with 3 mL of Wash Buffer 1 (50 mM sodium phosphate pH 7.4, 300 mM NaCl, 10 mM imidazole) and 3 mL of Wash Buffer 2 (50 mM sodium phosphate pH 7.4, 300 mM NaCl, 20 mM imidazole). The purified His-tagged TerW protein was eluted with 2 mL buffer (50 mM sodium phosphate pH 7.4, 300 mM NaCl, 250 mM imidazole). After that, the samples were detected by 12% SDS-PAGE.

Non-radiochemical electromobility shift assay (EMSA)

DNA fragments encompassing the PPRR of the *ter* operon were generated by the PCR method. Protein/DNA binding reactions were carried out in 20 μL of 20 mM Tris-HCl (pH 7.6), 75 mM KCl, 5 μg/mL of bovine serum albumin, 10% (v/v) glycerol, for 30 min at 37℃. The binding mixtures (protein/DNA) were applied on 5% non-denaturing PAGE, stained with ethidium bromide and visualised with UV alongside a 1 kb ladder (Fermentas). Primers SL1 (5'-GTACCGTCAGCCTGGTCAGTG-3') and LVr4 (5'-CGATTCCTTTGGAGCCGAAC-3') were designed to amplify a part of *terA* gene, which was used as a negative control, not expected to be recognised by *terW* gene product.

Non-radiochemical DNase I footprinting by capillary electrophoresis (CE)

Total DNA from the *E. coli* KL53 strain was used as a template to generate a probe that encompasses the potential promoter region for the tellurite resistance genes (*terZABCDE*). The first probe (585 bp) was generated by PCR using the primers DV3 and Len1-FAM (5'-(6-FAM) CACTCCATAACCACTGAAGT-3'). The second, shorter probe (100 bp) was generated by PCR using the primers PTforward (5'-TCCGGAACGCCATGCAAA-3') and Len1-FAM. The PCR was performed for 30 cycles under the following conditions: 94℃ for 30 s, 55℃ for 30 s, and 72℃ for 60 s, and fluorescein-labelled PCR products were used to prepare the binding reactions. The control reaction contained 25 μL of binding buffer (50 mM Tris-HCl pH 8.0, 100 mM KCl, 12.5 mM $MgCl_2$, 1 mM EDTA, 20% glycerol, 1 mM DTT), 10 μL of FAM-labelled probe (500 ng) and 10 μL of nuclease free water. The sample reactions contained 25 μL of binding buffer, 10 μL of FAM-labelled probe (500 ng) and 10 μL of the purified TerW protein (ranging from 0.1 to 20 μg). The binding mixtures were incubated for 1 hour at 37℃. Next, 50 μL of Ca^{2+}/Mg^{2+} solution (5 mM $CaCl_2$, 10 mM $MgCl_2$) were added and the reactions were incubated at room temperature for 1 min. Immediately prior to use, we diluted 5 μL of RQ1 RNase-Free DNase (Promega; 1u/μL) in 100 μL of cold Tris-HCl, pH 8.0. For the DNase I treatment, we used 3 μL of diluted RQ1 RNase-free DNase, and all reactions were then incubated at room temperature for 90 s. Each reaction was terminated by adding 90 μL of Stop Solution (Promega), incubated at 75℃ for 10 min and then extracted with 200 μL of phenol:chloroform:isoamyl alcohol (25:24:1). The upper, aqueous phase was transferred to a fresh tube and precipitated on ice using 500 μL of 100% ethanol for 20 min. After precipitation and centrifugation ($14{,}000\times g$), the DNA pellets were washed once with 70% ethanol, after which the samples were heated to 95℃ for 2 min to remove any residual ethanol. To prepare the samples for CE, the DNA pellets obtained as described in the above section were resuspended in 20 μL of nuclease-free water. Digested DNA, 1 μL, was added to 8.6 μL HiDi formamide (Applied Biosystems) and 0.4 μL GeneScan-600 LIZ size standards (Applied Biosystems). The samples were analysed using the 3130xl DNA Analyzer (Applied Biosystems) with the G5 dye set, running an altered default genotyping module that increased the injection time to 45 s and the injection voltage to 2.5 kV. The CE traces were examined for a loss of signal in the protein-containing samples compared to the samples without protein to determine the protein-binding regions within the DNA fragment being examined.

