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Isolation and Characterization of Chain-Terminating Nonsense Mutations in a Porin Regulator Gene, *envZ*

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We isolated four independent amber mutations in gene *envZ*, whose product is involved in the regulation of porin protein genes *ompF* and *ompC*. The *envZ* amber strains exhibit an $OmpF^{-/+}$ $OmpC^{-}$ porin phenotype and express other envelope protein genes at wild-type levels. This phenotype is clearly different from that of the previously isolated class of *envZ* mutants that exhibit an $OmpF^{-}$ $OmpC^{+}$ phenotype and a pleiotropic decrease in the expression of several exported protein genes, including *lamB* and *phoA*. The addition of the local anesthetic procaine to wild-type strains also causes a pleiotropic decrease in the expression of genes *ompF*, *lamB*, and *phoA*. However, procaine has no effect on the synthesis of LamB or PhoA protein in the *envZ* amber strains. Thus, although EnvZ protein is required for the full expression of *ompF* and *ompC*, it apparently is not normally involved in the expression of other envelope protein genes. One interpretation of these results is that the EnvZ protein can be altered either by mutation or by procaine to a form that interferes with the expression of several envelope protein genes other than *ompF* and *ompC*. Finally, complementation analysis with *ompR* insertion mutations supports the physical data of Mizuno et al. (J. Biol. Chem 257:13692–13698, 1982) that suggest that *envZ* is cotranscribed with *ompR* from a single promoter in the order *ompR envZ*.

The outer membrane of *Escherichia coli* contains a set of abundant proteins, the porins, that serve as channels for the passive diffusion of small hydrophilic molecules (18, 21). In *E. coli* K-12 strains, the two major porins are OmpF and OmpC. Although the functional and structural properties of OmpF and OmpC proteins appear to be similar, the relative amounts have been shown to vary as a function of growth conditions and to be influenced by such factors as the ionic strength and osmolarity of the growth medium (3, 15, 17, 29).

The porin regulon consists of at least three genetic loci. Porin structural genes *ompF* and *ompC* map at 21 and 47 min, respectively (6, 26). A third locus, *ompB*, mapping at 74 min (25), was identified by the isolation of mutants that show an altered pattern of porin regulation. Genetic analysis has defined at least two genes, *envZ* and *ompR*, within the *ompB* locus (10, 11). A model for the function of EnvZ and OmpR proteins in porin regulation has been proposed to account for the phenotypes of *ompB* mutations. Hall and Silhavy (11) suggested that OmpR protein serves as the positive transcriptional factor for *ompF* and *ompC*, whereas the product of *envZ* acts by an unknown mechanism to mediate the fluctuation of the porins in response to changes in the environment.

Members of one class of *envZ* mutations (*envZ473*, *tpo*, *perA*) have been isolated by several laboratories (30–32) by the use of several selection techniques. Phenotypically these mutants are $OmpF^{-}$ $OmpC^{+}$ and are depressed for the synthesis of several envelope proteins, such as LamB and PhoA. Recently we isolated two new classes of *envZ* mutations (27a). The phenotypes exhibited by these mutants are consistent with the designation of EnvZ as a mediator of porin fluctuation and suggest that EnvZ protein may have a role in the regulation of membrane proteins other than OmpF or OmpC. One class of *envZ* mutations, typified by the *envZ3* allele, conveys a phenotype essentially opposite of that of mutants carrying *envZ473*; that is, mutant *envZ3* strains are $OmpF^{+}$ $OmpC^{-}$. In addition, these mutants are unaffected by procaine, which causes decreased synthesis of several envelope proteins, including OmpF, LamB, and PhoA, in wild-type strains. The third class of *envZ* mutants is also unaffected by procaine but expresses OmpF and OmpC proteins independent of the growth conditions.

Strikingly, none of the previously isolated

classes of *envZ* mutations included a defined knockout such as an amber mutation, a deletion, or an insertion. This finding was particularly interesting because all of the mutations showed some degree of codominance with the wild-type allele and with each other, suggesting the synthesis of an altered EnvZ protein. The failure of several laboratories to isolate defined mutations conferring one of the three *envZ* phenotypes led to the suggestion that the product of *envZ* might be essential for cell viability. Alternatively, the lack of defined *envZ* mutations may be because none of the described classes of phenotype reflected the phenotype of an *envZ* knockout.

