In vivo and in vitro evidence that TtgV is the specific regulator of the TtgGHI multidrug and solvent efflux pump of Pseudomonas putida

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The TtgGHI efflux pump of Pseudomonas putida DOT-TIE plays a key role in the innate and induced tolerance of this strain to aromatic hydrocarbons and antibiotics. The ttgGHI operon is expressed constitutively from two overlapping promoters in the absence of solvents and at a higher level in their presence, but not in response to antibiotics. Adjacent to the ttgGHI operon is the divergently transcribed ttgVW operon. In TtgV-deficient backgrounds, although not in a TtgW-deficient background, expression of the ttgGHI and ttgVW operons increased fourfold. This suggests that TtgV represses expression from the ttgG promoters and controls its own. TtgW plays no major role in the regulation of expression of these promoters. Primer extension revealed that the divergent ttgG and ttgV promoters overlap, and mobility shift assays indicated that TtgV binds to this region with high affinity. DNeil footprint assays revealed that TtgV protected four DNA helical turns that include the −10 and −35 boxes of the ttgV and ttgG promoters.

In their natural habitats, living organisms are exposed to a wide range of natural and human-made toxic compounds, and survival in sites that have become hostile involves an extensive series of protective mechanisms. Multidrug efflux pumps are one of the most efficient tools that eukaryotic and prokaryotic cells use to extrude toxic chemicals and are very efficient in the removal of anticancer agents, antibiotics, dyes, superoxide-generating chemicals, organic solvents, and other toxic chemicals (31, 33, 34, 39). Efflux pumps of the resistance-nodulation-division (RND) family are very efficient in the removal of different drugs (33, 37, 45). These bacterial efflux pumps are made of three components (20, 28, 54): an efflux pump transporter located in the cytoplasmic membrane that recognizes substrates in the periplasm or in the outer leaflet of the cytoplasmic membrane (11, 28, 50), an outer membrane protein which forms a trimeric channel that penetrates into the periplasm (20) and contacts the efflux pump transporter, and a lipoprotein anchored to the inner membrane which expands into the periplasmic space and may serve as a bracket for the other two components (54).

Toluene, xylene, and styrene are among the most toxic chemicals that bacterial cells can be exposed to because they dissolve in the cytoplasmic membrane, disorganize it, and collapse the cell’s membrane potential. This, together with the loss of lipids and proteins, leads to irreversible damage, resulting in the death of the cell (8, 47). However, a number of Pseudomonas putida strains are able to grow in the presence of high concentrations of toluene and other aromatic hydrocarbons (7, 14, 18, 41, 52). Tolerance to these chemicals in these strains is achieved mainly by a series of RND efflux pumps, called Ttg (toluene tolerance genes) in P. putida DOT-TIE (15–18, 21, 27, 29, 42, 43). In P. putida DOT-TIE, three different efflux pumps, TtgABC, TtgDEF, and TtgGHI, function additively to prevent the accumulation of toluene and other aromatic hydrocarbons in the cell membrane (43). Two of these efflux pumps, TtgABC and TtgGHI, also extrude antibiotics such as chloramphenicol, tetracycline, ampicillin (40, 43), and, in the case of TtgABC, probably also heavy metals (P. Godoy and J. L. Ramos, unpublished data).

Some of these efflux pumps have a relatively high basal level of expression and confer so-called intrinsic resistance, whereas others are inducible by the transported product and confer induced resistance. In P. putida DOT-TIE, the ttgABC operon is expressed at a certain basal level, which increases slightly in response to solvents and significantly in response to chloramphenicol (9; W Terán, A. Felipe, A. Segura, A. Rojas, J. L. Ramos, and M. T. Gallegos, submitted for publication), whereas the ttgDEF operon is induced by aromatic hydrocarbons such as toluene or styrene (27). The ttgGHI operon seems to be expressed from two overlapping promoters at a certain basal level in the absence of solvents, and its expression increases severalfold in the presence of aromatic hydrocarbons (43). The RND efflux genes are often regulated by the gene product of an adjacent and divergently transcribed regulatory gene (1, 5, 9, 22, 24, 29, 30, 31, 33, 43, 45). In fact, the ttgR gene is adjacent to the ttgABC operon and is transcribed divergently from ttgA. In a TtgR-deficient background, expression from the ttgA promoter increased about 10-fold, suggesting that TtgR represses expression from the ttgA promoter (9). In this mutant background, expression from the ttgD and ttgG promoters followed the same pattern of expression as in the wild type, suggesting that other regulators are involved in the control of expression of the other two efflux pumps.