Fluorimetry

Conventional fluorescence microscopy was used to detect the promoter-GFPuv fusions. Promoter strength was measured with a fluorimetric assay. GFPuv reporter was chosen because tellurite toxicity prevented the use of the *lacZ* fusion as a reporter for regulatory studies (Toptchieva et al. 2003). *E. coli* BL21(DE3) containing pBAD-GFPuv with the *araBAD* promoter, P_{BAD}, from the arabinose operon, and the corresponding regulatory gene, *araC*, were used as positive and negative controls for fluorimetry that were dependent on arabinose addition/non-addition to the growth medium, respectively. Strains containing vector pBAD-GFPuv and vector derivatives carrying the PPRR of the *ter* operon promoter were grown in liquid media. Samples of these cultures were loaded into 96-well white microtitre plates and their fluorescence levels were measured on a fluorimeter (Tecan Safire^2-Basic) with 365/10 nm excitation and 509/10 nm emission filters. In all cases, the fluorescence level of each sample was divided by the OD_{600} of the sample.

Table 2. Primers used for qRT-PCR. All PCR products were 100 bp in length.

Gene	Forward primer sequence	Reverse primer sequence
terW	5'-ACCAGACAGGCCCGGATT-3'	5'-GTCGGCTCTGAGCATTCCA-3'
terZ	5'-TTCCGTGGTCAGTCGTTCAA-3'	5'-GAGCCCTGCTCAGTCAGCTT-3'
terB	5'-AATCGGCAAGGGCGAAAC-3'	5'-CACTTTTCGCAACGGCAAT-3'
terC	5'-TGTTTATGCACCGTGATGACAA-3'	5'-GTAGAGGAAACCGGCAAATGC-3'
tufA	5'-CCGCAGACTCGTGAGCACAT-3'	5'-AGCAGCTCTTCGTCATCAACCA-3'

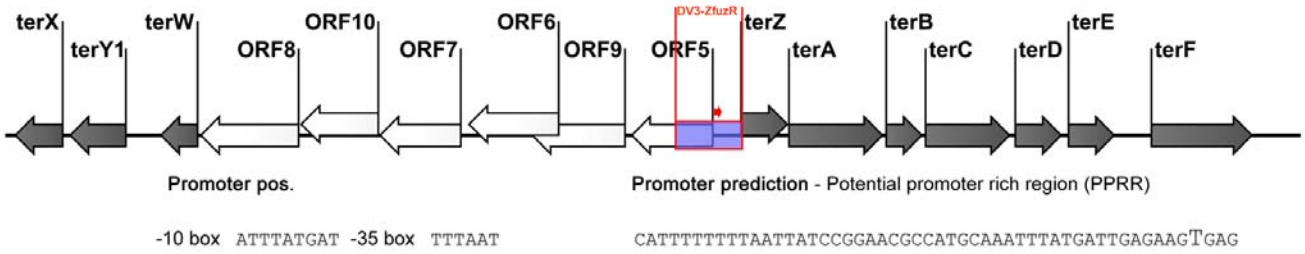

Fig. 1. Physical map and organisation of the *ter* operon from uropathogenic *Escherichia coli* KL53. The region represents the 15,719 bp assembly of sequences – GenBank Acc. Nos. AJ888883 and AJ238043. The PPRR is illustrated by the red arrow as a part of the DV3–ZfuzR DNA fragment (shaded violet). The basic promoter sequence was predicted by the Neural Network Promoter Prediction software for prokaryotic sequences with a score cutoff of 1.00 and by the Softberry-BPPROM software. The transcription start point is shown in a larger font.

