BioMetals (2006) 19:453–460
DOI 10.1007/s10534-005-4862-8

Analysis of the tellurite resistance determinant on the pNT3B derivative of the pTE53 plasmid from uropathogenic *Escherichia coli*

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Received 18 July 2005; Accepted 7 November 2005

Key words: *Escherichia coli*, expression of *ter* genes, horizontal gene transfer, real-time PCR, tellurite resistance

Abstract

We have found and sequenced a significant part of the previously described tellurite resistance determinant on mini-Mu derivative pPR46, named pNT3B, originally cloned from a large conjugative plasmid pTE53, found in *Escherichia coli*. This plasmid contains genes essential for tellurite resistance, together with the protective region bearing genes *terX*, *Y*, *W*, and the conserved spacing region bearing several ORFs of unknown function. Computer analysis of obtained sequence revealed a close similarity to the formerly described *ter* operons found on the *Serratia marcescens* plasmid R478 and the chromosome of *Escherichia coli* O157:H7. This finding confirms the presence of a whole region on the large conjugative plasmid that pTE53 originated from a uropathogenic *E. coli* strain, and suggests its possible role in horizontal gene transfer, resulting in the development of new pathogenic *E. coli* strains.

Introduction

Despite the rare occurrence of tellurite in nature, the determinant of tellurite resistance encoded by *ter* genes has been widely found in microbial flora, mostly within pathogenic microorganisms. It has been detected on the large conjugative plasmids of *Serratia marcescens* (Whelan *et al.* 1995), *Alcaligenes* sp. (Jobling & Ritchie 1988), *Klebsiella pneumoniae* (Chen *et al.* 2004), and was also incorporated into the chromosome of *Proteus mirabilis* (Toptchieva *et al.* 2003) and *Escherichia coli* O157:H7 (Perna *et al.* 1998). In this emergent hemorrhagic *E. coli* human pathogen the determinant is part of a large island of pathogenicity called TAI (tellurite resistance- and adherence-conferring island, Tarr *et al.* 2000). The latest study on the variability of this island in *E. coli* O157:H7 isolates (Taylor *et al.* 2002) gave us a strong impulse to intensify our studies of tellurite resistance encoded on the previously described pTE53 plasmid which was originally isolated from uropathogenic strain *E. coli* KL53 (Burian *et al.* 1990).

We have previously described and characterized pTE53 plasmid by *in vitro* and *in vivo* cloning approaches (Burian *et al.* 1998; Tu *et al.* 2001). The *in vitro* clone pLK18 contained the funtional part of the tellurite resistance operon, encompasing *terBCDEF*. The *terBCDE* genes were shown to be essential for the resistance by the Tn1737Km transposition mutagenesis approach (Kormut'áková *et al.* 2000). For *in vivo* cloning, a low copy mini-Mu derivative pPR46 was used resulting in the pNT3B plasmid containing the whole tellurite resistance operon together with surrounding DNA regions. A significant part of the *ter* operon from pNT3B has been sequenced

at present work [Acc. N. AJ888883] and it was analyzed together with the previously sequenced regions of the operon, found on pTE53 [Acc.N. AJ238043.1].

Escherichia coli represents in many cases a very useful model for phylogenetic as well as evolutionary studies, the sequences of several genotypes have been established to date and the evolution of pathogenic forms was very well documented, for example, in the case of the emergent human pathogen *E. coli* O157:H7 (Perna *et al.* 1998; Park *et al.* 1999; Wick *et al.* 2005). Our detection of the tellurite resistance determinant on a conjugative *E. coli* plasmid provides a missing piece in the puzzle, namely how do pathogenic forms of *E. coli* have the opportunity to arise *de novo* in nature. This fact is also important for detection of pathogens in clinical microbiology, since the commonly used commercial selection medium for verotoxigenic *E. coli* O157:H7 (Zadik *et al.* 1993) is based on tellurite resistance detection.

