Antibiotic-Dependent Induction of *Pseudomonas putida* DOT-T1E TtgABC Efflux Pump Is Mediated by the Drug Binding Repressor TtgR

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Received 19 May 2003/Returned for modification 13 June 2003/Accepted 8 July 2003

*Pseudomonas putida* is well known for its metabolic capabilities, but recently, it has been shown to exhibit resistance to a wide range of antibiotics. In *P. putida* DOT-T1E, the TtgABC efflux pump, which has a broad substrate specificity, extrudes antibiotics such as ampicillin, carbenicillin, tetracycline, nalidixic acid, and chloramphenicol. We have analyzed the expression of the *ttgABC* efflux pump operon and its regulatory gene, *ttgR*, in response to several structurally unrelated antibiotics at the transcriptional level and investigated the role of the TtgR protein in this process. *ttgABC* and *ttgR* are expressed in vivo at a moderate basal level, which increases in the presence of hydrophobic antibiotics like chloramphenicol and tetracycline. In vitro experiments show that, in the absence of inducers, TtgR binds to a palindromic operator site which overlaps both *ttgABC* and *ttgR* promoters and dissociates from it in the presence of chloramphenicol and tetracycline. These results suggest that the TtgR repressor is able to bind to structurally different antibiotics, which allows induction of TtgABC multidrug efflux pump expression in response to these antimicrobial agents. This is the first case in which the expression of a drug transporter of the resistance-nodulation-division family has been shown to be regulated directly by antibiotics.

The increasing antibiotic resistance in bacteria is creating a critical situation that may reverse the enormous medical gains experienced with the use of effective antibiotics. Resistance can be caused by diverse mechanisms involving decreased antibiotic accumulation, physical modification or destruction of the antibiotics, and alteration of the enzyme target of these agents. In recent years, a mechanism of resistance involving the active efflux of antibiotics by pumps has been elucidated. These efflux systems are broadly specific and able to accommodate a variety of structurally unrelated antimicrobial agents, such as antibiotics, biocides, dyes, detergents, fatty acids, and organic solvents (18, 26, 27, 30, 33).

Efflux systems capable of transporting multiple antimicrobials fall into six classes: the major facilitator superfamily, the ATP-binding cassette family, the resistance-nodulation-division (RND) family, the small multidrug resistance family (which is itself a member of the drug-metabolite transporter superfamily), the multidrug and toxic compound extrusion family, and the multidrug endosomal transporter family (37). The members of the RND family are the most relevant in respect to resistance to clinically important agents (30). Originally thought to be limited to gram-negative bacteria, members of the RND family have now been identified in all major kingdoms (44). In gram-negative microorganisms, RND transporters work in conjunction with a periplasmic membrane fusion protein (MFP) and an outer membrane protein, now called outer membrane factor (OMF) (47), which facilitates the removal of antibiotics from the periplasm or the outer leaflet of the inner membrane (7, 25, 43). RND/MFP/OMF-type multidrug efflux systems have been described in various microorganisms including *Escherichia coli*, *Salmonella enterica* serovar Typhimurium, *Haemophilus influenzae*, *Neisseria spp.*, *Pseudomonas aeruginosa*, *Pseudomonas putida*, *Burkholderia spp.*, *Campylobacter jejuni*, and *Stenotrophomonas maltophilia* (19, 30, 41, 47). The range and number of agents extruded vary from organism to organism, even for pumps with high degrees of amino acid sequence similarity, promoting resistance to a range of clinically relevant antimicrobials. Expression of the majority of the bacterial drug transporter genes is controlled by transcriptional regulatory proteins, either repressors or activators, that are often located upstream from efflux pump operons (12). Some of them (i.e., BmrR [45, 48], EmrR [5, 20], QacR [39, 40], and TetR [16, 28, 29]) are able to bind to substrates of their cognate multidrug transporters and promote an increase in their expression. The drug-binding properties of these well-described bacterial efflux pump regulators have provided new insights into the protein-drug recognition field and, thus, in the design of new and more effective ways to overcome this mechanism of resistance.

