Protein Science (1998), 7:1052–1056. Cambridge University Press. Printed in the USA.

FOR THE RECORD

The dimerization motif of the glycophorin A transmembrane segment in membranes: Importance of glycine residues

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(RECEIVED November 17, 1997; ACCEPTED January 14, 1998)

Abstract: The glycophorin A transmembrane segment homodimerizes to a right-handed pair of α-helices. Here, we identified the amino acid motif mediating this interaction within a natural membrane environment. Critical residues were grafted onto two different hydrophobic host sequences in a stepwise manner and self-assembly of the hybrid sequences was determined with the ToxR transcription activator system. Our results show that the motif LIxxGxxxGxxxT elicits a level of self-association equivalent to that of the original glycophorin A transmembrane segment. This motif is very similar to the one previously established in detergent solution. Interestingly, the central GxxxG motif by itself already induced strong self-assembly of host sequences and the three-residue spacing between both glycines proved to be optimal for the interaction. The GxxxG element thus appears to be the most crucial part of the interaction motif.

Keywords: glycine; glycophorin A; interaction; ToxR; transmembrane helix

Sequence-specific interactions between membrane-spanning α-helices support folding and/or oligomerization of a wide variety of integral membrane proteins (reviewed in Lemmon & Engelman, 1994; Dieckmann & DeGrado, 1997). A well-studied model-system is the glycophorin A (GPA) transmembrane (TM) helix whose homo-dimerization has originally been studied in detergent solution (Lemmon et al., 1992a). Based on saturation (Lemmon et al., 1992b) and insertion (Mingarro et al., 1996) mutagenesis, the motif ^{75}L^{76}Ixx^{79}G^{80}Vxx^{83}G^{84}Vxx^{87}T within the GPA TM-segment was proposed to constitute the helix-helix interface. The simultaneous replacement of all other residues within this motif by leucine preserved the ability of the motif to dimerize (Lemmon et al., 1994). Based on this motif, a model was developed where the GPA TM-segment forms a right-handed pair of α-helices (Adams et al., 1996). This model was confirmed by the NMR structure of the GPA TM-segment in detergent micelles. The helices cross at an angle of $-40°$ and make extensive backbone-backbone contact in the vicinity of glycines 79 and 83 (MacKenzie et al., 1997).

While all these experiments had been conducted in detergent solution, we recently developed a novel genetic approach which allows us to quantify interaction between TM-segments embedded in lipid membranes. This method is based on the ToxR transcription activator which was originally identified in *Vibrio cholerae*. The ToxR protein is anchored by a single TM-segment within the inner bacterial membrane where it exists in a monomer/dimer equilibrium. In its dimeric state, the ToxR molecule binds to the ctx promoter thereby initiating transcription of downstream genes (reviewed in DiRita, 1992). Transcriptional activity diagnostic of ToxR dimerization is conveniently measured in an *Escherichia coli* reporter strain which contains the lacZ gene under control of the *ctx* promoter (Kolmar et al., 1994; Kolmar et al., 1995). In a previous study, we engineered a tripartite protein (ToxR'GPA13) consisting of the cytoplasmic part of ToxR, the GPA TM-segment holding the above amino acid motif, and maltose binding protein (MalE). This protein elicited strong transcription activation indicative of dimerization of the GPA TM-helix in the bacterial membrane. The sequence specificity of this interaction was demonstrated by alanine-scanning mutagenesis which caused strong (^{79}G, ^{83}G: $\approx$70%), intermediate (^{76}I, ^{87}T: $\approx$40%), or small/no (^{75}L, ^{77}I, ^{78}F, ^{80}V, ^{81}M, ^{84}V, ^{85}I, ^{86}G, <25%) reductions in activity (Langosch et al., 1996). These results are consistent with the structure of the helix-helix interface in detergent solution (Lemmon et al., 1992b; Lemmon et al., 1994; MacKenzie et al., 1997).