Materials and methods

Bacterial strains, plasmids and media

All bacterial strains used in this study were from the collection of Department of Molecular Biology, Comenius University in Bratislava. Strain *E. coli* KL53 is the original clinical isolate from the Department of Urology, Faculty of Medicine in Bratislava (Burian *et al.* 1990), *E. coli* O157 KMB is the isolate from the State Veterinary and Food Institute in Bratislava. Plasmid construction and DNA manipulations were carried out with standard laboratory *E. coli* strains DH5α (Woodcock *et al.* 1989), XL1-blue (Bullock *et al.* 1987) and MC4100 (Silhavy *et al.* 1984). The plasmid pJS4 was prepared in this study by the cloning of the *Bgl*II fragment from plasmid pNT3B (Tu *et al.* 2001) into the cloning vector pBluescript II KS+ (Alting-Mees & Short 1989). The MC4100 *E. coli* has been transformed with the pJS4 generating the DV2 strain. *E. coli* MC4100 containing plasmid pNT3B was named DV3. All strains bearing *ter* genes determinant, O157 KMB including, have the same tellurite MIC as KL53 strain 5 mmol l^{-1}.

The H_2O_2 MIC was 45 mmol l^{-1} for strains DV2, DV3 and MC4100, 50 mmol l^{-1} for KL53 and 65 mmol l^{-1} for *E. coli* O157 KMB isolate.

Escherichia coli cells were cultivated overnight at 37 °C in Luria-Bertani (LB) medium, with addition of the appropriate selection agent. Antibacterial agent concentrations were used as follows: K_2TeO_3 (Biomark Laboratories) in range 100 μmol l^{-1}–10 mmol l^{-1} and H_2O_2 5–100 mmol l^{-1} (for MIC determination), ampicillin 100 μg ml^{-1} and chloramphenicol 34 μg ml^{-1} for recombinant cells selection. 1 mmol l^{-1} K_2TeO_3 or 5.3 mmol $l^{-1}H_2O_2$ were added to cell cultures in LB cultivation medium for induction of transcription and for following RNA isolation. The cells were pelleted 10 min after the induction.

Preparation of partial subclones of pNT3B and DNA sequencing

Plasmid DNA for sequencing was isolated with the commercial kit CONCERT^{TM} (GibcoBRL). Plasmid pNT3B was subcloned into the multicopy cloning vector pBluescript II KS+ (Stratagen) with the aim to obtain fragments suitable for DNA sequencing. DNA samples were sequenced by the automatic sequencer ABI 3100-Avant Genetic Analyser (Applied Biosystems) with the use of standard M13 sequencing primers. Finally, several gaps among contigs were closed either by primer walking on selected clones or by sequencing the DNA amplicons generated by PCR. The sequence was submitted to the EMBL Nucleotide Sequence Database with accession number AJ888883.

Bioinformatic analysis of sequences obtained

The 15,719 bp assembly of sequences [AJ888883] and [AJ238043.1] containing two parts of tellurite resistance determinant has been used for search for potential ORFs (with minimal length of 50 codons) based on the codon usage method (Staden & McLachlan 1982) by the Staden Package tool *Spin* (Staden 1996). The nucleotide and polypeptide sequences of predicted ORFs were compared with the non-redundant nucleotide and protein databases, using the BLAST tools (Altschul *et al.* 1990) available on the BLAST web page (http://www.ncbi.nlm.nih.gov/BLAST) at the NCBI of the United States National Institute of Health. For these searches the default settings of the BLAST ($E < 10$) were used. The

search for potential promoters was performed by the promoter prediction software freely available on the Berkeley Drosophila Genome Project web site (http://www.fruitfly.org/seq_tools/promoter.html). This server runs the 1999 Neural Network Promoter Prediction (NNPP) version 2.2 (March 1999) of the promoter predictor (Reese 2001). We specifically searched for prokaryotic type promoters, on both strands of the assembled sequence, with a minimum promoter score 0.9 for more reliable hits.

Polydot (Dot plot) analyses were performed using DotMatcher software, the EMBOSS tool publicly available on the SRS server of the Institute of Molecular Biology of SAS (http://srs.embnet.sk:8080/srs81/). For alignments windows size 40 and alignment threshold 75 were settled.

Extraction of total DNA, total RNA and preparation of cDNA

Total DNA was prepared by the use of DNeasy® Tissue Kit provided by Qiagen.

