TtgV Bound to a Complex Operator Site Represses Transcription of the Promoter for the Multidrug and Solvent Extrusion TtgGHI Pump

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The TtgGHI efflux pump of Pseudomonas putida extrudes a variety of antibiotics and solvents. We show that the ttgGHI operon is transcribed in vitro and in vivo from a single promoter and not from two overlapping promoters as previously proposed. The expression of this promoter is controlled by the TtgV repressor, whose operator expands through four helical turns that overlap the −10 region of the promoter. We also show that TtgV is released from its operator on binding of effectors such as aliphatic alcohols. Mutational analysis of the ttgGHI promoter revealed that substitutions at −13, −12, and −8 yielded promoters that were unable to drive transcription whereas certain mutations at −9, −11, and −6 to −3 increased expression in vivo. The cause of the increased expression was either a decrease in the affinity of the TtgV protein for its operator or an increase in the affinity of RNA polymerase for the mutant promoters.

Multidrug efflux pumps are widely distributed among prokaryotic and eukaryotic organisms (6, 16–18, 25, 29). In bacteria, resistance to a number of antibiotics, superoxide-generating agents, dyes, and organic solvents is mediated by different families of multidrug transporters. Toluene, styrene, and toxic solvents with log $P_{ow}$ values between 1.5 and 3.5 (i.e., the logarithm of the partition coefficient of the target compound in a mixture of octanol and water) partition preferentially in the cell membrane, leading to the disorganization of the inner membrane, which causes cell death. In solvent-tolerant gram-negative bacteria, these organic solvents are removed by pumps which belong to the resistance-nodulation-division (RND) family, so that the solvents are kept below their toxicity threshold (19).

RND efflux pumps are made of three components: an inner membrane transporter (13, 30), an outer membrane channel (8), and a lipoprotein anchored in the inner membrane that extends in the periplasm (31) and that is probably involved in the correct assembly of the other two elements and is needed for optimal functioning of the efflux pump. These efflux pumps are energized by a proton motive force (15).

Pseudomonas putida DOT-T1E is able to thrive in liquid medium containing toluene (21). This strain exhibits an innate high resistance to solvents, which increases when bacteria are preexposed to sublethal concentrations of toluene (2, 20, 23). This is achieved through the cooperative efflux of the solvent by three RND pumps called TtgABC, TtgDEF, and TtgGHI (12, 23). The expression of ttgABC is constitutive, and its level does not vary significantly in the presence of toluene (2). The ttgDEF operon is not expressed at all in the absence of solvents, and its expression is higher in the presence of aromatic hydrocarbons (23). The ttgGHI operon is expressed at a basal level in the absence of solvents, and its expression increases about threefold in response to toluene. The TtgGHI efflux pump plays a pivotal role in the innate and induced tolerance to solvents in this strain (12, 23), and a ttgH knockout mutant is extremely sensitive to solvent shocks (22).

We suggested that ttgGHI expression occurred from two overlapping tandem promoters (23) and that it was controlled by the ttgV gene product (22). In the present study we show that expression from the ttgGHI operon takes place from a single promoter and that solvents such as 1-hexanol trigger the release of the repressor from the operator site, allowing transcription.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture medium. The bacterial strains, cloning vectors, and plasmids constructed in this study are listed in Table 1. Bacterial strains were grown in Luria-Bertani (LB) medium at 30°C as described previously (22). Liquid cultures were shaken on an orbital platform operating at 200 rpm. Escherichia coli DH5α was used as the host strain to construct and maintain different plasmids (1). P. putida cultures were supplemented with solvents at 3 mM when indicated. When required, cultures were supplemented with the following antibiotics: ampicillin, 100 µg/ml; kanamycin, 50 µg/ml; rifampin, 20 µg/ml; and tetracycline, 20 µg/ml.

Nucleic acid techniques. DNA preparation, digestion with restriction enzymes, analysis by agarose gel electrophoresis, isolation of DNA fragments, ligations, and transformations were done by standard procedures (1). Plasmid DNA was sequenced on both strands with specifically designed primers, using an automatic DNA sequencer (ABI-PRISM 3100; Applied Biosystems). P. putida cells were electroporated done as described previously (23). RNA preparation and primer extension were done as described by Marqués et al. (11).

