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Evidence that TET Protein Functions as a Multimer in the Inner Membrane of *Escherichia coli*

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The inner membrane TET (TetA) protein, which is involved in Tn*10*-mediated microbial tetracycline resistance, consists of two domains, α and β, both of which are needed for tetracycline resistance and efflux (M. S. Curiale, L. M. McMurry, and S. B. Levy, J. Bacteriol. 157:211–217, 1984). Since tetracycline-sensitive mutants in one domain can partially complement sensitive mutants in the other domain and since some sensitive mutants show dominance over the wild type, a multimeric structure for TET in the membrane had been suggested. We have studied this possibility by using *tetA-phoA* gene fusions. We fused all but the last 40 base pairs of the *tetA* gene with the carboxy terminus of the *phoA* gene for alkaline phosphatase (PhoA), whose activity requires its dimerization in the periplasm. The *tetA-phoA* fusion protein was under control of the tetracycline-inducible regulatory system for the *tetA* gene. Induction led to the synthesis of a 78,000-dalton inner membrane protein. Tetracycline resistance was expressed at reduced levels, consistent with the terminal β domain deletion. Alkaline phosphatase activity was also present, but at low levels, suggesting that some, but not all, of the fusion proteins had their carboxy-terminal ends in the periplasm. When wild-type or mutant TET proteins were present in the same cell with the fusion protein, the tetracycline resistance level was affected (raised or lowered); however, phosphatase activity was reduced only when TET proteins with intact or near-intact β domains were present. These findings suggest that TET functions as a multimer and that intact β domains, on TET molecules in the heterologous multimer, either allow fewer PhoA moieties to project into the periplasm or sterically hinder PhoA moieties from dimerizing.

Five tetracycline resistance (Tc^r) determinants, designated classes A to E, have been defined by DNA hybridization studies with gram-negative enteric bacteria (20, 23). All have been found on transferable plasmids. Although the determinants are distinguishable, they appear to have evolved from a common ancestral gene(s), since each class specifies an energy-driven efflux of tetracycline (20, 22) and there is 50 to 70% homology at the DNA level among the four determinants whose sequence is known (7, 12, 25, 27, 28).

The most common Tc^r determinant among members of the family *Enterobacteriaceae* is that found on the transposable DNA element Tn*10* (21). Resistance to high concentrations of antibiotic is induced by growing the cells in a subinhibitory concentration of tetracycline (14, 30). A repressor protein, the product of *tetR*, has been identified (1, 30) which is a negative regulator of itself (1, 12) and of the single structural gene, *tetA*, associated with resistance (1, 8, 27, 29). The latter encodes the inner membrane protein TET (15) (which we also designate $TetA^B$ or TET^B as a means of distinguishing it as the structural protein for the class B determinant), which is required for resistance (5, 11). The sequences acted upon by the repressor have been located in an overlapping divergent regulatory region between *tetR* and *tetA* (2, 7, 25).

Two complementation groups have been found by genetic analysis of the *tet* gene region (4, 5). These two groups were found to represent two approximately equal domains (designated *tetA* α [proximal to promoter] and *tetA* β [distal to promoter]) of a single gene encoding the TET protein (6). The intragenic complementation data suggested that the TET protein exists as multimers in its inner membrane location. Using gene fusions of TET protein with alkaline phosphatase (PhoA), we have obtained further evidence for this multimeric state and for specific domain interactions.

MATERIALS AND METHODS

Bacterial strains and plasmids. *Escherichia coli* AW1061 [Δ(*brnQphoR*) *tsx*::Tn*5* *leu proC*], used for studies of alkaline phosphatase activity (9), and plasmid pCH40, which contained the carboxy terminus of *phoA* and was used for the fusion construction (9), were received from C. Hoffman and A. Wright. Strain MCL22 (provided by C. S. Rupert) was used for maxicell analysis (16). Plasmids containing the *tetA* gene are described in Fig. 1. Cultures were grown in either L broth or M9 minimal medium as indicated. Plasmids were introduced into strains by a standard $CaCl_2$ transformation protocol (17).

Enzymes and chemicals. Restriction enzymes, T4 DNA ligase, and *Pst*I linker DNA were obtained from New England BioLabs, Inc. Chloramphenicol, chlortetracycline, cycloserine, 5-bromo-4-chloro-3-indoylphosphate, *p*-nitrophenyl phosphate, and the protease inhibitors phenylmethylsulfonyl fluoride, *p*-toluenesulfonyl fluoride, and *N*-tosyl-L-lysl-chloromethane hydrochloride were obtained from Sigma Chemical Co. [^{35}S]methionine (1,086 Ci/mmol) was obtained from New England Nuclear Corp.