Total RNA was isolated from *E. coli* cultivated at different conditions by phenol-chloroform extraction. 6 ml of culture (OD_{600} 0.4–0.5) was pelleted by centrifugation. The cells were resuspended in 500 μl of lysation buffer (150 mmol l^{-1} sucrose, 10 mmol l^{-1} natrium acetate, pH 5.2 and 1% SDS) heated at 70–75 °C and the mixture was incubated at this temperature for 1 min. After the addition of 500 μl hot phenol solution (phenol with 100 mmol l^{-1} natrium acetate pH = 5.2) the mixture was vortexed and centrifuged. The water phase was extracted once with chloroform-izoamylalcohol (mixture 24:1) and total RNA was precipitated by ethanol. RNA was stored in RNase free water at −70 °C and the integrity of RNA was verified prior its use by agarose gel electrophoresis. About 10 μg of isolated RNA was purified by the commercial SV Total RNA Isolation System (Promega,) with dramatically increased the DNase treatment time (80 min) compared to manufacturer instructions. About 1 μg of total RNA was transcribed into cDNA by the standard protocol of the commercial ImProm-II™ Reverse Transcription System (Promega) and the use of universal hexaoligonucleotid primers.

Quantitative real time PCR

The primers used to detect the expression of particular *ter* operon genes as well as primer for the normative control *tufA* were designed based on known *Serratia marcescens* [Acc. No. NC005211] and *E. coli* K12 MG1655 [Acc. No. U00096.2] sequences in the database by the programme PrimerExpress (Applied Biosystems) and are shown in Table 1. All PCR products were of identical length 100 bp.

Real time PCR was performed in a thermocycler ABI Prism 9600 HT (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 programme consisted of an initial denaturation of 10 min at 94 °C and 40 cycles of 15 s at 94 °C, 1 min at 10 °C followed by a melting curve determination. The level of expression of particular genes in different cDNA samples was calculated based on a calibration curve, where the total DNA of *E. coli* KL53 in 10 fold dilutions was used as a template. Three parallel samples were measured for each calculation and the expression of *tufA* was used for normalizations.

Table 1. List of Real Time PCR primers.

Primer	5′–3′ Sequence
tufAF	CCGCAGACTCGTGAGCACAT
tufAR	AGCAGCTCTTCGTCATCAACCA
terZF	TTCCGTGGTCAGTCGTTCAA
terZR	GAGCCCTGCTCAGTCAGCTT
terAF	AGGGTGATGCGGACATGGT
terAR	GAGCGCGACAGTAAAGGTTGA
terBF	AATCGGCAAGGGCGAAAC
terBR	CACTTTTCGCAACGGCAAT
terCF	TGTTTATGCACCGTGATGACAA
terCR	GTAGAGGAAACCGGCAAATGC
terDF	CCGTCCCGTCTGATGTTGA
terDR	GCGAATAAACGCACCGGATA
terEF	GACAACGGCACGGAAATTG
terER	TTCACTCAGCGCCATGA
terFF	CGGACAAAACATACCGCTTCA
terFR	GCAGGAAGAGGCAGGTATCG
terWF	ACCAGACAGGCCCGGATT
terWR	GTCGGCTCTGAGCATTCCA
terXF	TCTCGGCAATGTGGAAAATG
terXR	CGCCCGTCAGCCAGATATT
terYF	AAGGATCCACACGCACTTGAA
terYR	GGGTAGAACGAGGCAATTTCG

Results

Preparation of DNA clones for sequencing and their identification

A restriction map of the *in vivo* cloned pNT3B was prepared for further DNA cloning. According to this map suitable DNA fragments were chosen and subcloned for sequence analysis. When necessary, additional PCR primers were designed from the sequence obtained for PCR sequencing. The whole sequence of 10,937 bp has been submitted to EMBL database with AJ888883 Access Number. Assembled sequences from *in vivo* and *in vitro* clones of the pTE53 determinant showed the same organization of the predicted operons. The comparison of our sequence with the sequences present in database revealed a close relationship to that of the IncHI2 plasmid R478 from *Serratia marcescens* and to chromosomal sequence of *E. coli* O157:H7 (96–99%). Apart from *terZABCDEF* region these similarities were found also in the presence of *terW*, *terY* and *terX* on pTE53 plasmid and spacing region between them (Figure 1).