To isolate defined mutations in *envZ*, we devised a procedure that allowed us to isolate recessive *envZ* null mutations regardless of the phenotype (including lethality) of the strain carrying the mutation. This was done by modifying a procedure first used to isolate amber mutations in *rpoB*, which encodes the β subunit of RNA polymerase (1). Starting with an *envZ*$^+$/*envZ3* merodiploid strain, we were able to detect the knockout of the wild-type allele by the unmasked phenotype of the recessive mutant allele. Here we report the isolation and characterization of four independent nonsense mutations in porin regulator gene *envZ*.

MATERIALS AND METHODS

Bacteria and bacteriophages. Bacterial and phage strains are listed in Table 1.

Media and chemicals. All media were prepared as described previously (19). Kanamycin was used at 40 μg/ml, and streptomycin was used at 100 μg/ml. Except where noted, tetracycline was used at 20 μg/ml.

Strains containing *asd* mutations were grown on rich medium supplemented with 0.1% diaminopimelic acid or on minimal medium containing 0.5% diaminopimelic acid plus 0.05% each of threonine and methionine. *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine was purchased from Sigma Chemical Co. and was used as a stock solution at 2.5 mg/ml in 95% ethanol.

Construction of strain SG608. The isolation of *ompR*$^+$ *envZ*$^+$ λ transducing phage λRT2 has been recently described (27a). Strain RT3 (MC4100 *envZ3*) was lysogenized with λRT2, and isolated colonies were scored for suppression of the recessive *envZ3* phenotype by the wild-type *envZ*$^+$ gene. Spontaneous phages released from the lysogen were checked for

TABLE 1. Bacteria and phages used

Bacterium or phage	Genotype or bacterial genes carried	Origin or reference
Bacterium		
MC4100	F$^-$ *araD139* Δ(*argF-lac*)*U169 rpsL150 relA1 flbB5301 ptsF25 deoC1*	(5)
MBM7014	F$^-$ *araC*(Am) *araD* Δ(*argF-lac*)*U169 trp*(Am) *mal*(Am) *rpsL relA thi supF*	(4)
SG263	MBM7014 *malA*::Tn*10*	This study
MH150	MC4100 *ompC12*::Tn*5*	(9)
MH760	MC4100 *ompB472*	(10)
MH1160	MC4100 *ompB101*	(10)
MH1471	MC4100 *envZ473*	(10)
MH225	MC4100 Φ(*ompC-lac*$^+$)10-25	(9)
MH513	MC4100 Φ(*ompF-lac*$^+$)16-13	(10)
SG608	MH225 (λRT2.3)	This study
TK821	MC4100 *ompR331*::Tn*10*	T. Palva
TK770	MC4100 Φ(*ompR-lac*) (Hyb)	T. Palva
RT3	MC4100 *malA*::Tn*5 envZ3*	R. Taylor
KLF41	F'141/*leuB6 hisG1 recA1 argG6 metB1 lacY1 gal6 xyl7 mtl12 rpsL104 tonA tsx1 supE44*	(16)
MS367	Hfr KL1699 *thi1 drm3 relA1 recA1 srl*::Tn*10*	(11)
AS.2	MC4100 (*phoA-lac*$^+$) *phoR*	P. Bassford
SG477	MC4100 *envZ22*	This study
Phage		
λ *vir*		Laboratory stock
λ *cI*		Laboratory stock
λ *cI h80*		Laboratory stock
λ *imm21 cI*		Laboratory stock
λ *imm21 cI h80*		Laboratory stock
K20		(12)
hy2		(3)
λRT1	*ompR*$^+$ *envZ'*	(27)
λRT2	λD69 *Bam* λ3::*envZ*$^+$ *ompR*$^+$	(27a)
λRT2.3	λRT2 *envZ3*	This study
φ80 p*supF*	*supF*	Laboratory stock
P1 *vir*		Laboratory stock

their ability to complement an *ompC-lac* fusion strain carrying *envZ3* (MH225 *envZ3*). Five phages that no longer complemented the *envZ3* mutation were then checked for their ability to complement an *ompF-lac* fusion strain carrying *ompR101* or *envZ473*. One phage that could complement the *ompR101* and *envZ473* alleles but could no longer complement the *envZ3* allele was assumed to be λ *imm*21 *ompR*$^+$ *envZ3* and was designated λRT2.3. Restriction analysis verified that this phage still carried the *ompB* locus (data not shown).