In this study, we report the identification of two genes, ttgV...
and ttgW, that form an operon and that are transcribed divergently from the ttgGHI operon. In the ttgV-deficient background, but not in the ttgW-deficient background, the ttgGIHI and ttgVW operons are expressed at a higher level. This suggests that TtgV is a repressor of the expression from the ttgGHI and ttgVW promoters. We overexpressed and purified TtgV and showed that this repressor binds to the ttgVW promoter region protecting a region covering four DNA helical turns where the overlapping ttgV and ttgG promoters lie.

**MATERIALS AND METHODS**

**Bacterial strains, plasmids, and culture medium.** The bacterial strains, cloning vectors, and plasmids constructed in this study are shown in Table 1. Bacterial strains were grown in Luria-Bertani (LB) medium (4). Escherichia coli cultures were incubated at 37°C, whereas *P. putida* cultures were incubated at 30°C. Liquid cultures were shaken on an orbital platform operating at 200 strokes per min. Under these culture conditions, the concentration of toluene via homologous recombination were selected on LB solid medium with kanamycin. Among these colonies, those that did not grow on LB medium with kanamycin. Among these colonies, those that did not grow

**Preparation of RNA, primer extension analysis, and RT-PCR.** *P. putida* DOT-T1E was grown overnight in LB medium. Cells were then diluted 100-fold in fresh medium, and aliquots were incubated in the absence or presence of 3 mM toluene, 1.5 mM styrene, or sublethal concentrations of antibiotics (chloramphenicol, 30 μg/ml; nalidixic acid, 30 μg/ml; tetracycline, 1 μg/ml; gentamicin, 0.25 μg/ml; and carbencillin, 120 μg/ml) until the culture reached a turbidity of about 1.0 at 600 nm. Cells (30 ml for primer extension and 1.5 ml for reverse transcriptase [RT] PCR) were harvested by centrifugation (5,000 × g for 10 min) and processed for RNA isolation according to the method of Marques et al. (23). Extracts were treated with RNase-free DNase I (50 U) in the presence of 3 U of an RNase inhibitor cocktail (Roche Laboratory). For primer extension analysis, we used as primers specific oligonucleotides complementary either to the ttgVW mRNA or to the ttgVW mRNA. Primers were labeled at their 5’ ends with [γ-32P] ATP and T4 polynucleotide kinase as described previously (4). About 10^6 cpm of the labeled primer was hybridized to 20 μg of total RNA, and extension was carried out using avian myeloblastosis virus RT as described previously (23). Electrophoresis of cDNA products was done in a urea-polyacrylamide sequencing gel to separate the reaction products. The relative intensity of the bands was quantitated using the Molecular Imager System GS 525 (Bio-Rad Laboratories) with the Multianalyst program.

