The roles of indoleglycerol phosphate and the TrpI protein in the expression of *trpBA* from *Pseudomonas aeruginosa*

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ABSTRACT

The TrpI protein belongs to the LysR-family of procaryotic regulatory proteins. Members of this family share a characteristic similarity of their N-terminal amino acid sequences, and many of them are activators of divergently transcribed genes or operons. In *Pseudomonas aeruginosa*, the genes for tryptophan synthase, *trpBA*, are regulated by indoleglycerol phosphate (InGP) and TrpI. We demonstrate here that in the absence of InGP, the binding site of TrpI is located in the -52 to -77 region of the *trpBA* promoter; in the presence of InGP, the binding region is extended to the -32 region. In addition, two major, slow moving protein-DNA complexes are seen in gel retardation assays: the faster moving complex is formed in the absence of InGP and the amount of the slower moving complex is greatly enhanced in the presence of InGP. These results suggest that the binding of a second TrpI protein molecule, promoted by InGP, plays a crucial role in activating the expression of the *trpBA* gene pair.

INTRODUCTION

In fluorescent pseudomonads, the seven *trp* genes responsible for tryptophan synthesis are found at four chromosomal regions (Fig. 1) (1, 2). Unlike the *trp* operon of *Escherichia coli*, the regulation of these genes' expression is not coordinately controlled by the level of the end product, tryptophan (1, 2). Early genetic studies showed that in *Pseudomonas putida* and *Pseudomonas aeruginosa* the genes for tryptophan synthase, *trpB* and *trpA*, are induced by the substrate, indoleglycerol phosphate (InGP), rather than repressed by L-tryptophan (1, 2). Recently the *trpBA* gene pair from *P. aeruginosa* strain PAC174, along with its regulatory gene, were transferred to *E. coli* by the broad host range plasmid R68.44 (3). Subsequently these genes were subcloned into an amplifiable pBR322 vector to facilitate deletion studies and DNA sequencing (4, 5, 6). The *trpI* regulatory gene modulating the induction of *trpBA* is located upstream of the *trpBA* gene pair (5). *TrpI* and *trpBA* are transcribed divergently; their translational start codons are separated by 103 bp (7). The *trpI* gene product belongs to the LysR-family of procaryotic regulatory proteins (7, 8). Up to 20 members of this family share a significant

similarity in the N-terminal portion of their amino acid sequences (7, 8, 9, 10, 11), and they frequently are transcribed in the opposite direction from the genes they regulate. The *trpI* and *trpBA* genes recently cloned from *P. putida* PPG1 exhibit the same arrangement (12); they are transcribed divergently and are separated by a small intergenic region of 110 bp.

Genetic studies indicate that both InGP and the *trpI* gene product are required to activate transcription of the *trpBA* gene pair (1, 5), but the precise mechanism of the activation event remains unclear. In this paper we have identified the transcriptional initiation sites of the *trpBA* and *trpI* genes, allowing prediction of the promoter regions. We also have identified the TrpI activator binding site, and examined the effect of InGP on the interaction of TrpI with DNA.

MATERIALS AND METHODS

Bacterial strains, plasmids and media

The bacterial strains and plasmids used or constructed in this research are shown in Table 1. The bacteria were cultured in Vogel-Bonner (V-B) minimal medium E (13), LB medium or on X-Gal plates (14). X-Gal plates were used to screen pUC plasmid derivatives transformed into JM107 cells. X-Gal (5-bromo-4-chloro-3-indoxyl-D-galactopyranoside) was obtained from Sigma. To ensure retention of recombinant plasmids, antibiotics were added in the following amounts: ampicillin 200 µg/ml, chloramphenicol 12.5 µg/ml, spectinomycin 50 µg/ml.

Plasmid DNA manipulation

The procedures for plasmid purification, restriction enzyme digestion, DNA ligation, agarose gel electrophoresis, polyacrylamide gel electrophoresis, phosphorylation of linkers, and Bal 31 nuclease deletion are described by Maniatis et al. (15). *E. coli* cells were made competent for transformation by the CaCl₂ method (16).

For DNA sequencing, DNA fragments were labeled with ³²P by one of two methods: 5' labeling with T4 polynucleotide kinase and [γ -³²P]ATP (15) or 3' fill-in labeling with the large fragment of *E. coli* DNA polymerase I and the appropriate radioactive deoxynucleotide triphosphate (15). Sequencing reactions were carried out by the Maxam and Gilbert method (17) and developed by 8% urea-polyacrylamide gel

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electrophoresis according to Sanger and Coulson (18). Sequence data were analyzed with the aid of the PCS computer program (19).