Here, we extended our results by reconstructing the interaction motif embedded in the membrane. Grafting the critical residues onto different host sequences in a stepwise manner allowed us to assess the relative contributions of different residue combinations to the GPA homo-dimerization motif. Interestingly, the central motif ^{79}Gxxx^{83}G appeared to be its most crucial part thus underscoring the importance of backbone-backbone interactions in a hydrophobic environment.

Results and discussion: *Characterization of host sequences:* Initially, we examined two hydrophobic sequences consisting of ei-

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ther 13 methionine (M13) or valine (V13) residues for their suitability to serve as hosts for the GPA dimerization motif. Expressed in the context of the ToxR protein (Fig. 1), these sequences formed artificial TM-segments since the chimeric proteins (ToxR′M13 and ToxR′V13) could not be extracted from bacterial membranes by NaOH treatment (Chen & Kendall, 1995, data not shown). To test whether these proteins were correctly integrated into the inner membrane with the ToxR moiety facing the cytoplasm and the MalE domain exposed to the periplasmic space ($N_{in} - C_{out}$, Fig. 1), they were tested for their ability to functionally complement a MalE deficient *E. coli* strain (PD28; Bedouelle & Duplay, 1988). This strain is unable to grow in minimal medium with maltose as the only carbon source. In cells expressing correctly inserted ToxR′ chimeric membrane proteins, the MalE domain allows utilization of maltose and thus cell growth. Upon expression in PD28 cells, the ability of ToxR′M13 to sustain cell growth in maltose-containing minimal medium was similar to that of ToxR′GPA13 whereas that of ToxR′V13 tended to be somewhat lower (Fig. 2). This reveals $N_{in} - C_{out}$ integration into the inner membrane. A control construct, whose TM-segment was deleted (ToxR′MalEΔTM), and which is therefore expected to reside in the cytoplasm, proved virtually unable to complement the MalE deficiency as expected (Fig. 2). The intrinsic self-assembly of the host sequences was assessed by expression in FHK12 indicator cells. ToxR′M13 and ToxR′V13 elicited only low levels of transcription activation (790 ± 121 and 357 ± 63 Miller units, means ± SD, respectively; Fig. 3) compared to that of ToxR′GPA13 (2,037 ± 313 Miller units). Thus, both host sequences self-associate only weakly in the bacterial membrane.

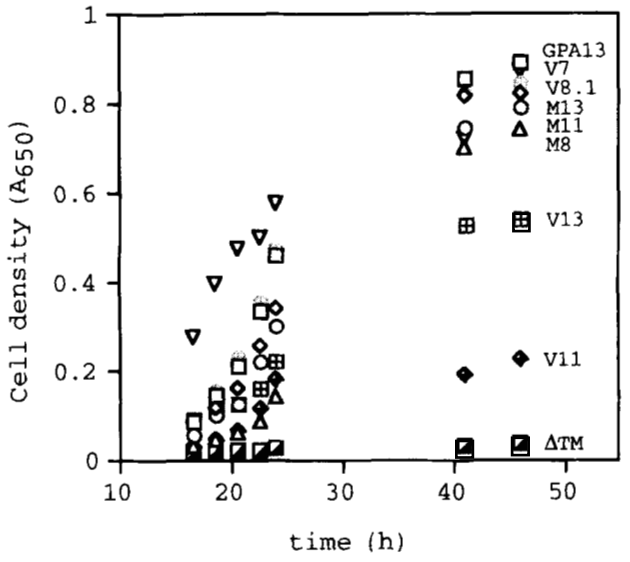

Fig. 2. Functional complementation of MalE deficiency to assess correct $N_{in} - C_{out}$ incorporation of the chimeric proteins into the inner bacterial membrane. Growth of expressing PD28 cells in minimal medium containing maltose was monitored by measuring cell density at the indicated time points. For the sake of clarity, only the growth curves of some key constructs are shown here. Note that most constructs allowed for similar rates of cell growth. The slower growth rate mediated by ToxR′V11 suggests that this protein is less efficiently or not correctly integrated into the membrane. A control construct whose TM-segment had been deleted (ToxR′ΔTM) did not support cell growth consistent with the expected intracellular accumulation of this protein. The values shown represent means from three to five independent experiments. ToxR′GPA13 (□), M13 (◇), M11, (○), M8 (△), V13 (⊞), V11 (⊕), V8.1 (⊕), V7 (▽), ΔTM (◪).