Analysis of obtained sequence

The search for potential promoters showed three potential promoter rich regions (PPRR in Figure 1) with high score, which is in accordance with our results of PCR reactions done on cDNA, namely that the structural genes *terZ-terE* are translated from a common transcript, different from *terF* gene (more details in *Transcriptional units identification* subchapter).

The Dot plot analyses performed using DotMatcher software showed a close relationship of the structural *ter* genes among themselves. The proposed proteins contain conserved domains TerD and TerZ (gnl|CDD\25899 and gnl|CDD|11989) ascribed to the bacterial stress protein family with common cAMP binding site. Appearance of these domains is shown in Figure 2.

Cloning of toxic genes and preparation of the two-plasmid co-expression system

While subcloning pNT3B, we were not able to obtain clones encompassing *terZ*, *terA* genes, together with the proposed promoter rich region of the whole *terZ-F* operon. We succeeded to clone this critical part of the determinant only in the presence of *terW* gene, which is known to be a protective factor against the toxicity of *terZ* and*terA* genes (Whelan *et al.* 1997).

For this purpose we cloned the *terW* gene into the multi copy cloning vector pBluescript II KS+. The *terZ* and *terA* genes were introduced into the medium copy plasmid pACYC184, to lower the

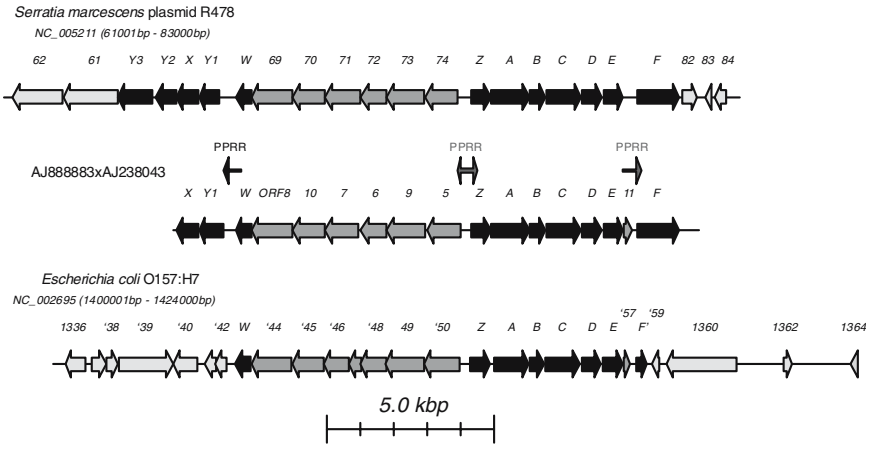

Figure 1. The alignment of tellurite resistance operon genes and their neighboring ORFs from three microbial sources: Uppes most, the 22 kbp region (61–83 kbp, ORFs from SMR0062 to SMR0084) of *Serratia marcescens* plasmid R478 [NC 005211], center, the 15,719 bp assembly of AJ888883 and AJ238043 from *Escherichia coli* plasmid pTE53, and bottom, the 24 kbp region (14–14.24 kbp, ORFs from ECs1336 to ECs1364) of the *Escherichia coli* O157:H7 strain Sakai complete genomic sequence [NC 002695]. In black color are the *ter* genes of the tellurite resistance operon and their associates; dark grey, the genes of the highly conserved tellurite resistance associated operon of unknown function and ligh grey, chromosomal genes of unknown function from close the vicinity of the tellurite resistance operon. The potential promoter rich regions (PPRR) in pTE53 sequence are shown by arrows.