**Construction of ttgV and ttgW knockout mutant strains.** To facilitate site-directed mutagenesis, the 4.5-kb BamHI-EcoRV fragment from pGG1 (43) harboring the ttgV and ttgW genes was ligated to pUC18Not digested with BamHI-SmaI to produce pANA82. Plasmid pANA82 was in turn digested with *Bgl*II (which cuts once in the plasmid within the ttgW gene), made blunt with the four deoxynucleoside triphosphates and Klenow enzyme, and then ligated a 2.25-kb BamHI fragment (blunted as above) from pH450-Km (37) that contained the kanamycin resistance cassette to yield pANA83 (Ap^r Km^r). About 600 ng of the suicide pANA83 plasmid was electroporated into *P. putida* DOT-T1E cells, and transformants that integrated the pANA83 plasmid into the host chromosome via homologous recombination were selected on LB solid medium with kanamycin and piperacillin. Successful integration was confirmed by Southern blot analysis (data not shown). A random merodiploid clone was grown overnight in LB medium to allow for a second recombination event in which the wild-type gene was replaced by the mutant allele. For this selection, colonies were plated again on LB medium with kanamycin. Among these colonies, those that did not grow in the presence of piperacillin were selected as putative resolved clones. The mutant clones were checked again by Southern blotting, and one of the clones, which exhibited the correct replacement (data not shown), was kept for further studies and named *P. putida* strain DOT-T1E-PS52.

To construct a ttgV polar mutant, we first amplified by PCR the ttgV gene by

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TABLE 1. Strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristic(s)^a</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. putida</em> DOT-T1E</td>
<td>Rif^r</td>
<td>41</td>
</tr>
<tr>
<td><em>P. putida</em> DOT-T1E-PS52</td>
<td>Rif^r Km^r ttgW::ΔttgKm</td>
<td>This study</td>
</tr>
<tr>
<td><em>P. putida</em> DOT-T1E-PS61</td>
<td>Rif^r Km^r ttgV::aph^R</td>
<td>This study</td>
</tr>
<tr>
<td><em>P. putida</em> DOT-T1E-PS62</td>
<td>Rif^r</td>
<td>This study</td>
</tr>
<tr>
<td>E. coli DH5αF^*</td>
<td>F’ rI ACP mutT conj gal dcm met auxotroph</td>
<td>Promega</td>
</tr>
<tr>
<td>pANA82</td>
<td>pUC18Not plasmid bearing a 4.5-kb BamHI-EcoRV insert with the ttgVW genes</td>
<td>This study</td>
</tr>
<tr>
<td>pANA83</td>
<td>ttgV::ΔttgKm, derived from pANA82</td>
<td>This study</td>
</tr>
<tr>
<td>pANA95</td>
<td>Promoter of ttgV cloned in pMP220</td>
<td>This study</td>
</tr>
<tr>
<td>pANA96</td>
<td>Promoters of ttgG cloned in pMP220</td>
<td>This study</td>
</tr>
<tr>
<td>pANA117</td>
<td>2.3-kb fragment cloned in pGEM-T, bearing ttgV and ttgW genes</td>
<td>This study</td>
</tr>
<tr>
<td>pANA118</td>
<td>ttgV::aph^R, derived from pANA117</td>
<td>This study</td>
</tr>
<tr>
<td>pANA119</td>
<td>ttgV::ΔttgKm, derived from pANA117</td>
<td>This study</td>
</tr>
<tr>
<td>pANA125</td>
<td>0.8-kb NdeI-BamHI fragment bearing ttgV in pGEM-T</td>
<td>This study</td>
</tr>
<tr>
<td>pANA126</td>
<td>ttgV::His^T tag cloned in pET28b (++)</td>
<td>This study</td>
</tr>
<tr>
<td>pET-28b(+)</td>
<td>Km^r, expression vector</td>
<td>Novagen</td>
</tr>
<tr>
<td>pGEM-T</td>
<td>Ap^r, cloning vector</td>
<td>Promega</td>
</tr>
<tr>
<td>pGG1</td>
<td>pUC18 bearing an 8-kb BamHI fragment with ttgGHI and ttgVW</td>
<td>Promega</td>
</tr>
<tr>
<td>pHP450-Km</td>
<td>Ap^r, carries the 2.25-kb flKm fragment</td>
<td>37</td>
</tr>
<tr>
<td>pMP220</td>
<td>Tc^r, promoterless lacZ vector</td>
<td>48</td>
</tr>
<tr>
<td>pSBkrapA</td>
<td>pSB plasmid with a nonpolar Km^r cassette</td>
<td>25</td>
</tr>
<tr>
<td>pUC18Not</td>
<td>Ap^r, cloning vector</td>
<td>32</td>
</tr>
</tbody>
</table>