ompC-lac operon fusion strain MH225 was lysogenized with λRT2.3, and the position of the λRT2.3 prophage was confirmed by transduction of the lysogens to tetracycline resistance (Tet^r) or kanamycin resistance with P1 *vir* grown on strain SG263 or MH150, respectively. Lysogens that lost the λRT2.3 prophage at a high frequency when transduced to *ompC*::Tn*5* but remained immune to λ *imm*21 *c*I when transduced to *malA*::Tn*10* were assumed to have integrated λRT2.3 through homology to λ sequences at the fusion site, rather than *ompB* homology at the *ompB* chromosomal site. One isolated strain, SG608, was used as the recipient *ompB* merodiploid in localized mutagenesis.

Localized mutagenesis. Samples containing 2 ml of a mid-logarithmic-phase culture of SG263 were added to 50 μl of an *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine stock solution and were incubated at 37°C for 10 min. The cultures were then centrifuged, washed once in minimal medium 63 (M63) (19), suspended in 5 ml of Luria (L) broth (27a), and grown overnight. The next day, the cultures were inoculated into L broth containing 5 mM $CaCl_2$ and were used to make P1 *vir* stocks. One drop of a P1 *vir* lysate was added to 100 μl of an overnight culture of SG608 and was adsorbed at 37°C for 20 min. Each mixture was then diluted eightfold with L broth containing 50 mM sodium citrate and was incubated at 37°C for 1 h to allow expression of the Tet^r phenotype. The transductant cultures were centrifuged for 5 min at 3,000 rpm, suspended in 200 μl of L broth, and then spread on lactose-tetrazolium indicator agar containing 8 μg of tetracycline per ml and 200 μl of 1 M sodium citrate. Strains that are able to metabolize the sugar lactose (Lac$^+$) form white colonies on these plates and can be easily differentiated from strains that are deficient in lactose metabolism (Lac$^-$) which form red colonies. After incubation for 1 to 2 days at 37°C, colonies that were Tet^r and Lac$^-$ were picked and restreaked on the agar for further characterization as potential *envZ* mutants. To verify that the recipient strain had retained the λRT2.3 transducing phage, mutants were cross-streaked against tester phages λ *imm*21 *c*I and λ *c*I. Colonies that retained λRT2.3 were finally cross-streaked on lactose-MacConkey agar against transducing phage φ80 p*supF* to test for suppression of the Lac$^-$ phenotype (Lac$^+$). Four independent Lac$^-$ amber mutants were saved and subjected to further genetic analysis.

Genetic mapping. To verify that the mutations were linked to the *ompB* locus, P1 *vir* lysates were made on the mutant strains and used to transduce the parent SG608 to Tet^r. All P1 transductions were done as described previously (19). Tet^r transductants of SG608 were scored for their Lac phenotype on lactose-tetrazolium agar and for their ability to grow on maltose (Mal$^+$) on maltose-MacConkey agar to ensure that Tn*10* had not transposed during localized mutagenesis. Mutants that transduced SG608 to Lac$^-$ about 50% of the time were assumed to contain a mutation linked to *malPQ* and possibly to *ompB*.

P1 *vir* lysates on these mutants were used to transduce strain MC4100, as well as *ompC-lac* and *ompF-lac* fusion strains MH225 and MH513, respectively, to Tet^r. Tet^r transductants of MC4100 were scored for their ability to express the OmpF or OmpC phenotype by being cross-streaked against phage K20 or hy2, which use the respective porins as their receptors (3, 12). Tet^r transductants of MH225 and MH513 were scored for porin expression by being streaked on lactose indicator agar or being tested for K20 or hy2 sensitivity. Strains were made isogenic with wild-type strains by transduction to Mal$^+$ from P1 *vir* lysates grown on MC4100. Loss of the Tn*10* insertion was also necessary to allow the subsequent introduction of amber suppressor *supF* by cotransduction with *zch*::Tn*10*.