For footprint assays, we used the TtgV protein prepared as described by Rojas et al. (22). DNase I footprint assays were carried out as described previously for
identification of the operator to which TtgV binds (22), whereas dimethyl sulfate (DMS) footprint analyses were carried out as described by Ausubel et al. (1).

Electric field mobility shift assay (EMSA). A 210-bp DNA fragment containing the sequence between the nucleotides before the first start codons of ttgV and ttgG was amplified by PCR from plasmid pGG1 using the following set of primers: 5’-GGAAATCTACTTCTCTGGGCTGCATC-3’ and 5’-AAGCTGCA GGCGGCTTTATCGTAATCTG-3’. Cycling parameters were 4 min at 96°C followed by 30 cycles at 96°C for 1 min, 60°C followed by 30 cycles at 96°C/96°C for 1 min, 60°C, and ending with 3 min at 72°C. These fragments were isolated from agarose gels and ended labeled with 32P as described previously (22, 27). About 1 nM labeled DNA was incubated with increasing amounts of purified TtgV for 10 min at 30°C in 10 μl of TGED binding buffer (10 mM Tris-HCl [pH 8.0], 5% [vol/vol] glycerol, 0.1 mM EDTA, 1 mM dithiothreitol) containing 20 μg of poly(dI-dC) per ml and 200 μg of bovine serum albumin per ml. Reaction mixtures were electrophoresed in a non-denaturing 4% (wt/vol) polyacrylamide gel in Tris-glycine buffer (0.2 M glycine, 0.025 M Tris-HCl [pH 8.8]). The results were analyzed with Molecular Imager FX equipment (Bio-Rad, Madrid, Spain).

Construction of $P_{ttgG}$ mutant promoters by PCR. The $P_{ttgG}$ mutant promoters were generated by overlap extension PCR mutagenesis, as described previously (22). The internal oligonucleotide primers used for mutagenesis exhibited one mismatch with the wild-type sequence. After DNA amplification, the resulting DNA was digested with EcoRI and PstI and the 210-bp EcoRI-PstI $P_{ttgG}$ mutant was inserted between the EcoRI and PstI sites of pMP220 to yield plasmid pMEx (the x indicates the plasmid number). All mutant $P_{ttgG}$ promoters generated in this study were confirmed by DNA sequencing.

Single-round in vitro transcription assays with supercoiled plasmid DNA. Reactions (20-μl reaction mixtures) were performed with STA buffer (10 mM Tris-acetate [pH 8.0], 8 mM magnesium acetate, 3.5% [wt/vol] polyethylene glycol, 10 mM KCl, 1 mM dithiothreitol) containing 10 and 100 nM amtoholo-enzyme (Epitcenc). 20 U of RNAase (Promega), and around 10 nM supercoiled plasmid DNA template. The reaction mixtures were incubated for 10 min at 30°C before the addition of the following elongation mixture: 0.1 mM each ATP, CTP, GTP, and UTP; 0.3 μCi of [α-32P]UTP (20 μCi/μl); and 100 μM of heparin per ml. After a further 10-min incubation at 30°C, the reactions were stopped by chilling to 4°C and the products were precipitated with 0.25 volume of 10 M ammonium acetate and 2.5 volume of ethan. The pellets were washed with 80% (vol/vol) ethanol. Dried pellets were resuspended in 8 μl of water plus 4 μl of formamide sequencing dye. Samples were electrophoresed in a 6.5% (wt/vol) polyacrylamide denaturing sequencing gel. The results were analyzed using Molecular Imager GS525 equipment with Quantity One software (Bio-Rad, Madrid, Spain).

RESULTS

In vitro evidence suggests that the $ttgGHI$ promoter is expressed from a single promoter rather than two overlapping promoters. Rojas et al. (23) suggested that in $P. putida$ DOT-T1E, the $ttgGHI$ operon was transcribed from two promoters, $P_{ttgG}$ and $P_{ttgH}$, since two potential transcription start points separated by 5 nucleotides were found in vivo regardless of the growth phase and growth in the absence or presence of tol-

e.

