Biogenesis and Topology of Integral Membrane Proteins: Characterization of Lactose Permease−Chloramphenicol Acetyltransferase Hybrids†

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ABSTRACT: Use of β-lactamase in gene fusions to study membrane protein topology permits exploitation of its biological activity to select for positive (external) hybrids on ampicillin agar plates. When the enzyme is attached to cytoplasmic loops of a membrane protein, it is not secreted and is therefore unable to confer ampicillin resistance. In this study, we examine the use of the cytoplasmic enzyme chloramphenicol acetyltransferase (Cat) as a complement to the use of periplasmic β-lactamase, in gene fusion studies. This enzyme is responsible for chloramphenicol resistance in *Escherichia coli*. We show that Cat confers substantial antibiotic resistance when fused to cytoplasmic loops of lactose permease. As expected, periplasmically exposed Cat is enzymatically active *in vitro* but unable to confer significant chloramphenicol resistance, presumably because of the absence of acetylcoenzyme A in the periplasm. Therefore, Cat may serve as a topogenic sensor in gene fusion studies. The new Cat fusion approach is discussed with regard to its potential use for selecting *E. coli* mutants which are defective in the assembly of membrane proteins.

In order to study structural aspects of integral membrane proteins in *Escherichia coli*, a genetic approach has been developed to promote *in vivo* analysis of their membrane topology [Manoil & Beckwith, 1985, 1986; Calamia & Manoil, 1990; reviewed in Traxler et al. (1993)]. Briefly, gene fusions encoding hybrid proteins composed of N-terminal fragments of the membrane protein attached to a cytoplasmic or a periplasmic reporter lacking its signal peptide are expressed in *E. coli*. Periplasmic reporters usually require export to the periplasm in order to be active and act as sensors for periplasmic location of the protein sequences to which they are attached (Calamia & Manoil, 1992). When the reporters are fused to cytoplasmic loops, they remain in the cytoplasm in an inactive form, and when fused to external loops, they are expected to be translocated across the membrane in an active form. In this respect, hybrids between N-terminal sequences of membrane proteins and the mature forms of alkaline phosphatase (Calamia & Manoil, 1990; Boyd et al., 1993) or β-lactamase (Broom-Smith & Spratt, 1986; Wang et al., 1991) have been used to determine the topology of polytopic integral membrane proteins in *E. coli*. Unlike alkaline phosphatase, β-lactamase provides a means to select for antibiotic resistance which is mediated only by periplasmically oriented hybrids. A different reporter that is sometimes used in gene fusion experiments is β-galactosidase (Manoil, 1990). This cytoplasmic enzyme is inactivated by partial membrane integration when fused to periplasmic loops of membrane proteins (Lee et al., 1989). However, the use of β-galactosidase may be problematic (Sarsero & Pittard, 1995), probably because of the toxic effect of the secreted form of this enzyme (Snyder & Silhavy, 1995). In the search for a cytoplasmic reporter for membrane protein topology studies, which also provides antibiotic resistance, the enzyme chloramphenicol acetyltransferase (Cat)[1] was selected. Cat catalyzes *O*-acetylation of chloramphenicol using acetylcoenzyme A (acetyl-CoA) as the acetyl donor (Shaw & Leslie, 1991). We reasoned that the enzyme should confer antibiotic resistance only in its native intracellular location since its activity requires a cytoplasmic cofactor, acetyl-CoA. If this hypothesis is correct, then the enzyme might serve as a useful probe for cytoplasmic domains in gene fusion experiments. This approach could be complicated by the fact that the active form of Cat is a homotrimer and three hybrids must associate in the correct fashion to achieve enzymatic activity. However, the successful use of another oligomeric reporter, alkaline phosphatase, which forms active homodimers in the periplasmic space is encouraging. Using the lactose permease (LacY) as a model polytopic membrane protein, we demonstrate that Cat is enzymatically active on both sides of the membrane *in vitro* but confers significant chloramphenicol resistance only when fused to cytoplasmic loops of LacY.

MATERIALS AND METHODS

Materials. Chloramphenicol, kanamycin, ampicillin, lysozyme, proteinase K, phenylmethanesulfonyl fluoride (PMSF), acetyl- or butyrylcoenzyme A, and protein A (cell suspension of *Staphylococcus aureus* cowan strain) were obtained from Sigma. Restriction enzymes were obtained from New England BioLabs and modifying enzymes from Boehringer Mannheim. Oligodeoxynucleotides were synthesized by the Scientific Services Unit at the Weizmann Institute of Science. [^{35}S]Methionine and [^{14}C]chloramphenicol were obtained from Amersham, and purified Cat and anti-Cat polyclonal antibodies were from 5 Prime-3

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[1] Abbreviations: Cat, chloramphenicol acetyltransferase; CoA, coenzyme A; LacY, lactose permease; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; PCR, polymerase chain reaction.

