Changes in the periplasmic linker and in the expression level affect the activity of ToxR and λ-ToxR fusion proteins in *Escherichia coli*

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Abstract In order to assess the potentiality of *Vibrio cholerae* ToxR protein and of bacteriophage λ repressor as indicators of the dimerization of periplasmic proteins in *Escherichia coli*, we have constructed a series of plasmids encoding transmembrane fusion proteins. The amino-terminal part, containing the DNA binding domain of either ToxR or λ repressor, is located in the cytoplasm and acts as reporter for dimerization. As models of periplasmic proteins we have used alkaline phosphatase (a dimer) and β-lactamase (a monomer). Both the expression level and the distance between the transmembrane segment and the periplasmic protein substantially affect the activity of the reporter domains.

Key words: Lambda repressor; ToxR; β-Lactamase; Alkaline phosphatase; Dimerization

1. Introduction

ToxR is the central regulator that co-ordinates the expression of several virulence factors in *Vibrio cholerae*. The protein acts at the transcriptional level, activating the cholera toxin genes located on the *ctx* operon (for a review, see [1]). ToxR is a single-spanning membrane protein containing a cytoplasmic amino-terminal domain which binds the *ctx* promoter (amino acids [aa] 1–182), a transmembrane segment (aa 183–198) and a periplasmic carboxy-terminal domain (aa 199–294) [2]. If ToxR is expressed in *Escherichia coli* from a strong promoter, such as that of the *tet* gene of the plasmid pBR322, activation by ToxR is 15–30-fold and does not depend on the ToxS protein [2,3]. However, if ToxR is expressed under the control of *toxR* promoter, it activates transcription 3–5 times and its activity depends upon ToxS, which interacts with ToxR at the periplasmic level [3,4].

When ToxR is fused to the enzyme alkaline phosphatase (PhoA) it can also activate the *ctx* promoter. The fusion ToxR210-PhoA, incorporating 12 ToxR aa residues past the transmembrane domain, when expressed from a strong promoter (i.e. the *tet* promoter of pBR322) and in absence of ToxS, highly activates *ctx* transcription [2]. However, if ToxR-PhoA fusions are expressed at a lower level, a short fusion behaves differently from a long fusion. Thus, under the control of *toxR* promoter, a fusion in which ToxR is truncated only five residues downstream of the transmembrane segment (ToxR203-PhoA), activates transcription at least as well as ToxR and independently of ToxS. In contrast, the activity of the hybrid ToxR286-PhoA, where the fusion joint is 88 aa residues from ToxR transmembrane domain (eight residues from ToxR C-terminus), requires ToxS for transcriptional activation [4]. It has been shown that ToxS protects ToxR286-PhoA from periplasmic proteolysis, which explains why in this circumstance the alkaline phosphatase dimer is unable to activate ToxR [4]. However, it is not known to what extent the distance between ToxR transmembrane segment and the PhoA domain is a critical factor for the activity of ToxR in absence of ToxS.

Because PhoA is enzymatically active as a periplasmic dimer, it has been suggested that ToxR active conformation might be dimeric [2]. Further support for this model derives from the fact that ToxR is able to dimerize the amino-terminal domain of λ phage repressor [5]. In agreement with the dimerization model are also the results obtained with other ToxR fusion proteins in which two domains with different tendencies to dimerize, PhoA, GCN4 leucine zipper domain and monomeric maltose binding protein have been fused to ToxR at the periplasmic level [6]. As a further development of this system, the ToxR activator has been used as an indicator for the association [7] and the folding stability [8] of dimers of immunoglobulin V_L domains and for the analysis of residues critical for the dimerization of the transmembrane segment of glycophorin A [9]. In contrast, another study has shown that hybrid proteins in which ToxR periplasmic domain was replaced with either PhoA, GCN4 leucine zipper domain or β-lactamase (Bla), all activate transcription from the *ctx* promoter at high level. Since Bla is a monomeric protein, the authors suggested that dimerization is not a requirement for ToxR activity in *E. coli* [10].

