Effector-Repressor Interactions, Binding of a Single Effector Molecule to the Operator-bound TtgR Homodimer Mediates Derepression

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The RND family transporter TtgABC and its cognate repressor TtgR from Pseudomonas putida DOT-T1E were both shown to possess multidrug recognition properties. Structurally unrelated molecules such as chloramphenicol, butyl paraben, 1,3-dihydroxynaphthalene, and several flavonoids are substrates of TtgABC and activate pump expression by binding to the TtgR-operator complex. Isothermal titration calorimetry was employed to determine the thermodynamic parameters for the binding of these molecules to TtgR. Dissociation constants were in the range from 1 to 150 nM, the binding stoichiometry was one effector molecule per dimer of TtgR, and the process was driven by favorable enthalpy changes. Although TtgR exhibits a large multidrug binding profile, the plant-derived compounds phloretin and querceatin were shown to bind with the highest affinity (K_D of around 1 μM), in contrast to other effectors (chloramphenicol and aromatic solvents) for which exhibited a more reduced affinity. Structure-function studies of effectors indicate that the presence of aromatic rings as well as hydroxyl groups are determinants for TtgR binding. The binding of TtgR to its operator DNA does not alter the protein effector profile nor the effector binding stoichiometry. Moreover, we demonstrate here for the first time that the binding of a single effector molecule to the DNA-bound TtgR homodimer induces the dissociation of the repressor-operator complex. This provides important insight into the molecular mechanism of effector-mediated derepression.

The active efflux of toxic compounds is a common mechanism employed by bacteria to protect themselves against the deleterious effects of toxic molecules they encounter in the environment. The extrusion of toxic compounds is mainly mediated by transmembrane multiple drug resistance (MDR) efflux pumps that are broadly specific and able to accommodate a variety of structurally unrelated antimicrobial agents (1–4). In Gram-negative bacteria, the majority of MDR efflux pumps belong to the RND family, which includes transporters able to expel clinically relevant antibiotics, dyes, biocides, detergents, fatty acids, heavy metals, or organic solvents (5–7). These MDR efflux pumps have recently become the object of special interest, not only because they are a cause of concern in the emergence of multidrug resistant pathogens such as Pseudomonas aeruginosa (8), Escherichia coli (9), Stenotrophomonas maltophilia (10), or Neisseria gonorrhoeae (11, 12), but also for their importance in bacterial tolerance to toxic xenobiotics like organic solvents (7). Besides the clinical and ecological relevance, the particular ability of these proteins to interact with a wide range of structurally different molecules is of fundamental interest in understanding protein-ligand specificity (13–17). Moreover, a number of studies have shown that the expression of some MDR transporters is controlled by transcriptional regulators with the same multidrug recognition properties, such as BmrR of Bacillus subtilis (18), EmrR of E. coli (19), QacR of Staphylococcus aureus (20), and TtgR of Pseudomonas putida DOT-T1E (21). In that sense, the recent determination of the three-dimensional structures of QacR and BmrR in complex with several ligands has provided important insight into the molecular mechanisms of multiple drug recognition (22–24).

However, one of the most intriguing issues concerning bacterial MDR systems is the physiological function they exert in their natural habitat (14, 25, 26). Some MDR efflux pumps present in soil-living or plant-associated bacteria have been shown to confer resistance to plant defense molecules and seem to play an important role in the efficient colonization of plant-influenced microenvironments by these microorganisms and/or in their virulence, in the case of plant pathogens (27–30).

P. putida is a non-pathogenic bacterium present in water and soil and able to colonize plant roots and seeds (31–33). The DOT-T1E strain was isolated for its particularly high resistance to toxic organic solvents (34) and three RND efflux pumps, TtgABC, TtgDEF, and TtgGHI, were found to be essential for this resistance (35). This study will focus on the TtgABC efflux pump and its cognate regulator TtgR. TtgABC has been shown to play an important role in the intrinsic tolerance of P. putida DOT-T1E to organic solvents (35, 36). In addition, TtgABC is able to extrude several structurally different antimicrobial compounds, such as antibiotics (chloramphenicol, tetracycline, nalidixic acid, norfloxacin, streptomycin, ampicillin, and cefotaxime), biocides (triclosan), and dyes (ethidium bromide) (35, 37, 38), and hence confers an MDR phenotype to P. putida DOT-T1E.

TtgR is a member of the TetR family of transcriptional repressors. Typically, these repressors have two functional domains, a highly conserved N-terminal DNA binding domain, and a less conserved C-terminal domain involved in both dimerization and effector binding (39). TtgR is a multidrug binding repressor that negatively controls the transcription of the ttgABC operon as well as its own expression. TtgR operates by an effector-mediated derepression mechanism (21), which implies that effector binding to DNA-bound TtgR causes protein dissociation and subsequently transcriptional activation of ttgABC and ttgR.