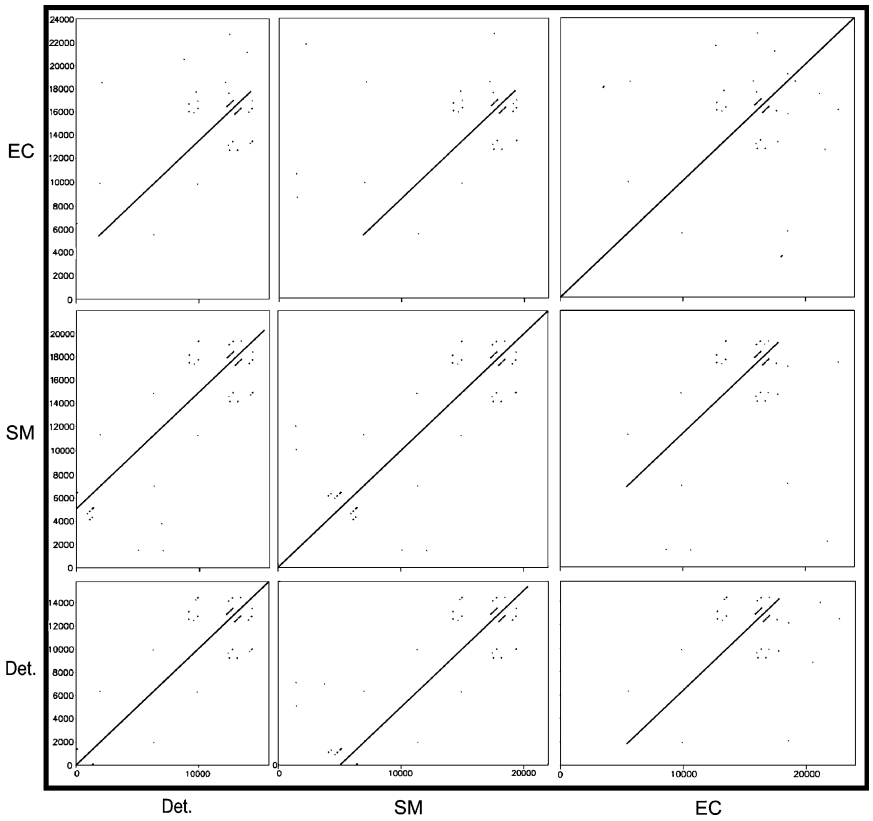

Figure 2. Polydot plot image of the tellurite resistance operon and the vicinity from the 15.7 kbp pTE53 determinant sequence, 22 kbp fragment of *Serratia marcescens* plasmid R478 [NC 005211], and 24 kbp genomic fragment of *Escherichia coli* O157:H7 strain Sakai [NC 002695]. A windows size of 40 and an alignment threshold 75 was used, obtained results were manually edited for better comparision.

gene dose of these toxic genes. By simultaneous transformation of both recombinant plasmids we developed a two-plasmid co-expression system, obtaining recombinant *E. coli* cells expressing *terZ*, *terA* genes in the presence of *terW* gene, and we confirmed the protective role of its gene product (data not shown).

Expression of ter genes in different cultivation conditions

We have determined the level of expression of the chosen *ter* genes in *E. coli* KL53, cultivated under several different conditions by real time PCR. For this purpose we designed primers for particular genes as well as for the *tufA* encoding translational elongation factor, which was used for the normalization of our results (Table 1). Firstly, we compared the basal expression of *ter* genes in LB medium, the obtained results are shown in Figure 3. It is clear that relatively high expression of *ter* genes is present without the addition of tellurite into the cultivation medium. The highest expression was detected for *terC*, however it represented only 1/30 of the level of *tufA* gene expression.

The level of *terZABD* gene expression was quite similar to the *terC*, suggesting the same transcript for entire genes, with a maximal twofold aberration. However, *terE* and *terF* genes located in the end of the operon showed lower level of expression, about 10 times less for *terE* and 30 times less for *terF*, when compared to the *terZABCD* genes. The *terWXY* genes are located 7 kbp upstream of the proposed *terZABCDE* promoter and have the opposite orientation. The level of *terW* gene expression is 4–5 times lower than that for *terZABCD* genes and similar level was detected also for *terX* and *terY*.

The possible influence of potassium tellurite or hydrogen peroxide on the expresion of the *ter*

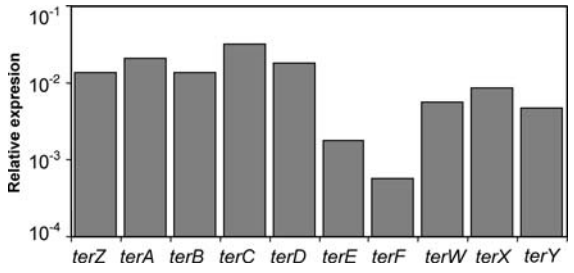

Figure 3. Relative expresion of *ter genes in E. coli* KL53. Expression was calculated as a ratio of the particular gene cDNA concentration to the concentration of *tufA* cDNA used as the normalization control. The *E. coli* KL53 was cultivated in LB medium (without tellurite) until a mild exponential growth.

