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Identification of a Conserved N-Terminal Sequence Involved in Transmembrane Signal Transduction in EnvZ

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To determine whether N-terminal sequences are involved in the transmembrane signaling mechanism of EnvZ, the nucleotide sequences of *envZ* genes from several enteric bacteria were determined. Comparative analysis revealed that the amino acid sequence between Pro41 and Glu53 was highly conserved. To further analyze the role of the conserved sequence, *envZ* of *Escherichia coli* was subjected to random PCR mutagenesis and mutant alleles that produced a high-osmolarity phenotype, in which *ompF* was repressed, were isolated. The mutations identified clustered within, as well as adjacent to, the Pro41-to-Glu53 sequence. These findings suggest that the conserved Pro41-to-Glu53 sequence is involved in the signal transduction mechanism of EnvZ.

In *Escherichia coli*, EnvZ is involved in sensing changes in the osmolarity of the external environment (2, 6, 8, 15). During adaptation to osmolarity stress, *E. coli* differentially regulates the genes encoding the outer membrane porin proteins, OmpF and OmpC. The response regulator OmpR controls the expression of the *ompF* and *ompC* genes. When cells are grown either under high-osmolarity conditions or in the presence of membrane-perturbing agents such as procaine, the level of OmpR-phosphate in the cell increases, which stimulates the expression of *ompC* and the repression of *ompF* (1, 4, 5, 9, 11, 16, 18). Modulation of the intracellular levels of OmpR-phosphate thereby controls the relative expression of the *ompF* and *ompC* genes (18, 24).

EnvZ functions as a dimer (17, 26) and undergoes transautophosphorylation on His243, using ATP as the phosphate donor (7, 19, 22, 27). The phosphate group is subsequently transferred to Asp55 of OmpR. EnvZ also possesses a phosphatase activity that stimulates the dephosphorylation of OmpR-phosphate. The sum of the kinase and phosphatase activities controls the level of OmpR-phosphate in the cell (5, 8, 15, 18). Hsing et al. recently presented a model in which the positioning of His243 relative to the ATP-binding domain determines whether EnvZ functions as a kinase or a phosphatase (8).

While the biochemical and structural properties of the cytoplasmic signalling domain have been extensively studied, the domains involved in the sensing function of EnvZ have not been elucidated. The periplasmic domain of EnvZ encompasses the region from Pro41 to Arg162 (3). It is flanked by two transmembrane sequences, TM1 (Leu16 to Leu40) and TM2 (Tyr163 to Ile179). It was found that replacement of the periplasmic region from Arg55 to Arg146 did not affect EnvZ function (12). Furthermore, EnvZ of *Xenorhabdus nematophilus*, which possesses a small periplasmic loop rather than the large domain found in the EnvZ proteins of most enteric bacteria, was able to complement an *envZ*-null strain of *E. coli* (20). These results raise the question of which regions of EnvZ are essential for sensing osmolarity signals. In the present study, we have taken both a comparative and a genetic approach to address this question.

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Bacterial strains and plasmids. The bacterial strains and plasmids utilized in this study are described in Table 1.

Identification of a conserved N-terminal sequence. In an attempt to find conserved sequences in the N-terminal region that may be involved in the sensing function of EnvZ, a comparative approach was taken in which EnvZ proteins from several genera within the *Enterobacteriaceae* family were analyzed. To this aim, the nucleotide sequences of the *envZ* genes of *Shigella flexneri*, *Enterobacter cloacae*, *Yersinia enterocolitica*, and *Proteus vulgaris* were determined. To obtain the nucleotide sequences of the various *envZ* genes, DNA fragments were PCR amplified directly from single bacterial colonies, using the following degenerate primer set: 5′GC(A/T)AA(C/T)GC(A/C/T)GA(A/G)CAGATG (Ala35 to Met40 of OmpR) and 5′CGG(C/G)GT(A/G)CG(C/T)AA(A/G)TC(A/G)TG (Pro248 to His243 of EnvZ). The nucleotide sequences of the different *envZ* genes were determined by a combination of direct sequence analysis of the PCR products and subcloning into M13.

