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Transcriptional control by two interacting regulatory proteins: identification of the PtxS binding site at PtxR

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ABSTRACT

The PtxS and PtxR regulators control the expression of the glucose dehydrogenase genes from the P_{gad} promoter in *Pseudomonas aeruginosa*. These regulators bind to their cognate operators, that are separated by ~50 nt, within the promoter region and interact with each other creating a DNA-loop that prevents RNA polymerase promoter access. Binding of the 2-ketogluconate effector to PtxS caused PtxS/PtxR complex dissociation and led to the dissolution of the repression loop facilitating the entry of the RNA polymerase and enabling the transcription of the *gad* gene. We have identified a hydrophobic surface patch on the PtxR putative surface that was hypothesized to correspond to the binding site for PtxS. Two surface-exposed residues in this patch, V173 and W269, were replaced by alanine. Isothermal titration calorimetry assays showed that PtxS does not interact with the mutant variants of PtxR. Electrophoretic mobility shift assay and DNAase I footprinting assays proved that both regulators bind to their target operators and that failure to interact with each other prevented the formation of the DNA-loop. *In vitro* transcription showed that PtxS *per se* is sufficient to inhibit transcription from the P_{gad} promoter, but that affinity of PtxS for its effector is modulated by PtxR.

INTRODUCTION

Pseudomonas aeruginosa is a ubiquitous opportunistic pathogen capable of infecting humans, animals and plants (1,2). We have recently shown that both glucose metabolism and the expression of the virulence factor exotoxin A, encoded by the *toxA* gene, are modulated by the concerted action of the PtxS and PtxR regulators (3,4). PtxR is a member of the LysR family of regulators (5), whereas the LacI family member, PtxS (6,7), is a repressor that specifically recognizes 2-ketogluconate as an effector. PtxR, but not PtxS, was found to bind to the P_{toxA} promoter. We have recently demonstrated that PtxS binds with high affinity to PtxR either when bound to DNA or in solution (8). More interestingly, PtxS–PtxR complex formation occurs only in the absence of 2-ketogluconate, whereas in its presence, both proteins fail to interact with each other (8).

PtxR bound adjacent to the −35 region of P_{toxA} promoter acts as a positive transcriptional regulator. The binding of PtxS to DNA-bound PtxR was found to prevent transcription of the *toxA* promoter by PtxR (8). The PtxS/PtxR complex also regulates the transcriptional activity of the P_{kgu} and P_{gad} promoters that drive the transcription of the *kgu* and *gad* operons, respectively, and that encode enzymes for glucose catabolism (7,8). At the P_{gad} promoter, PtxS and PtxR bind to two different operators that are separated by ~50 nt. PtxR binds at the −35 region for RNA polymerase (−42 to −33), whereas PtxS binds downstream of the transcriptional start point (+10 to +24) so that PtxS functions as a repressor blocking RNA polymerase progression if the initiation of transcription happens. Foot-printing assays showed that PtxS and PtxR interact with each other forming a DNA-loop when bound to P_{gad} and P_{kgu} promoters, which was visualized as a hypersensitive site in DNase I footprinting assays (8). This loop prevents the entry of the RNA polymerase within the promoter. This repression can be relieved through the binding of 2-ketogluconate to PtxS, which triggers PtxR–PtxS dissociation and the subsequent release of PtxS from DNA. Promoter-bound PtxR is an activator of transcription, but the binding of PtxS to this complex causes transcriptional repression.

Transcriptional control through protein–protein interaction of two regulatory proteins that belong to different families of regulators is unusual in prokaryotes, particularly when the action of one of the members can be exerted either in solution or bound to DNA. In this study, we

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report repression from P_{gad} promoter by PtxR and PtxS bound to their operator sequences. Based on 3D homology modelling, we have identified two hydrophobic residues (V173 and W269), putatively surface exposed at PtxR, which were hypothesized to be relevant for PtxS binding to PtxR. Using site-directed mutagenesis, we replaced these residues by alanine and analysed protein–protein interaction and the interaction of the mutant regulators with DNA via electrophoretic mobility shift assay (EMSA) and footprinting. Our data support that V173 and W269 are vital for PtxS–PtxR interaction and that the PtxR mutants recognize their specific operators, but fail to induce DNA bending. *In vitro* transcription results illustrate that PtxS *per se* prevents transcription from P_{gad}, but that the affinity for its effector 2-ketogluconate is allosterically fine-modulated by PtxR.