The bacteriophage λ repressor provides a sensitive in vivo assay for protein dimerization. It consists of an amino-terminal domain (1–92) which binds DNA, and a carboxy-terminal domain (93–236) which mediates dimerization. The repressor binds to DNA as a dimer, but the DNA binding domain alone dimerizes inefficiently and requires the carboxy-terminal domain to be functional (for a review, see [11]). The system using the repressor amino-terminal domain as a reporter for dimerization was first introduced to analyze the sequence requirements of GCN4 leucine zipper [12]. Since then it has been shown that the carboxy-terminal domain of the repressor can be functionally substituted by other cytoplasmic domains which also dimerize. Activity of the repressor can be easily tested by assaying sensitivity to λ phage infection of bacteria expressing the fusion protein.

The present work was originally undertaken with a view to using ToxR and λ repressor amino-terminal domains as ge-

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Abbreviations: aa, amino acid(s); Ap, ampicillin; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; Bgal, β-galactosidase; Bla, β-lactamase; Cm, chloramphenicol; HLPD, highest lysing phage dilution; Km, kanamycin; PhoA, alkaline phosphatase

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netic probes to detect the dimerization of periplasmic proteins. These turned out not to be as robust as expected. Here we show that: (i) a small variation in the length of the sequence between the periplasmic domain and ToxR transmembrane domain drastically affects the activity of the two reporter domains; (ii) the λ repressor activity correlates with the dimerization ability of the domain present in the periplasm, but only up to a certain level of concentration.

2. Materials and methods

Strains were propagated in LB medium. Antibiotics concentrations: chloramphenicol (Cm) 25–50 μg/ml; kanamycin (Km) 50 μg/ml; ampicillin (Ap) 200 μg/ml, unless differently indicated.

PhoA activity was inferred from the color of colonies after overnight growth at 37°C on LB plates containing 5-bromo-4-chloro-3-indolyl phosphate (BCIP) 30 μg/ml and 24 h incubation at 4°C.

In order to test ToxR transcriptional activity the plasmids were transformed in *E. coli* strain Q442λ2418. This strain was obtained by lysogenization of Q442 (*F*$^-$ *i*$^-$ *lac* amU281 *arg*Eam *rif nal*) with λ2418 containing the hybrid *pctx::lacZ*. β-Galactosidase (Bgal) activity was assayed according to the method of Miller [13].

Lambda repressor activity was assayed after transforming each expression vector in *E. coli* strains XL1-blue [14] or Q537 (*F*$^-$ *mcr*A *mcr*B $r_K{}^-$ $m_K{}^+$ *i lac* amU281 *arg*Eam *gal*$^-$ *rif nal sup*°). 24 h old transformant colonies were inoculated in LB supplemented with 1% maltose and 10 mM $MgSO_4$ Cm (50 μg/ml) or Km (50 μg/ml). Overnight cultures were tested by cross-streak test performed with a stock of λ2001, which carries the deletion KH54 in the *cI* gene that effectively prevents lysogenization. The protocol used to quantitate repressor activity is explained in the legend of Fig. 2.

Bla activity was scored determining the minimum inhibiting concentration of Ap for individual cells. 24 h old transformant colonies were inoculated in LB containing Cm (50 μg/ml). 25 μl of a 10^{-6} dilution of overnight cultures (about 50 cfu) was plated on LB agar containing serial concentrations of Ap and, as a control, on a Cm (50 μg/ml) plate. Plates were inspected for colony growth after 18 h incubation at 37°C.

Recombinant DNA procedures were performed according to standard methods [14,15].