Figure 1 shows the sequence alignment of amino acids Met1 to Asp244 of various EnvZ proteins. The recently determined sequence of EnvZ from *Vibrio cholerae* (23) is also shown in Fig. 1. The amino acid sequences of the EnvZ proteins of *S. flexneri* and *Enterobacter cloacae* were nearly identical to that of the *E. coli* protein (Table 2), so they were not included in the sequence comparison. This comparison revealed that a 13-residue sequence encompassing Pro41 to Glu53 of *E. coli* EnvZ was 100% identical to the corresponding *Proteus* sequence and 85% identical to that in *V. cholerae* (Table 2). We refer to this highly conserved sequence as the identity box or I box. The I box is located at the junction between TM1 and the periplasmic domain. In contrast to the highly conserved nature of the I box, the overall degree of identity in the transmembrane and periplasmic domains was low. In the TM1 domains of the various EnvZ proteins, 6 of the 25 residues were identical (24% identity), while the periplasmic and TM2 domains exhibited only 14 and 12% amino acid sequence identity, respectively.

The distinctive characteristics of the I box include the presence of a proline residue (Pro41), four polar residues (Ser42, Gln44, Gln45, and Asn47), and two charged residues (Lys48 and Glu53). Furthermore, the nonpolar residues Leu43, Phe46, and Leu50 are conserved in the EnvZ proteins. Based on secondary-structure predictions, the I-box sequence exists as an amphiphilic alpha helix in which Leu43 and Leu50 are positioned on the nonpolar face of the helix (26). The bio-

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant genotype	Source or reference
Strains		
S. flexneri	Wild type	ATCC 120022
Enterobacter cloacae	Wild type	ATCC 23355
Y. enterocolitica	Wild type	Lab strain
P. vulgaris	Wild type	ATCC 13315
E. coli		
AT142	F$^-$ *envZ*::Kan *lacU169 araD139 rpsL relA thiA flbB*	12
AT142*pcnB*	*pcnB*::Tn*10* of AT142	12
WH57	*ompF-lacZ* of AT142	7
LEO544	*pcnB*::Tn*10* of WH57	12
Plasmids		
pMRL25	*ompR-envZ* in pBR322	12
pKS2	pMRL25 with *envZ* H243N	19
pJW49	pMRL25 with a *Sal*I site at bp 5–10 of *envZ*	This study
pJW32P	pJW49 with *envZ* L32P	This study
pJW35A	pMRL25 with *envZ* L35A	This study
pJW35P	pJW49 with *envZ* L35P	This study
pJW43A	pMRL25 with *envZ* L43A	This study
pJW43P	pJW49 with *envZ* L43P	This study
pJW48E	pJW49 with *envZ* K48E	This study

chemical properties of this region of EnvZ of *X. nematophilus* were also conserved (20). EnvZ of *X. nematophilus* contains a proline residue (Pro50), three polar residues (Thr42, Ser44, and Ser47), and two charged residues (Glu41 and Asp46), as well as the invariant nonpolar residues mentioned above.

Finally, OmpR was found to be highly conserved. The OmpR proteins in the *E. coli-Proteus* group exhibited >89% amino acid identity while those from *V. cholerae* and *X. nematophilus* exhibited 82% (26) and 74% (20) identity, respectively, to OmpR of *E. coli*.