MATERIALS AND METHODS

Strains, plasmids and culture medium

Escherichia coli DH5α was used for gene cloning and *E. coli* BL21 (DE3) was used for protein expression. Cells were grown at 37°C with shaking in LB medium supplied with ampicillin (100 μg/ml) or kanamycin (25 μg/ml).

Site-directed mutagenesis of V173 and W269 residues in PtxR

PtxR mutants V173A and W269A were generated by amplification of the *ptxR* gene in plasmid pET28b(+)::ptxR using *pfu* turbo DNA polymerase (Stratagene) and 39 mer overlapping primers that incorporated appropriate mismatches to introduce the desired mutation(s), namely, 5′-GACTTCCAGCAGGCGGCCGTGGCGAC GCCGGGATACCTC-3′ annealed with 5′-CGTCGCCA CGGCCGCCTGCTGGAAGTCGCACAGCTTGCT-3′ for V173A and 5′-CTGCTGCCCGGTGCGCGCCTGCC GCAGGGCGGCATCTAT-3′ annealed with 5′-CTGCG GCAGGCGCGCACCGGGCAGCAGCCGGACCAGC CG-3′ for W269A, (9). The polymerase chain reaction (PCR) product, corresponding to the full plasmid bearing the required mutation(s), was digested with *Dpn*I and transformed in *E. coli* BL21 (DE3).

Overexpression and purification of His-tagged PtxS and PtxR variants

The corresponding plasmids bearing the gene encoding for PtxS, wild-type PtxR, PtxR-V173A and PtxR-W269A mutants were transformed in *E. coli* BL21 (DE3). Cells were grown at 30°C with shaking in 2-L Erlenmeyer flasks containing 250 ml of LB supplemented with kanamycin. When the cultures reached a turbidity of ~0.6 at 660 nm, 1 mM isopropyl-β-D-thiogalactopyranoside was added to induce the expression of the corresponding gene from the plasmid P_{lac} promoter, and from this point onward, cultures were incubated at 18°C overnight. Cells were harvested by centrifugation (30 min at 20.000*g*) and stored at −80°C until used for protein purification following the protocol described in (8).

Isothermal titration calorimetry

Microcalorimetric experiments were carried out at 20°C using a VP-microcalorimeter (Microcal, Amherst, MA). Wild-type PtxS, PtxR and PtxR-V173A and PtxR-W269A proteins were dialyzed against 50 mM Hepes, pH 7.9; 300 mM NaCl; 1 mM dithiothreitol (DTT); and 10% (v/v) glycerol buffer. For the protein–protein interaction assays, 3 μM of native or mutants PtxR was titrated with 3.2 μl aliquots of 50 mM of PtxS. All data were corrected using the heat changes arising from the injection of the ligand from the syringe into the buffer. The titration data were analysed using the 'one-binding site model' of the MicroCal version of ORIGIN. The parameters ΔH (reaction enthalpy), K_A (binding constant, $K_A = 1/K_D$) and n (reaction stoichiometry) were determined from the curve fit. The change in free energy (ΔG) and in entropy (ΔS) was calculated from the values of K_A and ΔH using the following equation: $\Delta G = -RT \ln K_A = \Delta H\text{-}T\Delta S$, where R is the universal molar gas constant and T is the absolute temperature (10).

Electrophoresis mobility shift assays

The P_{gad} promoter region of *P. aeruginosa* was amplified by PCR using pGEM-T:P_{gad} as a template and the amplified fragment was isolated from agarose gels and end-labelled with [γ-^{32}P] deoxy-ATP using the T4 polynucleotide kinase. A 10-μl sample containing ~2 nM of labelled DNA (1.5×10^4 cpm) was incubated with combined amounts of native PtxS, PtxR or PtxR mutants (20 μM) for 1 h in 10 ml of binding buffer [50 mM Tris–HCl, pH 7.5, 10 mM NaCl, 0.5 M magnesium acetate, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM DTT and 5% (vol/vol) glycerol] containing 20 mg/ml of polyd(IC) and 200 mg/ml bovine serum albumin. The DNA–protein complexes were resolved by electrophoresis in 4% (wt/vol) non-denaturing polyacrylamide gels in 1× TBE using BioRad electrophoresis equipment, as previously described (11,12).