Except for pAC103 and pAC105, which confer Km resistance, all the expression vectors used in this work confer Cm resistance. The *toxR* gene segments were PCR amplified from the plasmid pVM7 [16]. pAC1 and pAC2 (C.G. Koh, M.K. Trower and S. Brenner, unpublished), pMTC3, pMTC8 and pMTC9 [17] are derivatives of pACYC184 [18] and were constructed previously. pAC3 and pAC4 derive from pAC1 and pAC2 respectively, replacing the *Pst*I-*Kpn*I fragment with the *toxR* fragment amplified with oligonucleotides R1 and R2 and restricted with *Pst*I and *Kpn*I. pAC5 derives from pAC4 replacing the *Bst*EII-*Sal*I fragment with the *Bst*EII-*Sal*I fragment of pVM7. pAC6 and pAC7 derive from pAC1 and pAC2 respectively, replacing the *Pst*I-*Kpn*I fragment with the *toxR* fragment amplified with oligonucleotides R1 and R3 and restricted with *Pst*I and *Kpn*I. pAC11 originates from a derivative of pACYC184 [18], which contains the promoter of the *kan* gene of Tn*5* (Y. Mikawa and S. Brenner, unpublished), replacing the *Pst*I-*Sal*I fragment with the *Pst*I-*Sal*I fragment of pAC7. pAC15 and pAC16 derive from the same vector replacing the *Pst*I-*Sal*I fragment with the *Pst*I-*Sal*I fragment of pAC3 and pAC4 respectively. pAC13 derives from pAC11 replacing the *Kpn*I-*Sal*I fragment with the *Kpn*I-*Sal*I fragment of pAC1. pAC8 derives from pMTC8 replacing the *Kpn*I-*Sal*I fragment with the *Kpn*I-*Sal*I fragment of pAC2. pAC51 derives from pAC9 eliminating the *Xho*I-*Xho*I fragment. pAC117 derives from pAC116 eliminating the *Xho*I-*Xho*I fragment. pAC116 is a plasmid that, similar to pAC55, encodes the aa 1–101 of the repressor. pAC9 was obtained in a three-molecular ligation reaction and derives from pMTC8 replacing the *Sac*I-*Sal*I fragment with the following fragments: (i) the *toxR* fragment amplified by oligonucleotides R3 and R4 and restricted with *Sac*I and *Kpn*I; (ii) the *Kpn*I-*Sal*I fragment of pAC2. pAC19 derives from pAC9 replacing the *Bsp*HI-*Sal*I fragment with the *Bsp*HI-*Sal*I fragment of pAC4. pAC55 derives from pAC9 replacing the *Nsi*I-*Sal*I fragment with the *Nsi*I-*Sal*I fragment of pAC53, which was obtained replacing the *Pst*I-*Sac*I fragment with the *cI* fragment amplified with oligonucleotides R35 and R36 and restricted with *Pst*I and *Sac*I. pAC104 derives from pAC19 replacing the *Kpn*I-*Sal*I fragment with *Kpn*I-*Sal*I fragment of pAC1. pAC122 and pAC123 derive from pAC55 replacing the *Sac*I-*Xho*I fragment with the *Sac*I-*Xho*I fragment of pAC19 and pAC104 respectively. pAC125 derives from pAC122 replacing the *Sac*I-*Kpn*I fragment with the *toxR* fragment amplified with oligonucleotides R4-R90 and restricted with *Sac*I-*Kpn*I. pAC127 derives from pAC122 replacing the *Sac*I-*Kpn*I fragment with the *toxR* fragment amplified with oligonucleotides R4-R96 and restricted with *Sac*I-*Kpn*I. pAC124, pAC126 and pAC128 derive from pAC55, pAC125 and pAC127 respectively replacing the *Kpn*I-*Xho*I fragment with the *Kpn*I-*Xho*I fragment of pAC1. To obtain pAC103 and pAC105, first the *Pst*I-*Sal*I fragment of pAC19 was subcloned into pAC66, a Bluescript (Stratagene) derivative, to generate pAC102, which confers resistance to Ap. pAC103 derives from pAC76, a Bluescript derivative which confers resistance to Kan, but not to Ap, replacing the *Apa*LI-*Xba*I fragment with the *Apa*LI-*Xba*I fragment of pAC102. pAC105 derives from pAC103 replacing the *Kpn*I-*Sal*I fragment with the fragment *Kpn*I-*Sal*I of pAC1. Oligonucleotide sequences: R1 (5′-CACCTGCAGGGAGATACTGGGACATT-3′); R2 (5′-AGTGGTACCGCTGCCACTAGTTAGGGGTTTAAAGCTGGA-3′); R3 (5′-AGTGGTACCTGGGTTAGTGAGCAGTAATA-3′); R4 (5′-ATAGAGCTCGAAGAAGAGATGGCTCGCGA-3′); R35 (5′-GATCCTGCAGATCTAGGAGT-3′); R36 (5′-CACGAGCTCATACTCACTTCTAAGTG-3′); R90 (5′-TATGGTACCGCTGGATTGGCTTGGGTTAG-3′); R96 (5′-CACGGTACCTAGGGGTTTAAAGCTGGATT-3′).