^a Ap^r, Km^r, Na^r, Rif^r, and Tc^r, resistance to ampicillin, kanamycin, nalidixic acid, rifampin, and tetracycline, respectively.
expressed, would carry an N-terminal histidine tag. For His6-tagged N-TtgV operon promoter to ttgVW
Eco/H11032 BamHI restriction sites at their 5' ends, respectively, and cloned in LB medium with or without toluene in the gas phase. On the following day, cultures were diluted 1:100 and grown under the same conditions until the cultures reached a turbidity of about 0.8 at 660 nm. These cultures contained about 10^8 CFU/ml. The cultures were divided in two halves; to one we added 1.5 mM styrene. Assays were run in duplicate, with shaking, the cultures were incubated in 10 ml of LB medium with tetracycline, and then the cultures were diluted 100-fold in the same medium. After 1.5 h of incubation at 30°C with shaking, the cultures were harvested, and the RNA was isolated. 

**RESULTS AND DISCUSSION**

Identification of an operon upstream from the ttgGHI genes that is divergently transcribed with respect to the efflux pump genes. DNA sequencing upstream from the ttgGHI genes by using plasmid pGG1 as a template (43) revealed two ORFs, of 780 and 405 bp (Fig. 1A), separated from each other by 5 bp. These genes were transcribed divergently with respect to the ttgGHI operon. Sequence homology search of the deduced 259- and 134-amino-acid proteins with sequences deposited in several databases showed that the first ORF showed an overall 50 to 60% similarity with a number of transcriptional regulators belonging to the IclRI family (i.e., PsrA, IclRI, GltRI, PcaRI, PcaU, PbrRI, SrpRI, etc. [35, 36, 53]), whereas the second ORF showed similarity to members of the TetRI family of repressors (i.e., TetR, TtgR, AcrRI, MtrRR, SrpRI, and QacRI [6, 9, 12, 13, 19, 22, 24, 46, 53]). We hypothesized that these ORFs encoded proteins involved in the transcriptional control of the ttgGHI efflux pump operon, and we called the first and second ORFs ttgV and ttgW, respectively.

To test whether ttgV and ttgW were part of the same transcriptional unit, we prepared total RNA from P. putida DOT-TIE growing on LB medium and RT-PCR assays were done with primers based on ttgV and ttgW on the one hand and ttgV and ttgG on the other. Amplification was obtained only when the ttgV and ttgW primers were used, with the size of the amplified fragment being that predicted from the DNA sequence (Fig. 1B). This result confirmed the presumed operon structure of the cluster. Using primers based on ttgG, ttgR, and ttgI, we also showed that ttgGHI was transcribed as a single transcriptional unit (Fig. 1B).

Transcriptional analysis of the ttgW operon. The transcription initiation point of the ttgW operon was mapped, as de-
scribed in Materials and Methods, in cells growing in the absence or presence of toluene. Regardless of the growth conditions, the operon was transcribed from a single promoter (Fig. 2A). The $5'$ end of the ttgW mRNA starts at the G marked +1 in the sequence shown in Fig. 2A. The $-10$ and $-35$ regions of the ttgV promoter exhibit low similarity to promoters recognized by RNA polymerase with sigma-70 at the $-10$ region (5'-TGACGC-3') and the $-35$ region (5'-TG TAGC-3'). The location of the start sites of the ttgV and ttgG genes indicates that the promoters of the divergently transcribed operons overlap (Fig. 2B). In fact, the $-10$ and $-35$ boxes of the ttgVW promoter totally overlap the $-35$ and $-10$ boxes, respectively, of the P$_{G2}$ promoter (Fig. 2B).

