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The 3′ Conserved Segment of Integrons Contains a Gene Associated with Multidrug Resistance to Antiseptics and Disinfectants

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Nucleotide sequence analysis of ORF1 from the integron on the broad-host-range plasmid R751 revealed that the first 94 of 110 codons of ORF1 from R751 are identical to ORF4, an open reading frame from the 3′ conserved segment of other integrons found in gram-negative bacteria, after which point they diverged completely. The predicted products of both ORF1 and ORF4 share homology with the multidrug exporter QacC. Phenotypic analysis revealed that ORF1 specifies a resistance profile to antiseptics and disinfectants almost identical to that of *qacC*, whereas ORF4 specifies much lower levels of resistance to these compounds. ORF4, whose product lacks the C-terminal 16 amino acids of the ORF1 protein, may have evolved by the interruption of ORF1 from the insertion of a DNA segment carrying a *sulI* sulfonamide resistance determinant. Hence, ORF1 was designated *qacE*, and its partially functional deletion derivative, ORF4, was designated *qacEΔ1*. Fluorimetric experiments indicated that the mechanism of resistance mediated by QacE, the protein specified by *qacE*, is active export energized by proton motive force. Amino acid sequence comparisons revealed that QacE is related to a family of small multidrug export proteins with four transmembrane segments.

The evolution of a variety of multiresistance plasmids and transposons from gram-negative bacteria has been reported to have involved the site-specific integration of antibiotic resistance determinants (7, 39, 57, 63). This site-specific integration has been reported to be mediated by integrons, a family of potentially mobile DNA elements (54). Integrons consist of two conserved segments between which discrete units integrate as cassettes (Fig. 1). The 5′ conserved segment of integrons encodes Int (40, 54, 57), a site-specific recombinase of the integrase family (1). The precise content and extent of the 3′ conserved segment has not yet been clearly established (46), but as defined by Stokes and Hall (54), the 3′ conserved segment contains three open reading frames: *sulI*, a sulfonamide resistance determinant (57); ORF4, an open reading frame of unknown function whose 3′ end overlaps with the first two codons of *sulI*, although in a different reading frame (57); and ORF5, whose product has some similarity to puromycin acetyltransferase (4). In all cases reported, the cassettes have been inserted in the same orientation and are usually transcribed from a promoter in the 5′ conserved segment (54, 57, 59). The site-specific formation of plasmid cointegrates (30, 31) and their resolution (22) demonstrated that Int catalyzes recombination between short imperfect inverted repeats known as 59-bp elements which are located at the end of each cassette (22). Further Int-dependent site-specific rearrangements and deletions of inserted cassettes have been observed experimentally (9, 56). The integrated cassettes which have been reported in integrons include a variety of antibiotic resistance determinants specifying resistance to compounds such as aminoglycosides, penicillin, chloramphenicol, or trimethoprim (for a review, see reference 4).

The predicted product of ORF4, from the 3′ conserved segment of integrons, is a small polypeptide of 115 amino acids (aa) which has been reported to possess a high degree of homology with QacC, an antiseptic resistance protein from *Staphylococcus aureus* (27). QacC, which has also been referred to as QacD (27), Ebr (52), or Smr (20), is a highly hydrophobic protein of 107 aa which mediates resistance to a range of toxic cationic compounds including ethidium bromide and the quaternary ammonium compounds (27, 28). QacC has been reported to mediate resistance to these diverse compounds by an active efflux system energized by proton motive force (PMF) (28). Thus, QacC exhibits a multidrug resistance phenotype with a broad substrate specificity analogous to that of the mammalian resistance protein P glycoprotein, a multidrug exporter which uses ATP to energize transport (13). Other bacterial multidrug export systems which have been characterized include the antiseptic and disinfectant resistance proteins QacA (47, 61) and QacB (42) and the quinolone resistance protein NorA (36, 66) from *S. aureus*, the multidrug resistance protein Bmr from *Bacillus subtilis* (37), and the ethidium bromide resistance protein Ebr from *Escherichia coli* (44, 45). Ebr from *E. coli* should not be confused with the *S. aureus* antiseptic resistance protein Ebr (52), which is referred to as QacC in this report (see above).

R751 is a 51.4-kb broad-host-range plasmid which was originally isolated from *Klebsiella aerogenes* (26, 32). It contains the transposon Tn*402* (53) which has been suggested to be an integron (46). In the present study we report the sequence of ORF1 from the integron on the plasmid R751. The relationship between ORF1 from R751 and ORF4 from the 3′ conserved segment of integrons was analyzed,

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and the functions of their respective products were examined.

