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Phenotypic Revertant Mutations of a New OmpR2 Mutant (V203Q) of *Escherichia coli* Lie in the *envZ* Gene, Which Encodes the OmpR Kinase

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The *Escherichia coli ompR2* allele *ompR472* contains a valine-to-methionine point mutation at position 203, resulting in an OmpF-constitutive $OmpC^-$ outer membrane phenotype. In the present study, OmpR residue V-203 was replaced with glutamine (V203Q mutation), resulting in the same outer membrane phenotype. However, unlike the $OmpF^c$ $OmpC^-$ phenotype conferred by the OmpR(V203M) mutant protein, the $OmpF^c$ $OmpC^-$ phenotype produced by the OmpR(V203Q) mutation was suppressed by the envZ11(T247R) allele. Additional suppressors of OmpR(V203Q) were isolated by random mutagenesis. All suppressor mutations were found in the *envZ* gene and conferred an $OmpC^+$ $OmpF^-$ phenotype in the presence of the wild-type *ompR*. These *envZ11*-like mutations mapped to a region different from those previously reported and were incapable of suppressing the *ompR*(V203M) allele. Our results indicate that while methionine or glutamine replacements could cause similar effects on OmpF and OmpC expression, they conferred different abilities on the mutant proteins to be suppressed by *envZ*.

In *Escherichia coli*, two major outer membrane proteins, OmpF and OmpC, are differentially regulated in response to osmotic stress. OmpF is preferentially expressed under low-osmolarity conditions, while an increase in medium osmolarity results in repressed expression of OmpF and enhanced OmpC expression (8). OmpF and OmpC expression is controlled by the membrane receptor-cytoplasmic effector system consisting of EnvZ and OmpR (12, 13, 27). EnvZ is an integral inner membrane protein consisting of two transmembrane domains, a periplasmic receptor domain, and a cytoplasmic signaling domain (9). OmpR is a transcriptional regulator that binds to the promoter regions of *ompF* and *ompC* and has both activator and repressor functions (21, 25, 26). EnvZ acts as the sensor molecule and undergoes autophosphorylation with ATP at a histidine residue (17). Phosphorylated EnvZ is able to transfer the phosphate group to the regulator protein OmpR (2, 10, 15). Phosphorylation of OmpR is essential for transcriptional activation (1).

OmpR consists of 239 amino acid residues. The N-terminal portion serves as a substrate for phosphorylation by EnvZ and is thought to modulate the DNA-binding function of the C-terminal domain (4, 28). Within the activator domain, aspartate residues at positions 11, 12, and 55 represent the putative site(s) phosphorylated by EnvZ (5, 18, 24). Phosphorylation of OmpR enhances its affinity for the *ompF* and *ompC* promoter DNA (1, 15). Binding of OmpR to the *ompF* and *ompC* promoters has been characterized both in vitro with purified OmpR (21) and in vivo (28). OmpR binds to two different sequence motifs, the F box and the C box, located in the −40 to −100 regions of the *ompF* and *ompC* promoters (28). Binding of OmpR to the F boxes (between −60 and −100) in the *ompF* promoter activates *ompF* transcription under conditions of low osmolarity (1). In medium of high osmolarity, OmpR binds to a C box in the *ompF* promoter to repress *ompF* transcription (10, 26, 28) and presumably binds to the C boxes of the *ompC* promoter to activate *ompC* transcription.

Mutant OmpR molecules have been extensively characterized in an effort to understand how OmpR controls porin gene expression (18, 21, 22). One of the best-studied mutations has been the *ompR472* mutation (12, 25). Cells harboring the *ompR472* allele express high levels of OmpF protein regardless of medium osmolarity but do not express OmpC (12). This mutant *ompR* gene has been sequenced and shown to contain a G-to-A point mutation at position 607 (23). This creates a valine-to-methionine replacement at residue 203 in the amino acid sequence of OmpR (V203M mutation). DNase I footprinting of the purified OmpR472 protein has shown that it protects the −60 to −100 region but not the −40 to −60 region of the *ompF* promoter (21). In addition, the OmpR2 protein does not interact with the −40 to −80 region of the *ompC* promoter (21). In vivo dimethyl sulfate footprinting has confirmed these results (28). The occurrence of the V203M mutation in the C-terminal DNA-binding region (21, 23, 28) of OmpR, together with the loss of specific DNA binding, has indicated that the OmpR472 molecule is a DNA binding mutant. However, this interpretation is complicated by the observation that *ompR472* Δ*himA* or *ompR472* Δ*envZ* double mutants produce the OmpC protein to substantial levels (14, 25). It is difficult to reconcile the latter observations with a DNA-binding mutant.