I-box and TM1 mutations. The highly conserved nature of the I-box sequence suggested that it might be involved in perceiving osmolarity signals. High-osmolarity conditions stimulate an increased kinase-to-phosphatase ratio in EnvZ, which results in elevated OmpR-phosphate levels and the repression of *ompF* (5, 8, 26). Therefore, mutations that either enhance the kinase activity or decrease the phosphatase activity of EnvZ would generate a high-osmolarity-type signal. If the I-box region was involved in modulating the kinase-to-phosphatase ratio of EnvZ, mutations that stimulate elevated OmpR-phosphate levels, and the concomitant repression of *ompF*, would be predicted to occur in this region of the molecule. To test this prediction, a genetic screen was designed to isolate strains with mutations in the N-terminal region of EnvZ that cause repression of *ompF*. A PCR approach was used in which the DNA fragment encoding the N-terminal region of EnvZ (Met1 to Glu106) was amplified under mutagenic conditions (10). The resultant PCR fragments were ligated into a plasmid encoding OmpR and the C-terminal region of EnvZ (Phe107 to Gly450), and the recombinant plasmids were transformed into the *envZ*-null strain LEO544, which contains both an *ompF-lacZ* reporter gene fusion and the *pcnB80* allele, which is used to maintain plasmids at low copy numbers (7, 13). Since OmpR is phosphorylated by acetyl phosphate in *envZ*-null strains (7, 12), *ompF* is expressed at low levels in LEO544, and hence this strain forms red colonies on MacConkey-lactose agar. LEO544 transformed with *envZ* alleles that cause *ompF* to be completely repressed form white colonies (LacZ$^-$) on MacConkey-lactose agar. The formation of white colonies could result either from *envZ* mutations that stimulate higher OmpR-phosphate levels (i.e., kinase positive and phosphatase negative), resulting in the repression of *ompF*, or from mutations that reduce OmpR-phosphate to levels that are insufficient to activate *ompF* expression (i.e., kinase negative and phosphatase positive; see reference 18). These possibilities can be distinguished since in the former case, OmpC is produced, while in the latter instance it is not produced (see Fig. 2). In this screen, *envZ*-null alleles are not recovered since they produce red colonies. To ensure that this was the case, an *ompR-envZ*-containing plasmid carrying the *envZ*-null allele, H243N (19), was transformed into LEO544. As expected, the resultant strain formed red colonies.

Using this screen, seven white colonies containing single missense mutations in *envZ* were obtained. The following single-amino-acid substitutions were identified: Leu32 to Pro (L32P), Leu35 to Pro (L35P), Leu43 to Pro (L43P), and Lys48 to Glu (K48E). Mutants with the L35P allele were isolated three times, and mutants with the L43P allele were isolated twice. Leu32 and Leu35 are located in the TM1 domain adjacent to the I box, while Leu43 and Lys48 are located within the I-box sequence (Fig. 1). Thus, the mutations isolated clustered in the C-terminal end of TM1 and in the I-box sequence.

The effect that the mutations had on the kinase-to-phosphatase ratio was assessed by analyzing the production of OmpC in the mutant strains. If the kinase-to-phosphatase ratio was elevated, OmpC would be produced at increased levels. On the other hand, OmpC would not be produced in strains in which the kinase-to-phosphatase ratio was low (18). To distinguish between these possibilities, the mutant strains were grown in MacConkey medium and the outer membrane proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 2). The relative amounts of OmpF and OmpC in the outer membrane were then analyzed by densitometric scanning (Table 3). Figure 2 shows that OmpC was produced by all of the mutant strains. Densitometric scanning revealed that the levels of OmpC were elevated relative to that of the wild-type strain (Table 3). Thus, the mutations in TM1 and the I-box sequence caused an elevation of the intracellular levels of OmpR-phosphate, indicating that the kinase-to-phosphatase ratio in the mutant EnvZ molecules was increased.

To ensure that the mutant EnvZ proteins had properly assembled into the cytoplasmic membrane, vesicles were prepared (12) and EnvZ was detected by Western blot analysis, using enhanced chemiluminescence (Sigma Co.). Membrane vesicles prepared from the *envZ*-null strain harboring plasmids

TABLE 2. Amino acid sequence identity relative to the *E. coli* protein

Bacterium	% Identity for:	
	EnvZ[a]	I box
S. flexneri	99.6	100
Enterobacter cloacae	98.4	100
Y. enterocolitica	92.2	100
P. vulgaris	45.8	100
V. cholerae[b]	35.7	85

[a] Met1 to Ile179.
[b] Taken from reference 23.