Plasmid-encoded protein synthesis in maxicells. Maxicells (26) were prepared from plasmid-containing MCL22 cells. Cultures were grown at 37°C to an A_{530} of 0.4 in M9 minimal medium supplemented with 0.2% Casamino Acids (Difco Laboratories), 5% glucose, and thiamine and biotin (2 μg/ml each). Cultures were UV irradiated and subsequently incubated overnight at 37°C in the presence of cycloserine (200 μg/ml) and ampicillin (50 μg/ml). Cells were washed and suspended in M9 medium supplemented with 0.2% glucose,

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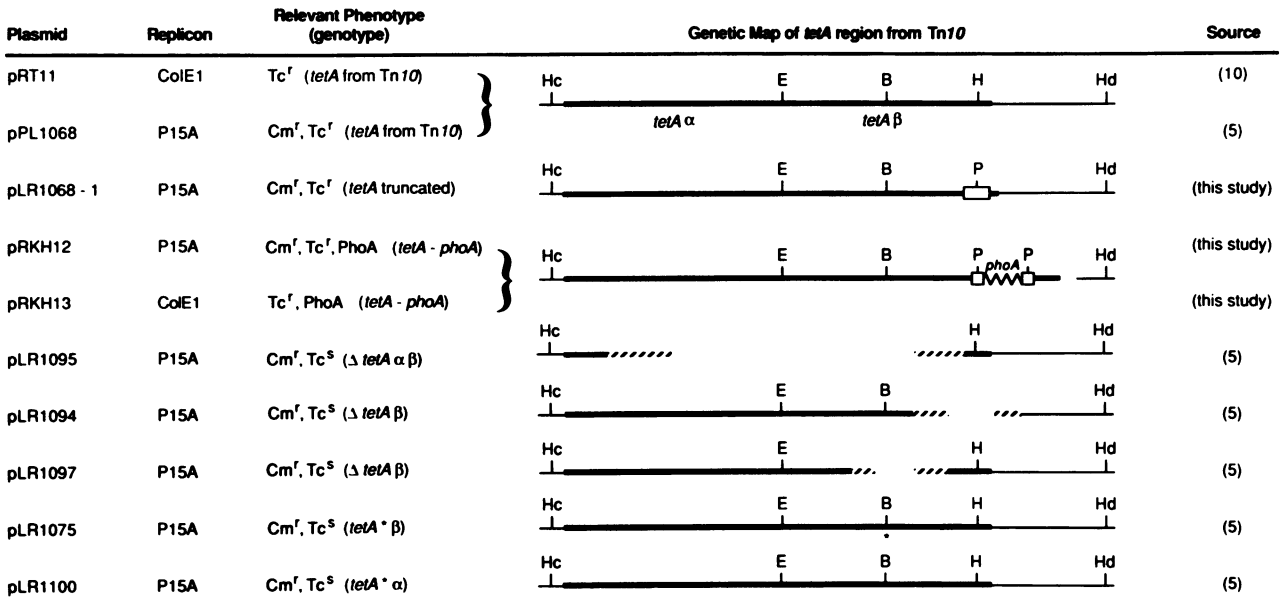

FIG. 1. Schematic map of gene fusions and deletion and point mutations of the tetracycline structural gene region of the Tn*10* tetracycline resistance determinant (not drawn to scale). The *Hin*cII-to-*Hpa*I fragment of *tetA* is 1,223 bp. The complete *tetA* gene is 1,203 bp (7). Symbols: ▬, Tn*10* DNA sequence encoding the structural protein TET; ▭, *Pst*I linker DNA; ∿∿, structural gene for alkaline phosphatase. ——, Sequences known to be present; spaces, sequences known to be deleted; ////, regions where the sequence is not known. Restriction endonuclease sites are abbreviated as follows: Hc, *Hin*cII; E, *Eco*RI; B, *Bgl*I; H, *Hpa*I; P, *Pst*I; Hd, *Hin*dIII. Although the precise genetic locations of point mutations (*) in pLR1075 or pLR1100 are unknown, they have been assigned to a domain on the basis of complementation studies of tetracycline resistance (6).