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Prime Inc. Antibodies to the C-terminal tail of LacY were kindly provided by Dr. H. R. Kaback (UCLA). Prestained protein molecular mass markers were purchased from Bio-Rad and DNA molecular mass markers from GIBCO-BRL. GeneClean and Mermaid glassmilk DNA purification kits were obtained from Bio 101 and Wizard Mini Prep kits from Promega. All other materials were reagent grade and obtained from commercial sources

Bacterial Strains. *E. coli* UT5600 (*ompT*$^-$) and *E. coli* Hfr 3000 X74 (Δ*lac*) were obtained from the *E. coli* Genetic Stock Center at Yale University (strain 7092 and strain 5261, respectively). *E. coli* UTZ [UT5600 (Δ*lacY*)] was prepared in the lab by conjugation of UT5600 with Hfr 3000 X74 and used for antibiotic resistance assays. *E. coli* UTZ expresses constitutively genes that are regulated by the *lac* promoter. *E. oli* HB101 [*hsdS20 (r-B, m-B), recA13, ara-14, proA2, lacY1, galK2, rpsL20 (Smr), xyl-5, mtl-1, supE44,* λ^-/F$^-$] was used for preparation of various plasmid constructs. *E. coli* T184 [*lacI*$^+$*O*$^+$*Z*$^-$*Y*$^-$*(A), rpsL, met*$^-$*, thr*$^-$*, recA, hsdM, hsdR/F′, lacI*^{q}*O*$^+$*ZD118(Y*$^+$*A*$^+$*)*] was used for specific metabolic labeling and immunoprecipitation experiments and for *in vitro* quantitative Cat assays.

Construction of lacY−Cat Fusions. Plasmid pT7-5 (Tabor & Richardson, 1987) containing a *lac* promoter (pT7-5/*lacY*, Bibi & Kaback, 1990) was used in these studies in order to allow expression from either the T7 promoter or the *lac* promoter (see below). The Cat-encoding gene from plasmid pACYC184 (New England Laboratories) was amplified by polymerase chain reaction (PCR), using the following oligodeoxynucleotides: a sense primer 5′AGGACTCGAGCCTGCAGAAAAAAATCACTG containing *Xho*I and *Pst*I sites and an antisense primer 5′ACTGCTCGAGAACTGCAGCGCCAGCGCCCCGCCCTGCC including the same sites. Using the *Xho*I site, the *cat* gene was inserted into pT7-5/*lacY* to form a sandwich construct, SanS194Cat, encoded by pTE/*SanS194cat*. The *Pst*I site was used in a similar manner to construct hybrid SanQ100Cat, encoded by plasmid pTE/*SanQ100cat*. *E. coli* SCS110 (*dam*$^-$, *dcm*$^-$) cells (Stratagene) were transformed with plasmid pTE/*SanQ100cat* to enable *Cla*I digestion, followed by Klenow treatment and self-ligation, thus forming an intermediate plasmid (pTE/*Q100cat-ClaI*). This plasmid was digested with *Sty*I and *Xmn*I and ligated with the complementary fragment from pTE/*SanS194cat* digested with *Sty*I and *Xmn*I to produce hybrid S194Cat encoded by pTE/*S194cat*. Hybrid Q100Cat encoded by pTE/*Q100cat* was constructed by ligating the complementary fragments of pTE/*S194cat* and pTE/*SanQ100cat* which were digested by *Sty*I and *Xmn*I. Deletion mutants of hybrids Q100Cat and S194Cat were constructed as follows. pTE/*Q100cat* was digested by *Acc*I and *Eco*RI, treated with Klenow, and ligated to itself to produce plasmid pTE/*Q100cat*Δ(37−71) encoding hybrid Q100CatΔ(37−71). To create hybrid S194CatΔ(144−163), plasmid pT7-5/*lacY* was digested by *Eco*RV and *Xho*I, ligated with pTE/*S194cat* which was digested with *BSSH*II, treated with Klenow, and then digested with *Xho*I. To construct a full-length LacY−Cat hybrid (V416Cat), plasmid pT7-5/*lacY-phoA* (Bibi et al., 1991) was digested with *Nhe*I and *Xmn*I to remove the *phoA* gene. The remaining fragment was ligated with a DNA fragment containing the *cat* gene which was previously constructed, using PCR, to carry a C-terminal extension identical to the nonrelated linker connecting LacY and alkaline phosphatase in pT7-5/*lacY-phoA*, and also the complementary *amp*^{R} gene which was deleted by the *Nhe*I-*Xmn*I digest of pT7-5/*lacY-phoA*. A deletion mutant of V416Cat was constructed by PCR-mediated deletion of the coding sequence of transmembrane segment 11 of LacY. The resulting plasmid, pTE/*V416cat*Δ(339−379) encodes hybrid V416CatΔ(339−379) in which Cat is fused to a deletion mutant of LacY missing transmembrane segment 11.