Chromosomal DNA cloning and restriction site mapping

High molecular weight chromosomal DNA from *P. aeruginosa* PAO1 cells was prepared essentially as described by Saito and Miura (20). Chromosomal DNA and pBR322 plasmid DNA were digested with *Bam*HI, ligated, and transformed into competent *E. coli* JM107 cells (21). Several hundred transformants were screened by colony hybridization (15). Southern hybridization (15) was used to determine the restriction enzyme map of the cloned *trp*BA genes. A *Kpn*I DNA fragment isolated from pZAZ202-489 (7), containing most of the *trp*I gene and the first

part of *trp*B gene from strain PAC174, was used as hybridization probe in these experiments. Because there are very few differences between the *trp*BA regions of strain PAO1 and PAC174, stringent conditions were used for colony hybridization and Southern hybridization.

Cellular RNA isolation

A *P. aeruginosa* PAO1 *trp*A strain was grown in 0.5% acid hydrolyzed casein, 1 mM MgCl₂ and 0.1 mg/ml tryptophan at 37°C to late exponential phase, harvested, and resuspended in the same medium without tryptophan. After the culture had reached the early stationary phase and indoleglycerol was detected in the medium (22), the cells were collected for isolation of total cellular RNA as described by Baker and Yanofsky (23).

S1 nuclease mapping

The transcription initiation site for the *trp*BA gene pair was determined by S1 nuclease mapping (24). A 235 bp *S*tyI DNA fragment was labeled at the 5' ends with γ -³²P ATP using T4 polynucleotide kinase (15), then the labeled strands were separated as described by Maniatis et al. (15). Total cellular RNA (175 μ g) isolated from *P. aeruginosa* PAO1 *trp*A cells was mixed with ³²P-labeled, single stranded DNA in 20 μ l of hybridization buffer (0.4 M NaCl, 0.04 M PIPES pH 6.4, 1.25 mM EDTA) and incubated overnight at 52°C. To the hybridization mixture was added 35 μ l of cold 2 $\times$ S1 nuclease buffer (60 mM sodium acetate pH 4.6, 100 mM NaCl, 2 mM ZnCl₂ and 10% glycerol) and 550 units of S1 nuclease (Pharmacia). Digestion was carried out in a final volume of 70 μ l of 1 $\times$ S1 nuclease buffer for 60 minutes at room temperature. The sample was then precipitated and electrophoresed alongside a sequencing ladder of the same DNA strand.

The transcriptional initiation site for the *trp*I gene was established similarly. The other single strand of the 235 bp *S*tyI DNA fragment obtained in the strand separation procedure was hybridized with 766 μ g of cellular RNA and incubated overnight at 65°C. To the hybridization mixture was added 100 μ l of cold 2 $\times$ S1 nuclease buffer and 1728 units of S1 nuclease. After 60 min of incubation at room temperature, the sample was precipitated and electrophoresed alongside standard size markers.

Figure 1. In *Pseudomonas aeruginosa* and *Pseudomonas putida*, the genes responsible for tryptophan synthesis are located in four chromosomal clusters. The *trpR* gene encodes a repressor acting on *trpE* and the *trpGDC* cluster. The *trpI* gene encodes an activator inducing the *trpBA* gene pair. *trpE* and *trpG* encode the large and the small subunits of anthranilate synthase, and *trpA* and *trpB* encode the α and β subunits of tryptophan synthase. The gene products and their participation in biosynthetic reactions are indicated by arrows. Abbreviations: AS, anthranilate synthase; PRT, anthranilate phosphoribosyltransferase; InGPS, indoleglycerolphosphate synthase; PRAI, N-phosphoribosylanthranilate isomerase; TS, tryptophan synthase; PRA, N-phosphoribosylanthranilate; CDRP, 1-(α -carboxyphenylamino)-1-deoxyribulose phosphate; InGP, indoleglycerolphosphate; TS-A, tryptophan synthase A reaction; TS-B, tryptophan synthase B reaction.