Tellurite susceptibility testing

The exposure agent K_2TeO_3 was serially diluted (0–10 mM) in a deep-well microplate (Sarstedt) containing 200 µL of LB medium supplemented with the required antibiotic(s) and 1 mM IPTG per well in case of His-TerW overproduction in the strains *E. coli* KL53-W1 and *E. coli* KL53-W1b. Cultures grown at 37 °C in LB medium with the appropriate antibiotic(s) to an $OD_{600} \sim 0.4$ were added to each well. Cells were incubated at 37 °C with shaking overnight and the OD_{600} was monitored to determine the minimal inhibitory concentration (MIC). The MIC was determined as the lowest concentration of K_2TeO_3 that totally inhibited bacterial growth.

Quantitative real time (qRT) PCR analysis

The strains *E. coli* KL53-W1 and *E. coli* KL53-W1b, which contain expression vectors for IPTG-induced TerW overproduction, were cultivated at 37 °C to an OD_{600} of ~ 0.5 in LB medium supplemented with 1 mM potassium tellurite. Overexpression of TerW protein was induced by the addition of 1 mM IPTG and cell cultures were further incubated with shaking at 37 °C for 1 h. Subsequently, RNA was isolated from the IPTG-induced and non-induced strains, *E. coli* KL53-W1 and *E. coli* KL53-W1b. The RNA was stabilised by mixing 3 mL of bacterial culture with 5.4 mL of RNAlater (Ambion). After 5 min at room temperature, the bacteria were harvested by centrifugation at $3{,}000 \times g$ for 10 min. RNA was isolated from the pellet using the commercial SV Total RNA Isolation System (Promega), with increased DNase treatment time (60 min) compared with the manufacturer's protocol. The RNA (1 µg) was reverse-transcribed to cDNA using a ImProm-II™ Reverse Transcription System (Promega) and universal hexaoligonucleotide primers. For each sample, a control to check for DNA contamination in the RNA preparation was included, from which reverse transcriptase was omitted. Real time PCR was performed in an ABI Prism 9600 HT thermocycler (Applied Biosystems) with the SybrGreen Master Mix (Applied Biosystems), 10 pmol of each primer and 25 ng of template cDNA in a total volume of 20 µL. The thermocycler program consisted of 40 cycles (15 s at 94 °C, 1 min at 60 °C) after an initial denaturation of 10 min at 94 °C. The primers, designed using PrimerExpress (Applied Biosystems), are described in Table 2. The expression levels of particular genes were quantified using the $\Delta\Delta Ct$ method (Livak & Schmittgen 2001) normalised to the expression of the *tufA* gene. For each experiment, qRT-PCR was used to determine the transcript levels in triplicate on three independent cDNA samples derived from three independent cultures. Error bars show standard deviations. Expression levels were relative to the relevant non-IPTG-induced samples.

Results

In our previous work (Vavrova et al. 2006), we measured the expression of the *ter* operon genes in uropathogenic *E. coli* KL53 under different cultivation conditions using qRT-PCR and transcription unit identification. Those experiments confirmed that *terZABCDE*, without *terF*, is transcribed as one mRNA unit, and similarly, the *terW* gene is transcribed separately from the *terXY* genes. Based on these results, we searched the *ter* operon sequence (GenBank Acc. Nos. AJ888883 and AJ238043) for potential promoters common to the same transcription unit as the *terZABCDE* genes. We used bioinformatics analysis to identify potential prokaryotic promoters. To increase the reliability of our predictions, we specifically identified potential promoters with a minimum promoter score of 1.0 (Fig. 1).