Antibiotic Resistance Assays. *E. coli* UTZ cells transformed with the appropriate *lacY−cat* constructs were divided, and one portion was plated on LB plates containing only ampicillin (50 μg/mL). The other portion was plated on LB plates containing different concentrations of chloramphenicol, in addition to ampicillin. The plates were incubated at 37 °C, and the number of colonies was counted after 24 h.

Chloramphenicol Acetyltransferase Activity Assays. CAT assays were carried out with whole *E. coli* UTZ cells expressing given LacY−Cat hybrids or with isolated membrane fractions obtained by sonication, removal of cell debris (by low-speed centrifugation), and membrane collection by two cycles of wash and high-speed centrifugation. The reactions were performed in 150 μL containing 30 μL of Tris-HCl (1 M, pH 7.8), 9.5 μL of chloramphenicol (1 mM), 0.5 μL of [^{14}C]chloramphenicol (0.1 mCi/mL, 59.7 mCi/mmol), 20−35 μL of the sample (whole cells) or 50 μg of protein (membranes), 10 μL of either acetyl-CoA (12.35 mM) or butyryl-CoA (11 mM), and 65−80 μL of water. After incubation at 37 °C, the reaction was stopped with 300 μL of cold ethyl acetate which was also used to extract the chloramphenicol and its acylated forms. The extraction was repeated twice, and the organic phases were pooled and dried under nitrogen. Organic extracts were dissolved in 30 μL of ethyl acetate, spotted on silica gel thin-layer plates, and separated by chloroform−methanol (95:5). Radioactive spots were visualized with a Bio Imaging Analyzer and by autoradiography.

Immunoblotting. *E. coli* UTZ cells transformed with the indicated plasmid were grown overnight at 37 °C in LB supplemented with ampicillin (100 μg/mL). Cultures were then diluted 1:100 and grown to an OD_{600} of 0.8. Cells were harvested, washed in buffer A [100 mM Tris-HCl (pH 8), 50 mM NaCl, 1 mM EDTA, and 1 mM PMSF], and resuspended in the same buffer. Cell suspensions (500 μL of 10 OD_{420} units) were sonicated, and cell debris was removed by centrifugation (2 min at 13 000 rpm). Membranes were collected by ultracentrifugation (30 min at 150000*g*). Pellets were resuspended in buffer A, and the protein concentration was measured according to a modified Lowry procedure (Markwell et al., 1978) in the presence of 2.5% SDS using bovine serum albumin as a standard. An aliquot of the resuspended membranes was then mixed with 2X sample buffer [according to Laemmli (1970)], incubated at 37 °C for 10 min, and subjected to SDS−PAGE (12%). Immunoblotting was carried out as described (Herzlinger et al., 1985) with anti-Cat or anti-LacY antibodies and horseradish peroxidase-conjugated goat anti-rabbit antibodies.

[^{35}S]Methionine Labeling and Immunoprecipitation. *E. coli* T184 cells harboring pGP1-2 were transformed with the fusion vectors, LacY encoding plasmid pT7-5/*lacY* or pT7-5 as a negative control, and grown overnight at 30 °C in M9 minimal media containing 0.5% glycerol, 100 μg/mL ampicillin, 30 μg/mL kanamycin, 1 μg/mL thiamin, and all the amino acids (15 μg/mL). Cells were diluted 1:20 and grown