MATERIALS AND METHODS

Bacterial strains and plasmids. The *E. coli* K-12 strains used in the present study were JM101 (65), BHB2600 (25), CSR603 (50), and AN418 (10). The plasmids used in the present study were R751 (26); pLMO20, an integron-containing plasmid with an inserted *dhfrV* cassette (57); pLKO603A (46), which consists of the 8.3-kb *Pst*I fragment from R751 cloned into pUC19 (65); and pLKO1B (58), which consists of the 3.5-kb *Bam*HI fragment from pLMO20 cloned into pBR322 (5).

General methods. The media used and the method for MIC determinations have been described previously (29, 60). MIC tests were performed at least in triplicate. Reserpine (4 μg/ml) was used as described by Neyfakh et al. (37) as an inhibitor in the MIC tests.

Recombinant DNA techniques. *E. coli* plasmid isolation and recombinant DNA techniques have been described previously (48). Tn*5* mutagenesis was performed as described by Weaver et al. (62) by using phage λ467 (3) as the source of the transposon.

DNA sequencing. Nucleotide sequencing was performed by the method of Sanger et al. (51). Single-stranded templates were obtained by use of the M13mp18/19 phage vectors (65). Chain extensions were performed with Sequenase (U.S. Biochemicals, Cleveland, Ohio) and with [α-^{35}S]dATP (New England Nuclear, Du Pont Scandinavia AB, Stockholm, Sweden) as the labeling component.

Maxicell analysis. The production of L-[^{35}S]methionine-labeled proteins in the maxicell strain CSR603 (50) and their separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis were performed as described previously (49).

Measurement of ethidium efflux. A fluorimetric assay was used to measure ethidium efflux. Cells were loaded with ethidium bromide by treatment with 20 μM carbonyl cyanide *m*-chlorophenylhydrazone, and substrate-driven efflux was assayed as described previously (33, 61).

Computer analysis. Sequence analysis was performed by using the package of programs developed by the University of Wisconsin Genetics Computer Group (12). Hydropathy analysis was performed by using the program PROFILEGRAPH (24) with the hydrophobicity scale of Engelman et al. (14). Pairwise alignments of amino acid sequences were performed with GAP (12, 35) by using the 250 PAM matrix of Dayhoff et al. (11). The statistical significance of the alignment was determined by comparison of the alignment with alignments between one of the sequences and random shufflings of the other sequence. This was calculated as significance $(Z) = (a - m)/\sigma$, where a is the alignment score, m is the mean of 100 alignment scores in which one of the sequences has been randomly shuffled, and σ is the standard deviation of m.

Nucleotide sequence accession number. The DNA sequence reported here appears in the EMBL data base under accession number X68232.

RESULTS

Sequence of ORF1 from R751. R751 is an IncP-1 plasmid (26, 32) which contains the trimethoprim resistance determinant *dhfrIIc* (16). Examination of the sequences flanking the *dhfrIIc* determinant has suggested that R751 contains an integron with an integrated trimethoprim resistance cassette (46). However, the integron on R751 does not contain the sulfonamide resistance determinant *sulI* found in the 3′ conserved segment of other integrons (Fig. 1). To investigate whether R751 contains ORF4, which is also located in the 3′ conserved segment of integrons, the nucleotide sequence of a 441-bp *Dde*I fragment downstream of the *dhfrIIc* resistance determinant from R751 was determined (Fig. 2).

Analysis of this sequence revealed the presence of a single open reading frame, designated ORF1, which is preceded by a potential Shine-Dalgarno ribosome-binding site. ORF1 was predicted to code for a 110-aa hydrophobic polypeptide of 12.5 kDa. Maxicell analysis demonstrated that the production of a polypeptide of approximately 12.5 kDa was correlated with the expression of ORF1 (data not shown). Nucleotide sequence comparisons indicated that the first 94 codons of ORF1 from R751 are identical to ORF4 from the 3′ conserved segment of other integrons, after which point they diverged completely (Fig. 2).

Comparison of the structure of the R751 integron with those of other integrons (Fig. 1) indicated that the 3′ conserved segment of the R751 integron lacks both *sulI* and ORF5. Bissonette and Roy (4) have previously proposed a model for the evolution of integrons in which they are descended from an ancestral integron which contained a shortened 3′ conserved segment and lacked *sulI* and ORF5. The structures of the R751 integron and the altered 3′ end of ORF1 compared with those of ORF4 from other integrons suggested that ORF4 may have evolved from ORF1 by the insertion of a segment of DNA carrying both *sulI* and ORF5 into the 3′ end of ORF1 between codons 94 and 95. This resulted in ORF4 consisting of a truncated ORF1 fused in frame with part of the inserted DNA segment. In this case it is possible that such an interruption of the terminal 15% of the ORF4 sequence compared with the ORF1 sequence may have caused changes in the phenotypic expression or the phenotypic manifestations of the protein.