DNaseI footprinting

The DNA fragment containing P_{gad} of *P. aeruginosa* was amplified by PCR and labelled with [γ-^{32}P] deoxy-ATP. For the footprinting assays, 10 μl samples containing 2 nM of probe were mixed with different amounts of PtxS and PtxR or its mutant derivatives (20 μM) in binding buffer. Samples were incubated at 30°C for 1 h, before being treated with DNase I (0.4 U; Roche Biochemicals) diluted in 10 mM Tris–HCl (pH 7) supplemented with 2.5 mM $MgCl_2$, 1 mM $CaCl_2$, 0.1 mM EDTA and 50 mM KCl. After incubation for 3 min, the reaction was stopped by adding 2 μl of 500 mM EDTA. DNA was extracted with phenol-chloroform, ethanol precipitated and dissolved in 10 μl of sequence loading buffer. Equal amounts of DNA were heated at 95°C for 5 min and electrophoresed through a 6.5% (wt/vol) DNA sequencing gel (13). Appropriate sequencing reactions were loaded onto the gels along with the footprinting samples and used as a size ladder for identification of the sequences of protected sites.

Homology modelling and identification of key amino acids for PtxR and PtxS interaction

The *P. aeruginosa* PtxR 3D model was generated using the EasyPred 3D server and the Swiss-Model server based on the crystal structure of CrgA of *Neisseria meningitidis* [pdb: 3hhg, (14)] as template, which exhibits 29% identity with PtxR. The potential PtxS binding site was identified by a visual inspection of the surface potential. The prediction of amino acids that appear to be more important for PtxS and PtxR interaction was identified by PyMOL and the PtxR homology model ViewerLite software, excluding the potential surface area predicted to be the PtxR dimer interface. In this way, a hydrophobic patch was identified as the potential PtxS binding site and the inspection of the molecular detail of this patch resulted in the identification of two surface-exposed hydrophobic amino acids (V173 and W269) which were assumed to play key roles in protein–protein interaction owing to their surface exposure and conservation in multialignments of homologous proteins (See later in the text).

In vitro transcription assays

The P_{gad} promoter region of *P. aeruginosa* was amplified by PCR using pGEM-T:P_{gad}. *In vitro* transcription reactions (20 µl) were performed in 50 mM Tris–HCl (pH 7.5) supplemented with 10 mM NaCl, 0.5 mM magnesium acetate, 0.1 mM EDTA and 1 mM DTT containing 50 nM *E. coli* σ^{70}-holoenzyme, 20 U of RNasin (Promega) and 5 nM linear P_{gad} DNA template that had been generated by PCR amplification, as described above. The mixtures were incubated for 30 min at 30°C before the addition of 0.1 mM ATP, CTP and GTP, 0.05 mM UTP and 3.6 µCi of [α-^{32}P]UTP (10 µCi/µl) (1 Ci = 37 GBq), to which wild-type PtxS was added with or without the different PtxR proteins. After 30 min of incubation, the reactions were chilled at 4°C, and 4 µl of formamide sequencing dye was added. Samples were separated in a 6.5% (wt/vol) polyacrylamide gel electrophoresis. When indicated, 2-ketogluconate was added to the reaction mixture at the indicated concentrations.

RESULTS

Identification at the PtxR protein of a region for the potential binding to PtxS

Because PtxR belongs to the LysR family of transcriptional regulators (5), a homology model for PtxR was generated using the structure of another LysR type regulator, CrgA of *Neisseria meningitidis* [pdb: 3hhg, (14)], as a template. Both proteins share 29% sequence identity and the resulting model was of satisfactory geometry as judged by its Ramachandran plot (Supplementary Figure S1). The model suggested that a canonical helix-turn-helix DNA binding domain was linked through a connecting helix to the effector and dimerization domain of the protein.

To determine the oligomeric state of PtxR, we purified PtxR to homogeneity and carried out gel filtration analysis. We found that PtxR behaves as a monomer in solution (Supplementary Figure S2), and because the PtxR operator is palindromic (8), we suggested that PtxR dimerizes on binding to DNA, as in CrgA [in CrgA, dimerization occurs through a back-to-back association of the effector binding domains (Supplementary Figure S3 and (14)]. We assumed that PtxR dimerizes in an analogous manner as CrgA, and the proposed dimerization interface of PtxR is highlighted in Figure 1.