Assay of β-galactosidase activity. β-Galactosidase was assayed essentially as described previously (19).

Diploid analysis. Two of the four mutants that contained amber mutations linked to *ompB* were subjected to diploid analysis by the use of porin fusion strains MH225 and MH513. The *ompB* alleles (see Table 3) were homogenotized onto an F'141 episome and then mated into *recA ompB asd ompC-lac* and *recA ompB asd ompF-lac* hosts as described previously (11). We tested exconjugants for the ability of two *ompB* alleles to complement each other by streaking them on lactose indicator agar or by cross-streaking them against phages K20 and hy2.

Polyacrylamide gel electrophoresis. Membrane samples were prepared by sonication as described previously (14), and electrophoresis was performed with 8 M urea in the separating gel. The acrylamide concentration was 12%.

RESULTS

Rationale and isolation of mutants. The *envZ3* allele confers an OmpC$^-$ OmpF$^+$ phenotype, whereas wild-type *envZ*$^+$ strains are OmpC$^+$ OmpF$^+$. Although *envZ3* is somewhat codominant with *envZ*$^+$, under the conditions described for mutant isolation (see above) a strain diploid for both alleles is phenotypically OmpC$^+$ OmpF$^+$. Thus, merodiploid *envZ*$^+$/*envZ3* strains, which acquire an *envZ* knockout mutation in the wild-type allele, will lose EnvZ protein function and unmask the OmpC$^-$ OmpF$^+$ phenotype of the *envZ3* strain. Moreover, since *envZ3* strains are viable, the *envZ*$^-$/*envZ3* merodiploid will survive even if *envZ* is an essential gene.

In practice, we chose localized mutagenesis (13) to generate knockout mutations in *envZ*. Donor strain SG263 carries the *supF* suppressor, so an *envZ* amber mutation acquired during mutagenesis will not be lethal if *envZ* is an essential gene. Transposon Tn*10* in the *malA* region provides a selectable marker ca. 50% linked by P1 transduction to the *ompB* locus (Fig. 1). To score for the loss of EnvZ protein

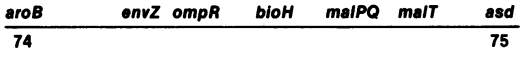

FIG. 1. Map of the *ompB* (*ompR envZ*) locus and relevant flanking genes.

wild-type function in the *envZ*$^+$/*envZ3* recipient strain, we made use of the fact that *ompC* expression is transcriptionally regulated by the EnvZ protein. Thus, an *envZ*$^+$ *ompC-lac* fusion strain is Lac$^+$, whereas an *envZ3 ompC-lac* strain is Lac$^-$. Strain SG608 is an *envZ*$^+$ *ompR*$^+$ *ompC-lac* operon fusion strain lysogenized with *envZ3 ompR*$^+$ transducing phage λRT2.3. As such, the strain is normally Lac$^+$. Since the λRT2.3 prophage is integrated by homology at the λ phage adjacent to the *ompC-lac* fusion, transduction of the strain to *malA*::Tn*10* replaces the chromosomal *ompB* locus but not the unlinked *ompC-lac* gene.

Donor strain SG263 was mutagenized with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and was used to generate P1 *vir* pools as described above. Using 20 independent pools, we transduced the recipient *envZ*$^+$/*envZ3* merodiploid SG608 to Tet^r on lactose-tetrazolium indicator agar containing tetracycline. We picked and scored 72 Lac$^-$ colonies for the presence of the λRT2.3 phage, as well as for suppression of the Lac$^-$ phenotype by transducing phage φ80 p*supF*. Four of the mutants, each from an independent P1 *vir* lysate, had retained λRT2.3 and were suppressible by φ80 p*supF*.