The intensity of the cDNA bands determined densitometrically in Fig. 2A was used to estimate variations in the expression of ttgV in the absence or presence of toluene. We observed that in the presence of toluene the level of expression of the ttgVW operon was three- to fourfold higher than in the absence of the aromatic hydrocarbon. Our group has shown before that expression from the ttgGHI promoters in the DOT-T1E strain is about threefold higher in DOT-T1E-PS62 than in the wild-type strain in the presence and absence of 3 mM toluene (Table 2), and we found that expression from the ttgGHI promoters was about four times higher in DOT-T1E-PS62 than in the wild-type strain in the absence of toluene (Table 2). This indicates that TtgV is a repressor that prevents the expression of the ttgGHI operon. Expression of the ttgVW promoter was about threefold higher in the ttgV mutant than in the wild-type strain in the absence of toluene, indicating that TtgV controls negatively its own expression.

FIG. 1. Physical organization of the ttgGHI-ttgVW cluster and evidence that ttgVW is an operon. (A) The sequence of the ttgGHI and ttgVW operons can be accessed from GenBank (accession no. AF299253.2). The approximate sizes of the products of each ORF are given in kilodaltons below each gene. (B) The products resulting from each RT-PCR were separated on agarose gels, as described in Materials and Methods. Lane 1, ttgV-ttgW amplification (expected size, 398 bp); lane 2, ttgV-ttgG amplification (expected size, 437 bp); lane 3, ttgG-ttgH amplification (expected size, 606 bp); lane 5, molecular weight markers, whose sizes are provided on the right.

The set of results presented above indicates that the pattern of inducibility of the ttgVW operon is similar to that of the efflux pump ttgGHI operon, probably because the two promoters are regulated in the same way.

The ttgV gene product, but not that of the ttgW gene, is a repressor of the ttgV and ttgG promoters. We constructed three different mutant strains in the ttgVW operon as described in Materials and Methods: DOT-T1E-PS62 (TtgV$^-$ TtgW$^+$); DOT-T1E-PS61 (TtgV$^-$ TtgW$^-$), andDOT-T1E-PS52 (TtgV$^+$ TtgW$^-$). To determine the effect of these mutations on the expression of the ttgGHI and ttgVW operons, we used transcriptional fusions of the corresponding promoters to lacZ. Plasmids pANA96 (P$_{tnG2}$::lacZ) and pANA95 (P$_{tnG2}$::lacZ) were transformed in the wild-type DOT-T1E strain and in the three mutant strains. β-Galactosidase activity was measured in the presence and absence of 3 mM toluene (Table 2), and we found that expression from the ttgGHI promoter was about four times higher in DOT-T1E-PS62 than in the wild-type strain in the absence of toluene (Table 2). This indicates that TtgV is a repressor that prevents the expression of the ttgGHI operon. Expression of the ttgVW promoter was about threefold higher in the ttgV mutant than in the wild-type strain in the absence of toluene, indicating that TtgV controls negatively its own expression.

Expression from the ttgG and ttgV promoters in the mutant background DOT-T1E-PS52 deficient in TtgW but proficient in TtgV was similar to that found in the wild-type strain (Table 2), which suggested that the TtgW protein, under our assay conditions, does not play a major role in the regulation of the expression from either of these two operons. The level of expression from the ttgVW and ttgG promoters in the DOT-T1E-PS61 mutant deficient in TtgV and TtgW was similar to that obtained in mutant DOT-T1E-PS62 (ttgV$^-$ ttgW$^+$) (Table 2).
These results suggest that the TtgV protein is a repressor of its own synthesis as well as of the expression of the ttgGHI operon.

To further corroborate that TtgV is a repressor of expression from ttgG and ttgV, transcription from pANA95 and pANA96 was assayed in the heterologous E. coli DH5α F’ host with or without pANA126 that overproduced TtgV. We found that in the constructions in which TtgV was overproduced the level of expression for P_{ttgG} and P_{ttgV} was 10 to 20% of the level found in the strain without TtgV, which confirms that TtgV is a repressor of the expression of both operons.