PtxS recognizes PtxR with high affinity *in vitro*. In general, protein–protein interactions are primarily mediated by the interaction of hydrophobic amino acids, and the inspection of protein–protein interfaces shows that these binding sites frequently form a hydrophobic patch (15). The inspection of the surface potential of PtxR revealed a hydrophobic patch (Figure 1A) located perpendicular to the proposed dimerization interface. We hypothesized that this patch could represent the region to which PtxS binds. The inspection of this hydrophobic patch showed that the side chains of two hydrophobic amino acids, V173 and W269 (Figure 1B), were entirely surface exposed and could play a role in a potential interaction with PtxS. In an alignment of PtxR homologues (Supplementary Figure S4), W269 was always present, and at position 173, an aliphatic amino acid (V, L, I) was conserved.

Mutation of PtxRV173 and PtxRW269 abolished interaction with PtxS but does not alter DNA binding

To verify the potential role of V173 and W269 in the interaction with PtxS, alanine replacement mutants were generated. Isothermal titration calorimetry (10) was used to determine the effect of the amino acid replacement on the PtxS–PtxR interaction. As shown previously (8), PtxS binds tightly to PtxR and the corresponding isothermal titration calorimetry curve is provided in Figure 2A. Binding was characterized by two events with K_D values of 12 ± 2 and 99 ± 8 nM and enthalpy changes of -27.3 ± 0.7 and -13 ± 0.8 kcal/mol, respectively. In marked contrast, the titration of PtxS with both PtxR-V173A and PtxR-W269A gave rise to small even peaks that can be entirely attributed to dilution heats (Figure 2B and C). We therefore concluded that mutation of both amino acids prevented a molecular interaction between PtxS and PtxR.

To study whether the PtxR mutants were able to bind to its target operators, we first carried out a series of EMSAs. Control assays revealed that the PtxR mutants did not bind to non-specific DNA, although they did bind to the P_{gad} promoter (Figure 3). In fact, the results presented in Figure 3 (lanes 3–5) revealed that migration of the target DNA fragment increased in the presence of PtxR, PtxR-V173A and PtxR-W269A, which suggests that the PtxR mutant protein can recognize its target operator. In the presence of PtxS, a similarly retarded band was obtained, as expected from the similarities of the molecular masses of PtxS (37 kDa) and PtxR (35 kDa). Furthermore, in assays containing both PtxS and PtxR, DNA mobility was further retarded, which is in agreement with the simultaneous binding of PtxR and PtxS. However, we noticed that when the PtxS and PtxR mutants were used, the

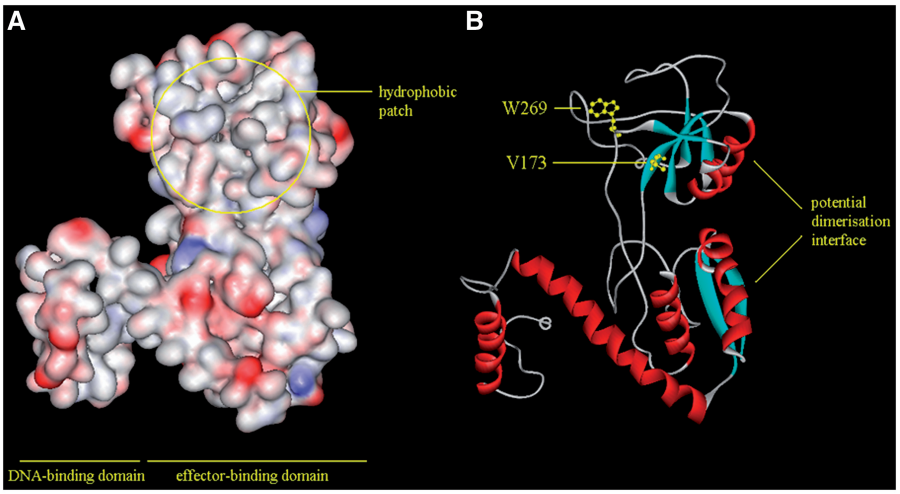

Figure 1. Homology model of PtxR. This model was generated using the CrgA structure of *Neisseria meningitidis* [pdb: 3hhg, and (14)] as template. Perpendicular view to potential dimerization interface. (**A**) Surface potential of the model. Highlighted is a hydrophobic patch. Red colour indicates negative charges, blue positive charges and grey uncharged residues. (**B**) Ribbon diagram of the model at the same orientation. Amino acids W269 and V173, which were replaced by alanine residues, are highlighted.