Lysates of P1 *vir* grown on each of the four merodiploid mutant strains were used to transduce the wild-type MC4100 *ompB*$^+$ strain to Tet^r, and transductants were cross-streaked against phage hy2 or K20 to test for the presence of their respective receptor, OmpC or OmpF. Between 50 and 60% of the Tet^r transductants from all four lysates were phenotypically OmpC$^-$ OmpF$^{-/+}$. Subsequent transductions placed the mutations between *aroB* and *malPQ* on the *E. coli* chromosome (2) and failed to separate the OmpC$^-$ phenotype from the OmpF$^{-/+}$ phenotype, suggesting that both phenotypes are conferred by the same mutation. As expected, introduction of amber suppressor *supF* into strains carrying each of the four *envZ* nonsense mutations restored the porin phenotype to that of a wild-type *ompB*$^+$ (OmpC$^+$ OmpF$^+$) strain. The results for one amber mutation, *envZ22*, are presented in Table 2. This allele, along with at least one other amber mutation, was used for further analysis. Since strains carrying nonsense mutations in *envZ* are viable (see below), *envZ* cannot be an essential gene. Furthermore, the OmpC$^-$ OmpF$^{-/+}$ phenotype conferred by such mutations is clearly different from the phenotypes of all other *envZ* mutant strains.

Although strains carrying the *envZ22* mutation grew more slowly than the isogenic *ompB*$^+$ parent, the growth defect was comparable to that conferred by the *ompR101* mutation. The growth disadvantage of an *ompR101* mutant strain is caused by the diffusion defect resulting from the absence of porins OmpC and OmpF (24). We have shown that the growth disadvantage of *envZ* amber strains is also the result of the diffusion defect, rather than the direct result of the loss of an unknown essential EnvZ function. Strains containing an *envZ* amber mutation grew normally when a gene specifying porin protein 2, which is not regulated by *ompB* (7), was introduced into the mutant cells (data not shown).

Porin phenotypes. To determine whether the OmpC$^-$ OmpF$^{-/+}$ phenotype of the MC4100 *envZ22* strain (SG477) was caused by a reduction in transcription from the porin structural genes, *envZ22* was moved into strains MH225 and MH513 by cotransduction with *malA*::Tn*10*. Strain MH225 is an *ompB*$^+$ *ompC-lac* fusion strain in which the *lac* operon is fused to, and is under the control of, the promoter for the *ompC* structural gene (5, 10). Strain MH513 carries a fusion of the *lac* operon to the *ompF* promoter and also carries the wild-type *ompB* region. Hall and Silhavy (9, 10) have previously shown that the levels of β-galactosidase expression in the porin fusion strains coincide with the regulatory phenomena of the porin proteins. Strains

TABLE 2. Porin expression in *ompB* strains[a]

Allele	OmpC expression		OmpF expression	
	hy2	Lac	K20	Lac
ompB$^+$	+	+	+	+
ompR101	−	−	−	−
ompR472	−	−	+	+
envZ473	+	+	−	−
envZ3	−	−	+	+
envZ22	−	−	−/+	−/+
envZ22 supF	+	+	+	+

[a] Strains carrying the indicated *ompB* mutations were MC4100 or the porin operon fusion strains MH225 or MH513. OmpC or OmpF expression was measured by cross-streaking against phage hy2 or K20 or by expression of the Lac phenotype in the appropriate fusion strain. Lac phenotype was judged by colony color on lactose-tetrazolium and lactose-MacConkey agar. (+) Sensitive or Lac$^+$ (red colonies on lactose-MacConkey agar or white colonies on lactose-tetrazolium agar); (−/+) partially resistant or Lac$^{-/+}$ (pink colonies on lactose-MacConkey agar or pink colonies on lactose-tetrazolium agar); (−) resistant or Lac$^-$ (white colonies on lactose-MacConkey agar or red colonies on lactose-tetrazolium agar).

MH225 and MH513, carrying the *ompB* alleles listed in Table 2 (including *envZ22*), were scored for their Lac phenotype (β-galactosidase synthesis) on lactose-tetrazolium and lactose-MacConkey indicator agars. The production of β-galactosidase by the *ompC-lac* and *ompF-lac* fusion strains reflects the amounts of OmpC and OmpF proteins for all *ompB* alleles tested (Table 2). Thus, the OmpC$^-$ OmpF$^{-/+}$ phenotype of an *envZ* amber strain is a result of reduced transcription from the respective structural genes.