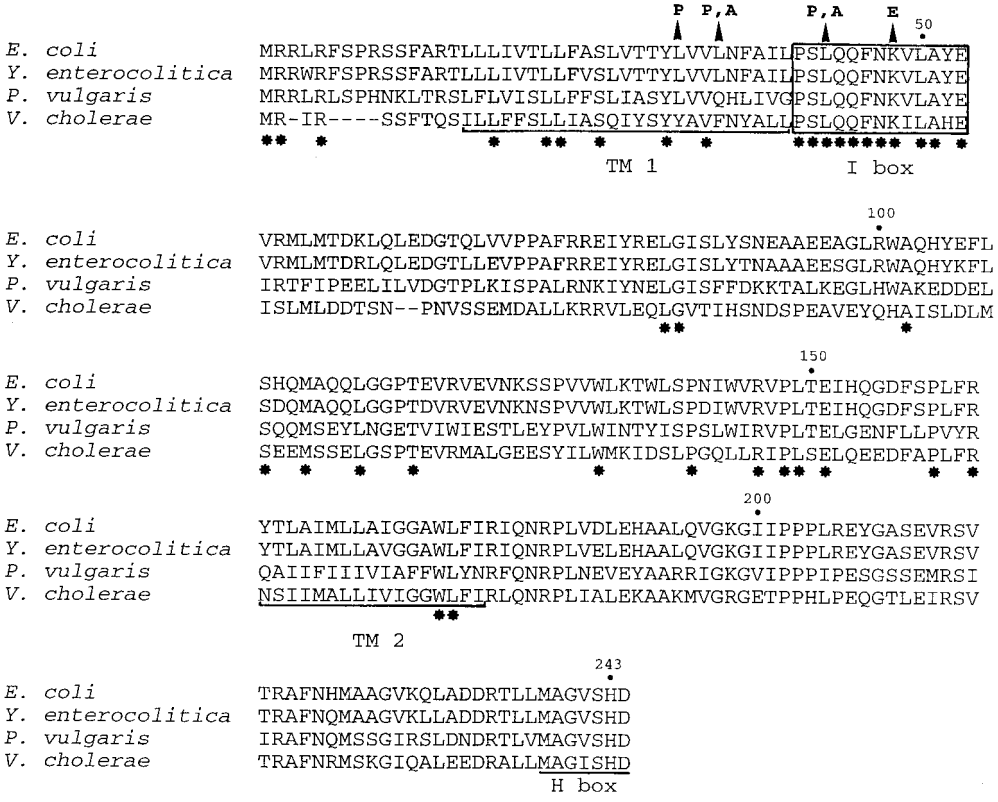

FIG. 1. Comparison of amino acid sequences of the Met1-to-Asp244 regions of various EnvZ proteins. Invariant residues in the N-terminal region (Met1 to Ile179) are indicated by stars beneath the sequence. The I box is enclosed in a box. The TM1 and TM2 domains and the sequence containing the H243 site of phosphorylation (H box) are underlined. Amino acid substitutions of mutants isolated in this study are indicated by arrowheads above the sequence.

encoding either the wild-type or a mutant EnvZ protein contained a protein with a molecular weight of 50,000, representing full-length EnvZ (data not shown). These findings indicated that the mutant EnvZ proteins were incorporated into the membrane.

Growth of mutant strains in nutrient broth. To further evaluate the effect that the TM1 and I-box mutations had on EnvZ function, we grew cells in nutrient broth. OmpR-phosphate is maintained at moderate levels, and OmpF is produced at high levels, in cells grown under nutrient broth conditions (5, 18). By growing the mutant strains under these conditions, we could determine the extent to which the kinase-to-phosphatase ratio had been reset in the mutant EnvZ proteins. Strains with the TM1 mutation L35P or the I-box mutation L43P were selected for this analysis. Table 4 shows that in wild-type cells grown in nutrient broth, OmpF was produced at approximately threefold-higher levels than OmpC. In contrast, OmpF production was markedly decreased, but not fully repressed, and OmpC production was increased in the mutant strains. These results indicated that the L35P and L43P mutations had elevated the kinase-to-phosphatase ratio of EnvZ sufficiently to trigger a switch in the relative amounts of OmpF and OmpC produced by the cell. However, the amount of OmpR-phosphate present in the mutant cells was apparently not large enough to reduce OmpF to low levels. To address the question of whether the mutant EnvZ proteins were able to sense high-osmolarity signals and further increase the levels of OmpR-phosphate, the mutant cells were grown in nutrient broth containing 20% sucrose. Table 4 shows that OmpF production was further reduced in the mutant strains grown under high-osmolarity conditions. This result suggested that the mutant proteins were still able to perceive high-osmolarity stimuli and set the kinase-to-phosphatase ratio to higher levels, resulting in an increase in OmpR-phosphate levels and a concomitant decrease in OmpF production.