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3 The abbreviations used are: MDR, multiple drug resistance; ITC, isothermal titration calorimetry; Km, kanamycin; LB, Luria-Bertani culture medium; MIC, minimal inhibitory concentration; RND, resistance nodulation cell-division; Pipes, 1,4-piperazineinediethanesulfonic acid; DTT, dithiothreitol.
TABLE 1
Bacterial strains and plasmids used in this study

| Strains and plasmids | Characteristics* | Ref. |
|----------------------|------------------|------|
| *P. putida* DOT-T1E | Rif*, Tol* | 34 |
| DOT-T1E18 | Rif*, Km*, ttgB::phoA-Km | 36 |
| DOT-T1E13 | Rif*, Km*, ttgR::dOhGr:Km | 37 |
| *E. coli* B834 (DE3) | pET29a (+) | Novagen |
| pWR12 | Km*, protein expression vector | Novagen |
| pED14 | Te*, phoA::lacZ fusion in pMP220 | 21 |

* Km*, Rif*, Te*, and Tol* stand for resistance to kanamycin, rifampicin, tetracycline and toluene, respectively.

As mentioned above, a number of structure-based studies on this type of multidrug binding regulators have provided important insights into ligand-induced conformational changes and protein-ligand contacts. However, only a few studies were aimed at identifying possible natural effectors for these regulators, which would help to ascribe a physiological function to a given MDR system. As *P. putida* is a very abundant soil bacterium found in close association with plant roots, we hypothesized that the TtgABC/TtgR system might be involved in the resistance to plant defense metabolites. To address this issue, two complementary approaches were used. Initial in vivo studies aimed at exploring the TtgABC-mediated resistance to plant defense metabolites, as well as the role of TtgR in the expression of the ttgABC operon in response to these compounds. Subsequently, an extensive in vitro characterization of TtgR binding to different plant secondary metabolites is carried out using ITC. Most importantly, the binding of different effectors to DNA-bound TtgR is also explored, which is an innovative approach we judge of general relevance for the study of transcriptional repressors.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains, Plasmids, and Culture Medium**

The bacterial strains and plasmids used in this study are shown in Table 1. Bacterial strains were grown in LB medium at 30°C as described before (21) or in 2×YT medium (40) for the production of the TtgR protein. When required, antibiotics were added to the culture medium to reach the following final concentrations: rifampicin, 20 μg/ml, and tetracycline, 20 μg/ml, pED14 is a low-copy number plasmid derived from pMP220 (41), which carries a transcriptional fusion of the *ttgA* promoter to the reporter gene *lacZ* (21).

**β-Galactosidase Assays**

Bacterial cells were inoculated from fresh LB agar plates in LB liquid medium supplemented with the appropriate antibiotics and grown overnight at 30°C with shaking (200 rpm). Cultures were diluted 100-fold in 15 ml of fresh LB or in the same medium supplemented with 1 mM of the inducing compounds, then incubated at 30°C with shaking until the culture reached an *A₀₅₀* of 1.0 when β-galactosidase activity was determined according to Miller (42). All the chemicals were used at concentrations below the minimal inhibitory concentration (MIC), therefore they did not affect growth. Results presented are the mean of four different experiments.

**Antimicrobial Agent Susceptibility Tests**

The MIC of the tested compounds was determined in LB medium by the microtiter broth dilution method (43). Microtiter plate wells containing each 100 μl of LB were inoculated with 10⁵ colony-forming units/ml and incubated for 16 h. The growth was then analyzed and the MIC corresponded to the minimal concentration at which growth was inhibited by at least by 90%.

**Overexpression and Purification of Native TtgR Protein**

A 651-bp fragment containing the *ttgR* gene was amplified by PCR from *P. putida* DOT-T1E chromosomal DNA using the primers TtgR5’Ndel (5’-NNNNNNCATATGGTCGCAGACCCAG-3’) and TtgR3’HindIII (5’-NNNNNNAGCTTCTACTATTTCCGGA-AGCCCGGCTC-3’), which contained Ndel and HindIII restriction sites, respectively (underlined), and the latest included also the *ttgR* stop codon (in bold). The PCR products were restricted with the above enzymes and subsequently ligated into the pET29a(+) vector (Novagen) previously digested with the same enzymes. The resulting plasmid was termed pWR12.