$MgCl_2$ substituted for $MgSO_4$, thiamine, and biotin. Induction of the *tet* operon was effected by the addition of autoclaved chlortetracycline (50 μg/ml) at the initiation of sulfur starvation for 1 h. Proteins were labeled by the addition of [^{35}S]methionine (10 μCi/ml). Maxicells were separated into supernatant and membrane fractions by centrifugation for 1 h at 40,000 × *g* after lysis with lysozyme-EDTA treatment in the presence of proteolytic inhibitors phenylmethylsulfonyl fluoride, *p*-toluenesulfonyl fluoride, and *N*-tosyl-L-lysyl-chloromethane hydrochloride (final concentration, 1 mM each). Immunoprecipitates were prepared with anti-PhoA antiserum (provided by C. Hoffman and A. Wright) as described by Ito et al. (10). Radiolabeled proteins were examined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (12% acrylamide) (6) and viewed by fluorography. Densitometric analysis of autoradiograms was performed on an LKB 2202 Ultrascan laser densitometer. The bands corresponding to fusion protein were compared with that for chloramphenicol acetyltransferase encoded by the pLR1068 derivatives.

Determination of tetracycline susceptibility. The MIC of tetracycline for strains was determined by the gradient plate technique as previously described (5). Cultures were grown to an A_{530} of 0.2 in L broth containing the gratuitous inducer autoclaved chlortetracycline and streaked with a cotton swab onto the surfaces of gradient plates. The plates were examined after 24 h at 37°C. The MIC refers to the estimated tetracycline concentration in the gradient plates at which confluent growth ceased.

Assay of PhoA activity. Alkaline phosphatase activity was measured by the production of *p*-nitrophenol from *p*-nitrophenyl phosphate (3, 9). Cultures of AW1061 bearing plasmids with the *tetA-phoA* gene fusion were grown to an A_{600} of 0.5 in L broth (50 ml) containing autoclaved chlortetracycline. Cells were washed and suspended in 50 mM Tris (pH 8.0) to which was added a 1/10 volume of 0.1 M EDTA–10 mg of lysozyme per ml. After 10 min at 0°C, the cells were lysed by sonication. Membrane fractions were collected by centrifugation for 1 h at 48,000 × *g* and then suspended in 1 ml of 1 M Tris (pH 8.0). Substrate (*p*-nitrophenyl phosphate 0.4 mg/ml final concentration) was added to the supernatant and membrane suspensions, which were then incubated at 37°C for 90 min. The reaction was stopped by the addition of 1/10 volume of 1 M K_2HPO_4, and samples were assayed by measurement of the A_{420}. Membranous material was removed by centrifugation, as described above, before the absorbancy was measured.

Construction of plasmids bearing a *tetA-phoA* gene fusion. Plasmid pLR1068 (6) was digested with *Hpa*I, which has a unique site 40 base pairs (bp) from the carboxy terminus of the *tetA* gene (Fig. 2). This *Hpa*I restriction site was converted to a *Pst*I restriction site by ligation with *Pst*I linker DNA to generate plasmid pLR1068-1. Subsequently, a 3.0-kbp *Pst*I fragment (encompassing the coding region for the enzymatically active carboxy terminus of PhoA) purified from pCH40 was ligated to *Pst*I-digested pLR1068-1 and used to transform *E. coli* AW1061. Chloramphenicol-resistant transformants were replica plated onto L plates containing 50 μg of autoclaved chlortetracycline per ml and 40 μg of 5-bromo-4-chloro-3-indolylphosphate (a chromogenic substrate for PhoA) per ml. Transformants showing PhoA activity, detected after 2 to 3 days of incubation, were screened for recombinant plasmids. One such recombinant plasmid selected was designated pRKH12 (Fig. 2). Restriction enzyme analysis of pRKH12 (data not shown) revealed that the *phoA* gene was oriented properly with respect to the direction of transcription of the *tetA* gene.