OmpR472 is a member of a broad class of *ompR2* mutant proteins that can be distinguished on the basis of their porin patterns at low and high osmolarities and their degree of suppression by *envZ473*, an *envZ11*-like mutation that confers an $OmpC^c$ $OmpF^-$ phenotype in the presence of wild-type OmpR. Unlike other *ompR2* mutant alleles, the *ompR472* allele is not suppressed by *envZ473* (25). In an

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TABLE 1. Plasmids

Plasmid	Relevant genotype
pTB0201	*ompR*$^+$ *envZ*$^+$
pTB0228	*ompR*$^+$ *envZ11*(T247R)
pHY1430	*ompR*(V203M) *envZ*$^+$
pHY1440	*ompR*(V203Q) *envZ*$^+$
pHY1431	*ompR*(V203M) *envZ11*(T247R)
pHY1441	*ompR*(V203Q) *envZ11*(T247R)
pHY1432	*ompR*(V203M) *envZ*(A195V)
pHY1433	*ompR*(V203M) *envZ*(E212K)
pHY1434	*ompR*(V203M) *envZ*(R253H)
pHY1442	*ompR*(V203Q) *envZ*(A195V)
pHY1443	*ompR*(V203Q) *envZ*(E212K)
pHY1444	*ompR*(V203Q) *envZ*(R253H)
pHY1402	*ompR*$^+$ *envZ*(A195V)
pHY1403	*ompR*$^+$ *envZ*(E212K)
pHY1404	*ompR*$^+$ *envZ*(R253H)
pHY1500	*ompR*(V203Q) *envZ*(W355termination codon)
pHY1445	*ompR*(V203Q) Δ*envZ*

effort to gain further insight to the nature of the *ompR472* mutant, we carried out studies in which the valine 203 residue was replaced with a glutamine rather than a methionine. The OmpR(V203Q) mutant produced an OmpF-constitutive porin phenotype identical to that of the OmpR (V203M) mutant. Surprisingly, the V203Q mutation was suppressed by the *envZ11*(T247R) mutation, which lacks phosphatase activity and, like *envZ473*, produces an OmpC^c OmpF$^-$ phenotype when expressed with wild-type OmpR (3). Experiments to isolate additional suppressors of OmpR (V203Q) resulted in the identification of more suppressors which mapped in *envZ*. In our discussion, we consider several ways these results could be interpreted within the context of a DNA-binding mutant.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains used in this study were W2 (MC4100 *ompR*::Tn*10* *ompC-lacZ*), GM33 (*dam*), SG480Δ76 [MC4100 Δ(*malT-ompB*)] (12), and AR137 (SG480Δ76 *pcnB80*). This last strain was created by P1 transduction with a lysate kindly provided to us by J. Beckwith. The *pcnB80* allele is tightly linked to a Tn*10* (19), and transductants were selected for tetracycline resistance. Plasmids are listed in Table 1.

Plasmid mutagenesis. The plasmid pTB0201 (5) contains the wild-type *ompB* locus cloned into pBR322 and was used for the construction and expression of *ompR* and *envZ* mutants. The *ompR*(V203M) (pHY1430) and *ompR*(V203Q) (pHY1440) mutations were created by using a site-directed mutagenesis kit (Amersham version 2). The amino acid at position 203 was changed from valine to glutamine in order to create a *Bcl*I restriction site for screening purposes. In addition, site-directed mutagenesis was used to create an *Sst*II site at bp 327 in *ompR* to allow subcloning of the region of *ompR* encoding the C terminus. No amino acids were changed in this latter mutation.