For the purification of native TtgR, pWR12 was transformed into *E. coli* B834(DE3). The cells were grown at 30°C in 2-liter Erlenmeyer flasks containing 1 liter of 2×YT culture medium (40) supplemented with 50 μg/ml Km. For TtgR production, protein expression was induced at an *A₀₆₆₀* of 0.5–0.6 by adding 1 mM isopropyl β-D-1-thiogalactopyranoside. Cells were grown for another 3 h at 20°C and subsequently harvested by centrifugation. The pellet resulting from a 1-liter culture was resuspended in 30 ml of buffer A (10 mM Tris-HCl, pH 6.4, 20 mM NaCl, 5% (v/v) glycerol, 0.1 mM EDTA, and protease inhibitor mixture (Complete®, Roche)) and broken by treatment with 20 μg/ml of lysozyme and French press. Following centrifugation at 13,000 × g for 40 min, the TtgR protein was predominantly present (more than 80%) in the soluble fraction. The supernatant was loaded onto a Hitrap Heparin HP column (5 ml, Amersham Biosciences) equilibrated with buffer A and eluted with a gradient of 0.02–0.5 M NaCl. Fractions containing TtgR were pooled, concentrated to 4 ml by ultrafiltration using an Amicon 8050 apparatus (Amicon-Millipore), and dialyzed against buffer B (20 mM Tris-HCl, pH 7.5, NaCl 250 mM, 10% (v/v) glycerol, 0.1 mM EDTA, and 1 mM DTT). The sample was then submitted to size exclusion chromatography using a Sephacryl HR-200 column (Amersham Biosciences). Eluted fractions of TtgR were pooled, concentrated using the above equipment, and dialyzed against buffer C (10 mM Tris-HCl, pH 7.5, 250 mM NaCl, 50% glycerol (v/v), 0.1 mM EDTA, and 2 mM DTT) for protein storage at ~70°C (long term storage) or ~20°C (short term storage). Protein concentrations were determined using the Bio-Rad Protein Assay kit.

**Electrophoretic Mobility Shift Assay**

Electrophoretic mobility shift assays were carried out as described previously (21). The DNA probe was a 189-bp fragment containing the *ttgABC*-ttgR intergenic region obtained from *P. putida* DOT-T1E chromosomal DNA by PCR. 1 nM of the radiolabeled probe (~10,000 cpm) was incubated with 0.75 μM purified TtgR in 10 μl of DNA binding buffer (10 mM Tris-HCl, pH 7.0, 250 mM NaCl, 10 mM Mg acetate, 10 mM KCl, 5% glycerol (v/v), 0.1 mM EDTA, and 1 mM DTT) supplemented with 20 μg/ml poly(dI-dC) and 200 μg/ml bovine serum albumin. Effectors (prepared in Me₂SO) were added to the binding reaction at a final concentration of 1 μM. Reactions were incubated for 10 min at 30°C and samples were run on 4.5% (w/v) non-denaturing polyacrylamide gels (Bio-Rad Mini-Protean II) for 2 h at 50 V at room temperature in Tris glycine buffer (25 mM Tris-HCl, pH 8.0, and 200 mM glycine). The resulting gels were analyzed using a Personal FX equipment and QuantityOne software (Bio-Rad).
**TtgR Multidrug Binding Repressor**

**ITC**

**Effectors Binding to TtgR**—Measurements were performed on a VP-microcalorimeter (MicroCal, Northampton, MA) at 30 °C. This instrument is equipped with a 1.437-ml sample cell and a 300-μl syringe. The protein was thoroughly dialyzed against effector binding buffer (25 mM Pipes, pH 7.0, 250 mM NaCl, 5% (v/v) glycerol, 10 mM Mg acetate, 10 mM KCl, 0.1 mM EDTA, and 1 mM DTT). The protein concentration was determined using the Bradford assay. Stock solutions of 1-naphthol, 1,3-dihydroxynaphthalene, butyl paraben, naringenin, quercetin, phloretin, apigenin, luteolin, coumestrol, and chlormphenicol were freshly prepared at 0.5 M in Me2SO, and subsequently diluted in dialysis buffer. The appropriate amount of Me2SO (0.1–0.3% (v/v)) was added to the protein sample in each assay. When indicated, analyses were carried out using 5% (v/v) Me2SO to facilitate the effector solubilization. All chemicals were manipulated in glass vessels and effector samples were neither degassed nor filtered, to avoid evaporation or nonspecific binding. Typically, an experiment involved a single 2 μl and a series of 4-μl injections of effector molecules into the protein solution.

**Effectors Binding to TtgR-DNA Complex**—A 40-bp DNA fragment containing the TtgR operator was used for these assays. It was obtained by assembling two complementary oligonucleotides (5′-CACCGAG-TATTTCACAACAACCATGAGTGAATCTTATCTTC-3′ and its complementary oligonucleotide). Annealing was carried out by mixing equimolar amounts (60 μM) of each oligonucleotide in an annealing buffer (10 mM Tris-HCl, pH 8.0, and 0.5 mM MgCl2). The mixture was incubated at 95 °C for 5 min, followed by slow cooling to room temperature. Both, DNA and TtgR protein were subsequently dialyzed in DNA binding buffer (10 mM Tris-HCl, pH 7.0, 250 mM NaCl, 5% (v/v) glycerol, 10 mM Mg acetate, 10 mM KCl, 0.1 mM EDTA, and 1 mM DTT). TtgR (typically 15–20 μM) was placed into the ITC cell and titrated by the addition of the 40-bp DNA fragment. When protein saturation was reached, i.e., when measured heat changes corresponded to those observed in the titration of buffer with DNA, the experiment was stopped and the cell content adjusted to a volume of 1.437 ml. The completeness of protein saturation with DNA was verified by native polyacrylamide gel electrophoresis. Subsequently, the DNA-TtgR complex was titrated with effector molecules as described above. The titration of unbound TtgR with effector molecules was carried out on the complex was titrated with effector molecules as described above. The titration of unbound TtgR with effector molecules was carried out on the same day using the same chemicals, exactly the same ligand concentrations and injection volumes, which guarantees that the absence/presence of DNA is the only difference between both assays.