To duplicate this fusion on a different compatible plasmid, we subcloned the *tetA-phoA* gene fusion into the ColE1 replicon of plasmid pRT11, which bears an intact *tetA* gene (Fig. 1). A 5-kbp *Xba*I-*Hin*dIII fragment from pRKH12,

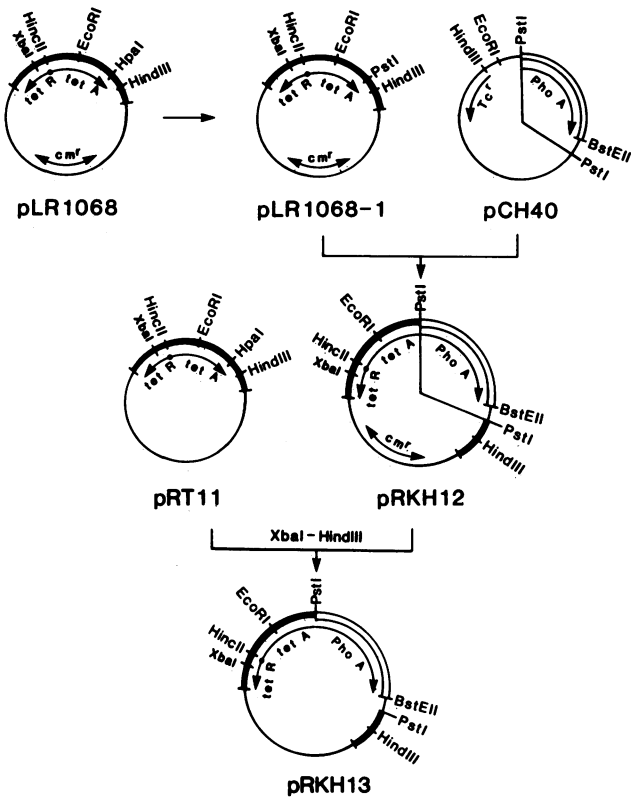

FIG. 2. In vitro construction of a *tet*A-*phoA* gene fusion. Abbreviations: TetR, Tn*10*-encoded repressor; TetA, Tn*10* structural gene for tetracycline resistance; PhoA, structural gene for alkaline phosphatase; Tc^r, class C tetracycline resistance determinant; Cm^r, chloramphenicol resistance. Symbols: ▬, Tn*10* tetracycline resistance determinant; ▭, structural gene for alkaline phosphatase.

containing the *tetA-phoA* gene fusion, was substituted for the analogous *tetA*-containing gene fragment in pRT11 to generate pRKH13 (Fig. 2).

RESULTS

Expression and stability of the *tetA-phoA* gene fusion product. All detectable PhoA activity in strains bearing the *tetA-phoA* gene fusion copurified with the membrane fraction (data not shown). This activity was fully extractable from membranes by Sarkosyl (0.5% [CIBA-GEIGY Corp.] in 50 mM Tris [pH 8.0]), suggesting an inner membrane location (15).

Besides membrane-bound PhoA activity, this fusion protein also produced Tc^r. The level of Tc^r, 36 μg/ml, was the same as that expressed by the wild-type *tetA* gene with a *Pst*I linker inserted at the same site as the fusion, 14 amino acids upstream from the carboxy terminus of the TET protein (at the terminus of the β domain) (Table 1). Resistance was, however, lower than that (103 μg/ml) expressed by the intact *tetA* gene on the same plasmid vector.

The level of PhoA activity in strain AW1061 carrying pRKH13 was less than 0.1% of a fully derepressed wild-type *E. coli* strain (3). To help explain this finding, we examined the stability of the fusion protein in maxicells. Proteins encoded by pRKH13 were labeled with [^{35}S]methionine in MCL22 maxicells containing the plasmid. A tetracycline-inducible protein of approximately 78,000 daltons was seen by SDS-polyacrylamide gel electrophoresis (Fig. 3). The size of this protein was consistent with the combined size of the two individual proteins present in the fusion, as analyzed separately in this SDS-polyacrylamide gel electrophoresis system: TET protein (43,300 daltons) migrates as a 36,000-dalton protein and PhoA migrates as a 47,000-dalton protein. The synthesis of a fusion protein of this size confirmed that the *Pst*I fusion junction was in frame, as predicted by the DNA sequences of the 3′ end of the *tetA* gene at the *Pst*I restriction site in pLR1068-1 and of the 5′ end of the *phoA* fragment from pCH40. Pulse-chase labeling experiments in which the reaction was stopped by heating cells in SDS buffer showed no breakdown of the fusion protein during chase periods of up to 60 min (Fig. 3). The nature of the broad band of polypeptides at 30 to 35 kilodaltons is unknown, but it is thought to be an artifact of the maxicell system (R. K. Hickman and S. B. Levy, unpublished results). The amount of the peptides remained unchanged during the chase, which suggests that they are not degradative products of the fusion protein.