Strains of the species *P. putida* are well known for their metabolic capabilities, but recently, their mechanisms of resistance to a wide range of antibiotics and organic solvents have become the object of study (6, 17, 32, 33). In *P. putida* strain DOT-T1E, three homologous efflux pumps belonging to the RND family of bacterial transporters (TtgABC, TtgDEF, and TtgGHI) are involved in solvent tolerance (6, 24, 35). Although these pumps are 70% identical at the protein level, they exhibit significant differences in substrate specificity, and so TtgABC and TtgGHI extrude antibiotics as well as solvents.
Only the TtgABC efflux pump has been shown to be essential for the antibiotic resistance of this strain, as a ttgB-knockout mutant experienced a significant decrease in resistance to an ampicluey of antibiotics (6, 32, 35). On the other hand, the ttgGH operon is expressed at high levels regardless of the growth conditions, and its expression is not increased in response to antibiotics in the culture medium (36). Divergent with respect to these structural genes are the putative regulatory genes: ttgR for ttgABC and an operon, ttgVR, in the case of the ttgGH operon (6, 35, 36). TtgR and TtgV downregulate the expression of ttgABC (6) and ttgGH (36), respectively, but the role of the ttgW gene product in the control of expression of ttgGH remains to be determined.

Given that TtgABC has been shown to extrude multiple antibiotics, we decided to analyze the expression of this pump at the transcriptional level in response to the antibiotics that the pump extrudes. We observed that, among the antibiotics tested, chloramphenicol and tetracycline were able to induce the expression of the ttgABC efflux pump and its regulatory gene, ttgR, and showed that TtgR is a drug-binding repressor involved in the antibiotic induction of this multidrug efflux pump.

**MATERIALS AND METHODS**

**Bacterial strains, plasmids, and culture medium.** The bacterial strain used in this study was *P. putida* DOT-TIE (31). This strain was routinely grown in liquid Luria-Bertani (LB) medium at 30°C (38). Antibiotics were added to the culture medium to reach final concentrations of 20 µg/ml for rifampin and 20 µg/ml for tetracycline. We constructed fusions of the promoters of the *ttgABC* and *ttgR* operons to a promoterless *lacZ* gene in the low-copy-number (two to four copies per cell) PM220 vector (42). The *ttg-ttg* intergenic region (300 bp) was amplified by PCR from *P. putida* DOT-TIE chromosomal DNA, which was isolated as described by Ausubel et al. (4) with primers incorporating EcoRI and PstI restriction sites (primer TTGAEcoRI [5'-CTTCTCTTTTGTTGACCCGGGATTTCG-3'] and primer TTGEcoRI [5'-AAACTGGAGAGAGACCCGGGATTTCG-3']) to create fusions of the *ttgABC* operon to *lacZ* with and without TTGREcoRI (5'-GCCGAATTCAGGAGTTTTAGCC-3') and TTGRPstI (5'-AAACCTGACCGCCTGCGGGG-3') to create a fusion of the *ttgABC* promoter to *lacZ*. PCR (50 µl) were carried out with 2.5 U of Taq polymerase (Amersham-Pharmacia) and consisted of 1 × PCR buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl), 0.2 µM each primer, 0.8 mM each deoxynucleoside triphosphates (Roche), and about 100 ng of template DNA. Cycling parameters were 2 min of incubation at 96°C, followed by 30 cycles of 96°C for 1 min, 55°C for 30 s, and 72°C for 1 min, before finishing with 10 min at 72°C. Upon amplification, DNA was digested with EcoRI and PstI and ligated to EcoRI-PstI-digested pMP220 to produce pED14 (P<sub>ttg</sub>-clacZ) and pED13 (P<sub>ttg</sub>-lacZ). Plasmids pED13 and pED14 were sequenced to make sure that no mutations were introduced in the corresponding promoter regions. These plasmids were electroporated in *P. putida* DOT-TIE as described by Enderle and Farwell (8). Cells in 0.2-ml cuvettes were subjected to a high-voltage pulse (3,000 V) for 5 ms by using a MicroPulse electroporation apparatus (Bio-Rad), and transformants were selected in LB agar plates supplemented with tetracycline.

**DNA techniques.** A Qiagen spin miniprep kit (Qiagen) was used for plasmid isolation. Both strands of the plasmid DNA were sequenced with universal reverse, or specific designed primers by using an automatic sequencing system (ABI-PRISM 310; Applied Biosystems, Inc.).