Alanine substitutions at Leu35 and Leu43. The mutations of strains isolated in this study consisted of either proline substitutions or a charge reversal. Proline substitutions could indirectly affect EnvZ function by inducing conformational alterations. For example, proline replacement in transmembrane domains has been shown to disrupt alpha-helical structure (14). To introduce conservative substitutions in TM1 and the I-box sequence, alanine residues were substituted for Leu35 and Leu43 by site-directed mutagenesis (Sculptor in vitro system; Amersham). The resulting L35A and L43A *envZ* alleles were transformed into the *envZ*-null strain AT142*pcnB*, and

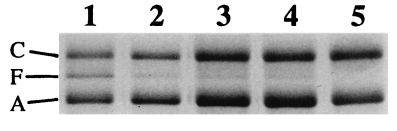

FIG. 2. Outer membrane protein analysis. AT142*pcnB* cells containing various *envZ* alleles were grown on MacConkey medium, and outer membrane proteins were prepared and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described previously (5). Lane 1, pJW49 (wild type); lane 2, pJW32P; lane 3, pJW35P; lane 4, pJW43P; lane 5, pJW48E. C, OmpC; F, OmpF; A, OmpA.

TABLE 3. Porin protein production on MacConkey medium

Strain	Relative % of porin protein produced[a]	
	OmpF	OmpC
Wild type	14	34
L32P	4	40
L35P	2	43
L43P	2	40
K48E	1	51

[a] The values represent the relative amounts of OmpF and OmpC, expressed as a percentage of the total absorbance of OmpA, OmpF, and OmpC, as determined by scanning densitometry of the outer membrane proteins.

porin production in cells grown in nutrient broth was analyzed. Table 4 shows that OmpF was repressed and OmpC was stimulated in cells containing the L43A form of EnvZ. In addition, OmpF production was further reduced in cells grown under high-osmolarity conditions. These results further support the idea that Leu43 is involved in the signal transduction mechanism of EnvZ. In contrast, cells containing the L35A form of EnvZ produced OmpF and OmpC at levels similar to those found in the wild-type strain (Table 4). Thus, the OmpF repression observed when Leu35 was replaced by a proline residue appeared to be due to an induced secondary-structure alteration in TM1.

Summary. The Pro41-to-Glu53 sequence of EnvZ, referred to as the I box, was found to be highly conserved in enteric bacteria and *V. cholerae*. The I box is located in the periplasmic domain of EnvZ, in close proximity to the cytoplasmic membrane. We showed that replacement of Leu43 with either a proline or an alanine residue stimulated a reduction in OmpF production under conditions in which OmpF is normally produced at high levels. Mutations in EnvZ that cause *ompF* to be repressed had been previously determined to occur at Pro41, Leu43, Gln44, and Leu50 (8, 22, 26). It was proposed recently that Leu43, Leu50, and Leu57 are involved in a dimeric leucine zipper-like structure and that this structure may play a role in osmotic signal transduction (26). Based on the present and previous information, we propose that the I box is directly involved in sensing osmolarity signals. Leu43 appears to be particularly critical in this function. We envision that the I box undergoes conformational alteration by sensing changes in the physical and/or chemical properties of the cytoplasmic membrane that are induced by osmolarity stress. Alternatively, the I box may directly sense changes in extracellular water activity (25). A conformational change in the I box would in turn affect the secondary structure of TM1, which has been proposed to be critical in maintaining the proper balance between the kinase and phosphatase activities of EnvZ (8, 21).

TABLE 4. Porin protein production in nutrient broth medium

Strain	Relative % of porin protein produced in[a]:			
	NB		NB + sucrose	
	OmpF	OmpC	OmpF	OmpC
Wild type	51	18	7	53
L35P	19	48	8	50
L43P	17	50	6	55
L35A	54	20	3	60
L43A	18	58	2	66

[a] The values represent the relative amounts of OmpF and OmpC as described in the footnote in Table 3. NB, nutrient broth.

Nucleotide sequence accession numbers. The partial sequences reported herein have been deposited in the GenBank DNA database under the following accession numbers: AF030314 (*ompR*) and AF030415 (*envZ*) for *S. flexneri*; AF030315 (*ompR*) and AF030416 (*envZ*) for *Enterobacter cloacae*; AF030316 (*ompR*) and AF030417 (*envZ*) for *Y. enterocolitica*; and AF030317 (*ompR*) and AF030418 (*envZ*) for *P. vulgaris*.

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