The TtgV mutant is more resistant to toluene than is the wild-type strain. It has previously been reported that the TtgGHI efflux pump is involved in both intrinsic and induced resistance to organic solvents of P. putida DOT-T1E (43). Given that expression from P_{ttgG} increased in the mutant deficient in the TtgV protein, we analyzed solvent tolerance in the three mutant strains by determining the survival rate of the cells after a sudden 0.3% (vol/vol) toluene shock when the cultures were pregrown on LB liquid medium with or without toluene in the gas phase. Strain DOT-T1E-PS52 (TtgV^{+} W^{-})
TABLE 2. Transcription from the ttgVW and ttgGHI operon promoters determined as β-galactosidase activity using fusions of the operon promoters to lacZ

<table>
<thead>
<tr>
<th>Fusion and strain</th>
<th>Genetic background</th>
<th>β-Galactosidase activity and growth conditions</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Without toluene</td>
</tr>
<tr>
<td>P_{tgVW}::lacZ</td>
<td>Wild type</td>
<td>V+ W+</td>
</tr>
<tr>
<td></td>
<td>PS62</td>
<td>V+ W+</td>
</tr>
<tr>
<td></td>
<td>PS61</td>
<td>V+ W+</td>
</tr>
<tr>
<td></td>
<td>PSS2</td>
<td>V+ W+</td>
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<tr>
<td>P_{tgGHI}::lacZ</td>
<td>Wild type</td>
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<tr>
<td></td>
<td>PSS2</td>
<td>V+ W+</td>
</tr>
</tbody>
</table>

The strains were transformed with pAN95 (P_{tgVW}::lacZ) or pAN96 (P_{tgGHI}::lacZ), and cells were grown in LB medium in the absence or presence of 3 mM toluene. β-Galactosidase activity was determined 4 h later in duplicate samples. The data are rounded values of the averages of at least three independent assays.

Survived the toluene shock to the same extent as wild-type DOT-T1E (Fig. 3A and C). The survival rate when the cells were not preinduced was around 1 cell per 10,000, whereas almost 1 in 10 cells survived the shock when the cultures were preinduced with toluene in the gas phase. These results correlate with the data presented above, i.e., since the expression of the efflux pump is not significantly increased in this mutant, the tolerance to toluene remains as in the wild type. However, in DOT-T1E-PS62 (ttgV− ttgW+) and in the double mutant DOT-T1E-PS61 (ttgV− ttgW+), we observed an increase in the survival rate of about 2 orders of magnitude, with the levels of survival being almost identical to the levels obtained when the cells were preinduced (compare Fig. 3A, B, and D). These results indicate that an increase in the expression of the TtgGHI efflux pump, such as that found in the mutant deficient in TtgV, increased the survival rate of cells that were suddenly exposed to toluene. Therefore, these results support the notion that TtgV is strongly involved in the solvent-dependent induction of the TtgGHI pump.

Rojas et al. (43) showed that the TtgGHI efflux pump contributed to resistance to ampicillin, chloramphenicol, and tetracycline only in a ΔttgABC mutant background. This indicated a modest role for the TtgGHI pump in antibiotic efflux from the cell. We have now compared the sensitivities of the wild-type and the DOT-T1E-PS52, -PS61, and -PS62 mutants to the three antibiotics cited above by determining the MICs that prevented growth. We found no difference in antibiotic resistance between each of the mutants and the wild-type strain (data not shown). These results suggest that the primary role of the TtgGHI efflux pump is to extrude solvents, whereas other antibiotic extrusion pumps operate efficiently in DOT-T1E (Terán et al., unpublished) so that the increased level of TtgGHI in the ttgV mutant backgrounds does not result in a significant increase in antibiotic resistance.