To verify that the OmpC$^-$ OmpF$^{-/+}$ phenotype accurately reflects porin expression, we prepared membrane preparations from SG477 and subjected them to electrophoresis on polyacrylamide gels with similar preparations from other *ompB* strains. Figure 2 shows the membrane profile of the amber strain, along with the profiles of strains MC4100 (OmpC$^+$ OmpF$^+$), MH1160 (OmpC$^-$ OmpF$^-$), and MH1471 (OmpC$^+$ OmpF$^-$). As expected, the *envZ22* strain lacks OmpC protein and has a reduced level of OmpF protein in the outer membrane.

Diploid analysis. Complementation analysis was performed by the introduction of an episome covering the *ompB* region into *recA ompB asd ompC-lac* and *recA ompB asd ompF-lac* fusion strains and by the determination of the phage sensitivity or Lac phenotypes of the diploid strains. All combinations of diploid fusion strains containing the *ompB* alleles listed in Table 3 (with the exception of the *ompR-lac* fusion, which was not homogenotized to the episome) were examined. This analysis showed that both classes of *ompR* mutations (*ompR101*-OmpF$^-$ OmpC$^-$ and *ompR472*-OmpF$^+$ OmpC$^-$) complement and are complemented by the amber mutation. Since neither *envZ473* nor *envZ3* complements the amber mutation, we concluded that the nonsense mutation is in *envZ*. Although both *envZ473* and *envZ3* are codominant with the wild-type *envZ* allele, under the conditions used for these tests the amber mutation is completely recessive to all other *envZ* alleles, including *envZ*$^+$.

In contrast to *ompR101* and *ompR472*, the *ompR-lac* fusion does not complement any of the *envZ* mutations listed in Table 3, including *envZ473*, *envZ3*, and *envZ22*. Subsequent diploid analysis confirmed (data not shown) that two Tn*10* insertions in *ompR* and an *ompR* amber mutation, isolated in this laboratory as part of the attempt to identify amber mutations in *envZ*, are also unable to complement any of the *envZ* alleles carried in *trans*. The most likely explanation for these results is that *ompR* and *envZ* are in an operon and that these *ompR* mutations prevent EnvZ protein expression by exerting a polar effect on the downstream gene *envZ*.

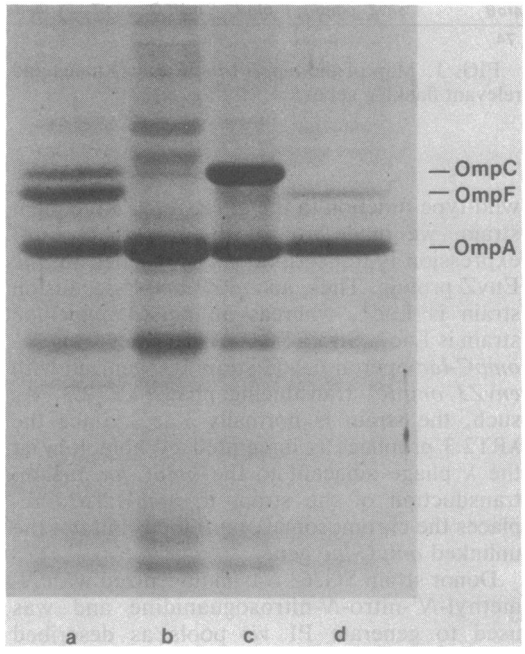

FIG. 2. Outer membrane proteins of *ompB* strains. All strains were grown overnight at 37°C in tryptone-yeast extract broth, harvested, and sonicated as described in the text. Electrophoresis of the membrane fractions was performed with a urea-sodium dodecyl sulfate-polyacrylamide gel. Lane: (a) MC4100 (*ompB*$^+$); (b) MH1160 (*ompB101*); (c) MH1471 (*envZ473*); and (d) SG477 (*envZ22*).