Table 1. Bacterial strains and plasmids

Strain/plasmid	Relevant characteristics	Reference/source
<i>E. coli</i> JM107	<i>endA1</i> , <i>gyrA96</i> , <i>thi</i> , <i>hsdR17</i> , <i>supE44</i> <i>relA1</i> , λ^- , Δ (<i>lac-proAB</i>), [F', <i>traD36</i> , <i>proAB</i> , <i>lac</i> ⁺ Δ M15] <i>thr</i> ⁻ , <i>trpR</i> ⁻ , <i>ter</i> ⁺	21
<i>E. coli</i> CY15071		25
<i>P. aeruginosa</i> PAO1		30
<i>P. aeruginosa</i> PAO1 #918	<i>trpA1</i> , <i>rif</i> -67	30
pUC18	Amp ^r	14
pMI602	Amp ^r , <i>trpI</i>	This work
pMI603	Amp ^r	This work
pMI604	Amp ^r , <i>trpI</i>	This work
ptacterm	Amp ^r	25
pMS421	<i>lac</i> ⁺ , <i>Spe</i> ^r	26
pMI657	deletion of the <i>trpB</i> region in pMI604	This work
pMI616	<i>Bam</i> HI- <i>Sa</i> I fragment from pMI657 subcloned into ptacterm	This work
pMI631	<i>Bam</i> HI- <i>Sa</i> I fragment from pMIB85 subcloned into ptacterm	This work
pMIB85	<i>trpI</i> from strain PAC174 cloned into pACYC184	7
pMIM5	<i>Bam</i> HI- <i>Xho</i> I fragment from pMI631 replaces the same region in pMI616	This work

RESULTS

Cloning and sequencing *P. aeruginosa* PAO1 *trpI*

Because the *trpIBA* region from *P. aeruginosa* PAC174 was available on a broad host range plasmid (R68.44) transferable from *P. aeruginosa* to *E. coli*, the activation of *trpBA* by TrpI was first studied in that system (3, 4, 5). *P. aeruginosa* strain PAO1 (30), however, is well mapped and several useful *trp* mutants are available, so we decided to clone its *trpIBA* genes. Chromosomal DNA digested with *Bam*HI was cloned into pBR322, and colony hybridization was used to screen this genomic library. The selected plasmid, pMI602, had three *Bam*HI-fragment inserts; one of them (about 16 kb) hybridized with the *Kpn*I-fragment probe from pZAZ202-489 containing the *trpI* gene of strain PAC174 (see Materials and Methods). Subsequently we subcloned a *Kpn*I fragment and a *Sal*I fragment from this large *Bam*HI fragment into pUC18 to generate pMI603 and pMI604, respectively, which are smaller in size and convenient for DNA manipulation and sequencing. The DNA sequence of the *trpI* gene and the first portion of the *trpB* gene of strain PAO1 was determined; the sequencing strategy was similar to that used for the *trpI* gene from PAC174 (7). Figure 2 presents the DNA sequence and the deduced amino acid sequence of *trpI* from strain PAO1; differences from the PAC174 sequence are shown in parentheses. We have corrected one part of the published DNA sequence, and the corrected version of the *trpI* gene product is 294 residues long.

Transcriptional initiation site for the *trpBA* gene pair and the *trpI* gene

The *trpBA* and *trpI* transcriptional initiation sites were located by S1 nuclease mapping. *TrpBA* and *trpI* are transcribed divergently; their translation initiation codons are separated by 103 bp. Therefore, their promoters are likely to be located within this intergenic region. A 235 bp *S*tyI fragment containing the intergenic region was labeled at both 5' ends and separated by the strand separation procedure. Each single strand of DNA was hybridized with cellular RNA isolated from InGP induced *P. aeruginosa* PAO1 *trpA* cells and subsequently subjected to S1 nuclease digestion. (The *trpA* mutant encoding the defective tryptophan synthase is unable to convert InGP to indole. Therefore InGP accumulates and induces the expression of *trpBA* in the tryptophan-starved culture) After digestion, the S1 nuclease-resistant DNA was electrophoresed adjacent to a sequencing ladder of the same DNA fragment. Figure 3A shows the transcriptional initiation site for *trpBA*. Two major bands are seen, separated by 7 nucleotides, with minor bands close by. Two bands might indicate multiple transcriptional initiation sites for *trpBA* or might result from nibbling by S1 nuclease (31). The most likely transcriptional initiation site for *trpBA* is indicated in Figure 4. This most distal site was chosen as the likely transcription initiation site for *trpBA*, since in the -9 to -14 region it had the characteristic -10 sequence of an *E. coli* σ^{70} promoter.