for 4 h at 30 °C. After three washes in M9 at room temperature, the cells were resuspended in M9 containing all the amino acids except for methionine and cysteine to an OD_{420} of 0.8. Following 1 h of incubation at 30 °C, the cultures were induced by heat shock at 42 °C for 20 min. Rifampicin was then added (40 mM final concentration), and cells were left at 42 °C for an additional 20 min. After 5 min of equilibration at 30 °C, cells were labeled with 15 μCi [^{35}S]methionine (1000 Ci/mmol) and incubated at 30 °C for 30 min. Labeled cells (0.5 mL) were treated and immunoreacted with anti-Cat antibodies as described (Manoil, 1991). Immunoprecipitated material was extracted in 50 μL of sample buffer (Laemmli, 1970) and separated on SDS−PAGE (12%), and the dry gel was exposed to film for at least 24 h. Prestained molecular mass markers were used to estimate the molecular mass of the different hybrid proteins. For limited proteolysis experiments, labeled cells were resuspended in cold sucrose buffer [40% sucrose (w/v) and 33 mM Tris-HCl (pH 8)] and stored at −80 °C until use.

Limited Proteolysis. Proper conditions for limited proteolysis were calibrated using a LacY−alkaline phosphatase hybrid, in which alkaline phosphatase is fused to the N-terminal transmembrane segment of LacY (data not shown). Labeled cells in ice-cold sucrose buffer (see above) were incubated with lysozyme (6.25 mg/mL) and EDTA (1 mM) on ice for 15 min. Proteinase K was then added (final concentration 0.8 mg/mL) with or without Triton X100 (2%), and the mixture was incubated for 1 h on ice. The proteolysis was stopped by PMSF (1 mM) followed by TCA (10% final concentration) precipitation. Pellets were solubilized and immunoprecipitated as described (Manoil, 1991).

RESULTS

Construction of lacY−cat Gene Fusions. In order to examine the applicability of Cat as a cytoplasmic reporter in gene fusion studies, we constructed hybrids between N-terminal portions of LacY and Cat lacking its N-terminal methionine. We chose the LacY of *E. coli*, because it is well-characterized both structurally and functionally. This prokaryotic polytopic membrane protein catalyzes the coupled translocation of β-galactosides and H^+ [cf. Kaback (1995) for a current review]. It is well-accepted that the LacY polypeptide has 12 hydrophobic membrane-spanning domains (Figure 1). Evidence for the general features of the model and the demonstration that both the N- and C-termini are on the cytoplasmic surface of the membrane has been obtained from a variety of experimental approaches, including extensive series of *lacY−phoA* (lactose permease−alkaline phosphatase) fusions (Calamia & Manoil, 1990). In this study, we directed the fusion joints, connecting LacY with the reporter protein, to known cytoplasmic or periplasmic locations according to the well-studied putative secondary structure of the permease which has been recently updated (Ujwal et al., 1995). Fusions were constructed in the second periplasmic loop (Q100Cat), the third cytoplasmic loop (S194Cat), and the cytoplasmic C-terminus of LacY (V416Cat) (Figure 1). In addition, to examine the ability of Cat to monitor topological changes that are independent of the local sequence at the fusion joints, we constructed deletion mutants missing one putative transmembrane segment other than the one that is directly connected to the reporter. The deletions are expected to reverse the orientation