Pleiotropic effects of *envZ* mutations. Since our method for isolation of *envZ* knockout mutations was not biased by selection for a particular haploid phenotype, we were interested in the effect the amber mutations would have on the expression of other envelope proteins known to be affected by *envZ* mutations, such as *envZ473*. Strains carrying the *envZ473* mutation are depressed for the synthesis of various envelope proteins, including LamB, MalE, and PhoA, and this effect is the result of decreased transcription of the respective structural genes. To test the effect of *envZ22* on the expression of the other envelope proteins, the mutation was transduced into strains that contained the genes of the *lac* operon fused to the promoter for the *lamB*, *malE*, or *phoA* gene. In these strains, β-galactosidase activity reflects transcription initiation at the respective promoters. The β-galactosidase activities of the *lamB-lac*, *malE-lac*, and *phoA-lac* fusion strains carrying the *envZ22* allele were compared with the activities of the same strains containing the wild-type *envZ*$^+$ gene by streaking each strain on the appropriate lactose indicator agar (see above). In contrast to the *envZ473*

TABLE 3. Diploid analysis with *ompB* alleles[a]

Allele	Expression of OmpF or OmpC porin											
	ompB$^+$		*ompB101*		*ompB472*		*envZ473*		*envZ3*		*envZ22*	
	F	C	F	C	F	C	F	C	F	C	F	C
ompB$^+$	+	+	+	+	+	+	+	+	+	+	+	+
ompR101	+	+	−	−	+	−	+	+	+	+	+	+
ompR472	+	+	+	−	+	−	+	+	+	+	+	+
envZ473	+	+	+	+	+	+	−	+	+	+	−	+
envZ3	+	+	+	+	+	+	+	+	+	−	+	−
envZ22	+	+	+	+	+	+	−	+	+	−	−/+	−
ompR-lac	+	+	−	−	+	−	−	+	+	−	−/+	−

[a] The *ompC-lac* and *ompF-lac* operon fusion strains were made diploid for the *ompB* region by use of F'141 carrying the various *ompB* alleles as described in the text. Results were scored on lactose-MacConkey or lactose-tetrazolium agar. F, OmpF; C, OmpC. (+) Lac$^+$ (red colonies on lactose-MacConkey agar or white colonies on lactose-tetrazolium agar); (−/+) Lac$^{-/+}$ (pink colonies on lactose-MacConkey agar or pink colonies on lactose-tetrazolium agar); (−) Lac$^-$ (white colonies on lactose-MacConkey agar or red colonies on lactose-tetrazolium agar).

allele, the *envZ22* mutation had no effect (data not shown) on the expression of the *lamB*, *malE*, or *phoA* gene. Therefore the *envZ* amber mutation, by itself, has no noticeable pleiotropic effects.

Procaine reduces expression of *ompF* and several other envelope protein genes, including *lamB* and *phoA* (8, 22, 23). The similarity of this phenotype to that of mutant *envZ473* strains, along with the fact that mutants resistant to the procaine effect define two new *envZ* classes (*envZ3* and *envZ6*), suggest that the response to procaine is mediated by the EnvZ protein (27a). If this is true, one might expect that mutants lacking the EnvZ protein would not respond to procaine. Since this effect is mediated at the level of transcription, we transduced a *phoA-lac* operon fusion strain to *envZ22* and measured the β-galactosidase activity of this and a *phoA-lac* *envZ*$^+$ strain with and without the addition of procaine. The strains were grown overnight in L broth and reinoculated in L broth and L broth containing 15 mM procaine. Cultures were incubated with shaking to mid-log phase, and β-galactosidase activities were measured as described previously (19). Activity of the *envZ*$^+$ strain with and without the addition of procaine was measured at 79 and 160 U, respectively. Activity of the *envZ22* strain with and without the addition of procaine was measured at 143 and 129 U, respectively. Therefore, addition of up to 15 mM procaine reduced the expression of *phoA* in the *envZ*$^+$ strain but had no effect on *phoA* transcription in the *envZ22* mutant.