The stability of the PhoA activity of the fusion protein in live cells was also examined. Chlortetracycline-induced cultures of AW1061 containing pRKH13 were centrifuged, washed, and suspended in 50 mM Tris (pH 8.0) in the presence of 100 μg of chloramphenicol per ml for 90 min at

TABLE 1. Effect of native and mutant TET proteins on PhoA activity of the *tetA-phoA* gene fusion product

Plasmid combination[a]		Tetracycline MIC[b] (μg/ml)	PhoA activity (U/ml)[b] (%)[c]
A	B		
None	pLR1068 (*tetA* wild type)	103.4 ± 4.7	ND[d]
None	pLR1068-1 (*tetA*-truncated)	35.8 ± 2.3	5.4 ± 2.3
pRKH13 (*tetA-phoA* gene fusion)	None	36.0 ± 2.5	66.6 ± 0.3 (100)
pRKH13	pRKH12 (*tetA-phoA*)	33.7 ± 2.5	87.3 ± 0.9 (131)
pRKH13	pLR1068 (*tetA* wild type)	100.0 ± 3.1	22.7 ± 0.5 (34)
pRKH13	pLR1068-1 (*tetA*-truncated)	38.8 ± 1.9	37.3 ± 0.3 (56)
pRKH13	pLR1100 (*tetA* α mutant)[e]	65.8 ± 7.1	32.9 ± 0.5 (49)
pRKH13	pLR1075 (*tetA* β mutant)[e]	30.3 ± 2.5	68.5 ± 0.4 (103)
pRKH13	pLR1094 (*tetA* β deletion)[e]	25.8 ± 1.9	67.5 ± 0.6 (101)
pRKH13	pLR1097 (*tetA* β deletion)[e]	21.9 ± 0.9	64.0 ± 0.8 (96)
pRKH13	pLR1095 (*tetA* α +β deletion)[e]	33.0 ± 3.0	62.4 ± 0.6 (93)

[a] Plasmids are described in Fig. 1.
[b] Mean ± standard deviation of three separate determinations.
[c] Compared with PhoA activity expressed by pRKH13.
[d] ND, Not determined.
[e] Specifies no Tc^r.

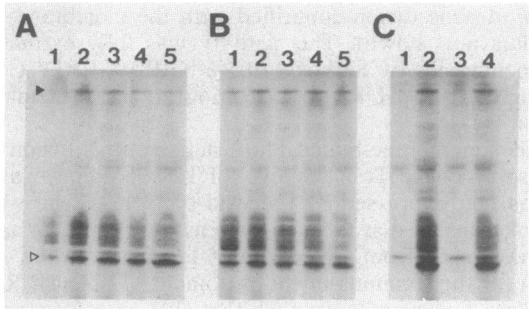

FIG. 3. Stability of TET-PhoA protein fusions. Maxicells containing pRKH13 were labeled with [^{35}S]methionine for designated periods. Subsequently, a 1,000-fold excess of cold methionine was added in a pulse-chase experiment. At various time intervals, portions of cells were immediately lysed in SDS treatment buffer. Results are from induced cultures unless otherwise stated. (A and B) Two- and five-minute labels, respectively: lanes 1, no chase; lanes 2, 10-min chase; lanes 3, 20-min chase; lanes 4, 30-min chase; lanes 5, 60-min chase. (C) Lane 1, uninduced, 30-min label, no chase; lane 2, induced, 30-min label, no chase; lane 3, uninduced, 60-min label, no chase; lane 4, induced, 60-min label, no chase. The solid arrowhead shows the location of the TET-PhoA fusion protein. The open arrowhead shows the location of the tetracycline-inducible repressor protein.

37°C. These cells were subsequently lysed by sonication and separated into membrane and soluble fractions. There was no loss of PhoA activity in the chloramphenicol-treated culture, and all activity was still associated with the membrane fraction (data not shown). These findings suggest that low phosphatase activity was not a result of breakdown, since the fusion protein had a half-life in vivo of greater than 60 min.

When ^{35}S-labeled maxicells were lysed for preparation of membrane and soluble fractions, two tetracycline-inducible fusion proteins were observed (Fig. 4): a major one of 78,000 daltons and a minor, slightly smaller one. Both of these tetracycline-inducible proteins were precipitable with anti-PhoA antiserum (data not shown). Both forms were found exclusively in the membrane (Fig. 4B, lane 1). Since these two TET-PhoA forms were not observed in labeled whole cells subjected directly to SDS-polyacrylamide gel electrophoresis, we attribute this doublet to proteolysis during lysis, despite the presence of proteolytic inhibitors. A doublet TET protein was also occasionally seen in maxicells lysed under the same conditions (Fig. 4B, lane 3).