Hydroxylamine mutagenesis of plasmid pHY1440 was carried out as described previously (6). The mutagenized DNA was used to transform W2 cells to ampicillin resistance on lactose-MacConkey indicator plates (containing 50 μg of ampicillin per ml). Dark red colonies (activation of the *ompC* promoter) were chosen as putative revertants and were restreaked onto a fresh plate. Plasmid DNA was then prepared and used to transform strain GM33 (*dam*). Plasmids prepared from this strain were digested with *Bcl*I to identify those revertants which still contained the original *ompR*(V203Q) mutation.

To identify second-site suppressor mutations, the *Sst*II fragment, encoding the *ompB* locus from amino acid 110 in OmpR to amino acid 392 in EnvZ, was subcloned into nonmutagenized pHY1440. W2 cells were transformed with these plasmids and were spread on MacConkey agar (containing 50 μg of ampicillin per ml). Plasmids were prepared from dark red colonies and sequenced within the subcloned region.

Outer membranes were prepared as described previously (7).

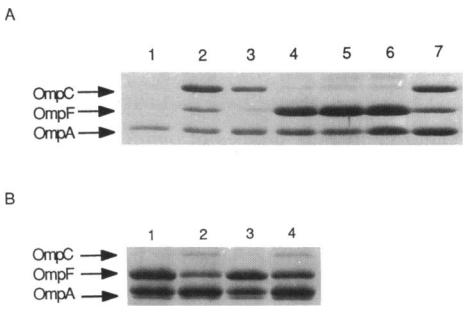

FIG. 1. Outer membrane protein profiles of the *ompB* deletion strain (AR137) harboring various plasmids. (A) Cells grown in L broth. Results for cells with plasmids pBR322 (lane 1), pTB0201 (*ompR*$^+$ *envZ*$^+$) (lane 2), pTB0228 [*ompR*$^+$ *envZ11*(T247R)] (lane 3), pHY1430 [*ompR*(V203M) *envZ*$^+$] (lane 4), pHY1440 [*ompR*(V203Q) *envZ*$^+$] (lane 5), pHY1431 [*ompR*(V203M) *envZ11*(T247R)] (lane 6), and pHY1441 [*ompR*(V203Q) *envZ11*(T247R)] (lane 7) are shown. (B) Cells grown in nutrient broth alone (lanes 1 and 3) or nutrient broth with 20% sucrose (lanes 2 and 4). Results for cells with plasmids pHY1430 (lanes 1 and 2) and pHY1440 (lanes 3 and 4) are shown.

RESULTS

Differences between OmpR(V203Q) and OmpR(V203M). To evaluate OmpF and OmpC production, plasmids containing various combinations of *ompR* and *envZ* alleles were used to transform strain AR137. The *pcnB80* allele present in AR137 reduces the copy number of plasmids with the ColE1 origin of replication (19). The AR137 strain, harboring pBR322, fails to produce either OmpF or OmpC (Fig. 1A, lane 1) because of the deletion of the *ompB* locus but could be complemented with any of the *ompB* constructs used in the present study (Fig. 1A, lanes 2 through 7). Plasmid pTB0201, expressing the wild-type OmpR and EnvZ proteins, resulted in production of both OmpF and OmpC (Fig. 1A, lane 2), while plasmid pTB0228, carrying *envZ11*, conferred an OmpC$^+$ OmpF$^-$ phenotype (Fig. 1A, lane 3). OmpR(V203M) (plasmid pHY1430) and OmpR(V203Q) (plasmid pHY1440) exhibited the same OmpF$^+$ OmpC$^-$ phenotype when cells expressing them were grown in L broth (Fig. 1A, lanes 4 and 5). In addition, the two mutants were identical in their responses to low- and high-osmolarity media (Fig. 1B, lanes 1 through 4), indicating that OmpR(V203Q) is a new OmpR2 mutant.