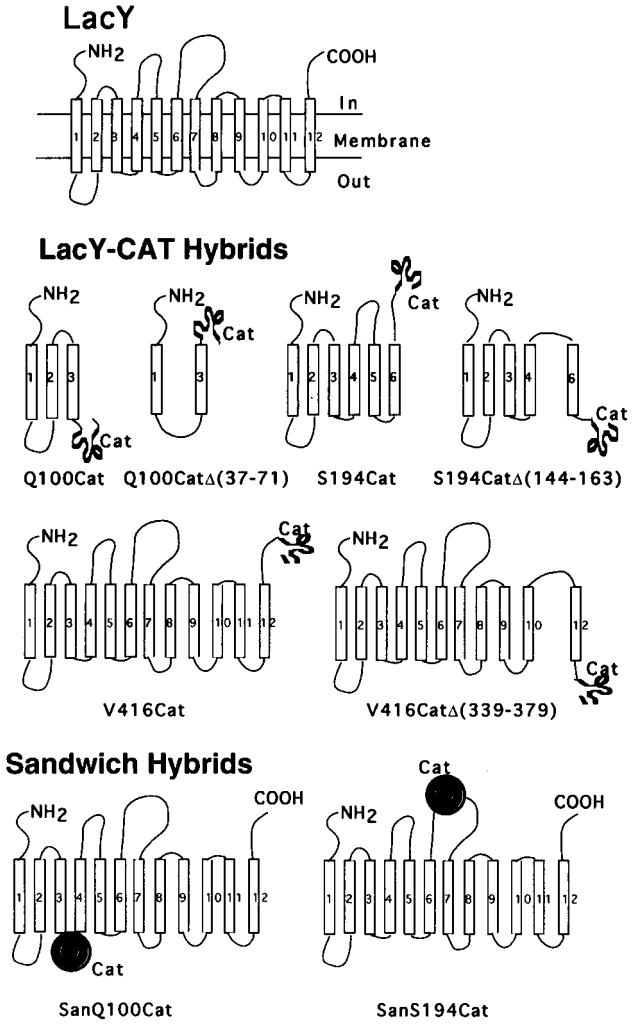

FIGURE 1: Schematic representation of the secondary structure model of LacY (upper panel), LacY−Cat hybrids (middle panel), and sandwich hybrids (lower panel).

of the transmembrane segment that is connected to the reporter and consequently the orientation of the reporter itself. The mutated hybrids are also presented schematically in Figure 1 and include Q100CatΔ(37−71), S194CatΔ(144−163), and V416CatΔ(340−376). Finally, to examine the ability of Cat to function when both its amino and carboxy termini are embedded within LacY, sandwich hybrids were constructed (Ehrmann et al., 1990). In these constructs, Cat was inserted into an intact LacY after Q100 (San100Cat) or S194 (San194Cat) (Figure 1). These specific insertion joints were selected on the basis of the previously described insertional mutagenesis studies on LacY (McKenna et al., 1992), where it was shown that six contiguous His residues can be inserted at these sites without a dramatic change in LacY activity. The protocols used for the DNA manipulations are described in Materials and Methods.

Expression of LacY−Cat Hybrids. Western blots were carried out using anti-Cat antibodies with membranes prepared from *E. coli* UTZ-harboring plasmids encoding given hybrid proteins. As frequently observed in gene fusion studies [cf. Sarsero and Pittard (1995) for a current example], the level of expression of the different hybrids is variable (Figure 2A). Fusions encoding a putative cytoplasmically oriented Cat [Q100CatΔ(37−71) and S194Cat] are expressed at somewhat higher levels (Figure 2A, lanes 2 and 3, respectively) than hybrids in which the reporter is expected

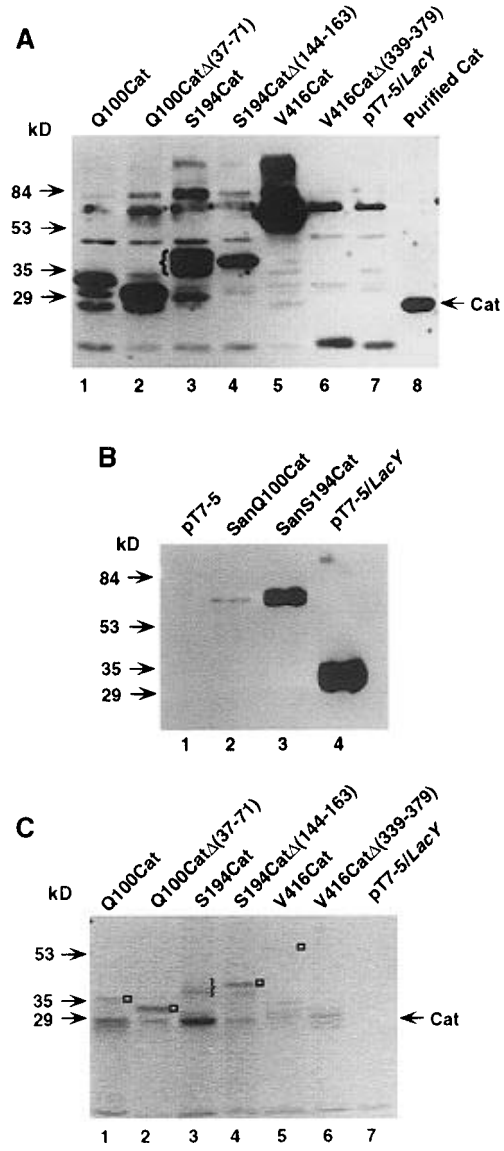

FIGURE 2: Expression of LacY−Cat hybrids. (A and B) Immunoblot of membrane fractions from *E. coli* UTZ harboring pTE*lacY-cat* constructs, pT7-5/*lacY*, and purified Cat (indicated by an arrow) after SDS−PAGE (12%). After electroblotting, the nitrocellulose paper was blocked and incubated with anti-Cat antibodies and with HRP-conjugated anti-rabbit antibodies. Immunoreactive species were detected by the ECL method. Hybrid S194Cat appears as a doublet indicated in lane 3. Prestained molecular mass standards are indicated by the arrows on the left. (C) Autoradiograph of whole cells labeled with [^{35}S]methionine after immunoprecipitation with anti-Cat antibodies and SDS−PAGE (12%). The full-length hybrids are indicated by white squares, and hybrid S194Cat is indicated as a doublet (lane 3, see also in panel A). Prestained molecular mass markers were used as indicated by the arrows on the left.