Competitive inhibition of PhoA activity by native and mutant TET proteins in the same cell. Previous complementation studies had suggested that TET protein exists as a multimeric complex within the inner membrane (5, 6). Since native PhoA must be dimerized in the periplasmic space to express enzyme activity (3, 9, 18, 19, 24), one explanation for the recovery of PhoA activity from a TET-PhoA fusion protein is that the fusion protein exists at least as dimers. Alternatively, membrane fluidity might permit the PhoA carboxy end to freely dimerize in the periplasm while the amino terminus of TET remains as a monomer in the membrane. We reasoned that if TET exists as multimers, wild-type TET protein might compete with TET-PhoA fusion proteins to lower PhoA activity. If TET were a monomer, such competition would not be expected.

Wild-type and mutant *tetA* genes on plasmid pLR1068 (p15A replicon) were introduced into AW1061 carrying the *tetA-phoA* fusion on the compatible plasmid pRKH13 (ColE1 replicon). Tc^r and PhoA activity levels in these strains were assayed (Table 1). Introduction of the wild-type *tetA* gene on pLR1068 into cells containing the *tetA-phoA* fusion resulted in a significant reduction (66%) of membrane-bound PhoA activity and an increase in Tc^r to a level close to that of the intact *tetA* gene. Plasmid pLR1068-1 bearing a partially active TET protein, missing the same carboxy-terminal 14 amino acids as the fusion, also lowered PhoA activity (44%), with minimal change in the Tc^r level (Table 1). These results showed that both types of TET molecules interfered with PhoA activity. Apparently, heterologous multimers had indeed formed owing to multimerization of the TET regions. The wild-type TET was evidently dominant for Tc^r over the terminally deleted β domain of the fusion protein. Plasmid pLR1068-1 coded for the same amount of Tc^r as did the gene fusion of pRKH12, and so Tc^r was not affected by the fusion of PhoA at the *Hpa*I site in TET.

Plasmids bearing point mutations in the α or β domain, both of which code for detectable but inactive TET proteins (6), were introduced into the same cell with the fusion protein. Plasmid pLR1100 (containing an α domain point mutation) caused a 51% lowering in PhoA activity, but an

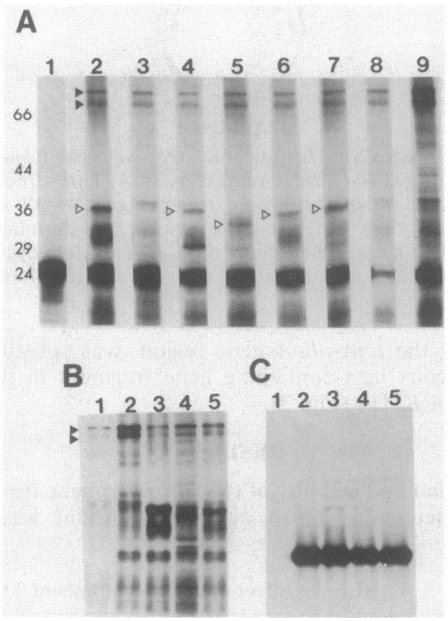

FIG. 4. Synthesis of [^{35}S]methionine-labeled TET proteins and TET-PhoA fusion protein in MCL22 maxicells. Plasmids bearing *tetA* point and deletion mutations were introduced into strain MCL22 carrying the *tetA-phoA* gene fusion on plasmid pRKH13. Cells were treated with lysozyme-EDTA, sonicated, and separated into membrane and supernatant fractions. Results are from cells induced with autoclaved chlortetracycline unless otherwise stated. Proteins radiolabeled for 30 min were separated on an SDS–12% polyacrylamide gel. (A) Lysed whole cells: lane 1, pRKH13 plus pRL1068, uninduced; lane 2, pRKH13 plus pLR1068; lane 3, pRKH13 plus pLR1095; lane 4, pRKH13 plus pLR1075; lane 5, pRKH13 plus pLR1094; lane 6, pRKH13 plus pLR1068-1; lane 7, pRKH13 plus pLR1100; lane 8, pRKH13; lane 9, pRKH13 plus pRKH12. (B and C) Membrane and supernatant fractions, respectively: lanes 1, pRKH13; lanes 2, pRKH13 plus pRKH12; lanes 3, pRKH13 plus pLR1068; lanes 4, pRKH13 plus pLR1094; lanes 5, pRKH13 plus pLR1100. Solid arrowheads show the locations of TET-PhoA fusion proteins. Open arrowheads show the locations of wild-type and mutant TET proteins (see text). The sizes (in kilodaltons) of molecular weight markers are given at the left in panel A.