In all cases, the mean enthalpies measured from the injection of the ligand into the buffer were subtracted from raw titration data before data analysis with ORIGIN software (MicroCal). Titration curves were fitted by a non-linear least squares method to a function for the binding of a ligand to a macromolecule (44). From the curve thus fitted, the parameters ΔH (reaction enthalpy), K_A (binding constant, K_A = 1/K_D), and n (reaction stoichiometry) were determined. From the values of K_A and ΔH, the changes in free energy (ΔG) and in entropy (ΔS) were calculated with the equation: ΔG = −RTlnK_A = ΔH − TΔS, where R is the universal molar gas constant, and T the absolute temperature.

**RESULTS**

**In Vivo Susceptibility of P. putida DOT-T1E to Plant Antimicrobials**—As mentioned above, the implication of the TtgABC/TtgR system in the organic solvent tolerance and multidrug resistance of DOT-T1E was previously reported (21, 35, 37). However, the particularly broad substrate specificity of this efflux system and the multidrug binding capacity of its cognate repressor TtgR raised the question concerning their physiological role(s). Based on the fact that the plant rhizosphere is one of the natural habitats of *P. putida,* and considering that the AcrB transporter from the plant pathogen *Erwinia amylovora,* involved in the resistance of this strain to various plant defense metabolites (27), is 60% identical to the TtgB transporter from *P. putida* DOT-T1E, we investigated the potential role of TtgABC in the resistance to plant-derived antimicrobials. To verify this hypothesis, the MICs of five plant secondary metabolites known to have antibacterial activities (45–47) were determined for both, *P. putida* DOT-T1E and its derivative mutant strain DOT-T1E18, deficient in the TtgABC efflux pump (Table 2). To different extents, the wild type strain was susceptible to all of the compounds tested. The susceptibility of DOT-T1E18 to phloretin, quercetin, and naringenin was significantly increased, suggesting that the TtgABC efflux pump confers resistance to these compounds. In contrast, no differences in the susceptibilities to coumestrol and berberine were detected, indicating that these compounds are probably not substrates of this pump. To corroborate this fact and given that antimicrobial activity of plant secondary metabolites is often masked by the concerted action of multiple MDR efflux pumps with similar substrate profiles (47), the MICs were determined in the presence of the MC207110 inhibitor, known to inhibit certain RND efflux pumps (48, 49). In the presence of this inhibitor, the susceptibilities of both strains to phloretin, quercetin, naringenin, and even coumestrol dramatically increased. Interestingly, the DOT-T1E18 mutant still showed increased susceptibilities to these compounds as compared with its parental strain. This indicates, first, that coumestrol is, in addition to phloretin, quercetin, and naringenin, also a substrate of the TtgABC pump and, second, that the TtgABC efflux pump is not (or not completely) inhibited by MC207110. This hence suggests the existence of other pump(s) that are impaired by MC207110 and expel the same compounds, accounting for the high level of resistance of *P. putida* DOT-T1E to these natural antimicrobials. Furthermore, the low MIC values obtained for coumestrol (0.78 μg/ml) or quercetin (4.2 μg/ml) are comparable with those found for clinically relevant antibiotics (35, 37), which points to the antibacterial potential of these natural compounds against *Pseudomonas* sp. strains when used in combination with MDR inhibitors. No MIC variation was detected for berberine, indicating that this plant alkaloid is not a TtgABC substrate. Taken together, these data confirm the role of this MDR pump in the high resistance of *P. putida* DOT-T1E to several plant antimicrobials present in the rhizosphere.

**TABLE 2**

**Susceptibilities of P. putida DOT-T1E and derivative mutant strain to different plant antimicrobials**

| Strain       | MIC (μg/ml) |
|--------------|-------------|
| DOT-T1E      | 8.2         |
| +            | 1.0         |
| DOT-T1E18    | 2.0         |
| +            | 0.13        |

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were also tested with any of the compounds tested, indicating that in the wild type back-
ttgR repressor (21), was used as a positive control. In the mutant DOT-
metabolites used above exert the same effect, electrophoretic mobility shift
of TtgR from its operator (21). To verify whether the plant secondary
levels that increases in the presence of antibiotics, such as chloramphenicol
was the most potent inducer (2.4-fold increase). Other aromatic com-
plant alkaloid. Other aromatic compounds that enhanced ttgABC expression in vivo (see above)
release TtgR from its operator, as expected from the in vivo ttgABC expres-
sion profile (Fig. 1), whereas no effect was obtained in the case of the organic
solvent styrene. Thus, these results confirm the multiple effector binding
capacity of TtgR, as it seems to recognize not only different classes of anti-
bio, but also aromatic solvents and plant secondary metabolites of very
different size and structure.