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristicsa</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>$P. putida$ DOT-T1E</td>
<td>Rif&lt;sup&gt;e&lt;/sup&gt;</td>
<td>21</td>
</tr>
<tr>
<td>$E. coli$ DH5α</td>
<td>recA1</td>
<td>1</td>
</tr>
<tr>
<td>pANA96</td>
<td>$Tc^r$, $ttgG$ promoter cloned in pMP220</td>
<td>22</td>
</tr>
<tr>
<td>pET28b(+) $ttgV$</td>
<td>Ap&lt;sup&gt;+&lt;/sup&gt;, vector use to produce TtgV</td>
<td>22</td>
</tr>
<tr>
<td>pUC18</td>
<td>pUC18 bearing an 8-kb BamHI fragment with $ttgGHI$ and $ttgW$</td>
<td>23</td>
</tr>
<tr>
<td>pMP220</td>
<td>$Tc^r$, promoterless $lacZ$ expression vector</td>
<td>26</td>
</tr>
<tr>
<td>pME103-P $ttgG$</td>
<td>Mutant $ttgG$ promoter cloned in pMP220</td>
<td>This study</td>
</tr>
<tr>
<td>pET103-P $ttgG$</td>
<td>Ap&lt;sup&gt;+&lt;/sup&gt;, promoter of $ttgG$ cloned upstream of the T7 terminator in pET103</td>
<td>This study</td>
</tr>
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</table>

<sup>a</sup> Ap<sup>+</sup>, Km<sup>+</sup>, Rif<sup>e</sup>, and $Tc^r$ stand for resistance to ampicillin, kanamycin, rifampin, and tetracycline, respectively.

<sup>b</sup> x indicates the plasmid number bearing different mutant promoters fused to $lacZ$. 

![Table 1. Strains and plasmids used in this study](http://jb.asm.org/)
ranged between 23 and 62% of that of the wild type). We also found that certain mutant promoters had higher activity than the wild type (between 1.9- and 9.3-fold that of the wild-type): C-11→T, C-9→A, T-6→C or G, C-4→A or G, and C-3→G (Table 2). Mutations at −14 and −16 had no significant effect on the level of expression of the promoter. Primer extension assays confirmed the results of the β-galactosidase assays; namely, when we found no β-galactosidase activity, we found no mRNA, and when we found lower or higher β-galactosidase activity than those in the wild type, the mRNA levels estimated by primer extension correlated with the results of the enzymatic assay (data not shown).

In vitro transcription assays with mutant promoters revealed that whereas mutant promoters altered at −13, −12, −8 and other mutants altered at −7 and −6 were not transcribed in vitro, promoters altered at any of the other positions were transcribed (data not shown).

Given that mutations at positions −13, −12, and −8 yielded no β-galactosidase activity (or very low levels of activity) and did not drive the expression of mRNA in vivo and in vitro, we considered that these positions delimited the −10 hexamer with the 5′-TACACT-3′ sequence. This sequence exhibits four of six identities to the −10 consensus of the promoters recognized by σ22 (5′-TATAAT-3′) (9).

**Why do certain point mutations in the ttgG promoter result in an increase in expression?** DNase I footprint assays showed that TtgV covered four DNA helical turns that overlap the ttgGHI promoter (22). The increase in expression of certain mutant promoters (i.e., C-3→G, C-4→G or A, C-9→A, and C-11→T) in the ttgG promoter may have resulted from either an improved −10 box for RNA polymerase or a decrease in the affinity of TtgV for its target sequences. We tested whether the mutant promoters that exhibited a higher activity than the wild type in vivo (i.e., C-4→A or G, C-3→G, C-9→A, and C-11→T) also exhibited increased expression in vitro with respect to the wild-type promoter. These assays were done with limiting amounts of RNA polymerase. The level of transcripts obtained in vitro from mutant promoters C-4→A or G and C-3→G was similar to that obtained from the wild-type promoters (see Fig. 3 for the C-4→A mutant), whereas with mutant promoters C-9→A and C-11→T, the level of expression was higher (Fig. 2). We suggest that the increased expression with C-9→A and C-11→T may have resulted from a promoter whose sequence, TATACT in C-11→T and TACAT in C-9→A, was more similar to the consensus sequence since only one mismatch was found in each mutant promoter.