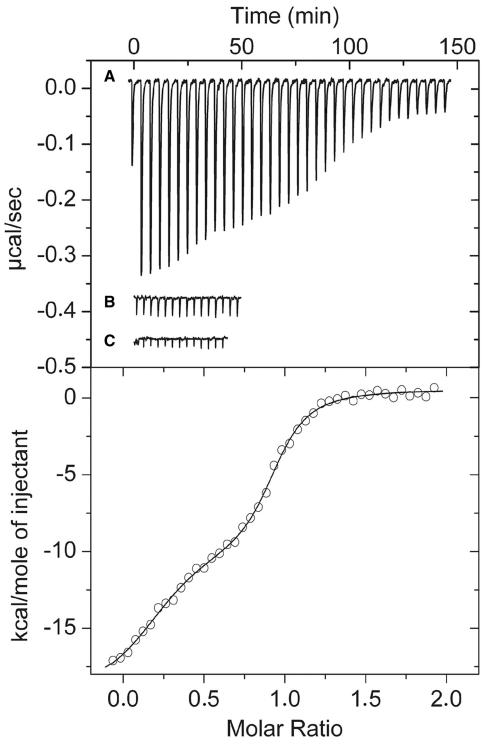

Figure 2. Microcalorimetric binding studies of PtxS to native and mutant PtxR. In all experiments, 3 µM of native or mutant PtxR was titrated with 3.2 µl aliquots of 50 mM of PtxS. (**A**) Titration of wild-type PtxR and PtxS. (**B**) Titration of PtxR(V173A) with PtxS. (**C**) Titration of PtxR(W629A) with PtxS. Top: Titration raw data. Bottom: Integrated and dilution corrected raw data for the titration of PtxR with PtxS. Data were fitted with the 'Two binding site model' of the MicroCal version of ORIGIN.

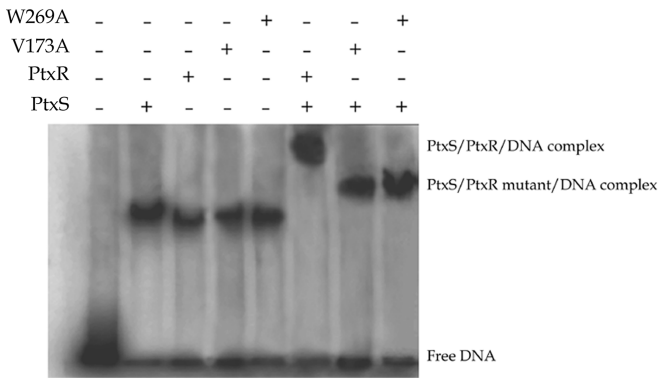

Figure 3. Evaluation of the change in electrophoretic mobility of P_{gad} DNA following binding to PtxS/PtxR or PtxS/mutant PtxR. EMSAs were conducted using 20 µM of purified protein with 2 nM of the P_{gad} DNA end-labelled with [γ-^{32}P] [from left to right: Free DNA, PtxS(WT), PtxR(WT), PtxR(V173A), PtxR(W269A), PtxS(WT)/PtxR(WT), PtxS(WT)/PtxR(V173A) and PtxS(WT)/PtxR(W269A)].

mobility of the band was retarded with respect to that of single proteins and was different from that with PtxS and the wild-type and PtxR protein (Figure 3, lanes 6–8). Because the electrophoretic mobility of DNA/protein complexes depends on the mass and the shape of the complex (16), we hypothesized that this could be due to the adaption of different shapes of DNA when bound to PtxS/PtxR or PtxS/PtxR mutants.

To further characterize this interaction, we carried out DNase I footprinting assays using a DNA fragment comprising the P_{gad} promoter and different combinations of the regulator proteins under study. As shown in Figure 4, PtxS (lane 2) and PtxR (lane 3) both bind individually to their promoter, as witnessed by clear zones of protection that were called PtxS and PtxR boxes, and which confirmed the EMSA results. In the presence of both PtxS and the wild-type PtxR, the size of protected zone increased and a strong band indicating DNase I hypersensitivity appeared in the area extending from the PtxS box to the PtxR box (lanes 4 and 5 in Figure 4). Such hypersensitivity is attributed to the induction of a strong