Reconstruction of the GPA TM-helix-helix interface: Starting with glycines 79 or 83, we now grafted the residues previously implied in GPA TM-helix-helix interaction (Langosch et al., 1996; Lemmon et al., 1992b) onto the host sequences in a stepwise manner. This yielded the hybrid sequences shown in Figure 3A. With the M13 host, introducing single glycines at positions equivalent to GPA glycine 79 (M12.1) or glycine 83 (M12.2) did not measurably affect ToxR activity, whereas the ^{79}Gxxx^{83}G motif increased activity (ToxR′M11, 1,795 ± 321 Miller units) more than twofold. Introducing isoleucine 76 + threonine 87 (ToxR′M9) or leucine 75 + isoleucine 76 + threonine 87 (ToxR′M8) in addition to both glycines resulted in further slight increases of mean activity (1,859 ± 407 or 2,014 ± 638 Miller units, respectively) up to the level exhibited by ToxR′GPA13 (Fig. 3B). The relative contribution of these amino acids to the GPA helix-helix interface seemed to be different with the ToxR′V13 host. Here, introduction of the ^{79}Gxxx^{83}G motif only slightly increased ToxR activity (ToxR′V11, 574 ± 156 Miller units). Activity strongly increased upon introducing isoleucine 76 + threonine (ToxR′V9) or leucine 75 + isoleucine 76 + threonine 87 (ToxR′V8.1) in addition to both glycines (1,267 ± 181 or 1,951 ± 331 Miller units, respectively). The latter sequence ^{75}L^{76}IVV^{79}GVVV^{83}GVVV^{87}T elicited an activity level indistinguishable from that of ToxR′GPA13 (Fig. 2B). Within this motif, the host valines at positions 80 and 84 correspond to the valines present within the original GPA TM-segment. In the solution NMR structure, these valines contact glycines 79 and 83 of the opposing helix (MacKenzie et al., 1997); mutating these residues decreased the level of GPA dimerization in detergent solution (Lemmon et al., 1992b) but not in membranes (Langosch et al., 1996). To clarify their potential contribution to dimerization of ToxR′V8.1

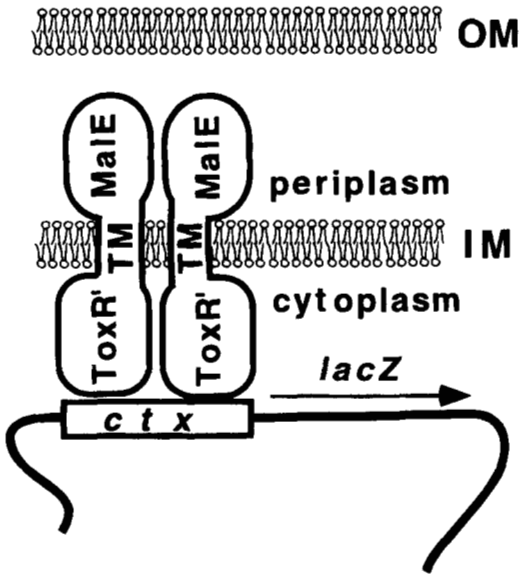

Fig. 1. Functional organization of ToxR′ chimeric proteins. The cytoplasmic domain ToxR′ is linked via a TM-segment of choice to the periplasmic MalE moiety. Upon dimerization, ToxR′ binds to the *ctx* promoter thus initiating *lacZ* transcription in the indicator cells. OM, outer membrane; IM, inner membrane.