Alignment of the amino acid sequences of the predicted polypeptides encoded by ORF1 and ORF4 with QacC suggested that they are both closely related to the multidrug exporter QacC (see below). This is supported by the comparison of the hydropathy profiles of each of these three proteins (Fig. 3). Hydropathy analysis of the products of ORF1 and ORF4 indicated that they both possess four strongly hydrophobic domains, as has been reported for QacC (27), that are likely to form transmembrane α helices. Interestingly, the ORF4 protein contains a long C-terminal hydrophilic tail compared with the sequences of the ORF1 protein and QacC (Fig. 3). The C-terminal tail of the ORF4 protein arose from the fusion of the truncated ORF1 with 21 aa encoded by the inserted DNA segment (see Fig. 4).

ORF1 and ORF4 specify multidrug resistance to antiseptics and disinfectants. The multidrug transporter QacC has been shown to mediate resistance to intercalating dyes and quaternary ammonium compounds (28). The QacA multidrug resistance protein also mediates resistance to these two classes of compounds as well as to the diamidines and chlorhexidine (28, 61). To investigate the potential roles of the products of ORF1 and ORF4 as mediators of resistance to antiseptics and disinfectants, MICs of a range of representative compounds were determined for strains containing ORF1 and ORF4 compared with those for a strain containing *qacC* (Table 1).

Strains containing either ORF1 or ORF4 were resistant to both intercalating dyes and quaternary ammonium compounds, but they were susceptible to the diamidines and the