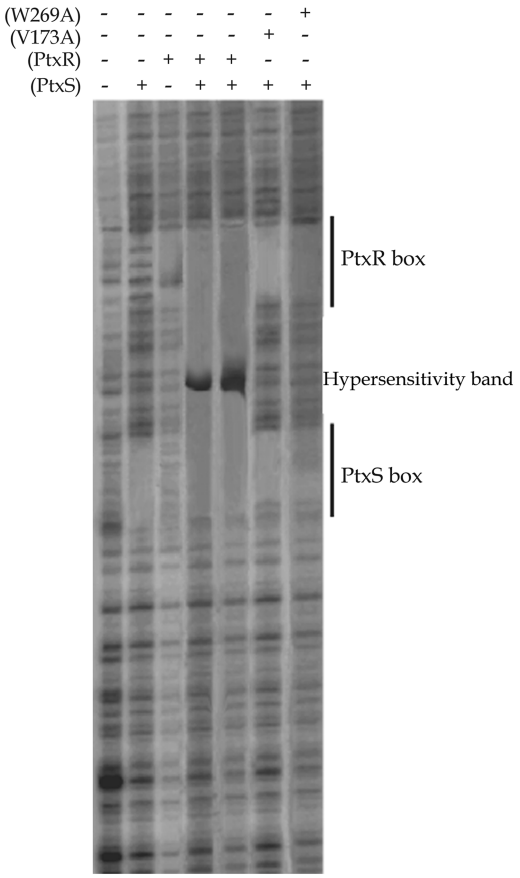

Figure 4. DNase I footprinting assays of promoter P_{gad}. Experiments were conducted as described in 'Materials and Methods' section. (**A**) Lane 1: free DNA, lane 2: DNA + 10 μM PtxS, lane 3: DNA + 10 μM PtxR, lane 4: DNA + 10 μM PtxS and PtxR, lane 5: DNA + 20 mM PtxS and PtxR, lane 6: DNA + 20 μM PtxS and 20 μM PtxR(V173A) and lane 7: DNA + 20 μM PtxS and 20 μM PtxR(W269A).

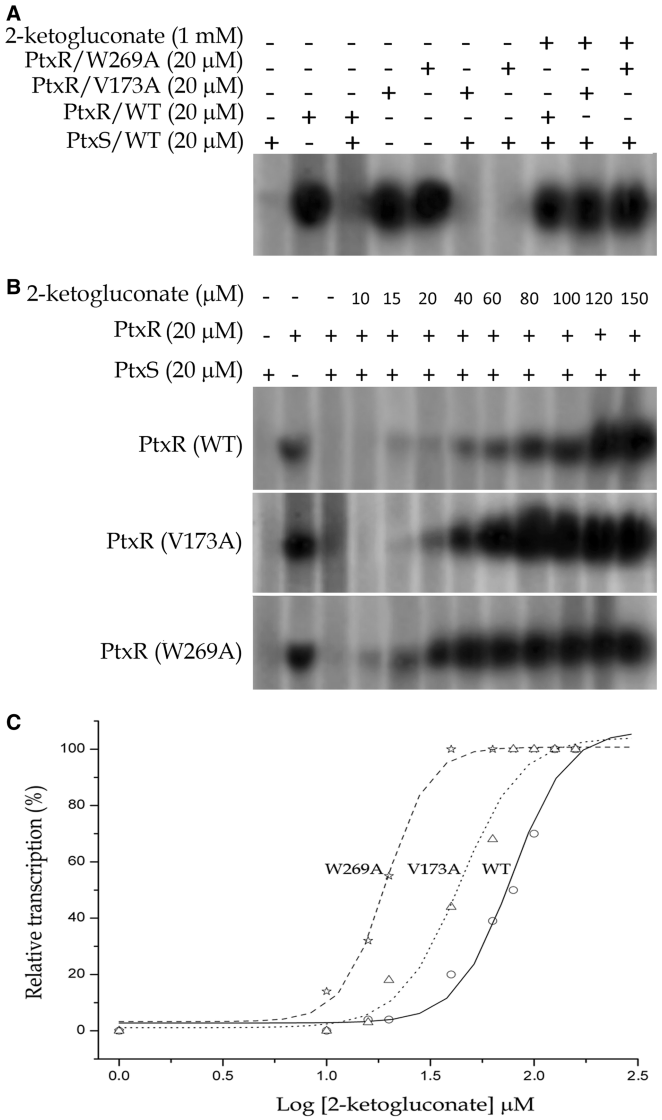

Figure 5. *In vitro* transcription from P_{gad}. Transcription assays were carried out as described in 'Materials and Methods' section. (**A**) The assay performed in the presence of 20 μM combined wild-type PtxS with wild type PtxR, PtxR(V173A) or PtxR(W269A) mutants. (**B**) *In vitro* transcription assays obtained by supplementation of increase concentration of 2-ketogluconate (0–150 μM). (**C**) The densitometric analysis of the *in vitro* transcription gels. Circles: WT PtxR protein, triangle: V173A PtxR protein and star: W269A.