to cross the membrane [Q100Cat and S194CatΔ(144−163); Figure 2A, lanes 1 and 4, respectively]. Significantly, V416Cat, with the Cat moiety fused to the C-terminus of LacY, exhibits the highest expression level (V416Cat; Figure 2A, lane 5), while the same fusion deleted of transmembrane segment 11 is hardly detected by Western blot [V416CatΔ(340−376); Figure 2A, lane 6]. A similar expression pattern is observed with sandwich constructs of LacY and Cat as shown by Western blot experiments using antibodies directed against the C-terminal tail of LacY. When Cat is inserted into a putative external hydrophilic loop (San100Cat), the level of expression is drastically reduced (Figure 2B, lane 2) compared to that of a hybrid containing Cat in a putative cytoplasmic stretch of the permease (San194Cat; Figure 2B, lane 3). To study the stability of LacY−Cat hybrids, specific [^{35}S]methionine labeling [using the T7 RNA polymerase system (Tabor & Richardson, 1987)] and whole cell immunoprecipitations with anti-Cat antibodies were conducted. The *E. coli* T184 strain was used for labeling because it is *met*$^-$, and therefore allows efficient methionine starvation and subsequent specific labeling of the induced proteins. The results are shown in Figure 2C and also reveal in addition to the full-length hybrids a significant amount of protein species with an apparent molecular mass corresponding to the free Cat protein (Figure 2C, indicated by an arrow). In fact, hybrid V416CatΔ(339−379) is completely degraded (Figure 2C, lane 6). In this respect, LacY−Cat hybrids behave in a manner similar to that of alkaline phosphatase hybrids in various topology studies (Sarsero & Pittard, 1995). Importantly, the proteolysis pattern does not depend on the location of the fused Cat moiety with respect to the membrane. More specifically, hybrids with opposite orientations, Q100Cat (out) and S194Cat (in), exhibit similar and very intense proteolytic products (Figure 2C, lanes 1 and 3, respectively). A comparison between the Western blot (Figure 2A) and the immunoprecipitation (Figure 2C) experiments reveals significantly different patterns of intensities of the respective full-length hybrids. An extreme example is the relatively high expression of the full-length hybrid V416Cat observed in the Western blot experiment relative to the amount immunoprecipitated which is particularly diminished. Notably, however, the immunoblot experiments were performed with *E. coli* UTZ, a strain deficient in the outer membrane protease OmpT, which probably accounts for the higher expression levels observed on immunoblot.

Table 1: Chloramphenicol Resistance Assay on Plates

LacY−Cat hybrids	predicted Cat location		chloramphenicol[a] (μg/mL) 4	6	12	26
Q100Cat	out		+	−	−	−
Q100CatΔ(37−71)	in		+	+	+	−
S194Cat	in		+	+	+	−
S194CatΔ(144−163)	out		+	−	−	−
V416Cat	in		+	+	+	+
V416CatΔ(339−379)	out		+	+	−	−
LacY	−	(2+)	−	−	−	−
SanQ100Cat	out		+	−	−	−
SanS194Cat	in		+	+	−	−

[a] + indicates formation of single colonies after incubation overnight at 37 °C. [b] − no growth.

Chloramphenicol Resistance Experiments. Acetyl-CoA, the acetyl donor for Cat, is found in the cytoplasmic compartment. To test the idea that chloramphenicol acetyltransferase is able to confer chloramphenicol resistance only when fused to the cytoplasmic loops of LacY, antibiotic resistance assays were performed. Cells transformed with plasmids harboring the *lacY−cat* constructs were plated on chloramphenicol-containing LB agar plates, and their ability to form single colonies was tested at different chloramphenicol concentrations. As shown in Table 1, the putative cytoplasmically oriented hybrids S194Cat and V416Cat confer resistance to at least 12 and 26 μg/mL chloramphenicol, respectively. In contrast, the putative periplasmically oriented hybrid Q100Cat confers resistance to only a very low level of chloramphenicol (4 μg/mL). As expected, the corresponding deletion mutants of the hybrids exhibit opposite phenotypes. Hybrid Q100CatΔ(37−71) facilitates