To explain the higher expression in vivo of the other mutant
producers (C-4→A or G, C-3→G), we hypothesized that the affinity of TtgV for its operator might be reduced in the C-4→A or -G and C-3→G promoters and that therefore it competes less effectively with the RNA polymerase for its binding site. To test this hypothesis we performed single-round transcription assays with the wild-type and mutant promoters. While the addition of 0.5 μM TtgV prior to RNA polymerase led to 70% inhibition in the in vitro transcription assay with the wild-type promoter, inhibition with the mutant promoters was only around 40%. Figure 3 shows the results obtained for the mutant promoter C-4→A. Similar results were obtained when the mutant promoters C-4→G or C-3→G were used (data not shown). In contrast, when EMSAs were done with the up mutant promoter C-9→A, the results were similar to those obtained with the wild-type promoter (Fig. 4), which supports the notion that increased expression in this mutant promoter could be the result of a higher affinity of the RNA polymerase for the mutant promoter.

1-Hexanol is an effector for TtgV and releases the repressor from its binding site in P\_ttgG. Rojas et al. (22) showed that the TtgGHI efflux pump expels a large number of aromatic hydrocarbons (toluene, styrene, and ethylbenzene), aliphatic alcohols (1-octanol and 1-hexanol), and antibiotics such as ampicillin, carbenicillin, nalidixic acid, and tetracycline. We determined the in vivo effector profile for TtgV by measuring β-galactosidase activity using P\_ttgG::lacZ fusions in the presence of different compounds. Our assays revealed that antibiotics were not inducers of the expression of the tggGHI operon, in accordance with the results of Rojas et al. (22). Aromatic compounds such as toluene, styrene, propylbenzene, m-xylene, and indole and aliphatic alcohols, i.e., 1-octanol and 1-hexanol, induced expression of the operon between 2.3- and 3-fold (data not shown). The in vivo assays did not reveal whether this was due to a direct or an indirect effect on the TtgV repressor. When we tested the effect of 1-hexanol on TtgV target binding in EMSA, we observed that hexanol had an in vitro effect on the binding of TtgV to the tggGHI-ttgG operator site. Figure 5 shows the increase in dissociation of TtgV from the operator.
containing the DNA fragment in EMSA when increasing concentrations of 1-hexanol were added to the binding region.

We also tested the effect of this compound in in vitro transcription assays using 20 to 200 μM 1-hexanol. In this range of concentrations, 1-hexanol did not interfere with RNA polymerase activity since the control P<sub>sp</sub> promoter yielded the expected 488-nucleotide mRNA. We then performed in vitro transcription assays with TtgV added before RNA polymerase and with increasing concentrations of 1-hexanol. We found that the higher the concentration of 1-hexanol, the higher the level of transcription from the wild-type P<sub>ttgG</sub> (Fig. 6).

**DMS footprint assays.** DNase I footprint assays revealed that TtgV protected the region in the P<sub>ttgG</sub> promoter corresponding to positions +13 to −29 from digestion (22). To characterize the binding region in greater detail, DMS methylation protection assays were carried out. Figure 7 shows that G at position −14 in the bottom strand was hypermethylated in the presence of TtgV. This suggests that binding of TtgV to target DNA triggered local DNA conformational changes. We observed that G's at positions +6, −15, and −27 were protected in the top strand and G's at positions −23, −11, −4, −3, and +10 were protected in the bottom strand, suggesting that they might be contacted by TtgV on binding to its operator. All these positions are in accordance with our previous DNase I footprinting since they are located within the proposed binding site. Furthermore, the importance of G-4 and G-3 in TtgV operator recognition is consistent with our results showing that G-4→A and G-3→C are less prone to be recognized by TtgV.

![FIG. 4](image)

**FIG. 4.** EMSAs with the wild-type and C-4→A and C-9→A mutant promoters. The promoters were amplified by PCR as 210-bp fragments and labeled at the 5' ends. About 1 nM DNA containing the ttgG promoter region was incubated with increasing concentrations of TtgV (from left to right, 0, 10, 50, 100, 200, 300, 400, 500, and 700 nM) for 10 min and electrophoresed as indicated in Materials and Methods. B, bound DNA; F, free DNA.