3. Results

PhoA and Bla are normally located in the periplasmic space and are not functional in the cytoplasm. Since the activity of these enzymes is easy to test, they have been fused to carboxy-terminally truncated versions of membrane proteins to assess their topology [19,20]. As one is a dimer and the other a monomer, we chose them since a difference between a PhoA chimera and a Bla chimera would validate the hypothesis that the activity of the ToxR and λ repressor moieties depends on periplasmic dimerization.

The structure and activity of ToxR fusion proteins are summarized in Fig. 1. All use ToxR as cytoplasmic reporter. In the upper part of the figure, expression is from a pACYC184 plasmid derivative which contains a tightly repressed copy of the 434 phage p_L promoter ensuring a constant low level of transcription [21]. The hybrid ToxR210-Bla, encoded by plasmid pAC3, conferred resistance to Ap, indicating that Bla is translocated into the periplasmic space. In the fusion ToxR210-PhoA, encoded by pAC4, PhoA replaces Bla. As inferred by the blue color of the colonies on an indicator plate containing BCIP, this fusion is able to translocate PhoA into the periplasm. In addition a vector producing the whole ToxR protein was constructed (pAC5, not shown). In order to test ToxR transcriptional activity these constructs were transformed in *E. coli* strain Q442λ2418 which contains the hybrid gene *pctx::lacZ* as a reporter. None of these three constructs activated transcription over the background value. However, a shorter fusion ToxR201-PhoA (pAC7) did, as shown by a two-fold increase in β-galactosidase activity (Fig. 1). These data indicate that the ability of a ToxR-PhoA fusion to activate transcription is susceptible to relatively small variations in the length of the periplasmic linker connecting the ToxR transmembrane segment to PhoA. They also confirm previous findings that in the absence of ToxS and at low levels of expression, a short ToxR-PhoA fusion has higher capacity in activating transcription than wild-type ToxR [4]. To determine if, in such conditions of expression, ToxR activity is

influenced by the dimerization capability of the domain present in the periplasm, we also generated the short fusion ToxR201-Bla (pAC6). However this construct did not confer proper resistance to Ap (i.e. it did not allow growth of isolated colonies), indicating that in this case the Bla moiety, if translocated, is not functional or unstable.

In order to express these gene fusions at a higher expression level, the same constructs have been subcloned into an other derivative of the plasmid pACYC184 [18] which contains the strong constitutive promoter of *km* gene (Y. Mikawa and S. Brenner, unpublished). These are summarized in the lower part of Fig. 1. As with pAC6, plasmid pAC13 encoding ToxR201-Bla displayed no resistance to ampicillin. Plasmid pAC11, directing the synthesis of ToxR201-PhoA, activated transcriptional activity at a very high level, similar to that elicited by the plasmid pVM7 [2] which encodes the wild-type ToxR protein and was used as a positive control (not shown). In addition this activation was substantially higher in comparison to the similar vector expressing the long construct ToxR210-PhoA (pAC16). These results confirm the influence of the periplasmic linker on ToxR activity. The long ToxR-Bla fusion (pAC15) activated transcription at a level very similar to the long ToxR-PhoA fusion (pAC16) which is an indication that, in a condition of elevated expression, ToxR activity is not dependent upon the dimerization capability of the domain attached on the periplasmic side of the membrane. In the next experiment we estimated the difference between the concentration of fusion protein expressed at high level and those expressed at low level. Because the vectors pAC3 and pAC15 direct the synthesis of identical products, the Bla activity is proportional to the amount of enzyme present in the periplasmic space. XL1-blue cells transformed with pAC3 and pAC15 were plated on agar containing concentrations of Ap 50, 100, 150 etc. (μg/ml). While pAC3 grew only at Ap concentration of 50 μg/ml, bacteria harboring pAC15 were able