**In vitro study of the interactions between the ttgV and ttgG promoter region and the TtgV protein.** The results presented above suggest that the ttgV and ttgG promoters overlap and that the TtgV protein is a transcriptional repressor of the expression of both promoters. A gel mobility shift assay was done to study protein-DNA interactions within the ttgV-ttgG promoter region. A 210-bp DNA fragment containing the sequence between the first nucleotide before the start codon of ttgV and ttgG was synthesized by PCR and labeled with 32P. When this fragment was incubated with increasing concentrations of TtgV, a single shifted band was found (Fig. 4). The retarded band was gradually lost in competition assays in which increasing concentrations of cold DNA probe were used, but not when unspecific competitor DNA [poly(dI-dC)] was used (data not shown). Based on the concentration of the protein that was used and on the degree of retard, we estimated that TtgV had a high affinity for its target, whose range is around 5 × 10⁻⁷ M.

Footprinting assays were carried out to define the region in which TtgV binds within the ttgV-ttgG intergenic region. A 228-bp DNA fragment, which contained the region between +134 and −94 of the ttgV promoter, was generated by PCR and labeled at each of its 5’ ends, incubated with or without His₆-TtgV, and then treated with DNase I as described in Materials and Methods. The digestion pattern for the two strands is shown in Fig. 5. The pattern revealed a 40-bp region where the abundance of certain fragments decreased as the TtgV concentration increased. The protected region covers the −10 and −35 regions of each of the ttgG promoters and the divergently oriented ttgV promoter (Fig. 1B). In fact, the pro-
The protected region extends from +7 to −34 with respect to the ttgG1 promoter (+12 to −29 for the overlapping ttgG2 promoter) and from −13 to −55 for the ttgV promoter (Fig. 1B). Given the overlapping organization of these promoters and the fact that TtgV represses the expression of both operons to the same extent, it is likely that the binding of TtgV to the intergenic region blocks the entry of RNA polymerase to transcribe these promoters (44). We found two inverted repeats within the protected region: one located between −12 and −34 with respect to the ttgG1 promoter (+12 to −29 for the ttgG2 promoter and −12 to −36 with respect to ttgV) (Fig. 1B), and the other located between −2 and −24 in P_{nsgG1} (+4 to −19 in P_{nsgG2}) and −24 to −46 in the ttgV promoter (Fig. 1B). If the first inverted repeat were the target for TtgV, then it is difficult to explain how TtgV protects the right-most region shown shaded in Fig. 2B. The position of the second inverted repeat is more central and could in principle explain why TtgV bound to this motif covers the adjacent region. It is also interesting to note the presence of three direct repeats within the protected region: one located between −12 and −34 with respect to the ttgG1 promoter. Examples of transcriptional regulators that recognize direct repeats have been described in the AraC/XylS family of positive transcriptional regulators (10, 51). Although at present we cannot discern which nucleotides are specifically recognized by TtgV, it is worth noting that the QacR regulator interacts with its cognate promoters by recognizing direct repeats within inverted-repeat sequences (46). We cannot, therefore, exclude the possibility that TtgV binds to the left-most inverted repeat and that it recognizes a half site in the right-most protected region. Therefore, further in vitro assays using mutants in the potential target sequences and a wide series of footprint analyses are needed to define the precise motif recognized by TtgV.

In the acrAB operon of *E. coli*, the AcrR protein functions as the local specific regulator (22), but it has been shown that its level of expression is influenced by global regulators such as MarA, SoxS, and SdiA (22, 38). The genome of *P. putida* KT2440, a strain highly similar to DOT-T1E, was recently sequenced and annotated (30). Our BLASTN search revealed the presence of a SdiA homolog, but no MarA or SoxS homologs were found. Results from our laboratory seem to rule out the participation of SdiA in the control of ttg efflux pumps, because the overproduction of SdiA did not alter the expression pattern of a transcriptional fusion of each of the ttg promoters to *lacZ* (E. Duque and J. L. Ramos, unpublished results). At present, we have no evidence that the ttgGHI/ttgVW system is integrated in any of the global regulatory circuits that operate in *Pseudomonas* sp., but future work with...
the tggGH/tggKV system should reveal more intimate details of its regulation.

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