pronounced DNA bend. However, when the latter experiment was repeated with PtxR-V173A and PtxR-W269A instead of PtxR, the protected areas corresponding to the PtxS and PtxR boxes appeared, but there was no band reflecting hypersensitivity (lanes 6 and 7). This suggests that both PtxR mutants bind to their operator sites, but owing to their incapacity to bind PtxS, no DNA bend is introduced.

Taking into account the EMSA and DNAase I footprinting data, it is reasonable to suggest that the DNA complexes with PtxS/PtxR and those of the PtxS/mutant PtxR share the same masses, but differ in shape. Data also suggest that mobility of DNA-loop containing protein complexes is inferior to the complexes with straight DNA and leads to the prevention of the DNA-loop with the mutants, which, in turn, results in apparently higher mobility in EMSA.

In vitro transcription assays

We have conducted *in vitro* transcription assays to assess the effect of PtxR mutation on the transcriptional readout. In the absence of a regulatory protein, basal transcriptional activity was observed (Figure 5A). The addition of PtxS resulted in complete repression (lane 1), whereas the addition of PtxR (lane 2) increased transcription as compared with the basal activity. Replacing PtxR with any of its two mutants (lanes 4 and 5) did not alter transcriptional activity. In the presence of both regulators (lane 3), complete repression was observed suggesting a dominating role of PtxS in the control of transcription. Again, replacement of PtxR with the PtxR mutants in this complex did not alter transcription (lanes 6 and 7). Lanes 8–10 show that the addition of saturating 2-ketogluconate concentrations to the PtxS/native or mutant PtxR complex stimulated transcription in a similar manner.

The above series of experiments were conducted with high enough 2-ketogluconate concentrations to saturate the PtxS protein present in the solution. To analyse in greater detail the role of 2-ketogluconate in the PtxS–PtxR interaction, we hypothesized that the PtxS–PtxR complex formation could alter the affinity of PtxS for 2-ketogluconate and thus modulate the sensitivity of the complex regulatory system. To verify this hypothesis, we conducted transcription assays with constant amounts of protein, but different effector concentrations (Figure 5B). Assays were conducted in the presence of PtxS containing either native or mutant PtxR. These experiments revealed significant differences in the onset of transcriptional activation, which occurred with the PtxR mutants at lower 2-ketogluconate concentrations as compared with wild-type PtxR. The bands obtained were quantified densitometrically and plotted against the logarithm of the effector concentration (Figure 5C). Data were fitted with the sigmoidal model of the ORIGIN software to determine the corresponding EC50. For the wild-type PtxR protein an EC50 of 78 μM was obtained, whereas the corresponding values for the PtxR-V173A and PtxR-W269A mutants were 44 μM and 19 μM, respectively, which were significantly lower. We had previously shown that 2-ketogluconate binds exclusively to PtxS but not to PtxR (8). Taken together, the data show that the DNA-loop containing complex is characterized by a DNA-bound PtxS–PtxR interaction that is less sensitive to 2-ketogluconate than in the absence of complex formation, which suggests that PtxR modulates the affinity of PtxS for its effector molecule.

DISCUSSION

In this study, we show that the mutation of two presumed surface-exposed amino acids (V173 and W269) of PtxR has a profound effect in the interaction with its pair regulator PtxS, although it has no relevant effect on the ability of the mutants to recognize their target operators. Valine and tryptophan residues are prone to be part of protein–protein interaction sites. A recent analysis of protein structures and protein–protein interaction surfaces has shown that ~1 and 4% of protein surface residues are tryptophan and valine residues (17). However, the same study has shown that the percentage of both amino acids present in protein–protein interaction surfaces is ~2 and 8% for tryptophan, while valine doubles its occurrence on protein surfaces. A statistical analysis was also presented to document the significance of the preferential presence of tryptophan and valine in protein–protein interaction interfaces (16). After leucine, valine is the second most abundant amino acid in protein–protein interaction surfaces and has shown to have a strong preference to interact with isoleucine, leucine, phenylalanine and tyrosine of the partner protein (17). Tryptophan and valine residues were found to have high residue interaction energies of approximately −52 and −20 kcal/mol, whereas that of alanine was significantly lower with approximately −10 kcal/mol. This may explain why single substitution of any of these amino acids abolished binding.