Because *trpI* mRNA was expected to be present in low

Figure 3. S1 nuclease mapping of *trpBA* (A) and *trpI* (B,C) transcriptional initiation sites. S1 nuclease-resistant DNA fragments were electrophoresed adjacent to a sequencing ladder of the same strand of DNA (A and B). AG and CT indicate the Maxam and Gilbert A+G and C+T sequencing ladders. For the *trpI* gene, the sample was also loaded on a denaturing gel with standard size markers (C). The standard was pBR322 plasmid DNA digested with the *Hin*FI restriction enzyme and labeled with [α - 32 P] deoxyadenosine triphosphate.

amounts, more total cellular RNA was hybridized with the labeled opposite strand of DNA. After digestion with S1-nuclease, the sample was run with standard size markers (Fig. 3C). We estimated the size of the S1 nuclease-resistant DNA fragment

Figure 4. *trpI* and *trpBA* promoters and the TrpI binding sites in the 103 bp intergenic region between the two translational start codons (underlined). The transcription initiation sites for *trpI* and *trpBA*, determined by S1 nuclease mapping, are numbered +1 on each strand. The region covered by solid brackets, designated site I, is protected by the TrpI protein from DNase I digestion in the absence of InGP. The region covered by dashed brackets, designated site II, is protected in the presence of InGP. The regions protected from hydroxyl radical cleavage by the TrpI protein are indicated by crosses. The predicted -10 and -35 regions for the promoters are underlined.

Figure 5 Construction of plasmid pMIM5. The *Bam*HI-*Sal*I fragment of pMI657 was subcloned into ptacterm, between the *tac* promoter and the *rpoC* terminator. Subsequently, the region between *Bam*HI and *Xho*I in pMI616 was substituted for the *Bam*HI-*Xho*I fragment from pMI631 to generate pMIM5. Restriction Enzyme Abbreviations: B, *Bam*HI; S, *Sal*I; X, *Xho*I.

at about 150 bp. In Figure 3B this fragment was run alongside a sequencing ladder to locate the transcriptional initiation site of the *trpI* gene, which is indicated in Figure 4.

Overproduction of the *trpI* gene product

Plasmid ptacterm is a small, high copy number ColE1 plasmid containing the *tac* promoter/operator and the *rpoC* terminator; between them are *Bam*HI and *Sal*I sites useful for cloning (25). Since no convenient restriction enzyme sites were available to subclone the entire *trpI* gene into ptacterm, *Bal* 31 nuclease was used to remove the *trpB* region from pMI604. The ends of the *Bal* 31-digested molecules were made blunt by incubation with the Klenow fragment of DNA polymerase I in the presence of all four deoxynucleotide triphosphates, then ligated to *Bam*HI linkers (CGCGGATCCGCG). Plasmid pMI657 constructed in this way has a *Bam*HI site located 75 nucleotides ahead of the presumed *trpI* start codon. Subsequently, the *Bam*HI-*Sal*I DNA fragment from pMI657 was subcloned into ptacterm to generate pMI616.

E. coli CY15071 transformed with pMI616 and pMS421 was cultured and induced by IPTG (see Materials and Methods). The cells from 1 ml were collected and heated in boiling water for 10 min. in SDS-PAGE buffer before loading onto SDS-polyacrylamide gels; this clone showed only a low level of TrpI (data not shown). Plasmid pMI631, constructed in a similar way from the PAC174 *trpI* gene, showed a higher productivity. We thought the somewhat different untranslated region might be influencing the expression of the TrpI protein from pMI616; therefore the region between the *Bam*HI and *Xho*I sites in pMI616 was replaced by the corresponding region from pMI631 to generate pMIM5 (Fig. 5). The amino acid sequence of the TrpI segment exchanged is identical in strains PAO1 and PAC174. *E. coli* cells transformed with pMIM5 and pMS421, induced by IPTG, had a high level of the TrpI protein.

The DNA binding activity of the TrpI protein

The TrpI-containing crude extract from pMIM5-transformed cells was tested for DNA-binding activity by the gel retardation assay. The *Bam*HI-*Bss*HII DNA fragment of pMI657, containing the intergenic region believed to have the activator binding site for the *trpBA* gene pair, was 3' end-labeled by the Klenow fragment of polymerase I. We prepared a TrpI-containing crude extract from pMIM5-transformed cells and a control extract from an identical amount of ptacterm-transformed cells. Both crude extracts in serial eight fold dilutions were incubated with a fixed amount of labeled DNA fragment. Results (Fig. 6A) showed that the DNA-binding activity was specific to the TrpI protein, because the crude extract from ptacterm-containing cells did not shift the mobility of this DNA fragment. There are four slow-moving complexes in the gel retardation assay. Fried and Crothers noted a similar pattern, having eight protein-DNA complexes, with the *lac* repressor (32). They propose that bands 1,2,3,...,n contain 1,2,3,...,n moles of *lac* repressor tetramer per mole of DNA. Although we did not investigate the ratio of protein to DNA in each band, we assume that the slower moving complexes have an increased number of TrpI molecules bound to the DNA fragment. The additional TrpI molecules may bind nonspecifically or to low-affinity TrpI binding sites.