As a means of examining possible differences between OmpR(V203Q) and OmpR(V203M), the effect of *envZ11* on these mutants was tested. The OmpR2 phenotype, OmpF^c OmpC$^-$, of *ompR*(V203M) is known not to be suppressed by the *envZ11*-like mutant *envZ473*. We show that OmpR

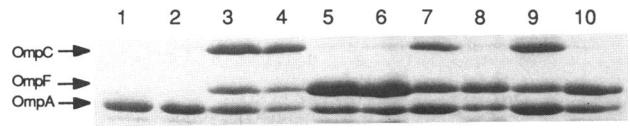

FIG. 2. Outer membrane profiles of *ompB* deletion strains harboring multiple (SG480Δ76, lanes 1, 3, 5, 7, and 9) and low (AR137, lanes 2, 4, 6, 8, and 10) copy numbers of plasmids pBR322 (lanes 1 and 2), pTB0201 (*ompR*$^+$ *envZ*$^+$) (lanes 3 and 4), pHY1440 [*ompR*(V203Q) *envZ*$^+$] (lanes 5 and 6), pHY1500 [*ompR*(V203Q) *envZ*(W355termination codon)] (lanes 7 and 8), and pHD1445 [*ompR*(V203Q) Δ*envZ*) (lanes 9 and 10).

(V203M) is not suppressed by *envZ11* either, as shown in Fig. 1A, lane 6. However, when *ompR*(V203Q) was placed *cis* to *envZ11*, unexpectedly the OmpR2 phenotype was suppressed, and both OmpF and OmpC were expressed (Fig. 1A, lane 7). Although *ompR*(V203M) and *ompR* (V203Q) represent mutations at the same residue and generate the same porin phenotype, they differ with respect to *envZ* suppression. This indicates that the nature of the amino acid substitution (methionine versus glutamine) at position 203 plays an important role in the DNA-binding property of OmpR.

Isolation of second-site suppressors of OmpR(V203Q). Next we attempted to isolate second-site suppressor mutations for OmpR(V203Q). If OmpR(V203Q) was a DNA-binding mutant, the majority of second-site suppressors would be within the *ompR* gene. On the other hand, if the V203Q mutation resulted in a defective interaction with EnvZ, second-site suppressors may be obtained in the *envZ* gene. The OmpR(V203Q)-producing plasmid, pHY1440, was subjected to in vitro random mutagenesis with hydroxylamine. The mutagenized plasmids were screened for suppressor mutations by using the W2 (MC4100 *ompR*::Tn*10* *ompC-lacZ*) strain. W2 cells, which lack a functional OmpR, appeared as white colonies on MacConkey agar. When transformed with pTB0201 (*ompR*$^+$ *envZ*$^+$), the *ompC-lacZ* fusion gene was activated to produce red colonies. Transformation with pHY1440 [*ompR*(V203Q) *envZ*$^+$] produced white colonies. W2 cells were thus transformed with mutagenized pHY1440, and dark red colonies were then identified as OmpR(V203Q) revertants. Of 19 revertants, 10 were found to be pseudorevertants. These were further examined by exchanging a fragment encoding the 3′ region of *ompR* and the 5′ region of *envZ* with nonmutagenized pTB0201. Of the constructs that transformed W2 cells to an OmpC$^+$ revertant phenotype, six were sequenced to identify suppressor mutations. None of these suppressor mutations were found in *ompR*; they were all located in *envZ*.

Three mutations were found within a short sequence (28 amino acid residues) in the cytoplasmic regions of EnvZ between the second transmembrane domain and the phosphorylation site, His-243. These were P185L (base change, C-554 to T), A193V (base change, C-578 to T), and E212K (base change, G-634 to A). Another suppressor mutation was R253H (base change, G-758 to A) in EnvZ.