Calorimetric Analysis of the Interaction of TtgR with Multiple
Effectors—The thermodynamic parameters for the interaction of TtgR
with a wide range of structurally different effectors were determined by
ITC. Initial experiments involved the titration of TtgR with ligands
belonging to three different classes: aromatic solvents (styrene, butyl
paraben, 1-naphthol, 1,3-dihydroxynaphthalene), antibiotics (chloram-
phenicol, and plant antimicrobials (phloretin, quercetin, berberine,
and naringenin). Typical data exemplified for phloretin and naringenin
are shown in Fig. 3. ITC data were analyzed using the “one set of sites”
algorithm and satisfactory curve fits were obtained. The corresponding
thermodynamic parameters are shown in Table 3. In all cases the titra-
tion was characterized by exothermal heat changes, indicating favorable
enthalpy changes. With the exception of butyl paraben, entropy changes
were unfavorable, which is generally attributed to a loss of conforma-
tional flexibility of the protein upon effector binding (50). Dissociation
constants were found to be between 1 and 150 μM, which are values
similar to those obtained for the effectors of other multidrug binding
transcriptional regulators (22, 23). Phloretin and quercetin were found
to bind with the highest affinity with $K_D$ values close to 1 μM (Table 3).
The titration profiles of these effectors were sigmoidal (exemplified for
phloretin in Fig. 3A), thus allowing a precise determination of the effec-
tor binding stoichiometry, which was found to be one effector molecule
for TtgR dimer. Naringenin, chloramphenicol, and butyl paraben exhib-
itiated an intermediate affinity ranging between 10 and 20 μM, giving rise
to hyperbolic titration profiles (exemplified for naringenin in Fig. 3B). It
should be noted here that the curve shape (hyperbolic or sigmoidal) is
determined solely by the ligand concentration in the ITC cell and the
binding affinity. Both, high ligand concentrations and high affinity, favor
sigmoidal curves. Therefore no conclusions concerning the binding
mechanism can be draw from differences in the curve shape. The
aromatic solvents 1-naphthol and 1,3-dihydroxynaphthalene had only
modest affinities (around 150 and 60 μM, respectively). It should be
noted that the additional hydroxyl group of 1,3-dihydroxynaphthalene
as compared with 1-naphthol, led to a 2-fold increase in both the bind-
ing affinity to TtgR and the enthalpy term (Table 3), which is consistent
with a direct recognition of the hydroxyl group at position 3 of 1,3-
dihydroxynaphthalene by TtgR.
TtgR Multidrug Binding Repressor

![Graph showing ITC data](image)

**FIGURE 3. ITC of the binding of different effector molecules to TtgR.** ITC experiments were carried out as described under “Experimental Procedures” at 30 °C in effector binding buffer. Heat changes (upper panels) and integrated peak areas (lower panels) for the injection of a 2-μl and a series of 4-μl aliquots of 200 μM phloretin (A) and 250 μM naringenin (B) into a solution of 8 μM TtgR are presented. Data were fitted with ORIGIN using the one set of sites algorithm.

**TABLE 3**

**Thermodynamic parameters derived from the calorimetric titration of TtgR with structurally different effectors**

| Effector                  | $K_a$ $M^{-1}$ | $K_D$ μM | $\Delta G$ kcal/mol | $\Delta H$ kcal/mol | $T\Delta S$ kcal/mol |
|---------------------------|----------------|-----------|---------------------|---------------------|-----------------------|
| Phloretin                 | 9.00 ± 0.38×10^2 | 1.11 ± 0.05 | −8.26 ± 0.03       | −15.70 ± 0.19       | −7.44 ± 0.17          |
| Naringenin                | 1.07 ± 0.04×10^2 | 9.35 ± 0.35 | −6.97 ± 0.02       | −27.2 ± 1.7         | −20.1 ± 1.7           |
| Chloramphenicol           | 9.17 ± 1.14×10^4 | 10.9 ± 0.3   | −6.88 ± 0.01       | −16.3 ± 0.19        | −9.42 ± 0.19          |
| Butyl paraben             | 5.07 ± 0.18×10^4 | 19.7 ± 0.7   | −6.52 ± 0.02       | −4.49 ± 0.07        | 2.03 ± 0.07           |
| 1,3-Dibydroxynaphthalene | 1.65 ± 0.11×10^4 | 61 ± 4      | −5.85 ± 0.04       | −19.3 ± 5.7         | −13.5 ± 5.8           |
| 1-Naphthol                | 6.73 ± 0.16×10^3 | 148 ± 4     | −5.31 ± 0.02       | −10.9 ± 0.2         | −5.6 ± 0.2            |
| Styrene                   |                | No binding |                     |                     |                       |
| Berberine                 |                | No binding |                     |                     |                       |

**Structure-Function Studies**—Although the TtgR effector profile was found to be large, the absence of styrene and berberine binding as well as important differences in affinity for the variety of effectors tested prompted subsequent studies that were aimed at exploring the structure-function relationship of TtgR effectors. Compounds selected for analysis were plant secondary metabolites that have a structure related to those of phloretin and naringenin, shown to bind tightly in the initial studies (Table 3). However, an obstacle encountered in this series of ITC assays was the reduced solubility of some of the compounds that did not permit their analysis by ITC using the initial buffer system. This problem was overcome by the addition of 5% Me2SO to the buffer, an approach typically used if the affinity of poorly soluble compounds is to be determined by ITC (51).