The introduction of DNA bends and loops is a widespread mechanism for transcriptional control (18). However, several evolutionary paths have been followed. Proteins, like the IHF or HU, induce bends by binding to DNA (19). In the case of Integration Host Factor (IHF), it has been shown that an IHF-induced bend was critical for the interaction of RNA polymerase bound at the downstream part of the promoter with regulators bound to upstream activation sequences (20–22). An alternative strategy is the evolution of promoters that contain multiple binding sites for the same regulatory protein (21). In this context, the regulation of the *lac* promoter by the LacI repressor is a representative example. This promoter contains three LacI operator sites (O1, O2 and O3). Binding of LacI dimers to these operator sites is followed by dimer association/tetramerization, causing the formation of loops (23). Our results support another strategy that is based on DNA-loop formation by hetero-association of different transcriptional regulators that bind to different operator sites.

In evolutionary terms, it is more complex to engineer a protein hetero-association than a homo-association, for which complementary 2-fold symmetries can be exploited. What could then be the evolutionary advantages of DNA bending caused by a hetero-association of transcriptional regulators? Our data support that PtxR/PtxS dissociation occurs in response to 2-ketogluconate, which acts as an effector for PtxS, but not for PtxR. The effector-mediated release of PtxS induces transcription from the regulated promoters (8). PtxR contains an effector binding domain; however, the cognate ligand remains to be identified. It appears plausible that binding of cognate effectors to PtxR also causes PtxS/PtxR complex dissociation and transcriptional modulation. As a result, transcriptional control could be achieved by two different signal molecules that are recognized by two different regulator proteins, allowing the fine-tuning of the regulatory output. However, such multifactorial regulation could be achieved by two regulator proteins that do not interact with each other, which hence raises the question of the usefulness of the molecular interaction between these two proteins. This is even more intriguing, as complete transcriptional inactivation is achieved through the binding of only PtxS, suggesting that the DNA-loop formation in the presence of PtxR does not have any additional influence on the transcriptional activity.

We showed previously that PtxR and PtxS bind to the P_{kgu} promoter and that in P_{kgu}, as in P_{gad}, the PtxR protein binds at the −35 region where it recognizes an inverted repeat, whereas PtxS binds to the −10 region (8). As in P_{gad}, in P_{kgu}, PtxR/PtxS induce loop formation, but in the presence of 2-ketogluconate, the loop is dissolved. Therefore, our current results support a similar mechanism of gene regulation for P_{kgu} and P_{gad}. It should be noted that at P_{toxA} only PtxR binds to its target DNA, which overlaps the −35 region, and although PtxS does not bind to this promoter, PtxS recognizes PtxR bound to DNA favouring the formation of a complex that prevents PtxR-dependent transcription of P_{toxA}. Consequently, the PtxR/PtxS complex seems to

work differently in the glucose degradation pathway and in the control virulence genes.

In all sequenced genomes of strains belonging to different species of *Pseudomonas,* PtxS has been found, whereas PtxR is only present in pathogenic *P. aeruginosa* strains (6,24). In *Pseudomonas putida*, del Castillo *et al.* (25) showed that repression of P_{gad} is achieved through PtxS and that with 2-ketogluconate expression from P_{gad} took place. This further supports that PtxR is not essential for P_{gad} transcription. The question that rises is the potential advantage of this enhanced complexity in regulation of P_{gad} in *P. aeruginosa*. One possible explanation of the use of this protein complex may lie on our demonstration that the molecular interaction between both proteins modulates the affinity for the effector 2-ketogluconate, although the molecular mechanism is at present unknown. Whether PtxR is capable of recognizing effectors also remains unknown, but if this were the case, we cannot rule out the possibility that the affinity of PtxR for its effectors being modulated by PtxS. Thus, the evolutionary advantage of DNA-loop formation by a hetero-association of different regulator proteins would rest on the fine-tuning of transcriptional regulation by the incorporation of different signal molecules and the possibility of a mutual modulation of effector affinities by protein complex formation.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online, including [26].

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Conflict of interest statement. None declared.

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