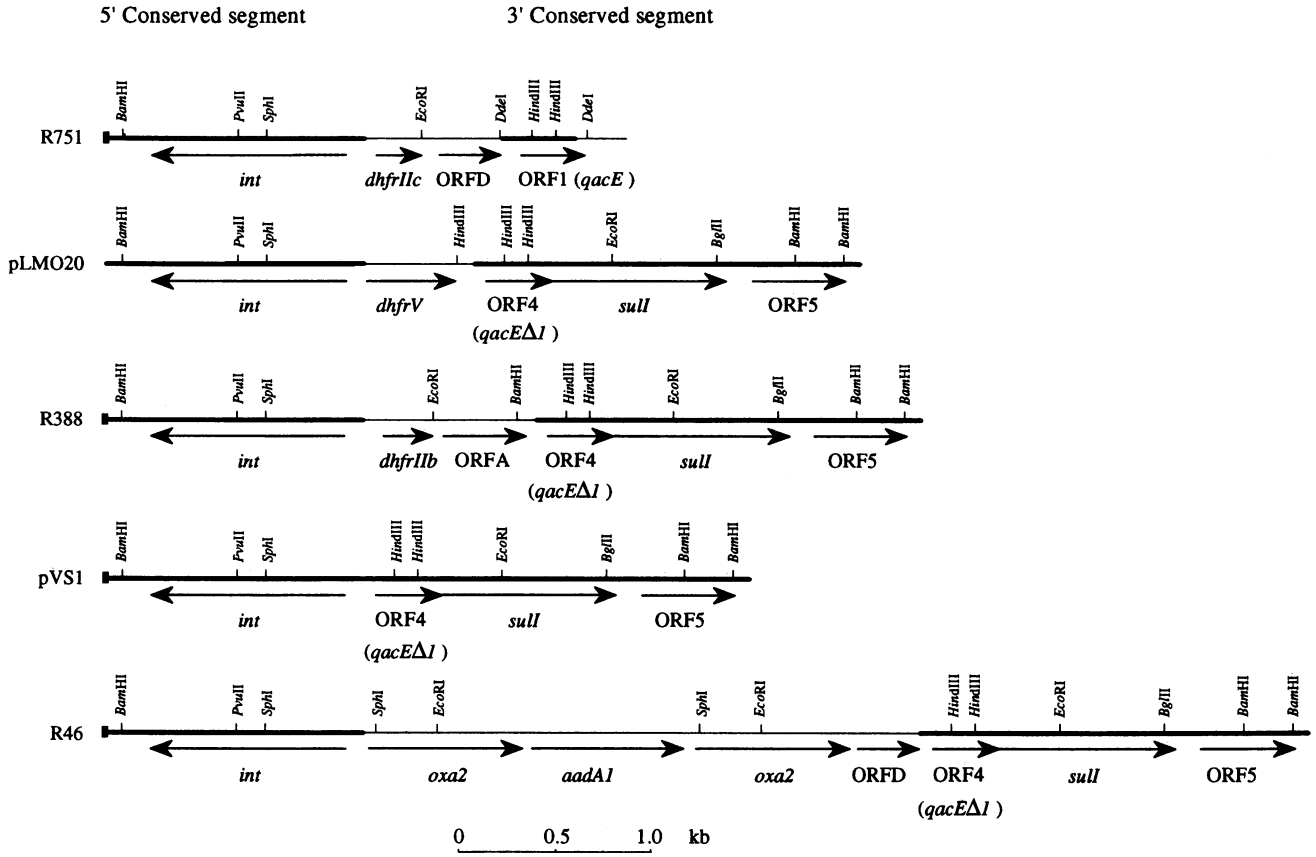

FIG. 1. Physical maps of representative integrons from the indicated plasmids. The 5' and 3' conserved segments are represented by thick lines and are as defined by Stokes and Hall (54). Selected restriction sites are shown above each line. The region between the two *Dde*I sites corresponds to the sequence shown in Fig. 2. The following genes are represented: *int*, site-specific integrase; *dhfr*, trimethoprim-resistant dihydrofolate reductases of types IIb, IIc or V; ORFA, D, and 5, open reading frames with unknown function (note that the ORFD shown here is unrelated to the ORFD from *P. vulgaris* [8] which is shown in Fig. 4); *qacE* (otherwise referred to as ORF1 from R751), the multidrug resistance gene investigated in the present study; *qacEΔ1* (otherwise referred to as ORF4 from other integrons), the multidrug resistance gene investigated in the present study; *sulI*, a sulfonamide resistance gene; *oxa2*, oxacillin resistance gene; *aadA1*, streptomycin and spectinomycin resistance gene. The shaded boxes located at the 5' end of each integron represent one of the terminal inverted repeats of 25 bp which flank some integrons (4, 6, 46, 54). The inverted repeat at the end of the 5' segment of pLMO20 is displaced by the insertion of an IS*6*-like element (46). The maps of the following are derived from the indicated sources: R751 (46), pLMO20 and R388 (57); pVS1 (4), and R46 (55).

biguanidine chlorhexidine (Table 1). Strains containing ORF1 were observed to possess a resistance phenotype almost identical to that specified by *qacC* in *E. coli*, whereas strains containing ORF4 were resistant to much lower levels of the compounds examined. It should be noted that *qacC*, when expressed in *E. coli* (Table 1), mediated resistance to a similar spectrum of compounds as that reported previously in *S. aureus* (28). However, in general, resistance levels in *E. coli* to the antiseptics examined were higher than levels previously observed in *S. aureus* (28) presumably because of, at least in part, the ethidium resistance determinant *ebr*, which is located on the *E. coli* chromosome (44, 45).

To confirm that expression of ORF1 or ORF4 was responsible for the observed resistance phenotypes, Tn*5* mutagenesis was performed on the plasmids pLKO603A and pLKO1B. The location of insertions of the kanamycin resistance transposon Tn*5* were determined by restriction mapping. Insertion of Tn*5* in either ORF1 or ORF4 was found to abolish resistance, whereas Tn*5* inserts outside of these open reading frames had no effect on resistance to quaternary ammonium compounds or intercalating dyes (data not shown). Thus, ORF1 encodes a phenotype of multidrug resistance to intercalating dyes and quaternary ammonium compounds, and consequently, we designated ORF1 from R751 as *qacE* and the 110-aa protein it encodes as QacE. Since both nucleotide sequence data and resistance studies indicate that ORF4 is a partially functional derivative of *qacE*, we designated this deleted derivative *qacEΔ1* and its product QacEΔ1.

Mechanism of resistance encoded by *qacE*. QacC has previously been reported (28) to mediate resistance by active export energized by PMF. A fluorimetric assay (33) was used to examine the mechanism of resistance specified by *qacE*. The plasmids pLKO603A and pLKO1B, which encode *qacE* and *qacEΔ1*, respectively, were transformed into AN418, an *uncA E. coli* strain which cannot produce ATP by oxidative phosphorylation (10). Cells containing *qacE* were loaded with ethidium, and the addition of formate resulted in the rapid export of ethidium (data not shown). This rapid export of ethidium was not observed in the absence of a carbon source and was also observed to be abolished by the addition of the protonophore carbonyl cyanide *m*-chlorophenylhydra-

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DdeI
CTAAGCACATAATTGCTCACAGCCAAACTATCAGGTCAAGTCTGCTTTTATTATTTTTAA    60