Initial experiments involved the analysis of the binding of phloretin and naringenin to TtgR in the Me2SO containing buffer system (Table 4) and a comparison of the obtained thermodynamic parameters with those obtained in the initial buffer system (Table 3, naringenin and phloretin were the only two effectors with a sufficient solubility to be analyzed in the absence of Me2SO). In the presence of Me2SO, favorable entropic and enthalpic contributions were observed, whereas the binding was strongly enthalpy driven and counterbalanced by unfavorable entropy in the absence of Me2SO. However, for both phloretin and naringenin, enthalpy changes as well as binding constants decreased proportionally following the inclusion of Me2SO, which justified the use of this solvent to compare thermodynamic parameters of different effectors.

The thermodynamic mode of binding of all effectors was found to be similar. In all cases binding was driven by moderately favorable enthalpy and entropy changes (Fig. 4, Table 4). As illustrated in Fig. 4, naringenin, luteolin, phloretin, and genistein can be compared with apigenin, which represents a core structure. The most dramatic change in binding parameters, a 4-fold decrease in binding affinity, was observed for the transition of apigenin to naringenin, characterized solely by the reduction of a double bond that makes the molecule non-planar and allows free rotation of the C-ring. The increased flexibility of phloretin, as a consequence of the B-ring opening, was accompanied by a slight increase in the binding affinity ($K_D$ around 11 μM). Comparing the flavone apigenin with its isomer the isoflavone genistein generated an almost 2-fold decrease in affinity. Only modest changes in binding affinity were observed for coumestrol. However, it is important to note that the additional presence of a hydroxyl group of luteolin with respect to apigenin resulted in a 2-fold increase of the binding constant.

It can be concluded that TtgR recognizes a wide range of plant secondary metabolites with physiologically relevant affinities. Nevertheless, two major determinants in the effector molecule seem to be essential for TtgR recognition, i.e. the presence of at least one aromatic ring, which reflects the hydrophobic nature of the compound, and the presence of hydroxyl groups. The latter appears to be a major feature
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Thermodynamic parameters derived from the calorimetric titration of TtgR with structurally related plant signal molecules

| Effector    | Type of compound | $K_d$ (µM) | $K_d$ (µM) | $ΔG$ (kcal/mol) | $ΔH$ (kcal/mol) | $ΔS$ (cal/mol) |
|-------------|------------------|------------|------------|-----------------|----------------|--------------|
| Luteolin    | Flavone          | 13.80 ± 0.65 | 7.20 ± 0.34 | −7.13 ± 0.03 | −3.05 ± 0.09 | 4.08 ± 0.10 |
| Phloretin   | Dihydrochalcone  | 9.14 ± 0.35 | 10.9 ± 0.4 | −6.88 ± 0.02 | −1.99 ± 0.03 | 4.89 ± 0.04 |
| Apigenin    | Flavone          | 6.45 ± 0.53 | 15.5 ± 1.3 | −6.67 ± 0.05 | −1.28 ± 0.04 | 5.39 ± 0.06 |
| Coumestrol  | Coumestan        | 5.49 ± 0.69 | 18.2 ± 2.3 | −6.57 ± 0.08 | −1.68 ± 0.25 | 4.89 ± 0.27 |
| Genistein   | Isoflavone       | 3.66 ± 0.17 | 27.3 ± 1.3 | −6.33 ± 0.03 | −1.93 ± 0.06 | 4.40 ± 0.07 |
| Naringenin  | Flavanone        | 1.54 ± 0.10 | 64.9 ± 4.2 | −5.81 ± 0.04 | −3.45 ± 0.50 | 2.36 ± 0.51 |

FIGURE 4. Molecular structures of plant-derived effectors of TtgR and their respective dissociation constants for the binding to TtgR. ITC experiments were carried out as described under “Experimental Procedures” at 30 °C in effector binding buffer to which 5% (v/v) Me2SO was added. Structures are positioned as pairs (double arrows) to illustrate the corresponding small changes in their structure. Additional thermodynamic parameters are listed in Table 4.

because the highest binding affinities were observed for the only two effectors (luteolin and phloretin) with four hydroxyl groups (Fig. 4 and Table 5).