![FIG. 5](image)

**FIG. 5.** EMSAs with the wild-type promoter in the presence of 1-hexanol. Conditions are as described in the legend to Fig. 4, except that DNA corresponded to the wild-type ttgG promoter and a fixed concentration of TtgV (500 nM) was used. Samples were incubated for 10 min with the indicated 1-hexanol concentration before electrophoretic separation.

![FIG. 6](image)

**FIG. 6.** TtgV repression of the transcription of P<sub>ttgG</sub> is alleviated by 1-hexanol. Single-round in vitro transcription assays were performed as described in the legend to Fig. 1A in the absence and presence of 0.5 μM TtgV added before the addition of RNA polymerase. Increasing concentrations of 1-hexanol were added as indicated. Other conditions as are described in Materials and Methods.
DISCUSSION

Our results establish that the \texttt{ttgGHI} operon is probably transcribed from a single promoter rather than from two tandem promoters, as proposed before (23). This assumption is supported by in vitro transcription analysis, which revealed a single transcription start point, and mutational analysis of the region upstream from the +1 position. Mutational studies support the notion that the most probable −10 hexamer of the promoter lies between positions −8 and −13 since a number of mutations in this stretch resulted in mutants that could not be transcribed either in vivo or in vitro. In contrast to these mutants, which lacked activity, we found that certain mutations at −9 and −11 increased promoter activity. This seems to be the result of a better −10 region that is recognized more efficiently by RNA polymerase.

DNase I footprint analysis revealed that TtgV covers four helical turns that cover the promoter region between positions +13 and −29 (22), which suggests that TtgV represses transcription from the \texttt{ttgG} promoter by competing with the RNA polymerase for promoter binding. To gain insight into the TtgV repression mechanism, we performed in vitro transcription assays. When TtgV was incubated with the \texttt{ttgG} promoter before the addition of the RNA polymerase, transcription from \texttt{P}_{\text{ttgG}} was repressed but that from a reference promoter, \texttt{P}_{\text{sp}}, was unaffected (Fig. 1A). The repression level of \texttt{ttgG} transcription correlated with the dose of TtgV added. However, TtgV did not repress \texttt{ttgG} transcription significantly when it was added after the formation of the RNA polymerase-\texttt{ttgG} promoter open complex (Fig. 1). This indicates that TtgV represses \texttt{ttgG} transcription by physically competing with the RNA polymerase for promoter binding, as in the case of the well-established classic model of repressor action (4, 7, 24, 28).

DMS methylation protection assays revealed that in the presence of TtgV, G-14 in the bottom strand becomes hypermethylated. This probably indicates that TtgV binding to the operator region provokes a distortion immediately upstream from the −10 region, which may prevent proper recognition of the promoter by RNA polymerase. DMS footprinting, however, did not help us to discern whether TtgV was able to recognize a relatively highly conserved direct repeat [5′-(A/C)T(G/A)N (C/T)NCA-3′] that appeared in four consecutive helical turns (Fig. 1B) or whether TtgV recognized some of the imperfect inverted repeats in the protected region. Analysis of mutant promoters revealed that mutations C-4→A or G and C-3→G resulted in increased expression in vivo but not in vitro and correlated with a decrease in the affinity of TtgV for the target operator as determined in EMSAs, as well as the protection of the bases at these positions in DMS footprint assays.

The TtgV protein belongs to the IclR family of regulators (10, 14). Members of this family repress the transcription of specific cognate genes in the absence of the target chemical and detach from the operator in response to the presence of a specific signaling molecule. A similar mechanism seems to operate for TtgV: EMSAs revealed that in the presence of increasing concentrations of 1-hexanol, TtgV dissociated from its target operator. This was corroborated by in vitro transcription assays showing that when TtgV was present, there was no mRNA synthesis, but in the presence of 1-hexanol, mRNA levels increased. This suggests that in the presence of 1-hexanol, TtgV was released from its operator site and RNA polymerase was able to access and transcribe the \texttt{ttgG} promoter.

These are the first experimental indications of a compound able to directly promote TtgV dissociation from its operator.
site. Future work on the tgtGHI-ttgV system should reveal more intimate details of the molecular interactions between the regulator, its effectors, and its target DNA.

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REFERENCES