GCGTGCATAATAAGCCCTACACAAATTGGGAGATATATCATGAAAGGCTGGCTTTTTCTT   120
                          --SD -      M  K  G  W  L  F  L        7

GTTATCGCAATAGTTGGCGAAGTAATCGCAACATCCGCATTAAAATCTAGCGAGGGCTTT   180
 V  I  A  I  V  G  E  V  I  A  T  S  A  L  K  S  S  E  G  F     27
DdeIHindIII
ACTAAGCTTGCCCCTTCCGCCGTTGTCATAATCGGTTATGGCATCGCATTTTATTTTCTT   240
 T  K  L  A  P  S  A  V  V  I  I  G  Y  G  I  A  F  Y  F  L     47

TCTCTGGTTCTGAAATCCATCCCTGTCGGTGTTGCTTATGCAGTCTGGTCGGGACTCGGC   300
 S  L  V  L  K  S  I  P  V  G  V  A  Y  A  V  W  S  G  L  G     67
                                  HindIII.
GTCGTCATAATTACAGCCATTGCCTGGTTGCTTCATGGGCAAAAGCTTGATGCGTGGGGC   360
 V  V  I  I  T  A  I  A  W  L  L  H  G  Q  K  L  D  A  W  G     87
                          DraI
TTTGTAGGTATGGGGCTCATAGTTAGTGGTGTAGTAGTTTTAAACTTGCTTTCCAAAGCA   420
 F  V  G  M  G  L  I  V  S  G  V  V  V  L  N  L  L  S  K  A     107

       DdeI
AGTGCCCACTAATAAACTCAG                                          441
 S  A  H  *                                                     110
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FIG. 2. Nucleotide sequence of the region of R751 containing ORF1 (*qacE*). The sequence shown (base pairs 1 to 441) corresponds to the *Dde*I fragment of R751 indicated in Fig. 1. The predicted amino acid sequence of QacE is shown in italics beneath the nucleotide sequence. Restriction sites *Dde*I, *Hin*dIII, and *Dra*I are indicated above the nucleotide sequence. A potential Shine-Dalgarno ribosome-binding site (SD) is shown. The region in boldface type represents the sequence of *qacE* which differs from that found in ORF4 (*qacEΔ1*) in other integrons.

zone. Similar rapid export of ethidium was not observed in the host strain AN418 under comparable conditions, although a very slow rate of ethidium export which was attributable to the *E. coli* chromosomal ethidium resistance determinant *ebr* was observed. These results suggested that the mechanism of resistance to ethidium specified by *qacE* is active export energized by PMF. Cells containing *qacEΔ1* (pLKO1B) were also examined fluorimetrically for ethidium transport (data not shown). Only a slow rate of ethidium export was observed, and this could not be differentiated from the background level of ethidium export owing to the chromosomal *ebr* determinant. Thus, because of the low