Calorimetric Analysis of the Effector Interaction with TtgR-DNA Complex—As reported previously (21), TtgR represses the ttgABC operon by binding to its operator site located in the ttgR-ttgA intergenic region. Transcriptional activation is achieved by an effector-mediated dissociation of DNA-bound TtgR, as shown in Fig. 2. ITC has thus been employed to study the binding of effector molecules to the DNA-bound form of TtgR to compare effector binding parameters to those obtained with the free protein. Initial experiments intended to find a buffer in which the protein-DNA complex was soluble and stable. The optimal buffer (see “Experimental Procedures”) slightly differed from the one used above (Tables 3 and 4). The DNA-TtgR complex (generated as detailed under “Experimental Procedures”) was titrated with the four effector molecules with the highest affinity for the unbound protein, namely phloretin, quercetin, naringenin, and chloramphenicol (Table 3). No effector binding to free DNA was observed in control experiments (data not shown).

Titrations of free and DNA bound TtgR with phloretin showed that peak widths of both raw data traces were comparable (Fig. 5). Moreover, aliquots of the ITC cell content were taken before and after the experiments were analyzed by native polyacrylamide gel electrophoresis, which confirmed the absence of free protein at the beginning of the experiment and the absence of DNA-bound protein at the end of the experiment (not shown). These observations are consistent with an immediate dissociation of the protein following effector binding. Under those circumstances, observed enthalpy changes for the effector binding to the protein-DNA complex correspond to heat changes because of effector binding and protein dissociation. Fig. 5 demonstrates unequivocally that the binding of TtgR to its operator DNA does not alter the effector binding stoichiometry of 1 effector molecule per TtgR dimer.

Most interestingly, phloretin was found to bind to free TtgR and to the DNA-bound protein with an affinity of around 1 and 0.5 µM, respectively, which was accompanied by a slight increase in the enthalpy change for the binding to the DNA-protein complex (Table 5). A similar increase in affinity and the negativity of the enthalpy change was observed for quercetin (Table 5). However, this behavior was not a general feature for all effectors, because naringenin bound with comparable affinities to free and DNA-bound protein, whereas chloramphenicol had a slightly reduced affinity for the TtgR-DNA complex (Table 5).

DISCUSSION

The TtgABC efflux pump and its cognate transcriptional regulator TtgR were previously characterized in the framework of both organic solvent tolerance and MDR phenotypes of P. putida DOT-T1E (35–37). The ttgABC operon showed a relatively high basal level of expression, which was not significantly altered by the presence of organic solvents (37), but was enhanced in response to some of the antibiotics expelled by the pump (21). These two facts raised the question concerning the physiological function of the TtgABC/TtgR system.

Plant defense metabolites such as certain flavonoids (quercetin, genistein) were previously shown to possess antibacterial activity (45–47), which is because of the inhibition of DNA gyrase (52). In the present work, we demonstrate that this system is involved in the resistance to plant antimicrobials like phloretin, quercetin, naringenin, or coumestrol. Therefore, the TtgABC efflux pump may be a critical element in the competitive colonization of plant roots by P. putida, similarly to other MDR transporters found in soil- and plant-associated bacteria (28–30). In addition, the fact that a mutant E. amylovora deficient in the MDR efflux pump AcrAB (60% identical to TtgAB) was impaired in its virulence against plants (27), emphasizes our hypothesis.

In vitro results demonstrate that the ttgABC-specific regulator TtgR recognizes a number of plant defense metabolites with high affinity. These compounds were shown to promote the dissociation of the TtgR-operator complex and thus appear to be among the effectors that possess a biologically relevant function for the survival of the bacterium in its natural habitat. ITC assays revealed that TtgR binds to multiple structurally unrelated molecules. Dissociation constants obtained ranged from 1 to 150 µM, which are biologically relevant and similar to those obtained for other multiple effector binding repressors of bacterial transporters as QacR (23, 53), EmrR (19), or TtgV (54). Although this confirms the particularly large multidrug binding profile of the TtgR repressor, it is interesting to note that the effectors with the high-


**TtgR Multidrug Binding Repressor**

**TABLE 5**

Thermodynamic parameters derived from the calorimetric titration of free TtgR and TtgR bound to its operator with different effectors

| Effector     | Receptor | $K_a$ ($10^6$ M$^{-1}$) | $K_d$ (μM) | $ΔG$ (kcal/mol) | $ΔH$ (kcal/mol) | $TΔS$ (kcal/mol) |
|--------------|----------|-------------------------|------------|-----------------|-----------------|-----------------|
| Phloretin    | TtgR     | 9.75 ± 0.49             | 1.03 ± 0.05| −8.30 ± 0.03    | −17.02 ± 0.18   | −8.72 ± 0.35    |
|              | TtgR-DNA | 2.05 ± 1.4              | 0.49 ± 0.03| −8.75 ± 0.04    | −19.20 ± 0.21   | −10.45 ± 0.22   |
| Quercetin    | TtgR     | 2.20 ± 0.03             | 4.54 ± 0.06| −7.41 ± 0.01    | −4.75 ± 0.25    | 2.66 ± 0.25     |
|              | TtgR-DNA | 5.42 ± 0.77             | 1.85 ± 0.16| −7.95 ± 0.09    | −5.13 ± 0.21    | 2.82 ± 0.23     |
| Naringenin   | TtgR     | 1.15 ± 0.04             | 8.70 ± 0.26| −7.02 ± 0.02    | −9.21 ± 0.16    | −2.49 ± 0.16    |
|              | TtgR-DNA | 1.06 ± 0.03             | 9.43 ± 0.23| −6.97 ± 0.02    | −14.20 ± 0.17   | 7.23 ± 0.21     |
| Chloramphenicol | TtgR     | 0.63 ± 0.03             | 15.8 ± 0.7 | −6.65 ± 0.03    | −13.40 ± 0.30   | −6.75 ± 0.31    |
|              | TtgR-DNA | 0.40 ± 0.01             | 24.8 ± 0.7 | −6.38 ± 0.02    | −11.30 ± 0.16   | −4.92 ± 0.16    |