Two other suppressor mutations were nonsense mutations, at residues 292 and 355. However, the reversion of OmpR(V203Q) by these null mutants was due to a multicopy effect (Fig. 2). Plasmids pBR322 (Fig. 2, lanes 1 and 2), pTB0201 (Fig. 2, lanes 3 and 4), and pHY1441 (Fig. 2, lanes 5 and 6) produced the expected phenotypes in both the SG480Δ76 and the AR137 strains. However, in the case of a nonsense mutation in *envZ* (W-355 changed to a termination

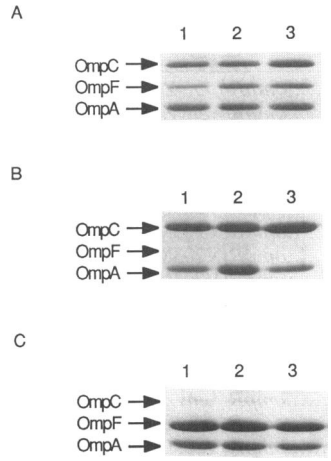

FIG. 3. Outer membrane protein profiles of the *ompB* deletion strain (AR137) harboring various plasmids. Cells were grown in L broth. (A) Suppression of *ompR*(V203Q) by *envZ* mutants carrying pHY1442 [*ompR*(V203Q) *envZ*(A195V)] (lane 1), pHY1443 [*ompR* (V203Q) *envZ*(E212K)] (lane 2), and pHY1444 [*ompR*(V203Q) *envZ* (R253H)] (lane 3). (B) Outer membrane phenotypes in the presence of wild-type *ompR* of *envZ* mutants carrying pHY1402 [*ompR*$^+$ *envZ*(A195V)] (lane 1), pHY1403 [*ompR*$^+$ *envZ*(E212K)] (lane 2), and pHY1404 [*ompR*$^+$ *envZ*(R253H)] (lane 3). (C) Expression of *envZ* mutants with *ompR*(V203M), carrying pHY1432 [*ompR* (V203M) *envZ*(A195V)] (lane 1), pHY1433 [*ompR*(V203M) *envZ* (E212K)] (lane 2), and pHY1434 [*ompR*(V203M) *envZ*(R253H)] (lane 3).

codon), the production of OmpC was seen only in SG480Δ76 (Fig. 2, cf. lanes 7 and 8), in which multiple copies of the plasmid exist. In addition, construct pHD1445, containing *ompR*(V203Q) and an *envZ* gene with two termination codons inserted 28 bp downstream of the translation initiation site, produced OmpC when expressed in SG480Δ76 (Fig. 2, lane 9). No OmpC was produced when pHD1445 was expressed in AR137 (Fig. 2, lane 10). At present, the suppressor mechanism of the nonsense mutations under multicopy conditions is unknown.

Characterization of the *envZ* suppressor mutations. Since *ompR*(V203Q) was suppressed by *envZ11*, which is known to lack phosphatase activity, the suppressor mutations described here are also considered to be similar to the *envZ11* mutation in terms of the EnvZ function. Therefore, the effects of suppressor mutations A193V, E212K, and R253H on *ompF* and *ompC* expression in the presence of wild-type OmpR were examined.

In strain AR137, *envZ* suppressors allowed OmpC production to wild-type levels when they were expressed along with the *ompR*(V203Q) mutation (Fig. 3A, lanes 1 through 3). These results were consistent with our original observations with MacConkey plates, demonstrating that the *ompR* (V203Q) mutation can be suppressed by *envZ* mutations. When these *envZ* genes were expressed along with the wild-type *ompR* gene in strain AR137, the outer membrane profile of all three mutants was OmpF$^-$ OmpC$^+$ (Fig. 3B, lanes 1 through 3). This indicates that these *envZ* suppressors are phenotypically similar to several well-known *envZ* alleles, such as *envZ473* and *envZ11*. It should be noted that none of the three EnvZ11-like mutants were able to suppress the OmpR(V203M) mutant (Fig. 3C, lanes 1 through 3). These results thus indicate that the mutant EnvZ proteins may function like EnvZ11 with respect to suppression of the OmpR2 phenotype.