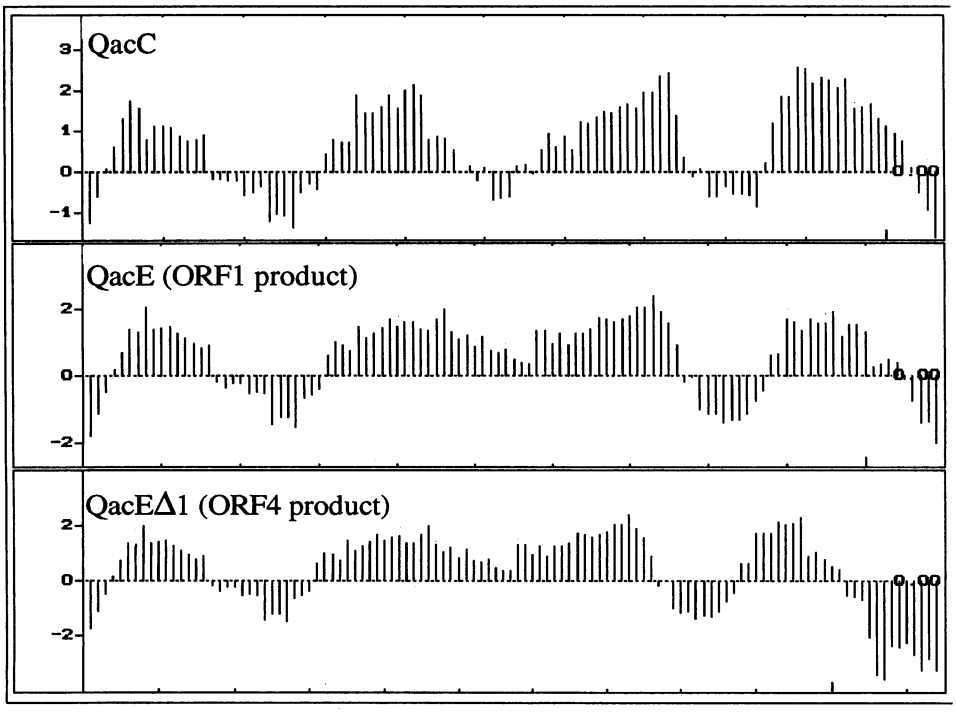

FIG. 3. Hydropathy profiles of QacC, QacE (the product of ORF1 from R751), and QacEΔ1 (the product of ORF4 from other integrons). The profiles were prepared by using the algorithm of Engelman et al. (14) with a window of 11 amino acids.

TABLE 1. MICs of antiseptics and disinfectants for the *E. coli* strains examined in this study

Plasmid[a]	*qac* determinant	MIC (μg/ml)[b]												
		Dyes				QAC					BIG	Diamidines		
		Eb	Pf	Cv	R6	Ct	Bc	Cc	Dc	Tp	Ch	Dd	Pi	Pt
—[c]	—	50	20	5	600	40	20	20	20	600	<1	100	100	100
pLMO20	ORF4 (*qacEΔ1*)	75	20	10	>800	60	20	30	40	600	<1	100	100	100
pLKO1B	ORF4 (*qacEΔ1*)	100	40	10	>800	60	20	40	40	600	<1	100	100	100
R751	ORF1 (*qacE*)	800	60	30	>800	100	80	80	40	600	<1	100	100	100
pLKO603A	ORF1 (*qacE*)	800	60	30	>800	100	80	80	40	600	<1	100	100	100
pSK534	*qacC*	800	60	20	>800	100	80	80	40	600	<1	100	100	100

[a] Plasmids were transformed into the *E. coli* host strain BHB2600 for MIC testing.
[b] The following dyes were tested: Eb, ethidium bromide; Pf, proflavine; Cv, crystal violet; and R6, rhodamine 6G. The following quaternary ammonium compounds (QAC) were tested: Ct, cetyltrimethylammonium bromide; Bc, benzalkonium chloride; Cc, cetylpyridinium chloride; Dc, dequalinium chloride; and Tp, tetraphenylarsonium chloride. BIG, biguanidine (Ch, chlorhexidine diacetate). The following diamidines were tested: Dd, diamidinodiphenylamine dihydrochloride; Pi, propamidine isethionate; and Pt, pentamidine isethionate.
[c] The data in this row represent a negative control with BHB2600 containing no plasmids.

level of ethidium resistance specified by *qacEΔ1*, it was not possible to establish whether the QacEΔ1 protein mediates resistance by active export.

Reserpine, an inhibitor of the mammalian multidrug exporter P glycoprotein (2), has also been shown to inhibit the function of the bacterial multidrug resistance proteins Bmr (37) and NorA (36). The addition of reserpine as described in Materials and Methods was found to have no effect on the levels of resistance specified by *qacC*, *qacE*, or *qacEΔ1* as assessed by MIC tests. This suggests that the multidrug resistance proteins specified by these genes are not inhibited by the presence of reserpine.

QacE is a member of a family of multidrug export proteins. Littlejohn et al. (27) have previously reported that QacC shares homology with ORF4(*qacEΔ1*) from integrons and to the product of ORFD, an open reading frame located adjacent to the fumarate reductase operon on the chromosome of *Proteus vulgaris* (8) (ORFD from *P. vulgaris* should not be confused with the unrelated ORFD from R46 and R751 shown in Fig. 1). The *E. coli* Ebr resistance protein, which has also been referred to as MvrC (34), has also been reported to be homologous to QacC (44). The proteins QacC (28), Ebr (33), and QacE (this study) have all been suggested to be multidrug exporters that use PMF to energize transport. The amino acid sequence similarities between these proteins were assessed by pairwise alignments by using GAP (12, 35) (data not shown). QacE was observed to share 40, 46, and 31% identity with QacC, Ebr, and ORFD, respectively. The statistical significance (Z) of these alignments was determined as described in Materials and Methods. Significant homologies were observed between all these proteins ($Z > 10$ in all cases), and thus, they form a family of homologous transmembrane proteins on this basis. These homologies suggest that the product of ORFD from *P. vulgaris* may also be a PMF-dependent multidrug exporter. A search of the SWISSPROT, GenBank, and EMBL data bases failed to identify any other proteins related to this family.