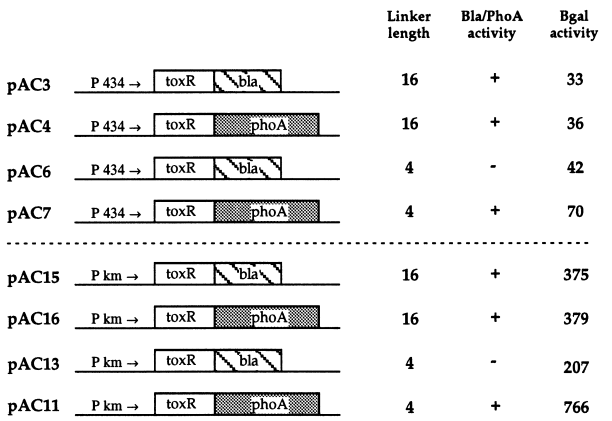

Fig. 1. Vectors encoding ToxR fusion proteins. The dashed line separates the constructs expressed at low level from those expressed at high level. Linker length: number of aa residues intervening between the end of ToxR transmembrane segment (L198) and either Bla or PhoA. For the vectors expressing the fusions at low level the Bgal activity values are the means for three independent experiments. pAC3 and pAC15 encode ToxR aa residues 1–210 followed by the sequence SGSG, fused to Thr4 of the mature form of Bla. pAC4 and pAC16 are identical to pAC3 and pAC15 respectively, except that the mature sequence of PhoA, starting from Thr2, replaces Bla. pAC6 and pAC13 encode ToxR aa residues 1–201 followed by a single Gly, fused to Bla. pAC7 and pAC11 are identical to pAC6 and pAC13 except that PhoA replaces Bla.

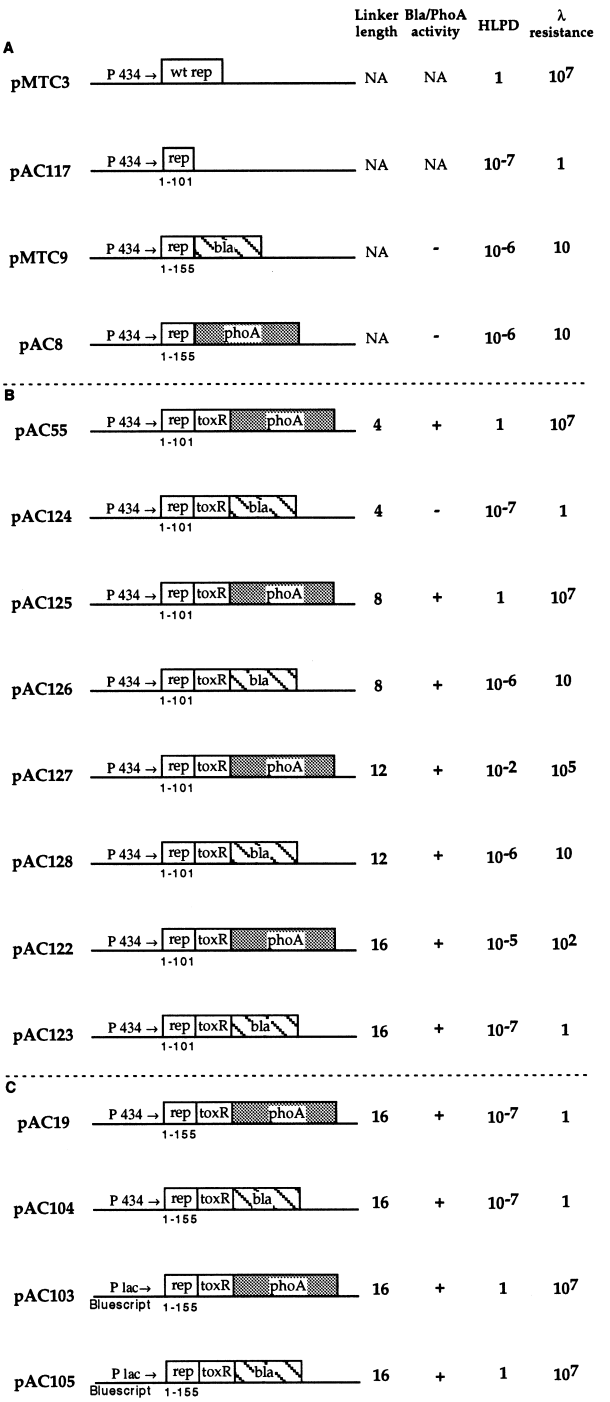

Fig. 2. Vectors encoding λ-ToxR fusion proteins. NA: not applicable. Linker length: see legend of Fig. 1. Except pAC103 and pAC105, all vectors are derivatives of plasmid pACYC184. Repressor activity was quantitated as follows: 5 μl of 10-fold serial dilutions of a 10^{10} pfu/ml phage stock containing $5–5\times10^{7}$ pfu/ml were spotted on bacterial lawns poured on λ plates containing Cm (50 μg/ml) or Km (50 μg/ml). Highest lysing phage dilution (HLPD) is the highest phage dilution that produces visible bacterial lysis. The level of λ resistance conferred by each recombinant protein is expressed as the ratio of HLPD that causes lysis in the strain producing that protein, and HLPD that causes bacterial lysis in the untransformed strain.