To assess the native *terZABCDE* promoter activity, we substituted the *araBAD* promoter and part of the *araC* regulatory gene in the pBAD-GFPuv cloning vector with a DNA fragment (DV3–ZfuzR) generated by PCR. In this way, we confirmed the existence of one common functional promoter for the *ter* genes by cloning a probable promoter region into the pBAD-GFPuv vector. The GFPuv reporter variant was ideal

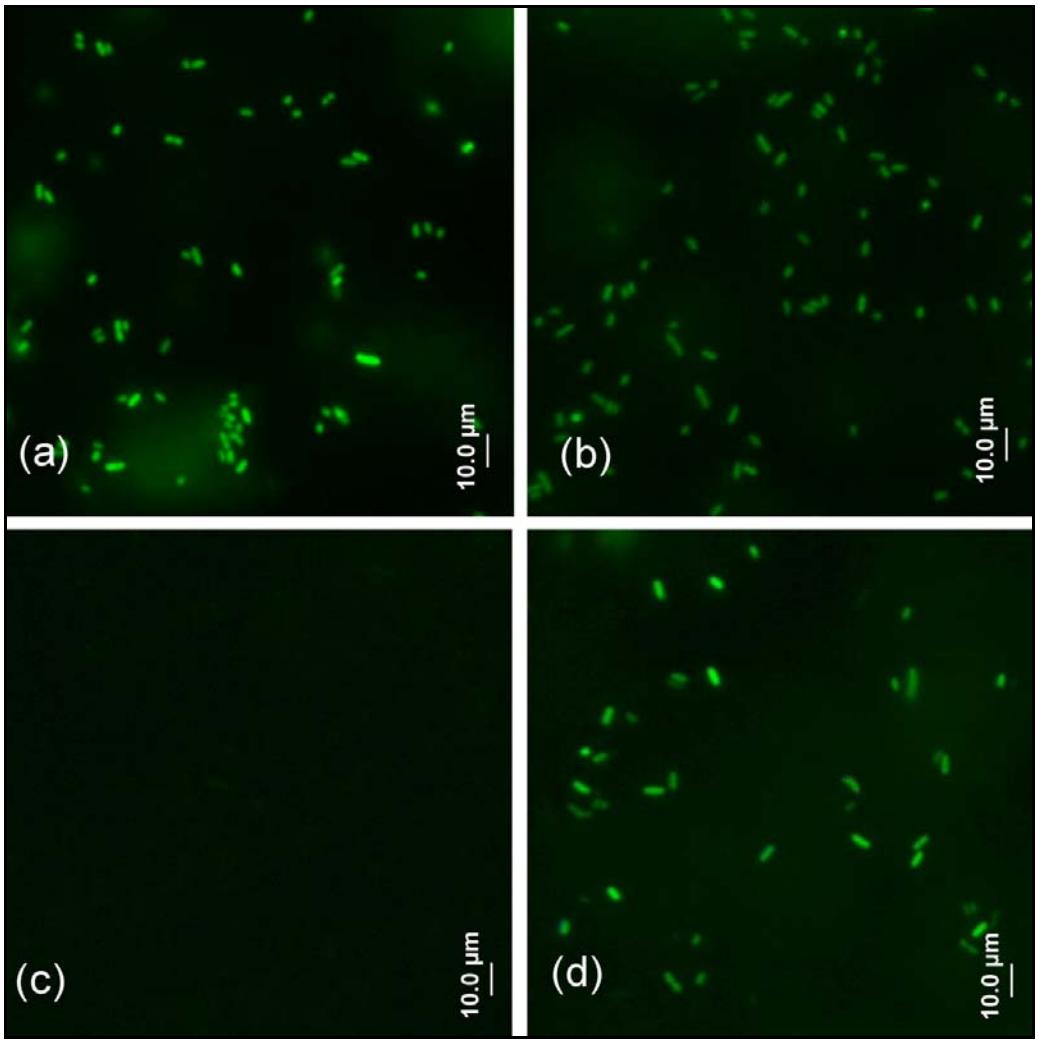

Fig. 2. *E. coli* with a *ter* promoter-*gfpUV* fusion. (a) *E. coli* cells containing the pMBAD1 plasmid expressing GFPuv. (b) *E. coli* cells containing the pMAC1 plasmid expressing GFPuv. (c) and (d) *E. coli* cells carrying the pBAD-GFPuv plasmids used as negative/positive controls in dependence on arabinose non-addition/addition to growth medium, respectively.