Indoleglycerol phosphate, the substrate of tryptophan synthase, functions as the inducer of the *trpBA* gene pair. Addition of InGP to the gel retardation assay enhances the formation of a slower moving complex, complex 2 (Fig. 6B) Although small amounts

Figure. 6 The DNA binding activity of the TrpI protein. TrpI-containing crude extract was tested for DNA binding activity by the gel retardation assay. The DNA fragment used in the assay was the *Bss*HII-*Bam*HI fragment labeled by radioactive deoxynucleotide triphosphates. (A) The DNA fragment was incubated with a series of dilutions of the crude extract from pMIM5-containing cells or ptacterm-containing cells. (B) The DNA fragment was incubated with a 1:5120 dilution of both crude extracts in the presence of 10^{-3} or 10^{-4} M InGP. I: the crude extract from the pMIM5-transformed cells. T: the crude extract from the ptacterm-transformed cells. F: DNA fragment only.

of complex 2 can form in the absence of InGP, InGP greatly increases their amount, suggesting that the inducer may promote occupancy of a second, lower affinity TrpI binding site. Both 10^{-3} M and 10^{-4} M InGP in the DNA-protein reaction mixtures give the same result, suggesting that at both levels InGP is in excess.

DNA footprinting analyses of the TrpI protein binding sites

Initially DNase I footprinting was carried out to locate the TrpI binding site. A 172 bp *Bss*HII-*Eco*O109 fragment containing the intergenic region of *trpI* and *trpBA* was used for all footprinting studies. Labeled DNA fragments were incubated with various amounts of the TrpI-containing crude extract, subjected to DNase I digestion and analyzed by electrophoresis in polyacrylamide/urea gels adjacent to the DNA sequence ladder. The footprints obtained with the coding and noncoding strands are shown in Fig. 7A and 7C, respectively. The protected regions are marked with solid brackets in Fig. 4.

In the gel retardation assay, InGP promotes the appearance of a second, slower moving protein-DNA complex, complex 2. We therefore examined the effect of InGP on DNase I protection. InGP was incubated with diluted crude extract and the DNA fragment before DNase I digestion. The digestion products were analyzed in polyacrylamide/urea gels, with the results shown in Fig. 7B for the coding strand and Fig. 7D for the noncoding strand. In the presence of InGP, the protected region extends toward the *trpBA* promoter. The second protected region, designated site II, is indicated by the dashed bracket in Figure 4. The DNase I footprinting result correlates nicely with the mobility patterns in the gel retardation assay. The formation of the slower-moving complex (complex 2) enhanced by InGP may be due to an additional TrpI molecule binding to site II of the TrpI binding region.

We used hydroxyl radical footprinting to define sites I and II of the TrpI binding region. TrpI-containing crude extract in various amounts was incubated with radioactive labeled DNA

Figure 7. DNase I footprint analyses of the TrpI binding sites. A 172 bp *Bss*HII-*Eco*O109 restriction fragment was labeled with radioactive deoxynucleotide triphosphate at the *Eco*O109 site (A) & (B) or the *Bss*HII site (C) & (D), and then incubated with crude extracts from pMIM5-transformed cells. After DNase I digestion, samples were analyzed by electrophoresis in 8% polyacrylamide/urea gels. (A) & (C), the DNA fragments were incubated with serially diluted TrpI-containing crude extract in the absence of InGP. (B) & (D), the DNA fragments were incubated with a 1:80 dilution of the TrpI-containing crude extract in the presence of InGP. AG and CT indicate the Maxam and Gilbert A+G and C+T sequencing ladders. I: the crude extract from pMIM5-transformed cells. T: the crude extract from pterm-transformed cells. F: DNA fragment only. The protected regions shown on the *Eco*O109-labeled strand are marked above the DNA sequence and those shown in the *Bss*HII-labeled strand are marked below the DNA sequence in Fig. 4.