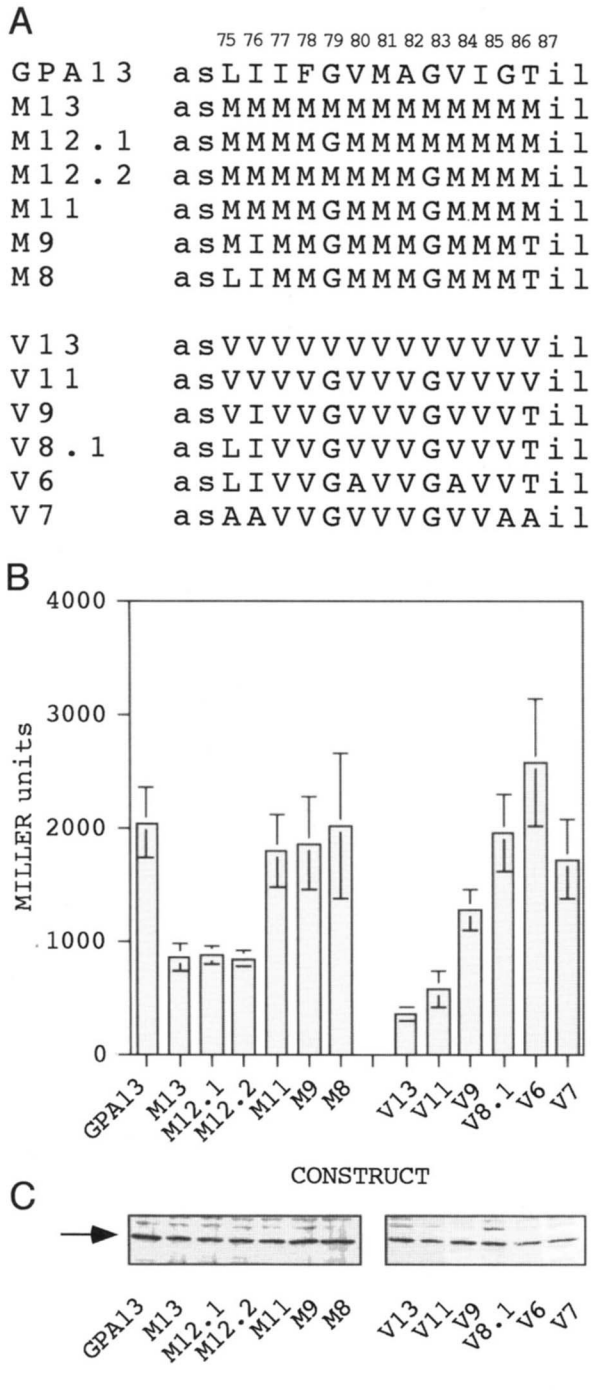

Fig. 3. Transcription activation and expression of the different constructs. **A:** Amino acid sequences of the different TM-segments as expressed in the context of the different ToxR′ chimera. The residue numbers of the GPA TM-segment are given above its sequence. Invariant flanking residues are in lower case letters. **B:** Transcription activation determined upon expressing the constructs in the indicator strain FHK12. The bars represent mean values and error bars denote standard deviation. **C:** Expression level of the different constructs. Western blotting of transformed FHK12 cells followed by densitometric evaluation established that the intensity of the relevant bands (arrow) ranged from 76% (pToxR′M13) to 88% (pToxR′M8) of the wild type (pToxR′GPA13) for the blot shown in the left panel while they ranged from 77% (pToxR′V6) to 123% (pToxR′V9) of the control (pToxR′GPA13, not shown) for the blot shown in the right panel. This indicates similar expression levels of the expressed 65 kDa pToxR′ chimeric proteins. Additional bands correspond to unspecific binding of the polyclonal antiserum.

in membranes, both valines were simultaneously mutated to alanines. Surprisingly, this resulted in a significant increase of ToxR activity indicating more efficient helix-helix packing (ToxR′V6, 2,575 ± 562 Miller units) (Fig. 3B). This corroborates the participation of positions 80 and 84 to the GPA dimer interface. In the membrane-embedded state, however, they need not be occupied by valine residues for optimal interaction possibly reflecting a subtle structural difference between the membrane-bound and the detergent solubilized helix-helix dimer.