DISCUSSION

Genetic analysis of the porin regulon has been complicated by the absence of defined *envZ* mutations. Previously isolated alleles exhibit three different combinations of porin phenotypes (9, 10, 27a, 31, 32), and in addition they exert pleiotropic effects on the expression of many other envelope proteins. To circumvent these problems, we designed a selection procedure using merodiploids that permitted us to isolate four independent *envZ* nonsense (amber) mutations. The phenotypes conferred by these mutations are identical; however, they differ from the phenotypes conferred by other *envZ* mutations. Thus, these alleles define a new class of *envZ* mutations.

Phenotypically, strains containing these amber mutations most resemble the class of *ompR* mutations that we have designated OMPR1 (11). The *envZ* amber strains are reduced in the expression of both porin genes, *ompF* and *ompC*, as shown by their resistance to phages that use their products as receptors and by the decreased β-galactosidase activity in mutant strains containing the *ompF-lac* or *ompC-lac* operon fusion. However, the expression of *ompC*, and in particular *ompF*, in the *envZ* amber strains is not as low as in OMPR1 strains. Thus, we suggest that the EnvZ protein is required for the full expression of *ompF* and *ompC*.

The pleiotropic nature of many *envZ* mutants suggests that the gene might be essential for cell viability. Our results do not support this idea. Although strains lacking the EnvZ protein grow somewhat slowly, the slow growth is probably the result of the paucity of diffusion pores.

One possible function of the *envZ* gene product is as a positive activator for several envelope protein genes other than *ompF* and *ompC*. This theory is based upon the following findings. First, previously isolated *envZ* mutations, such as *envZ473*, reduce the transcription of *lamB* and *phoA*, as well as porin gene *ompF*. Second,

wild-type strains grown in the presence of procaine exhibit a phenotype similar to that conferred by *envZ473* (28). Several laboratories have shown that procaine affects transcription (8, 22, 24). Recently we suggested (27a) that the *envZ* gene product is directly involved in mediating the response to procaine. This conclusion was based on the observation that mutations that block this response map in the *envZ* gene. One explanation for these results is that both procaine and the *envZ473* mutation inhibit the EnvZ protein from functioning as a positive regulatory element for such envelope protein genes as *lamB* and *phoA*. This hypothesis predicts that defined knockout mutations in *envZ* would confer a LamB$^-$ PhoA$^-$ phenotype. Our results with *envZ* amber strains do not support this prediction. Introduction of these amber mutations into strains carrying the *lac* operon fused to the envelope protein genes *lamB*, *malE*, and *phoA* showed no appreciable reduction in the expression of these genes.

Based on these results, we suggest a different model of the function of the EnvZ protein. The *envZ* amber mutations decrease the expression of both *ompF* and *ompC* but do not affect the expression of other envelope protein genes. This finding suggests that the EnvZ protein does not function as a positive activator for genes *lamB* and *phoA*. Thus, we suggest that the EnvZ protein can be changed to a form that interferes with the transcription of the structural genes for several exported proteins, either by mutation (*envZ473*) or by the addition of procaine. This model predicts that defined knockout mutations in *envZ* will eliminate the effect of procaine on gene expression. Our results indicate that this is indeed the case. Further support for this interpretation is provided by the results of our complementation analysis. In contrast to the *envZ* amber mutations, which are recessive in diploid analysis, the previously isolated *envZ* alleles exhibit codominance. The most likely explanation for this finding is that the other *envZ* alleles, including *envZ473*, result in the production of an altered EnvZ protein, whereas the amber mutations result in the complete absence of a functional gene product.

Conceivably, the *envZ* amber mutations allow production of a partially functional truncated peptide; however, we think that this is unlikely. Mizuno et al. (20) recently suggested that the *envZ* gene is cotranscribed with *ompR* and is distal to *ompR*. The basis for this assignment comes from DNA sequence analysis and studies with cloned regions of the *ompB* locus (20, 33). Our results from complementation analysis support this claim. Insertions within *ompR* are polar on *envZ*. Merodiploid strains that contain these insertions in the chromosomal *ompR* gene exhibit the porin phenotype conferred by the *ompB* allele on the episome. Indeed, using the described selection procedure for isolation of defined knockout mutations, we isolated two Tn*10* insertions in *ompR* and one polar *ompR* amber mutation. These results provide further evidence to support the conclusion that the *envZ* gene product is not essential for cell viability.

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