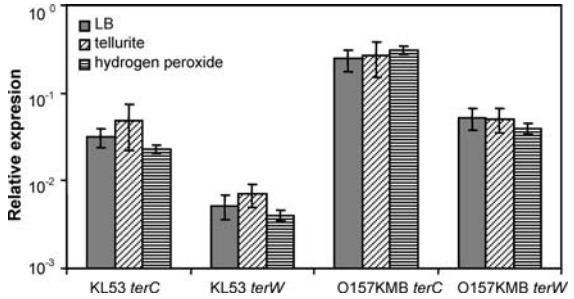

Figure 4. Expression of *ter C* and *ter* W genes in different growth conditions. *E. coli* strains KL53 and O157:H7 KMB were grown in LB medium until the mild exponential growth phase, then the medium was supplemented with 1 mmol l^{-1} potassium tellurite (shaded columns) or with 5.3 mmol l^{-1} H_2O_2 (hatched columns) or no addition was used (LB, filled columns) and cultivation continued for next 10 min. After this time the level of *terC* and *terW* expression was measured by real time PCR using the *tuf*A gene for normalisation.

operon was evaluated. The *terC* was chosen as a representative of the *terZABCDEF* operon together with the protective *terW* gene in this experiment. The level of *ter* expression in strain *E. coli* KL53 (the original clinical isolate containing plasmid pTE53) was compared with the expression of the same genes in *E. coli* O157 KMB strain (Figure 4). We detected that the level of *terC* and *terW* mRNAs was not influenced by the presence of either oxidative agent. The same results were obtained after the overnight induction of these strains in LB medium containing 1 mmol l^{-1} K_2TeO_3 or 5.3 mmol l^{-1} H_2O_2 (data not shown). The expression of *ter*C and *ter*W genes in the *E. coli* O157 KMB was 8–10 times higher than the expression of the same genes in the *E. coli* KL53 strain, however both strains had the same the minimal inhibitory concentration of 5 mmol l^{-1} K_2TeO_3.

Transcription units identification

Our previous findings were in agreement with our assumption that the *terZABCDE* genes are transcribed from a common promoter that is located upstream to these genes. To confirm this prediction we used the PCR primers designed for the real time PCR to amplify the neighboring genes on the cDNA and we were able to confirm that *terZABCDE* but not *ter*F are transcribed on one mRNA unit (Figure 5). Similary, the *terW* gene is transcribed separately from the *terXY* (data not shown) that is in accordance with the map of *ter* operon from *E. coli* KL53 (Figure 1).

Discussion

Both approaches to the cloning of the tellurite resistance determinant from clinical isolate *Escherichia coli* KL53 have their advantages and disadvantages. The previously characterized *in vitro* clone pLK18 revealed the minimal clonable part of the operon, essential for the tellurite resistance phenotype (Kormut'áková *et al*. 2000). A main disadvantage of this approach was that only a part of a large island of mutualy interacting genes was described. The tellurite resistance operons with intensive sequence homology to the genes from pTE53 were also described in IncPa plasmid RK2, isolated from *Pseudomonas aeruginosa* (Bradley *et al*. 1988), IncHI-2 plasmid pMER610 from an *Alcaligenes sp*. (Jobling & Ritchie 1987), IncHII plasmid pHH1508a from *Klebsiella aerogenes* (Walter & Taylor 1989), IncHI2 plasmid pR478 from *Serratia marcescens* (Whelan *et al*. 1997) as well as on chromosome of *E. coli* O157:H7 (Perna *et al*. 2001) and *Proteus mirabilis* (Toptchieva *et al*. 2003). This determinant provides *Escherichia coli* with protection from the toxic effects of potassium tellurite as well as some bacteriophages (Phi) and pore-forming colicins (PacB). In spite of its intensive studies the mechanism of tellurite resistance remains unknown, clear is only the result of the process – reduced metal tellurium deposited inside the resistant bacterial cells (Lloyd-Jones *et al*. 1994). However, the more recent study by Whelan *et al*. (1997) had shown that part of *ter* operon, especially genes *terZ* and *terA* were toxic to host cell, this effect could be reversed by the simultaneous expression of *terW* protective gene.