for these experiments because the expression of *gfpUV*-fusion proteins have been successfully detected using UV light and could easily be checked by visual inspection of growth plates. The functional fusion of the proposed PPRR with GFPuv in the generated plasmids (pMBAD1 and pMAC1) resulted in the expression of green fluorescent protein. Its expression was analysed on an Olympus BX50 fluorescence microscope using a 100×1.25 objective, Olympus C-35AD-4 camera system and BXTA WU/SWB filter. The *E. coli* cells in which the *gfpUV* gene was under the control of the *terZABCDE* promoter are shown in Figure 2. We measured the fluorescence with two different assays: (i) using a conventional fluorescent microscopy to visually detect the fluorescence emitted from bacteria; and (ii) collecting cells from a liquid growth medium and suspending them in PBS for the measurement using fluorimeter (Fig. 3).

Because we suspected that the product of the *terW* gene (17,263 Da) could be a key participant in the regulation of the *terZABCDE* loci at the transcriptional level, we generated plasmids expressing recombinant His-tagged TerW protein for an interaction study.

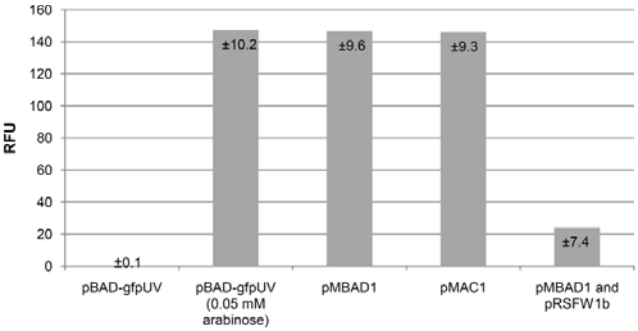

Fig. 3. Fluorimetric assay to determine the relative *ter* promoter activity using the reporter gene *gfpUV*. For each *E. coli* strain bearing the corresponding plasmids, the fluorescence (RFU) was averaged from 15 separate measurements and the standard deviations were calculated.

The first positive interaction between TerW protein and PPRR was confirmed by incubating purified His-TerW with a fragment containing the *ter* promoter region; the interacting mixture was resolved by native PAGE and identified by non-radiochemical EMSA. The gel band was gradually retarded and smeared following the addi-