to grow with a concentration as high as 100 μg/ml. This indicates that in the two conditions of expression, the number of

hybrid proteins present in the cell differs by a factor which is smaller than 3.

In the second part of this work we replaced the DNA binding domain of ToxR with the amino-terminal domain of λ repressor. The structure and activity of the fusion proteins are outlined in Fig. 2. As in the previous case, protein fusions were first produced at low level. Plasmids pMTC3 and pMTC8 [17] served as positive controls (Fig. 2A). *E. coli* strain XL1-blue synthesizing intracellularly the wild-type repressor molecule (pMTC3) and a variant with a 9 aa polylinker sequence between position 155 and position 158 (pMTC8, not shown), displayed very high level of phage resistance. In contrast, bacteria producing two truncated versions of the repressor, aa 1–101 in pAC117 (Fig. 2A) and aa 1–155 in pAC51 (not shown) were as sensitive as the parent XL1-blue strain. Plasmids directing either the intracellular synthesis of λ155-Bla (pMTC9) [17] or λ155-PhoA (pAC8) (Fig. 2A), or the periplasmic secretion of wild-type Bla or PhoA (not shown), displayed also high sensitivity to phage lysis.

In the fusions ToxR201-PhoA the first 126 aa residues have been replaced by aa residues 1–101 of λ repressor (pAC55). This construct translocated the PhoA moiety into the periplasmic space and conferred a level of resistance to λ phage as high as the complete repressor molecule (Fig. 2B). Since the repressor molecule acts as a dimer and because the protein produced by pAC55 appeared to be a fully functional transmembrane form of repressor, we decided to verify the phenotype contributed by a similar Bla fusion (pAC124) (Fig. 2B). However such a chimera as well as the short ToxR201-Bla fusion constructed before, did not confer ampicillin resistance to single bacterial cells and cannot be compared to the PhoA fusion. We therefore constructed the PhoA fusion pAC125 and the Bla fusion pAC126 which add four ToxR aa residues at the linker level. The new Bla fusion conferred resistance to Ap and indicated a high level of phage sensitivity. In addition, the new PhoA fusion still displayed a very high level of repressor activity which suggests that, at the level of expression used, repressor activity is definitely dependent upon the dimerization capability of the domain attached to the membrane (Fig. 2B).

In order to evaluate the effect of the length of the periplasmic linker on repressor activity, we have constructed two longer λ-ToxR-PhoA fusions. The longest fusion pAC122, incorporating a 16 aa linker conferred a level of phage resistance 10^5 lower than the 8 aa linker fusion pAC125. pAC127 encoding a 12 aa linker gave a level of resistance intermediate between pAC122 and pAC125. Two similar λ-ToxR-Bla fusions, encoded by the vectors pAC128 and pAC123 displayed sensitivity to λ phage (Fig. 2B). As with ToxR-PhoA fusions, these data indicate that the activity of the repressor is sensitive to small variations in the length of the periplasmic linker. We also determined the effect of shortening the ToxR sequence on the cytoplasmic side. However, in the context of the long periplasmic linker fusion, a 50 aa residue deletion of the ToxR cytoplasmic spacer separating the repressor DNA binding domain from the transmembrane domain did not increase the phage resistance (not shown).