**Primer extension analysis.** *P. putida* DOT-TIE was grown overnight in LB medium. The cells were then diluted 25-fold in fresh medium, and aliquots were incubated in the absence or presence of sublethal concentrations of antibiotics (carbenicillin [120 µg/ml], chloramphenicol [30 µg/ml], gentamicin [0.25 µg/ml], nalidixic acid [30 µg/ml], streptomycin [8 µg/ml], and tetracycline [1 µg/ml]) until the culture reached a turbidity of 1.0 at 660 nm. The cells (30 ml) were pelleted and processed for RNA isolation by the method of Marque et al. (22). mRNAs of the *ttgBC* pump operon and its corresponding regulator were reverse transcribed and analyzed as described by TtgR-[<sup>32</sup>P]ATP (38). The final reaction mixtures were run on 4.5% (wt/vol) native polyacrylamide gels (Mini-Protean II; Bio-Rad) and sequenced to make sure that no mutations were introduced in the corresponding promoter regions. These plasmids were electroporated in *P. putida* DOT-TIE as described by Enderle and Farwell (8). Cells in 0.2-ml cuvettes were subjected to a high-voltage pulse (3,000 V) for 5 ms by using a MicroPulse electroporation apparatus (Bio-Rad), and transformants were selected in LB agar plates supplemented with tetracycline.

**Overexpression and purification of TtgR.** A 651-bp fragment containing the *ttgR* gene was amplified from *P. putida* DOT-TIE chromosomal DNA by PCR with primers TtgRS/Ndel (5'-AAAAAATGTGGTCCGTCGAACAAACG-3') and TtgRS/hr (5'-AAAAAATGTGGTCCGTCGAACAAACG-3'), which generated Ndel and Xhol restriction sites, respectively. PCRs were carried out as described above. After digestion with these restriction enzymes, the PCR products were ligated into vector pET28a (+) (Novagen), which had previously been digested with Ndel and Xhol. The resulting plasmid, pTGF1, contained the *ttgR*-coding sequence in frame with a DNA sequence encoding a His tag, at its 3' end.

For TtgR-His<sub>6</sub> purification, pTGF1 was transformed into E. coli (b) (B834(DE3)). The cells were grown in several 1-liter batches at 30°C in 2 × YT culture medium (38) with 50 µg of kanamycin per ml to an *A<sub>600</sub>* between 0.5 and 0.7 and then induced with 1 mM isopropyl-β-D-thiogalactopyranoside. Cells were harvested after 3 h of induction at 20°C, resuspended in 25 mM sodium phosphate (pH 7.0)–0.5 M NaCl–5% (vol/vol) glycerol–protease inhibitor cocktail (Complete; Roche), and broken by treatment with 20 µg of lysozyme per ml and a French press. Following centrifugation at 20,000 × g for 30 min, the protein was found to be predominantly (more than 80%) in the soluble fraction. TtgR-His<sub>6</sub> was purified by nickel affinity chromatography with a HiTrap chelating Sepharose column (Amersham-Pharmacia) and eluted with an imidazole gradient. Peak fractions were pooled and dialyzed against TGED (10 mM Tris-HCl [pH 8.0], 5% [vol/vol] glycerol, 0.1 mM EDTA, 1 mM dithiothreitol) to which 50% (vol/vol) glycerol and 30 mM NaCl were added and stored at −70°C (long-term storage) or −20°C (short-term storage). Protein concentrations were determined using the Bio-Rad Protein Assay kit.

**Gel mobility shift assays.** The DNA probe was a 189-bp fragment containing the *ttgBC*-ttgR intergenic region obtained from DOT-TIE chromosomal DNA by PCR with the TtgABC1 (5'-AAGGCGCTCGGCGGCGGCGG-3') and TtgABC2 (5'-GGGAGGGCTCGTCCAGG-3') pair of primers. PCRs were carried out as described above; and cycling parameters were 1 min at 98°C, followed by 30 cycles of 96°C for 1 min, 58°C for 30 s, and 72°C for 30 s, before finishing with 5 min at 72°C. The PCR product was isolated from an agarose gel by using the QIAquick Gel Extraction kit (Qiagen) and was radiolabeled with its 5' ends with [<sup>32</sup>P]ATP and T4 polynucleotide kinase. The labeled probe (3 nM; ∼10,000 cpm) was then incubated with 0.75 µM purified TtgR-His<sub>6</sub> in 10 µl of TGED supplemented with 8 mM MgCl<sub>2</sub>, 20 µg of poly(dI-dC) per ml, and 200 µg of bovine serum albumin per ml. When present, chloramphenicol and tetracycline were added to the binding reaction mixture at concentrations ranging from 0.1 to 1.2 mM. The reaction mixtures were incubated for 10 min at 30°C, and samples were run on 4.5% (wt/vol) native polyacrylamide gels (Mini-Protein II; Bio-Rad) for 2 h at 50 V and room temperature in Tris-glycine buffer (25 mM Tris-HCl [pH 8.0], 200 mM glycine). The results were analyzed by using a Molecular Imager system (GS525 equipment; Bio-Rad).
2.6-fold increases in the levels of expression of both promoters were relatively high (40 arbitrary units (basal level) of expression of these promoters in the absence of antibiotics) before being treated with 40 μg/ml. The quantities of the ttgABC promoters in the presence of several structurally unrelated antibiotics, such as carbenicillin, chloramphenicol, gentamicin, nalidixic acid, streptomycin, and tetracycline, which had previously been reported to be substrates for the TtgABC efflux pump operon and the rpoS gene (an internal control) was carried out as described in Materials and Methods. The quantities of the corresponding cDNA bands are expressed at the bottoms of the gels in arbitrary units (basal level = 100 U).