fragments in the presence or absence of InGP. After adding Fe^{++} and hydrogen peroxide to the mixtures, hydroxyl radicals were generated, cleaving the unprotected region of DNA. The products of the cleavage reaction were separated on polyacrylamide/urea gels. Figure 8A and 8B show footprints of the coding and noncoding strands, respectively. DNA sequence of the protected regions are marked by crosses in Fig. 4. In the absence of InGP, three regions are protected, all falling within site I located by DNase I footprinting. In the presence of InGP, two more regions are protected within site II. These five protected

regions are separated from each other by ca. 10 bp, the distance of one turn of the DNA helix. This pattern suggests that the TrpI molecules are spatially oriented relative to the promoter, on one face of the DNA helix.

DISCUSSION

P. aeruginosa PAO1, isolated and studied extensively by Holloway, has been well mapped (30). *P. aeruginosa* PAC174 was isolated by Patricia H. Clarke. The sequence of the *trpI* gene

Figure 8. Hydroxyl radical footprint analyses of the TrpI binding sites. A *Bss*HII-*Eco*O109 restriction fragment, the same one used for the DNase I footprint analyses, was labeled with radioactive deoxynucleotide triphosphate at the *Eco*O109 (A) or *Bss*HII (B) site, and incubated with crude extract from pMIM5-containing cells. After the hydroxyl radical cleavage reaction, samples were analyzed by electrophoresis in 8% polyacrylamide/urea gels with a sequencing ladder in the center lanes. I: the crude extract from pMIM5-transformed cells. F: DNA fragment only. The samples obtained from the reaction in the absence of InGP were loaded to the left of the sequencing ladder and those generated from the cleavage reaction in the presence of InGP were loaded to the right. AG and CT indicate the Maxam and Gilbert A+G and C+T sequencing ladders. The regions protected by the TrpI-containing crude extract shown in (A) are indicated above the sequence and those shown in (B) are marked below the sequence in Fig. 4.

-10 +1
TTTACCCGGCGGGGGATTAGATT-CTGCCAT-ACG---TCATTT---CCTGGAGCCCTCCATG
CAGCCGGGCTTGCCGTGGTTAGAGTAAGCCCATCACTCATTCATTTACGCCTGGAGCGCCCCATG

with its flanking regions is now known in both strains, allowing us to analyze the differences between these two strains. In the *trpI* coding region, 17 out of 882 bp are different; among them, 7 are nonsynonymous changes and the rest result in synonymous codons. Within the intergenic region, 5 out of 103 bp are different. Even with these differences, the *trpI* gene from PAO1 is able to activate the *trpBA* gene pair from PAC174 in growth rate tests (data not shown). The *trpI* gene from *P. putida*, recently sequenced in this laboratory, has 298 codons, 4 codons more than that of *P. aeruginosa* (I. P. Crawford and L. Eberly, unpublished results), and 89 of them are different from *P. aeruginosa* PAO1. The *P. putida* intergenic region, between the start codons of *trpI* and *trpB*, is 110 bp long (12); alignment of this region from *P. aeruginosa* and *P. putida* is shown in Fig. 9. There are 41 nucleotide differences, but the TrpI binding region is well conserved, suggesting that it may be interchangeable between these two species. The *trpBA* promoter region from *P. aeruginosa* fused to the *xylE* gene can be expressed and regulated in *P. putida*. This means that the TrpI binding site from *P. aeruginosa* can be recognized by the TrpI proteins from both species (E. Han and I. P. Crawford, unpublished results).

the DNA, located at the edge of the DNase I footprint, is due to a shadow effect. By analogy to the cI repressor binding site, we looked for a symmetrical sequence within the central protected regions. Extending the comparison to the analogous TrpI binding region from *P. putida*, it appears that there is a symmetrical sequence within site I, TGTNAGNNNNNCTNACA, shown below the sequences in Fig. 9.

Genetic studies have indicated that the *trpI* gene product and accumulation of InGP are both required for the activation of the *trpBA* gene pair. The footprinting analyses show that in the absence of InGP the TrpI protein does bind to site I, but in the presence of InGP TrpI also binds to site II, in which position it partially overlaps with the -35 region of the *trpBA* promoter. Therefore it is likely that the binding of TrpI to site II, promoted by InGP, plays a crucial role in activating the expression of the *trpBA* gene pair. It is also possible that the binding of TrpI to site I represses transcription of the *trpI* promoter in an autoregulatory fashion, but this remains to be tested.

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