95% increase in Tc^r levels. These data implied multimer formation of TET leading to complementation of Tc^r and possible interference with PhoA dimerization. However, pLR1075 (containing a point mutation in the β domain) led to a 17% decrease in Tc^r without a detectable effect on PhoA activity. These differences showed that this β domain point mutation on the competing TET molecule was different from the α domain mutation in affecting Tc^r but not PhoA activity.

To investigate these findings further, we introduced plasmids bearing internal deletions in the β domain into cells producing the fusion protein. The presence of pLR1094 or pLR1097, both of which make an inactive gene product (6), led to a decrease in Tc^r levels (28% and 39%, respectively), while again having no detectable effect on the levels of PhoA activity. As a control for any effect of the p15A replicon, we introduced plasmid pLR1095 (deleted in both the α and β regions and with no identifiable gene product [6]) into the cell and found no effect on either PhoA activity or Tc^r levels. In these and previous complementations, the presence of both plasmids in the cell was verified by antibiotic resistance phenotype and agarose gel analysis of DNA preparations of the cultures used for these tests.

These results confirmed that TET proteins with mutated inactive β domains decrease the Tc^r specified by the TET-PhoA fusion protein. This was consistent with the formation of heterologous multimers between the fusion protein and the mutant TET protein. However, the presumed multimerization had no effect on the PhoA activity of the TET-PhoA fusion protein.

Effect of intact and mutant TET proteins on the synthesis and location of TET-PhoA fusion protein. We examined maxicells to determine whether the presence of mutant or wild-type TET proteins affected the relative amounts of fusion protein produced and/or its location in the cell (Fig. 4). We performed densitometric analysis of the fusion protein bands in lanes 2 to 7 and 9 of the autoradiogram (Fig. 4A) and compared their amounts relative to the prominent chloramphenicol acetyltransferase band (24 kilodaltons) for the pLR1068 derivatives as an internal standard. (The small amount of material seen at this location in lane 8 [pRKH13, a ColE1 derivative] is an artifact in the gel). In all maxicells having both a plasmid bearing the TET-PhoA fusion and another plasmid, which did (Fig. 4A, lanes 2 and 4 to 7) or did not (lane 3) cause the synthesis of a mutant TET protein, the relative amount of fusion protein was unchanged (range, 0.8 to 1.2). When two compatible plasmids, each specifying a fusion protein, were in the same cell (lane 9), 2- to 2.5-fold more fusion protein was detected. Examination of membrane fractions (Fig. 4B, lanes 1 to 5) and cytoplasmic fractions (Fig. 4C, lanes 1 to 5) of maxicells revealed that in all these cells the fusion protein remained membrane bound in the presence of membrane-associated wild-type or mutant TET protein.

Although cells with two plasmids bearing the same fusion protein had a twofold-higher amount of fusion protein located in the membrane (Fig. 4A, lane 9; 4B, lane 2), PhoA activity was only 30% higher and Tc^r levels remained largely unchanged (Table 1). To confirm that the maxicell data reflected the status in normal, growing cells, we examined the amount of fusion protein in ^{35}S-labeled cells bearing pRKH13 or both pRKH13 and pRKH12 by immunoprecipitation with anti-PhoA antiserum. The results were the same. Metabolically labeled normal cells bearing both plasmids contained two- to threefold more fusion protein than did cells bearing only pRKH13 (data not shown).

DISCUSSION

Using a bifunctional protein which specifies both Tc^r and PhoA activity, we have examined the self-interactions of the integral inner membrane protein TET. Dual functions of the fusion protein allow us to assay the interactions with other TET protein molecules by measuring two independent activities, those of Tc^r and PhoA.