Grinius et al. (20) also reported that two smaller proteins, each of which consists of only 83 aa and which are predicted to contain only two transmembrane segments (TMSs), from *Agrobacterium tumefaciens* (18) and *E. coli* (7) are related to this family of multidrug exporters. Our examination of the DNA sequences of these two putative proteins revealed that they are identical to *qacEΔ1* (ORF4) except for a 1-bp deletion in the case of the protein encoded on pDGO100 from *E. coli* (7) and a small number of changes in the case of the protein reported to be encoded on *A. tumefaciens* T-DNA (18). In addition, we found that the sequences surrounding these open reading frames included *sulI*, *int*, and other features characteristic of integrons (data not shown). This strongly suggests to us that both of these open reading frames are in fact copies of *qacEΔ1* which have been misidentified as encoding smaller proteins because of errors in their published sequences which resulted in reading frame shifts. This has been confirmed experimentally in the case of the open reading frame on pDGO100 (21), in which an extra guanine has been located at base pair 2111 in the sequence reported by Cameron et al. (7). This change makes this open reading frame identical to *qacEΔ1*. It should be noted that the open reading frame reported to be from *A. tumefaciens* (20) is not actually from this genus, but is located on an artificial cloning vector whose DNA derived originally from *E. coli* (23).

Sequence alignment of this family of exporters revealed that there are a number of regions in the proteins which are highly conserved and that may therefore be essential for the function of these transporters (Fig. 4). The consensus sequences of these conserved motifs are L x x A i x x E v i (which occurs in TMS I), l K x s e G F t r-k l (which occurs in the loop between TMSs I and II), P S x x x I i (which occurs in TMS II), and a Y A x W x G x G x V (which occurs in TMS III), where x represents any amino acid, capital letters indicate an absolutely conserved amino acid, and lowercase letters indicate a high frequency of occurrence (at least 80%).

DISCUSSION

Bissonette and Roy (4) proposed a model for the evolution of integrons in which they are descended from an ancestral integron which contained a shortened 3′ conserved segment and lacked *sulI* and ORF5. The results reported here indicate that the integron on R751 includes *qacE* (ORF1), which differs at its 3′ end compared with the sequence of *qacEΔ1* (ORF4) and does not contain *sulI* or ORF5 (Fig. 1). This report also provides evidence that strains containing QacE are resistant at much higher levels to a range of toxic compounds compared with strains containing QacEΔ1. This suggests that *qacEΔ1* evolved from *qacE* by the insertion of a segment of DNA carrying both *sulI* and ORF5 into the 3′ end of *qacE* between codons 94 and 95. In fact, it appears

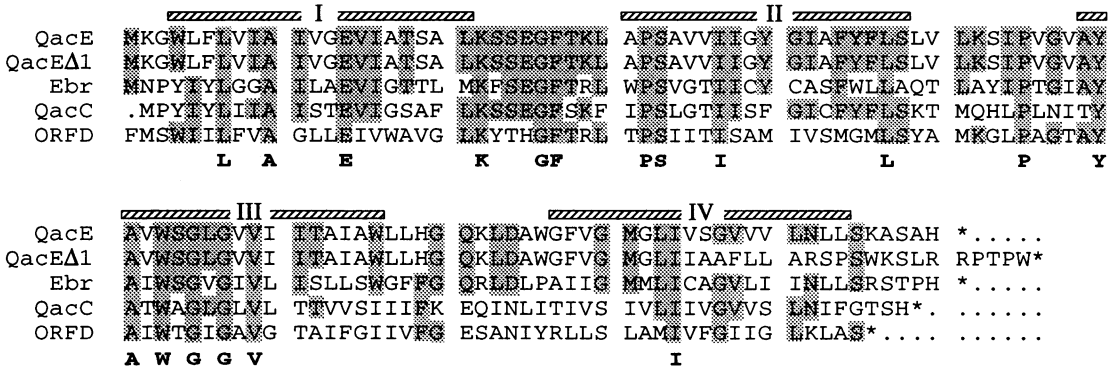

FIG. 4. Sequence similarities between QacE and other small membrane proteins. The predicted amino acid sequences shown are QacE (product of ORF1 from R751) (this study); QacEΔ1 (product of ORF4 from other integrons) (57); Ebr (44), which is identical to MvrC (34); QacC (27), which is identical to QacD (27, 28), Ebr (52), and Smr (20); and the product of ORFD from *P. vulgaris* (8). Identical amino acids present in more than two sequences are shaded, and absolutely conserved residues are shown in boldface undertype the sequences. The positions of putative transmembrane α helices are indicated by the hatched boxes above the sequences.

that *qacE* is a cassette (46) interrupted by the insertion of the segment mentioned above. This resulted in *qacEΔ1*, a truncated form of *qacE* fused in frame to sequences which code for an unrelated 21 aa from the inserted segment. This longer protein, QacEΔ1, appears to be only partially functional as a multidrug exporter. These results are consistent with the model of the evolution of the 3′ conserved segment proposed by Bissonette and Roy (4).