growth on 12 μg/mL chloramphenicol, similar to the resistance achieved by the cytoplasmic hybrid, S194Cat. Accordingly, when the cytoplasmically oriented hybrids S194Cat and V416Cat are deleted of one transmembrane segment [S194CatΔ(144−163) and V416CatΔ(340−376)], they do not support growth on high levels of chloramphenicol (in contrast to their parental constructs S194Cat and V416Cat). These phenotypes are expected if the orientation of the Cat moiety is reversed due to the deletion of one transmembrane segment. The observations support the suggestion that Cat is able to confer resistance at higher concentrations of chloramphenicol only when it is exposed to the cytoplasm. Importantly, a similar chloramphenicol resistance pattern was observed when the sample was assayed in the same minimal medium which was used for [^{35}S]methionine labeling experiments.

When Cat is inserted as a sandwich in given sites of the permease, it is unable to confer significant chloramphenicol resistance. This conclusion is derived from the low and almost indistinguishable levels of resistance conferred by the sandwich constructs San100Cat and San194Cat (4 and 6 μg/mL chloramphenicol, respectively; Table 1). The small difference in resistance between these two sandwiches probably reflects their level of expression (Figure 2B), since San194Cat is expressed much better than hybrid San100Cat. A possible explanation for the observation that Cat is not functional when expressed as a sandwich inside LacY is that the enzyme cannot form its functional conformation or its trimeric complex. This interpretation is consistent with recent studies showing that the carboxy terminus of Cat is important for correct folding (Robben et al., 1993).

In Vitro Cat Assays. Hybrids Q100Cat, S194CatΔ(144−163), and V416CatΔ(340−376) do not confer resistance to high levels of chloramphenicol. A possible explanation for this phenotype is that outwardly directed Cat constructs are inactivated when inserted into the membrane, as observed with β-galactosidase (Lee et al., 1989). To examine this possibility, *in vitro* Cat assays were used to evaluate the total cellular acetyltransferase activity of the hybrids. Cat activities in extracts of *E. coli* UTZ cells expressing the LacY−Cat hybrids were tested using [^{14}C]chloramphenicol in the presence of acetyl-CoA or butyryl-CoA. After incubation, the products were analyzed by thin layer chromatography (Figure 3I). All of the hybrids exhibit significant and comparable Cat activity. This included hybrids harboring Cat fused to periplasmic domains of LacY (Figure 3I, lanes 1, 4, and 6). Kinetic studies of the Cat activity in the hybrids (not shown) support the conclusion that they exhibit comparable acetylation rates (compared to 0.02 unit of purified Cat, V416Cat exhibits the lowest rate, approximately 50%, and Q100Cat exhibits the highest rate, approximately 110%). It is possible however that the enzymatic activity observed in cell extracts reflects the activity of free Cat since a few hybrids appear to be unstable. Therefore, the activity of a subset of hybrids was tested also in isolated membrane preparations, where only intact hybrids exist. The results, shown in Figure 3II, demonstrate that the fused Cat is functional. Interestingly, the two external constructs, Q100Cat and V416CatΔ(340−376), exhibit higher levels of acetyltransferase activity than the corresponding cytoplasmic hybrids Q100CatΔ(37−71) and V416Cat, respectively (Figure 3II, compare lanes 1 and 4 to 2 and 3, respectively). To assure accessibility of the reagents (chloramphenicol and