The binding stoichiometry of small ligands to a protein homodimer is 2:1 (Ref. 52), but for QacR (23) and BmrR (22) it was observed that two molecules of effector bind to each of the proteins.

**FIGURE 5.** ITC of the binding of the effector phloretin to free and DNA-bound TtgR. ITC experiments were carried out as described under “Experimental Procedures” at 30 °C in DNA binding buffer. Heat changes for the injection of a 2-μmol aliquots of 700 μM phloretin into 20 μM free TtgR (A) and TtgR-operator complex (TtgR at 20 μM in its DNA-bound form) (B). C, lower panel, integrated peak areas and non-linear regression of the above data using the one set of sites algorithm of Origin. Closed symbols, free TtgR; open symbols, DNA-bound TtgR.

For the TtgR operator complex the values of around 1 μM were determined from stoichiometry experiments at 30 °C in DNA binding buffer. Heat changes for the injection of a 2-μmol aliquots of phloretin into 20 μM free TtgR (A) and TtgR-operator complex (TtgR at 20 μM in its DNA-bound form) (B). C, lower panel, integrated peak areas and non-linear regression of the above data using the one set of sites algorithm of Origin. Closed symbols, free TtgR; open symbols, DNA-bound TtgR.

For the TtgR regulator with tetracycline (56). The 1:2 stoichiometry observed for the binding of effector molecules to TtgR can thus be regarded as unusual and raises the question whether a similar stoichiometry is observed when the protein is bound to the DNA, which is an essential aspect in understanding the molecular mechanism of effector-mediated derepression. ITC assays revealed that one effector molecule bound to the operator-TtgR dimer complex (Fig. 5). Mechanistically, this result indicates that the entry of a single effector molecule into the binding pocket of one of the monomers in the TtgR dimer bound to its operator is sufficient to promote the dissociation of the TtgR-operator complex. The analysis of effector binding to the DNA-bound form of TtgR presented in this work constitutes a novel approach for the functional study of a transcriptional repressor, because it reproduces more closely in vitro the biological process occurring in vivo, namely the binding of an effector molecule to DNA-bound protein. This aspect in the study of transcriptional repressors is yet relatively unexplored, as the majority of the biochemical analyses of repressor-effector interactions are carried out with free protein presuming that those interactions are identical when repressors are bound to their operators. Whereas this can be true for some systems, as we have already reported for the TtgV repressor in a previous analysis (54), our present results establish that significant differences exist between effector binding to free and DNA-bound protein (Table 5). Interestingly, for phloretin and quercetin, the two effectors found to bind most tightly to the free protein (Table 3), an increase of 2.1- and 2.5-fold in affinity was observed when TtgR was bound to the DNA (Table 5). In contrast, chloramphenicol bound with lower affinity to the protein-DNA complex than to the free protein (Table 5). Differences in repressor-effector interaction caused by DNA binding have been described recently for the VceR regulator of *Vibrio cholerae*, another member of the TetR family (57). Authors found that...
the affinity of VceR for its effector (carbonyl cyanide m-chlorophenylhydrazide) decreased when bound to DNA, and proposed an equilibrium model in which effector and DNA compete for VceR in a mutually exclusive manner. However, this model is not applicable to the system under study because clear evidence has been obtained for the binding of effector to DNA-bound protein.

The effector specificity of TtgR could be the result of an evolutionary process during which that P. putida has been exposed to a variety of compounds in the plant rhizosphere, where phloretin and quercetin are abundant plant secondary metabolites that are synthesized and secreted by plants either constitutively or in response to pathogens or stress (58). It is thus likely that TtgR has evolved to detect these compounds at concentrations below their toxicity threshold, so that induction of the efflux pump prevents the accumulation of these antimicrobials and their deleterious effects. The optimization of this property might have been achieved by increasing the affinity of TtgR for these plant antimicrobials, particularly in its DNA-bound form. It is then suggested that multidrug-binding proteins (regulators or transporters) have evolved to acquire a specific natural function and that in some cases recognition and/or expulsion of certain drugs or xenobiotic compounds is an accidentally occurring but intrinsic side effect of these systems.

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