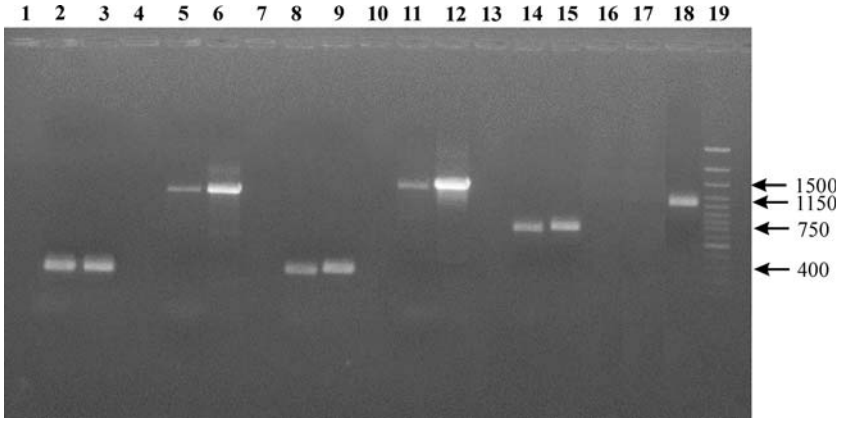

Figure 5. PCR products joining the neighboring *ter* genes. Real time PCR primers were used to amplify the neighboring fragments of DNA as follows: terZF-terAR for lanes 1–3, terAF-terBR for lanes 4–6, terBF-terCR for lanes 7–9, terCF-terDR for lanes 10–12, terDF-terER for lanes 13–15, terEF-terFR for lanes 16–18. Three different types of templates were used: RNA template for lanes 1,4,7,13 and 16; cDNA for lanes 2,5,8,11,14 and 17; total DNA of KL53 *E. coli* strain for lanes 3,6, 9,12,15 and 18. Lane 19 contains 100 bp Ladder, MWS provided by Fermentas; the arrows on the right indicate the size of amplified DNA fragments given in bp.

We had observed the same finding and therefore we developed the two-plasmid co-expression system to clone the toxic genes together with their natural regulatory upstream region for further characterization. Although the previous authors have not found any homology between these toxic genes and known cell division elements, we detected weak similarity of one internal probe of *terZ* gene designed for RT-PCR with the *ftsZ* gene, responsible for the site of septation localization. If this is true, it may explain why the cells expressing *terZ*/*terA* genes lose normal cell division ability, resulting in a long filamentous phenotype observed in many of our clones, expressing these genes. However the protective function of TerW remains unclear.

Toptchieva *et al*. (1999) have published the oxidative stress inducibility of the *ter* operon from *Proteus mirabilis* but this operon has its own promoter, different from the determinant encoded on pTE53. Using the real time PCR we have shown that transcription of *ter* genes is independent from the presence of any inducer (both tellurite of hydrogen peroxide were tested). This finding is in accordance with the expression of *ter* operon in *E. coli* O157:H7 presented by Taylor *et al*. (2002).

The tellurite resistance determinant represents a deceptive biological paradox phenomenon itself. It provides the bacterial cell with resistance against tellurium although tellurium is very rare in nature. However, tellurite salts are strong oxidative agents and it is likely that resistance to oxidative agents provides bacterial cells, especially pathogenic microorganisms, with a selective advantage in the mammalian host. Our present experiments are aimed to show that the introduction of *ter* operon into non-pathogenic or uropathogenic *E. coli* resulted in a substantial increase in survival inside macrophages (data unpublished).

Acknowledgements

This work was supported by APVT-20-017102 project and a grant from Comenius University Nr. 150/2003/UK. We would like to thank Dr. Philip Brewer for his valuable suggestions and critical review of the manuscript.

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