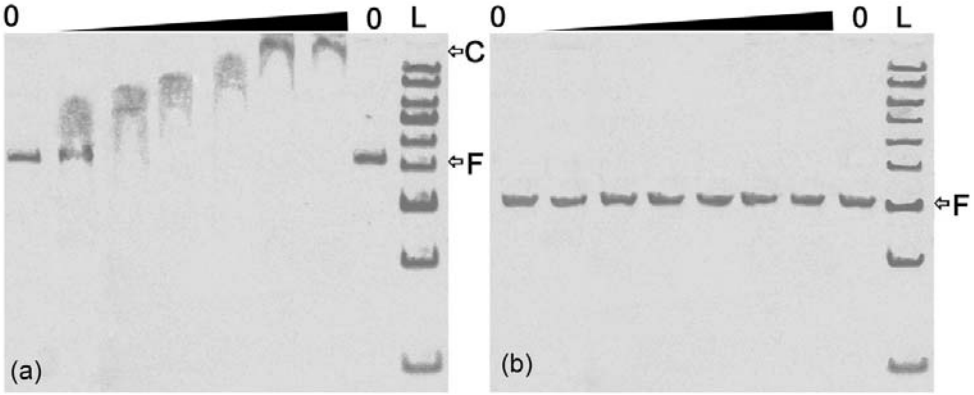

Fig. 4. Gel retardation assay. (a) In electrophoretic mobility shift experiments performed in the presence of an 838–base pair DNA fragment (DV3-ZfuzR), purified His-TerW at a concentration of 0.1–20 μg was bound to the *terZABCDE* promoter region. (b) A negative control performed with a 521-base pair DNA fragment (SL1–LVr4) not expected to be recognised by His-TerW (0.1–20 μg). The lanes marked L contained a molecular weight standard. Capital letters C and F represent protein-DNA complexes and free DNA fragment, respectively.

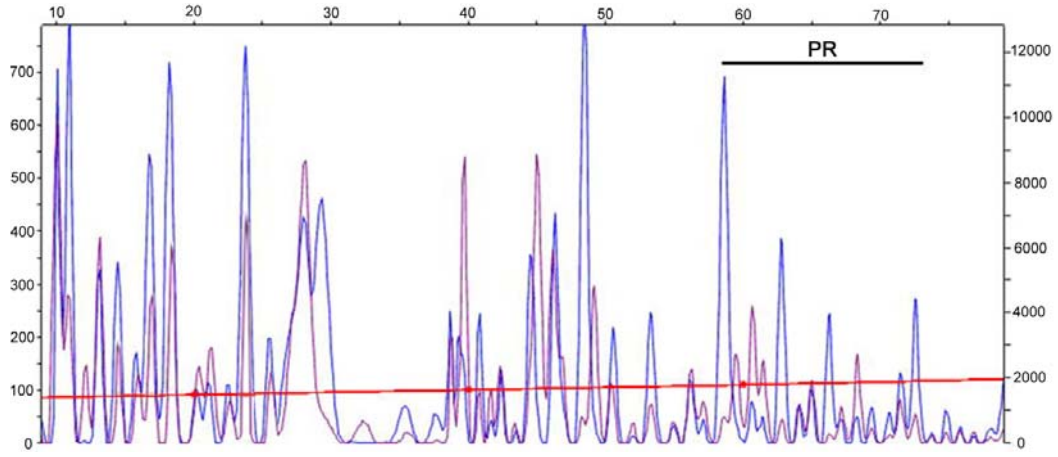

Fig. 5. Footprint analysis. The fragment profile showing the protection pattern of the *terZABCDE* PPRR fragments resulting from digestion with DNase I after an incubation in the presence (violet peaks) or absence (blue peaks) of TerW protein. The area showing significant differences in the peak profiles is remarked as PR. The fluorescence intensity of FAM-labelled DNA fragments (y axis) is plotted against the sequence length of the fragment (PTforward-Len1-FAM probe) (x axis).

tion of increasing protein concentrations (Fig. 4). Such behaviour suggested that TerW bound to the potential DNA promoter region and a native DNA/protein complex was formed. Regulation of this promoter was also confirmed by the presence of GFPuv fluorescence in the *E. coli* BL21(DE3) strain expressing the plasmid pMBAD1 (or pMAC1) but not in cells that also contained the expression plasmid pRSFW1b. In the latter case, bacteria with pMBAD1 (or pMAC1) decreased the GFPuv fluorescence due to the repression of the *terZABCDE* promoter by TerW overproduction (Fig. 3).

To confirm the DNA binding function of TerW protein using traditional methods, i.e., fluorescence measurement and gel retardation, we verified the non-radiochemical DNase I footprint assay using fluorescent dyes and CE. Peak Scanner software allowed us to superimpose electropherograms from CE and to compare the fragment patterns. Negative control reactions were included in each assay; these reactions contained no protected regions and showed uniform peaks across the whole potential promoter DNA fragment after cleavage because the TerW DNA binding protein was replaced with BSA. The resulting DNA fragment profiles after digestion in the absence and the presence of the DNA-binding protein TerW were compared. Dissimilarities between the protected and unprotected fragments were simultaneously examined by data assimilation in the same genotyping file. The protected DNA region of interest on the FAM-labelled strand matched our predicted TerW binding function and partially overlapped with the hypothetical promoter (Fig. 5).