We found that PhoA was anchored to the membrane as a result of its fusion to the TET protein, with at least some of the PhoA moieties extending into the periplasm, since PhoA requires dimerization in the periplasm for activity (3, 9, 18, 19, 24). A Kyte-Doolittle analysis (13) of the TET protein showed that the final 15 amino acids of the carboxy terminus have a negative hydropathy (25), suggesting that this portion of the protein is not membrane bound and thus could have either a cytoplasmic or a periplasmic location. The level of PhoA activity induced in cells carrying the *tetA-phoA* gene fusion was low (about 0.5 to 1%) in comparison with that of other gene fusion constructions with a PhoA carboxy terminus, e.g., a β-lactamase–PhoA gene fusion on pBR322 (18). Several days of incubation were required before the blue-green color was detected on the assay medium for the *tetA-phoA* gene fusion, in contrast to detection overnight for cells bearing the *phoA* chromosomally or in other *phoA* fusion constructions reported (9, 19). We could not attribute the low activity to the fusion's being out of frame, or to instability, since PhoA activity assayed in live cells and labeled fusion protein examined in maxicells were stable for at least 60 min. Recently we fused *lacZ* at the same *Hpa*I junction site and found that the synthesis of this fusion protein gave high, easily detectable levels of β-galactasidase activity (Hickman and Levy, unpublished experiments). On a mole-to-mole basis, the specific activity of the *tetA-lacZ* fusion was 20-fold higher than that of the *tetA-phoA* fusion. The high LacZ activity and low PhoA activity suggest that the carboxy terminus of TET at this fusion site is normally a cytoplasmic domain, not a periplasmic one (19). If this is the case, the low PhoA activity presumably results from a small proportion of the fusion proteins with PhoA translocated to the periplasm. Although cytoplasmic alkaline phosphatase is reportedly unstable (9, 19), the fusion of PhoA to an apparent cytoplasmic domain of the TET protein appears to protect the fusion protein from proteolysis. Stability has been reported previously for PhoA fusions to a cytoplasmic domain of the Tsr chemoreceptor protein of *E. coli* (19). Likewise, PhoA moieties in TET-PhoA fusions extending into the periplasm are apparently also protected from periplasmic proteolytic enzymes.

Full Tc^r is dependent on a complete β domain. Removal of the last 14 amino acids of the carboxy terminus of the TET protein lowered the Tc^r level to one-third that found in the wild type. This result confirmed previous findings (11) that a small deletion of the carboxy terminus of TET drastically decreased resistance. The addition of PhoA at the same *Hpa*I site in the carboxy terminus of the TET protein did not affect this lowered Tc^r level. This would suggest that the PhoA addition to the molecule did not alter its intrinsic tetracycline efflux activity and presumably its membrane orientation.

Introduction of wild-type and mutant TET proteins into the same cell bearing the *tetA-phoA* gene fusion resulted in significant changes in Tc^r. Only TET proteins with an intact or nearly intact (active) β domain (wild type, pLR1068-1 and pLR1100) reduced PhoA activity as well. The tetracycline-sensitive α domain mutation (on pLR1100) with an intact β domain both lowered PhoA activity and raised the low Tc^r

specified by the fusion protein. These results strongly suggest that these TET proteins are forming heterologous multimers with the TET-PhoA fusion protein in the cell. Combinations in which Tc^r was lowered, but PhoA activity was not, involved TET proteins inactivated by mutation in the β domain (pLR1075, pLR1094, and pLR1097). To explain these results, we postulate that only TET protein with an intact (i.e., active) β domain inhibits PhoA activity either by interfering with translocation of the carboxy terminus of the fusion protein to the periplasm or by sterically hindering the dimerization of PhoA moieties extending into the periplasm. Mixed multimers consisting of fusion protein and a TET protein that does not have an intact (active) β domain will not affect PhoA activity.

The presence of a second tetracycline-inducible operon and synthesis of additional TET protein did not appear to interfere with the expression of the fusion protein (Fig. 4). However, increasing the level of synthesis of the fusion protein by an increase in gene dosage (Fig. 4A, lane 9; Fig. 4B, lane 2) did not result in higher levels of Tc^r, and PhoA activity was increased only 31%. These results suggest that limited numbers of functionally active membrane sites or other additional factors may be required for functional activity of TET protein and/or there may be limited numbers of sites for PhoA to translocate to the periplasm. Although limited sites may exist, the complementation experiments in which Tc^r and PhoA activity did not decrease simultaneously must be explained in some other way. These findings are best interpreted as multimer formation between TET molecules and the fusion protein. They add further evidence that TET protein probably acts as multimeric units in its native state to efflux tetracycline. The α and β domains of this protein appear to be distinguishable functionally by their effects on PhoA activity in heterologous multimers.

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