In comparing the results obtained with both host sequences, the question arises as to why they responded differently to insertion of the ^{79}Gxxx^{83}G pair, which sharply increased the signal in the context of the methionine but not the valine host. This differential behavior may be ascribed to (a) a different ability of the methionine or valine host residues to cooperate with the GxxxG motif in helix-helix interaction, or (b) an abnormal low concentration of the ToxR′V11 protein in the bacterial membrane. Indeed, the ToxR′V11 protein was not as potent in complementing the MalE deficiency of PD28 cells and thus may not be integrated as efficiently and/or correctly into the membrane as the other constructs investigated here (Fig. 2). As exemplified by constructs ToxR′V9 and V8.1, exchanging residues at the TM-segment's termini by nonvaline residues corrected this aberrant behavior (Fig. 2). To uncover a potential contribution of the ^{79}Gxxx^{83}G motif to self-assembly of a valine host, we converted four terminal positions to alanine residues which are not expected to contribute to homophilic interaction due to their small side-chains. Indeed, the construct with the sequence AAVVGVVVGVVAA (ToxR′V7) appeared to be correctly integrated into the membrane (Fig. 2) and gave a signal (1,716 ± 350 Miller units) more than three times that of ToxR′V11 (Fig. 3B). It appears therefore that the ^{79}Gxxx^{83}G pair also exerts a strong effect in the context of a valine host sequence which may be masked by aberrant membrane insertion in our experimental system.

Control experiments showed that all other constructs complemented the MalE deficiency of PD28 cells with similar efficiencies (Fig. 2; data not shown) and all proteins were expressed at similar levels in the FHK12 indicator cells (Fig. 3C). Thus, except for the case of ToxR′V11, the different levels of ToxR activity are unlikely to result from different concentrations and/or orientations of these proteins in the bacterial inner membrane.

Importance of spacer length between glycine residues: Recognizing the role of the ^{79}Gxxx^{83}G pair for homo-dimerization, we examined the importance of spacer length between both glycines for self-recognition. To this end, the glycines within the motif LIVVGVVVGVVT (ToxR′V8.1) were individually shifted by one position toward the N- or C-terminus. This yielded the constructs ToxR′V8.3 to ToxR′V8.6 (Fig. 4A) where the spacing between the glycines is either two or four residues. Interestingly, the ToxR activities elicited by these proteins were decreased by about 50% as compared to the parental ToxR′V8.1 construct (Fig. 4B). Thus, a three residue spacing between both glycines appears to be optimal for self-interaction of the motif representing the GPA TM-helix-helix interface.

Western blot analysis indicated that constructs pToxR′V8.3–6 were slightly overexpressed relative to pToxR′V8.1, suggesting that their actual degree of dimerization may be even lower than indicated by the β-galactosidase activities (Fig. 4C). Functional complementation of MalE deficient PD28 cells (data not shown)