The possible influence of TerW overproduction on the expression of the *ter* operon was evaluated by qRT-PCR analysis. The *terZ*, *terB* and *terC* genes were chosen as representatives of the *terZABCDE* operon, together with the protective *terW* gene. We detected that the mRNA level of *terZ*, *terB* and *terC* was up-regulated by the presence of IPTG-induced TerW overproduction (Fig. 6). The results, also suggested the up-regulation function of the *terW* gene product, were obtained by the Te^R susceptibility testing. The MICs of potassium

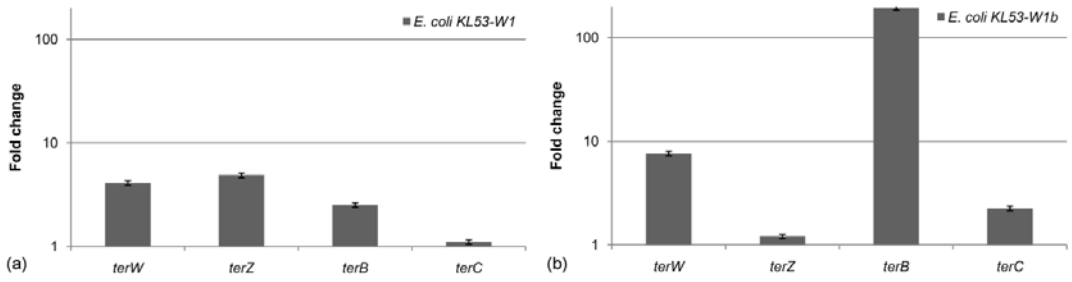

Fig. 6. The effect of TerW overproduction on *ter* gene expression using qRT-PCR. The housekeeping gene encoding *tufA* was used to normalise the RNA input and the level of *ter* expression was determined in the following IPTG-induced strains: (a) *E. coli* KL53-W1 and (b) *E. coli* KL53-W1b. Gene expression was calculated relative to the results obtained from the relevant non-IPTG-induced samples.

tellurite for the *E. coli* KL53-W1 and *E. coli* KL53-W1b strains were increased to 9 mM and 5 mM, respectively, whereas the original uropathogenic isolate *E. coli* KL53 had an MIC of 4.5 mM potassium tellurite.

Discussion

The regulation of heavy metal resistance is usually mediated by specific activators or repressors (Silver & Phung 1996). The precise molecular mechanisms used by cells to cope with tellurite have not been fully elucidated. Previous studies have shown that tellurite exerts various effects on the physiology of several enteric pathogens, leading to increased concentrations of intracellular oxygen reactive species and triggering oxidative stress (Perez et al. 2007; Tremaroli et al. 2007). The interaction of tellurite with the membrane redox systems and thiol compounds has been suggested to play an important role in tellurite reduction and its detoxification (Zannoni et al. 2008). Although the plasmid-borne or chromosomally located *ter* clusters have been extensively studied, the real functions of the Te^R genes are not well understood. The introduction of the *ter* operon from uropathogenic *E. coli* KL53 into non-pathogenic and uropathogenic *E. coli* (*ter*-gene free) showed that the presence of the *ter* loci improved the fitness of bacterial strains by enabling them to survive inside macrophages (Valkova et al. 2007). Deletion of the Te^R region reduced the ability of the strain to adhere to epithelial cells *in vitro* and in ligated pig ileal loops (Yin et al. 2009). In spite of the rare occurrence of tellurite in nature, the acquisition of the *ter* operon could offer some selective advantage to pathogens in natural environments, such as the urinary tract of humans, and facilitate colonisation despite immune assault.