RESULTS
Expression of P. putida DOT-T1E TtgABC efflux pump and its regulator, TtgR. We have analyzed the expression of the ttgABC efflux pump operon and the ttgR gene under different conditions by measuring the relative amount of mRNA expressed in P. putida DOT-T1E grown in the absence and in the presence of several structurally unrelated antibiotics, such as carbenicillin, chloramphenicol, gentamicin, nalidixic acid, streptomycin, and tetracycline, which had previously been reported to be substrates for the TtgABC efflux pump (6). We observed increases in the levels of the ttgABC and the ttgR mRNAs with respect to the basal level in response to chloramphenicol (4-fold) and tetracycline (2.6-fold) but did not observe increases in response to the other antibiotics (see the results for the ttgABC operon in Fig. 1). We determined the expression of rpoS as an internal control to ensure that the differences observed were not due to variability in RNA isolation and/or the primer extension technique. In Pseudomonas spp. rpoS gene expression is unaltered in the exponential phase under different growth conditions (9, 34).

The expression from the ttgA and ttgR promoters was also determined by using fusions of the corresponding promoters to lacZ in pED14 (P\textsubscript{ttgA}:lacZ) and pED13 (P\textsubscript{ttgR}:lacZ). The levels of expression of these promoters in the absence of antibiotics were relatively high (40 ± 2 U for the P\textsubscript{ttgA} promoter and 120 ± 10 U for the P\textsubscript{ttgR} promoter), but consistent 2.2- to 2.6-fold increases in the levels of expression of both promoters (105 ± 5 U for the P\textsubscript{ttgA} promoter and 265 ± 15 U for the P\textsubscript{ttgR} promoter) were observed in response to chloramphenicol at 30 μg/ml. However, no increase (<1.1-fold) at all was observed in response to the other antibiotics (data not shown). Note that because pED13 and pED14 are derivatives of Tet' plasmid pMP220, it is not feasible to analyze the induction mediated by tetracycline. Therefore, the β-galactosidase assays validated the mRNA levels. It is also worth noting that the expression patterns obtained from the ttgR and ttgA promoters were almost identical, which suggests a common mechanism of regulation for both promoters, also consistent with the fact that both promoters fully overlap.

TtgABC and TtgR expression are correlated with antibiotic concentration. As chloramphenicol was the best inducer of the expression of the TtgABC pump, we decided to analyze how the antibiotic dose influenced the expression of the pump and the regulatory gene. We assayed the β-galactosidase activities from the ttgABC operon and ttgR promoters in wild-type strain DOT-T1E bearing the corresponding promoter fusions on plasmids pED13 and pED14 and grown in the absence and presence of increasing sublethal concentrations of chloramphenicol (6) (Fig. 2). We observed a parallel increase in the levels of expression of both the pump and the regulator with increasing concentrations of chloramphenicol, which suggests that the antibiotic induction is dose dependent and confirms that both operons are regulated in the same way. Therefore, in P. putida DOT-T1E, the expression of ttgABC and its regulatory gene, ttgR, seems to be correlated with cell antibiotic exposure.

TtgR binds to the ttgABC-ttgR intergenic region and is released in the presence of chloramphenicol and tetracycline. It had been reported previously that the efflux pump operon is negatively regulated by the adjacent ttgR gene product, as the basal level of ttgABC expression exhibited a 6- to 10-fold increase in a ttgR-knockout mutant. In this mutant, the basal level of activity of the ttgR promoter was also much higher than that in the wild-type background (6). According to these observations, we expected that an increase in TtgR expression would inhibit transcription from both the ttgABC and the ttgR promoters. However, we have shown here that the levels of expression of both the efflux pump and TtgR are enhanced by the presence of certain antibiotics (Fig. 1 and 2), which could be achieved only if the effectors that induce transcription from those promoters have a direct effect on TtgR repressor func-