Next we analyzed the response of λ-ToxR fusions in a condition of more elevated expression. As a starting point we may consider the vectors pAC19 and pAC104 (Fig. 2C). pAC19 expresses at low level a λ-ToxR-PhoA construct which is identical to pAC122 except that it encompasses the first 155 aa residues of the repressor (vs. aa 1–101 of pAC122). This region extends well beyond the DNA binding domain (1–92). This was originally done to include in the fusions the sequence required for RecA mediated cleavage of the repressor. In the same way, pAC104 encodes a λ-ToxR-Bla fusion similar to pAC123. Both pAC19 and pAC104 supported growth of single plaques of λ at the lowest phage dilution indicating a high level of phage sensitivity. To explore the effect of a higher expression level these constructs have been subcloned in various expression vectors. We found that as a consequence of the synthesis from a high copy number vector and under constitutive expression of *lac* promoter, not only the PhoA fusion (pAC103), but also the Bla fusion (pAC105) conferred a very high level of resistance to phage lysis (Fig. 2C). Bacteria expressing the truncated version of the repressor in similar conditions, were still as sensitive as the parent strain (not shown). Next, the two vectors expressing the Bla fusion at lower level (pAC104) and at higher level (pAC105) were transformed in Q537 and plated on LB-agar containing various concentrations of Ap. It was determined that transformants containing pAC104 could grow at a concentration of Ap of 175 μg/ml but not at one of 200 μg/ml. In contrast bacteria harboring pAC105 were able to grow with at a concentration of 500 μg/ml but not at one of 600 μg/ml. These data indicate that pAC105 directed the synthesis of a fusion protein whose amount is between 2.5 and 3.4 times that produced by pAC104.

4. Discussion

The DNA binding domains of ToxR and λ repressors have been applied as genetic indicators of the dimerization of both periplasmic and membrane proteins [5–9]. It is important to validate the applicability of these reporter systems to different experimental conditions. We have found that the length of the periplasmic linker between the transmembrane segment of ToxR and PhoA, introduced as a model of dimeric protein, is critical; incremental changes of as few as 4 aa residues substantially affect λ sensitivity. One possibility is that the proximity of PhoA to the membrane enhances its capacity to dimerize the DNA binding domain. Alternatively a shorter linker might affect the level of full length fusion protein present in the membrane in view of a lower sensitivity to periplasmic proteases [4]. It should be noted that in some of the ToxR-PhoA hybrid proteins formerly constructed [2,4] a 17 aa residue linker, generated by the insertion of Tn*PhoA* [19], was interposed between the last ToxR aa residue and PhoA amino-terminus, Pro6 of the mature protein. In contrast, in the hybrid protein constructed by Kolmar et al. [6] ToxR is fused directly to Pro6. It follows that the fusion ToxR210-PhoA [6] is in fact 10 aa residues shorter than ToxR203-PhoA [4]. This observation may account for the higher transcriptional activation displayed by the first fusion in comparison to the latter, but this conclusion is limited by the fact that the expression conditions of these two fusions were not identical.

Two previous studies based on ToxR fusion proteins [6,10] have reached opposite conclusions about periplasmic dimerization as a requirement for ToxR activation (see Section 1). A possible explanation for this divergence is the fact that although in both studies the constructs were expressed from

medium copy number plasmids, the ToxR fusion proteins were expressed at quite different levels due to the presence of different promoters, *toxR* in one instance [6], *tet* in the other case [10]. Although in case of the fusions incorporating the ToxR DNA binding domain our experiments at a lower expression level were not conclusive (although pAC7 displayed ToxR transcriptional activity, the vector pAC6 encoding the similar Bla fusion did not conferred Ap resistance), at a higher level of expression the dimerization of the periplasmic domain is not required for the activation of ToxR. In case of the fusions incorporating λ repressor, we have found that at a low expression level a PhoA fusion, but not a Bla fusion, displays repressor activity. However following an increase in the expression level, both fusions confers a high grade of protection to phage lysis. Dimerization appears to be critical only within a narrow concentration range, because bacteria harboring the plasmid pAC105 have a level of λ-ToxR-Bla fusion protein that is only about three times higher than those containing plasmid pAC104. We have provided evidence that repressor activity does depend on the dimerization ability of the domain present in the periplasm, but only within certain levels of concentration. Our result is consistent with the data from a recent study [22] that has shown that when ToxR is overexpressed in *V. cholerae*, the amount of protein increases 2.7 times above the normal level. A biochemical cross-linking analysis indicated that when ToxR is overexpressed, it forms both ToxR homodimers and ToxR-ToxS heterodimers; however, at normal expression levels only ToxR-ToxS heterodimers are found [22].

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