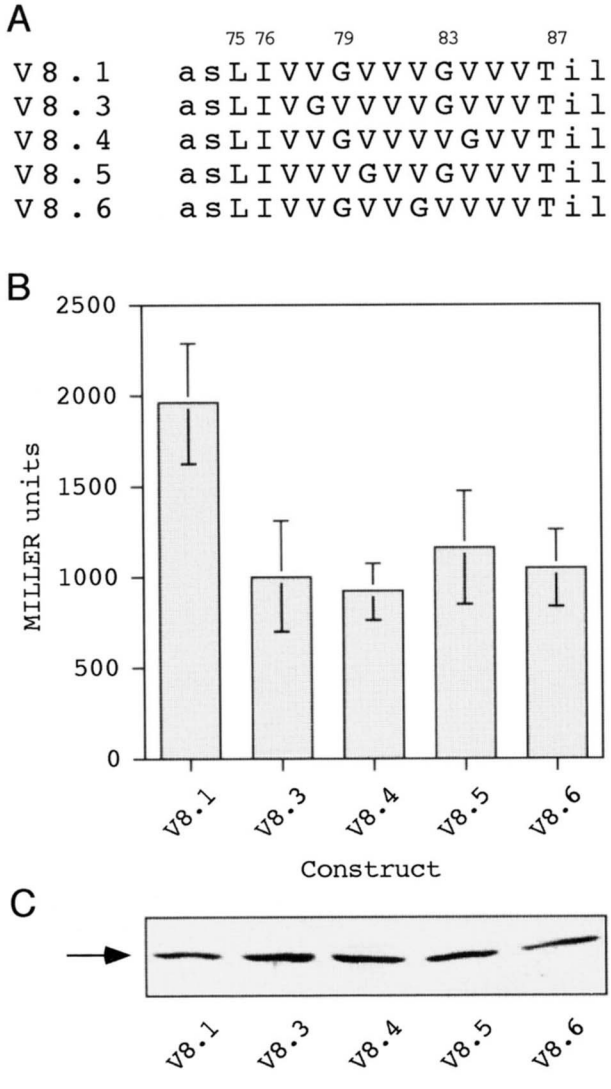

Fig. 4. Importance of spacer length between glycines 79 and 83 for dimerization of construct ToxR′V8.1. **A:** Amino acid sequences of the analyzed mutants. The numbering of the GPA residues is given above the V8.1 sequence. Invariant flanking residues are in lower case. **B:** Transcription activation elicited by the constructs in FHK12 cells. The bars represent mean values and error bars denote standard deviation. **C:** Expression level of the mutants. Western blotting followed by densitometry revealed staining intensities of the different constructs (arrow) ranging from 118% (pToxR′V8.6) to 168% (pToxR′V8.3) of the parental construct (pToxR′V8.1) indicative of slight over-expression of constructs pToxR′V8.3-6.

demonstrated correct orientations of these proteins in the inner membrane.

Taken together, our results suggest that the ^{79}Gxxx^{83}G motif constitutes the most crucial part of the GPA TM-helix-helix interface. Residues leucine 75, isoleucine 76, and threonine 87 contribute to helix-helix recognition, albeit to a smaller degree. This is supported by several lines of evidence. (a) Glycines 79 and 83 are those residues whose mutagenesis disrupts GPA dimerization to the largest degree (Lemmon et al., 1992b; Langosch et al., 1996). (b) Residues leucine 75 and isoleucine 76 where not required for the minimal dimerization motif grafted onto the protooncogene neu TM-segment (Lemmon et al., 1994). (c) Alanine insertion between isoleucine 76 and glycine 79 of the GPA wild-type sequence was tolerated surprisingly well (Mingarro et al., 1996). (d) Increasing or decreasing the residue spacing between both glycines perturbed dimerization (this study, see also Mingarro et al., 1996). (e) In the solution NMR structure of the GPA TM-segment pair, the helix backbones closely approach each other in the vicinity of glycines 79 and 83. This results in a smooth dimer interface proposed to aid in dimerization (MacKenzie et al., 1997).

A GxxxG motif has also been found in several other TM-segments capable of homophilic interaction. For example, self-association of the TM-segment is important for dimerization of phage M13 coat protein and considered essential for phage viability. Here, two glycines separated by three residues are among those amino acids displaying only limited mutability in a mutant screen (Williams et al., 1995). Oligomerization of the heparan sulfate proteoglycan N-syndecan also requires the TM-segment and simultaneous mutation of the glycines in its GxxxG motif partially abolished complex formation (Asundi & Carey, 1995). A GxxxG motif was also detected in TM-segment VI of the β_2-adrenergic receptor (β_2AR) which was shown to homo-dimerize. Addition of a synthetic version of TM-segment VI reduced dimerization whereas a peptide with both glycines (plus a C-terminal leucine) mutated to alanine had no effect (Hebert et al., 1996). The alignment of these TM-segments as given below is based on the conserved GxxxG motif; those residues implicated in self-assembly are in bold face letters.