QacE has been shown to be a member of a family of multidrug export proteins. Because of their dependence on PMF, the molecular mechanism of the action of these proteins is probably a multidrug-proton antiport system, as has been proposed for QacA (47) and Bmr (37). This suggests that the conserved motifs may be involved in proton translocation, substrate binding, or the opening and closing of a transport channel, because these features have been suggested to be essential for the function of a substrate-proton antiporter (64). Site-directed mutagenesis of Cys-42 in QacC resulted in mutants which displayed altered resistance phenotypes, which indicated that this residue may be located at or near a substrate recognition site (41). This cysteine residue is located directly adjacent to the conserved motif in TMS II (Fig. 4), which may implicate this conserved region in substrate binding. Speculatively, another region which may be involved in substrate recognition is the motif within TMS III, because this is the most highly conserved feature within this family of proteins. The motif located within TMS I contains a glutamic acid residue, which is the only charged residue which is highly conserved within a transmembrane segment. This suggests that this region may be involved in proton translocation as proposed previously (20). The other conserved motif is located in the loop between TMSs I and II. The location of this motif outside of the membrane suggests that it may be involved in a structural role in stabilizing protein conformation.

Ethidium efflux was not observed in strains containing *qacEΔ1*, and these strains were resistant to only low levels of the intercalating dyes and quaternary ammonium compounds in comparison with strains containing *qacE*. These phenotypic differences are presumably due to the C-terminal alteration between QacE and QacEΔ1. However, the changes between QacE and QacEΔ1 occur only in the fourth transmembrane segment and the C-terminal tail of these proteins, and there are no highly conserved residues located within these regions. The small sizes of these transport proteins compared with the sizes of other transporters, which often possess at least 12 transmembrane segments, suggest that the small export proteins examined in the present study may function as multimeric complexes. Thus, it is possible that the longer C-terminal tail of QacEΔ1 may interfere with the process of multimerization, which may explain the lower levels of resistance mediated by QacEΔ1. Alternatively, it is possible that the changes in the fourth transmembrane segment between QacE and QacEΔ1 result in reducing the affinity to a range of compounds of the substrate recognition site, or the presence of a longer C-terminal tail in QacEΔ1 may affect the gating mechanism involved in the opening and closing of a transport channel.

At least three different families of multidrug exporters have now been identified. The first is a family of ATP-dependent efflux proteins including mammalian P glycoprotein (13) and Pmd1, a multidrug exporter from *Schizosaccharomyces pombe* (38). These proteins are typically large, consist of 12 transmembrane segments, and are susceptible to inhibitors such as reserpine. They also share homology with a variety of other transport proteins, including the bacterial hemolysin exporter HlyB (15), and a protein implicated in chloroquine resistance in *Plasmodium falciparum* (17).

The second is a family of PMF-dependent efflux proteins (43) with either 14 transmembrane segments such as QacA (47) or 12 transmembrane segments such as Bmr (37) and NorA (36, 66). Some of these multidrug exporters have been shown to be inhibited by reserpine. These proteins also share homology with a range of other antibiotic and antiseptic exporters (43) and with sugar importers and other transport proteins (19).

The present study characterized a third family of multidrug exporters that included QacC, QacE, and Ebr. These proteins are typically small with only four transmembrane segments. In addition, they are unaffected by inhibitors such as reserpine and they do not display homology with any other known transport systems. Thus, this family possesses a number of unique characteristics not observed in other multidrug export systems. Members of this family appear to be widely distributed among prokaryotic organisms. *qacC* is located on a variety of plasmids from clinical strains of *S. aureus* and other staphylococci, and *ebr* appears to be stably maintained on the chromosome of *E. coli*. The present study revealed that additional family members *qacE* and *qacEΔ1*

are widely spread throughout various gram-negative bacteria because of their presence on integrons. The wide distribution of this family of multidrug resistance determinants may be due to the selective pressure imposed by the clinical use of agents such as benzalkonium chloride, cetyltrimethylammonium bromide, and acriflavine as antiseptics and disinfectants. Support for this is the observation that *qacC* and *qacE/qacEΔ1* are frequently found in conjunction with various antibiotic resistance determinants.

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