DISCUSSION

The present study was undertaken to further characterize the nature of the valine-to-methionine replacement in the carboxyl portion of OmpR472 [OmpR(V203M)]. Previous footprinting studies have shown that this mutant does not interact with a subset of DNA-binding sites in either the *ompF* or the *ompC* promoter (21, 28). As an *ompR472* strain exhibits an OmpF-constitutive $OmpC^-$ porin phenotype, these binding sites may be important for *ompF* repression and *ompC* expression (21, 25, 28). While *ompC* activation is associated with elevated levels of phosphorylated OmpR (3, 29), the *envZ11* and *envZ473* mutations which produce high levels of phosphorylated OmpR cannot suppress *ompR472* for *ompC* expression (this study and reference 25). These studies have led to the idea that OmpR472 is a DNA-binding mutant. This is consistent with the general view that the carboxyl terminus of OmpR contains the DNA-binding domain.

We created the related gene *ompR*(V203Q) and found that this mutation produced a porin pattern identical to that caused by *ompR*(V203M). On the basis of the matching phenotype as well as the fact that we altered the same residue, OmpR(V203Q) is related to OmpR(V203M) as a DNA-binding mutant. Unlike the OmpR(V203M) mutant, OmpR(V203Q) activated *ompC* expression in association with one of several *envZ* mutants, including *envZ11*. These results demonstrate that suppression of OmpR(V203Q) requires interactions with EnvZ.

Our study could be rationalized by considering that glutamine and methionine replacements affect the DNA binding of OmpR at the *ompC* promoter. They could prevent other amino acids from making favorable DNA contacts in the *ompC* promoter or, alternatively, they themselves could make unfavorable contacts with particular (but not necessarily the same) base pairs. Another possibility is that valine 203 itself makes a favorable DNA contact and the loss of this residue leads to an $OmpC^-$ phenotype. This defect would be partially compensated for by the glutamine replacement in association with an EnvZ mutation. However, OmpR472 [OmpR(V203M)] can activate the *ompC* gene in some instances, such as in an *ompR472 himA* double mutant (14), indicating that OmpR(V203M) interacts with the *ompC* promoter despite the valine replacement. It is difficult to rationalize these results if valine 203 makes direct contact with the *ompC* promoter.

Conformational defects are an alternative way of rationalizing the V203M and V203Q mutations. It is generally believed that OmpR exists in at least two DNA-binding conformations (11, 16): a low-osmolarity form (phenotypically $OmpF^+$ $OmpC^-$) and a high-osmolarity form (phenotypically $OmpF^-$ $OmpC^+$). The glutamine and methionine residues may allow OmpR to adopt a structure needed for the low-osmolarity form but may influence further conformational transitions leading to the high-osmolarity form. As a result, these residues lock OmpR into one type of DNA-binding mode. This model differs from those mentioned above, as amino acid residues at position 203 do not have to be directly involved with DNA-binding activity.

The suppression mechanism by which EnvZ11 overcomes putative DNA binding or conformational defects of OmpR(V203Q) is not clear. Since EnvZ11 is known to be unable to dephosphorylate OmpR (3), the mechanism may involve altered dephosphorylation activity. As phosphorylation is correlated with changes in OmpR DNA-binding activity (11, 16), EnvZ11 could elevate levels of phosphorylated OmpR to compensate for the true nature of the OmpR(V203Q) mutation.

All of our *envZ* mutants were phenotypically identical to *envZ11*. That is, they caused OmpC-constitutive expression in a wild-type *ompR* background and were unable to suppress the *ompR*(V203M) mutation. Three of our EnvZ mutants had amino acid replacements that clustered between the second transmembrane region and the putative phosphorylation site, His-243 (10). The occurrence of mutations in this region is different from the occurrence of other *envZ11*-like mutations which are located near His-243 at amino acid positions 240 (5), 241 (29), and 247 (20). Nevertheless, the general similarities we observed indicate that these mutants may also be altered in some aspect of OmpR phosphorylation and could define an additional region important for phosphate relay between OmpR and EnvZ.

The relationship between our study and another in which the *envZ473* allele was shown to suppress several *ompR2* genes (25) is interesting. Since none of these suppressible *ompR2* mutations which were studied in reference 5 were sequenced, the relationship between them and the (nonsuppressible) *ompR472* mutant was unclear. That is, these suppressible *ompR2* mutants could have either different amino acid replacements at valine 203 or mutations at some other position. Our study shows that different amino acid replacements at the same position can determine the degree of suppression by *envZ*.

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