FIG. 1. Effects of different antibiotics on the expression of the ttgABC promoter. P. putida DOT-T1E cultures were grown for 4 h in the absence and presence of different antibiotics at sub-MICx streptomycin (Sm), 8 μg/ml; chloramphenicol (Cm), 30 μg/ml; nalidixic acid (Nal), 30 μg/ml; tetracycline (Tc), 1 μg/ml; carbenicillin (Cb), 120 μg/ml; and gentamicin (Gm), 0.25 μg/ml. Primer extension analysis of the ttgABC operon and the rpoS gene (an internal control) was carried out as described in Materials and Methods. The quantities of the corresponding cDNA bands are expressed at the bottoms of the gels in arbitrary units (basal level = 100 U).

FIG. 2. Effect of chloramphenicol (Cm) concentration on expression of ttgABC and ttgR promoters. The β-galactosidase activities of the P. putida DOT-T1E ttgABC promoter (plasmid pED14; white bars) and the ttgR promoter (plasmid pED13; gray bars) in cultures grown until the turbidity at 600 nm was 1 were determined in the absence (−) and in the presence of 25, 50, 75, and 100 μg/ml of chloramphenicol per ml.
This prompted us to purify the protein and to study in vitro the effect of chloramphenicol and tetracycline in TtgR binding to the ttgABC-ttgR intergenic region.

Gel mobility shift assays were carried out to show that TtgR binds specifically to a DNA fragment from position +91 of ttgR to position +68 of ttgABC, corresponding to the ttgR-ttgABC intergenic region (Fig. 3). It should be noted that only excess unlabeled intergenic competitor DNA and not excess control unspecific DNA was able to partially titrate out TtgR from its target DNA in gel mobility shift binding reactions (data not shown). Moreover, as for the in vivo induction of expression, chloramphenicol and tetracycline were able to show an in vitro effect on the binding of TtgR to the ttgABC-ttgR operator site. Figure 3 shows the dissociation of TtgR from the operator-containing DNA fragment in gel mobility shift experiments when increasing concentrations of either chloramphenicol (Cm) and tetracycline (Tc) were present at 1.5 mM. The regions protected by TtgR in both the top and the bottom strands are indicated with respect to the transcriptional start point. Moreover, as for the in vivo induction of expression, chloramphenicol (Cm) and tetracycline (Tc) were present at 1.5 mM. The regions protected by TtgR in both the top and the bottom strands are indicated with respect to both divergent promoter regions is consistent with the coregulation of these promoters observed in vivo. In agreement with the gel mobility shift assays, this protection was lost when chloramphenicol or tetracycline was added to the binding reaction (Fig. 4A), confirming that, in the presence of these antibiotics, TtgR dissociates from its operator site. The addition of several other antibiotics like nalidixic acid, carbenicillin, gentamicin, or streptomycin to the assay did not cause TtgR dissociation (Fig. 4B), even at a high concentration of 3 mM (data not shown). These results are consistent with those of the in vivo experiments, as the antibiotics that did not induce the expression of ttgABC (Fig. 1) also did not affect TtgR binding, in contrast to the results for the antibiotics shown to be inducers. This provides further evidence for the direct involvement of TtgR in the antibiotic-dependent induction of the ttgABC multidrug efflux pump and strongly accounts for the hydrophobic antibiotic specificity of the TtgR repressor.

Similar to QacR (10, 40), TtgR seems to possess a multidrug-binding site able to accommodate two very structurally different drugs like chloramphenicol and tetracycline. Moreover, the TtgABC efflux pump was shown to extrude organic solvents like toluene and styrene as well as antibiotics (35). Also, TtgABC expression is induced by the presence of styrene or 1-naphthol (W. Terañ, J. L. Ramos, and M. T. Gallegos, unpublished data), which suggests that TtgR is also probably involved in organic solvent binding.
DISCUSSION

This paper provides the first experimental evidence to demonstrate that the antibiotic-dependent induction of the *P. putida* DOT-T1E multidrug efflux pump TtgABC and its regulatory gene, *ttgR*, is controlled by the repressor protein TtgR. This is the first case in which the expression of an RND drug transporter has been shown to be regulated directly by antibiotics via its local regulator. Other RND transporters regulated by diverse drugs have been described, but in these cases the regulation involved one of the so-called global stress regulators, MarA, Rob, or SoxS (1, 2, 21, 46). Addition of the structurally dissimilar antibiotics chloramphenicol and tetracycline, which are also substrates of the TtgABC transporter, has been demonstrated to result in derepression of the *ttgABC* and *ttgR* promoters in vivo (Fig. 1 and 2). By both in vitro gel mobility shift (Fig. 3) and DNase I protection (Fig. 4) experiments, we have identified the operator site for TtgR in the *ttgABC-ttgR* intergenic region and have shown that TtgR is released from its operator DNA site by binding to chloramphenicol and tetracycline.