Glycophorin A:	^{75}**LI**IF**GV**MA**GV**IG**T**
M13 coat protein:	^{30}**VV**IV**GA**TI**GI**KLF
Syndecan 3:	^{388}AVIV**G**GVV**G**ALFA
β_2AR-TM6:	^{272}LKTL**G**IIM**G**TFT**L**

In analogy to the structure of the GPA TM-helix pair, we speculate that their self-assembly is partially driven by a close apposition of the helix backbones close to the pair of glycines. This may either result in extensive backbone interactions or facilitate the packing of adjacent side-chains. A similar mechanism may operate in heterodimerization of class II MHC α or β chains which has been shown to depend on glycine-rich faces of the respective TM-segments (Cosson & Bonifacino, 1992). It is presently not clear to which extent sequence context influences the proposed role of the GxxxG motif. By randomizing the surrounding sequence this issue may be resolved in future studies.

Materials and methods: *Plasmid constructs:* Construction of plasmid pToxR′GPA13 was described previously (Langosch et al., 1996). The constructs pToxR′M13, M12.1, M12.2, M11, M9, M8, and V7 were made by synthesizing sense oligonucleotides encoding the desired sequences including a 5′ NheI and a 3′ MboI site plus a 15 base 3′ extension. After hybridizing a corresponding antisense 15mer to the 3′ extension, the antisense strand was synthesized with 2 units of Klenow polymerase in the presence of 0.15 mM dNTPs at 30°C for 30 min. After heat-inactivating the Klenow enzyme, the resulting cassettes were cut with NheI and MboI, gel-purified and ligated into the plasmid pHKT$_{ox}$R′(TM^{II4})MalE (Kolmar et al., 1995) previously cut with NheI and BamHI. The mutants pToxR′V11, V9, V8.1, V6 or V8.3, V8.4, V8.5, V8.6 were obtained by oligonucleotide-directed mutagenesis using the single-stranded pToxR′V13 or pToxR′V8.1 plasmid, respectively, as template (Kunkel et al., 1987). The construct pToxR′ΔTM was obtained by cutting pHKToxR′(TM^{II4})MalE with

NheI and BamHI, refilling the 5′ overhangs with Klenow polymerase, and religation. All constructs were verified by dideoxy sequencing.

ToxR activity assays: Transcription activation was determined upon expression of the different constructs in the indicator strain FHK12 (Kolmar et al., 1994). The measurements were done as described (Langosch et al., 1996) by quantitating β-galactosidase activities in crude cell lysates. Specific β-galactosidase activities are expressed as Miller units. For each construct, the reported numbers represent mean values of data derived from several independent measurements (n = 16 to 56 data points for individual constructs).

Western blotting: Aliquots corresponding to ca. 100 μg of total bacterial protein were subjected to 10% SDS-PAGE, transferred to nitrocellulose and probed with a rabbit antiserum directed against the MalE moiety of the fusion protein (New England Biolabs), the immunoblot was developed with an alkaline phosphatase coupled anti-rabbit secondary antibody (Promega) and 5-bromo-4-chloro-3-indolylphosphate plus nitro blue tetrazolium (Applichem) as chromogenic substrates. The intensity of the bands was quantitated by densitometry (NIH image software, version 1.6) after digitizing photographic images of the blots with a video camera (Cybertech CS1 imaging system).

Complementation of MalE deficiency: PD28 cells (Bedouelle & Duplay, 1988) transformed with the different constructs were cultured overnight in Luria-Bertani-medium with 30 μg/mL chloramphenicol. The cells were washed twice with phosphate buffered saline and used to inoculate M9 minimal medium including 0.4% Maltose, 80 μg/mL leucine and threonine, 2 mM $MgSO_4$, and 30 μg/mL chloramphenicol at a 200-fold dilution. Cells were grown in duplicate samples with shaking at 37 °C. The ability of the different constructs to complement the MalE deficiency of the PD28 cells was monitored by measuring cell density at different time points after inoculation. Cell density was determined at 650 nm in 96-well plates (7 mm light path length) using a Molecular Devices microplate reader.

Acknowledgments: We thank Prof. H. Bedouelle for providing strain PD28, our colleage Rico Laage for critically reading the manuscript and Dr. W.B. Huttner for continuous support. This work was supported by the Deutsche Forschungsgemeinschaft (Grant La699/4-1 and Heisenberg Program).

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