Determination of the expression level of the *ter* genes in uropathogenic *E. coli* KL53 revealed that *terW* showed 4–5 times lower expression when compared to the *terZABCDE* loci (Vavrova et al. 2006). The real time PCR assay also confirmed that the *ter* gene cluster in *E. coli* KL53 and its regulatory region are not inducible by potassium tellurite and/or hydrogen peroxide (Vavrova et al. 2006). This observation is in contrast to the regulation of the *ter* operon in the urinary pathogen *Proteus mirabilis*, in which transcription is induced by oxidative stress (Toptchieva et al. 2003). The original intention of this work was to characterise the genetic basis for the potential promoter region of the *ter* operon genes (*terZABCDE*) encoding Te^R in clinical isolate *E. coli* KL53. The re-cloned promoter sequence of the *ter* operon exhibited lack of homology to the *Proteus mirabilis* regulatory region, which is in agreement with the previously published failure to identify induction conditions necessary to achieve higher than basal expression of the *ter* genes (Vavrova et al. 2006). Based on these assumptions, we can conclude that the differences between inducible and constitutive *ter* determinants may be connected with the loss of a transcriptional repressor and changes in an unrelated promoter part of the *ter* operons. The present study demonstrates the potential regulatory function of overexpressed TerW protein, which is the first known function of the *ter* gene products involved in Te^R pathway.

The cloning of the *ter* genes has previously revealed their paradoxical nature. These genes protect many Enterobacteria from the toxic effects of potassium tellurite but parts of the *ter* operon, especially *terA* and *terZ*, have also been shown to be toxic to host cells when overexpressed or expressed without the protective *terW* gene product. The unusual filamentous morphology of *E. coli* cells harbouring the *ter* genes cloned into the pUC13 plasmid (plasmid pKFW4A) was described by Whelan et al. (1997), who divided the operon into protective (upstream of *terZ* gene) and toxic (downstream of, and including, the *terZ* gene) regions. They found that chromosomal DNA replication was not inhibited and proposed that the unusual filamentous morphology was caused by the inhibition of cell division. Using Tn1000 transposon mutagenesis they have shown that insertions in the *terZ*, *terA* and *terB* genes inhibited the filamentation, and the cells expressing these derivatives of pKFW4A had normal cell length. We have observed the same elongated phenotype in our *in vivo* sub-clone of a large conjugative *E. coli* plasmid pTE53, pNT3B (Tu et al. 2001), and also in an *in vitro* sub-clone, pJS4 (Vavrova et al. 2006). The pJS4 sub-clone expresses the *ter* genes in the presence of *terW* cloned into the pBluescript SK(+) phagemid. The plasmid pLK18, which is also an *in vitro* clone of pTE53, bearing only the essential *ter* genes (*terB*, *terC*, *terD*, *terE*, Δ*terF)*, did not show the elongated phenotype. Together with our many unsuccessful attempts to express a larger part

of the promoter, including more than 36 bp of the *terZ* gene, we can conclude that the *terZ* gene is the most toxic one. This is comparable to the phenotype of the other Te^R operon, which is found on a broad-host-range IncPα plasmid RK2 and known as *KilA-klaAB*. A derivative of RK2Ter, namely the *klaAB* gene, has been shown to mediate Te^R and λ phage development inhibition and also interfere with cell division (Saltman et al. 1991, 1992; Turner et al. 1994). The analogue of *terW* in this case might be *korA* (kill-override), which regulates transcription from the *kilA* promoter. However, the mechanism behind this biological control is not clear and the *kilA/klaAB* operon genes exhibit no similarity with our *ter* determinant. We suppose that the mechanism of their biological control is similar to that of the *terW/terZ(A)* genes, and the *ter* operon (*terZABCDE*) is naturally controlled by the *terW* gene product.

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