TtgR is a member of the TetR family of transcriptional regulators (3), all of which share a highly homologous N-terminal DNA-binding domain of ~45 residues. The structures of TetR and QacR have revealed that this region forms a three-helix bundle that contains a helix-turn-helix DNA-binding motif (16, 29, 40). These conserved DNA-binding domains are connected to diverse ligand-binding domains in the TetR family of proteins (3). Within the TetR family members, there are proteins which bind to ~15-bp operator sites, like TetR (16, 29), and others, like QacR, which bind to an unusually long (36-bp) operator consisting of 15-bp half sites separated by a 6-bp spacer region (10). TtgR binds to an imperfect inverted repeat operator sequence which overlaps the *ttgABC* and *ttgR* promoters (Fig. 4C); in fact, the −10 and −35 regions of the *ttgABC* promoter and the −10 region of the *ttgR* promoter are protected (Fig. 4). This operator site is also large (36 bp), comprising 12-bp half sites separated by 4 bp, which suggests that each half site would probably receive one dimer of TtgR, as reported for QacR (11, 40) and in accordance with the limited 6-bp DNA recognition capacity described for an HTH motif (14). The binding of TtgR to its operator prevents transcription from the *ttgABC* and *ttgR* operons, probably by blocking RNA polymerase access to the promoters. Most other members of the family of regulatory proteins that share homology with TtgR and that are divergently transcribed with respect to the operons that they control appear to regulate the expression of their own genes, such as TetR (15); CamR, a repressor of o-camphor degradation in *P. putida* (3); and TcmR, the repressor of the *Streptomyces glaucescens* tetracytonycin C resistance gene, *tcmA* (13).

The repression of *ttgABC* and *ttgR* transcription by TtgR was able to be overcome by the addition of the structurally dissimilar antibiotics chloramphenicol and tetracycline, resulting in the induction of *ttgABC* and *ttgR* expression (Fig. 1 and 2). Gel mobility shift assays (Fig. 3) suggested that for these two antibiotics, induction of *ttgABC* and *ttgR* expression involved the direct interaction of TtgR with the substrates. Direct recognition of structurally dissimilar compounds rather than the involvement of a secondary messenger has also been shown for a limited number of multidrug pump regulators like QacR (10) and BmrR (45, 48) and probably appears to be a more general feature in bacterial multidrug transporter regulation. Why do these antibiotics induce the efflux pump genes while other antibiotic substrates of TtgABC fail to do so? Chloramphenicol and tetracycline probably resemble the natural effectors of the regulatory protein or the natural substrates of the efflux pump.

On the other hand, Duque et al. (6) showed that the levels of expression in the wild-type strain never reached the ones observed in the *ttgR* null mutant, even in the presence of effectors, which suggests that, in strain DOT-T1E, *ttgABC* expression is always repressed in such a way that it never achieves the maximum level of expression or at least never achieves the levels reached in the TtgR mutant background. Because of the overlapping nature of the *ttgABC* and *ttgR* promoters, TtgR adjusts its own levels of expression in response to the different concentrations of effectors (Fig. 2). Therefore, TtgR seems to be constantly downregulating the expression of the *ttgABC* efflux pump promoter and, in doing so, prevents the overexpression of the pump under any conditions which could be harmful for the cell. This modulating role is similar to the one proposed for the AcrR repressor of the AcrAB pump of *E. coli* (21). Thus, TtgR functions as a multidrug-binding regulator that specifically modulates the expression of the *ttgABC* efflux pump in response to the presence of effectors. Future work on the *ttgABC-ttgR* system should reveal more intimate details of the molecular interactions between the regulator with its effectors and its target DNA.

ACKNOWLEDGMENTS

This work was supported by grants QLRT-2001-00435 and BIO4-CT97-2270 from the European Commission to J.-L.R. and grant RGY0021/2002 from the Human Frontier Science Programme to M.T.G.

We thank E. Duque for the gift of pED13 and pED14, Ana Hurtado for DNA sequencing, and Carmen Loret for improving the language of the manuscript.

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