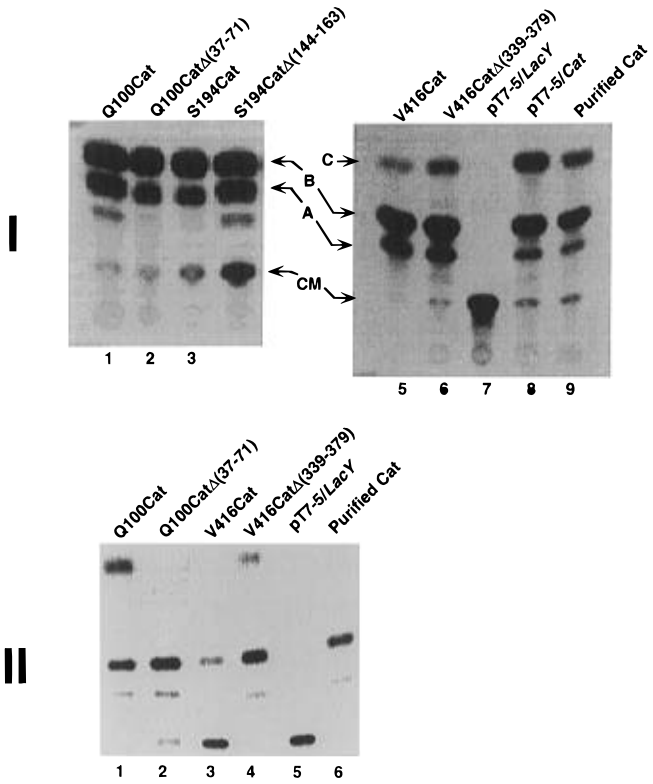

FIGURE 3: Chloramphenicol acetyltransferase assay with (I) whole cells and (II) membrane preparations. (I) Overnight cultures of UTZ cells harboring the different *lacY−cat* fusions, pT7-5/*Cat*, or pT7-5/*lacY* were resuspended in 50 mM potassium phosphate buffer (pH 7.5) containing 10 mM magnesium sulfate to an OD_{420} of 10. (II) Isolated membranes were prepared as described in Materials and Methods and suspended in the same buffer as in part I. Aliquots (130 μL) were subjected to Cat assays with [^{14}C]chloramphenicol and either butyryl-CoA (I, right panel) or acetyl-CoA (I, left panel, and II). [^{14}C]Chloramphenicol and its acylated forms were separated by TLC and detected by autoradiography as described in Materials and Methods. Commercial Cat (0.002 μg, 0.02 unit) was included as a control: CM, chloramphenicol; A, 1-*O*-acetyl- or butyryl-CM; B, 3-*O*-acetyl- or butyryl-CM; C, 1,3-*O*-dibutyryl-CM.

acetyl-CoA) to both sides of the membranes, the same experiments were performed in the presence of 0.1% Triton X100 which does not affect the Cat activity. The relative activities of the various hybrids (not shown) are essentially the same with or without the detergent. These observations thus rule out the possibility that Cat is inactivated by membrane integration during translocation and suggest that the periplasmic Cat hybrids are unable to confer chloramphenicol resistance due to their subcellular location, rendering acetyl-CoA inaccessible.

Topological Studies Using Limited Proteolysis. To obtain direct evidence for the location of Cat moieties in the hybrids and for the argument that the periplasmically oriented hybrids are in fact translocated across the plasma membrane, topological studies were conducted using limited proteolysis. Cells treated with EDTA and lysozyme (to permeabilize the outer membrane) were prepared from metabolically labeled cultures of *E. coli* T184 expressing a subset of the hybrid constructs. Proteinase K was added to the samples in the absence or in the presence of Triton X100. Proteinase K does not cross the intact membranes and therefore is expected to digest only periplasmic proteins. Under these conditions, Triton X100 permeabilizes the membranes, thus rendering cytosolic proteins accessible to proteinase K. After proteolysis was terminated, the solubilized pellets were immu-

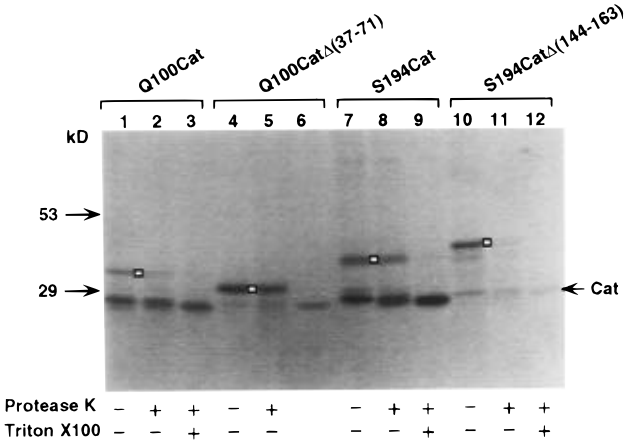

FIGURE 4: Proteolysis of the LacY−Cat hybrids. [^{35}S]Methionine-labeled T184 cells harboring the indicated LacY−Cat constructs were converted into spheroplasts as described. Equal portions of each sample were incubated with or without proteinase K, in the absence or in the presence of Triton Xx100 for 1 h on ice. Following proteolysis, proteins were immunoprecipitated with anti-Cat antibodies and the dry gel was autoradiographed. Full-length hybrids are indicated by white squares, and prestained molecular mass markers are indicated on the left.

noprecipitated with anti-Cat antibodies and analyzed by SDS−PAGE (shown in Figure 4). All the hybrids exhibit some degree of endogenous proteolytic instability in the absence of proteinase K and release significant but varying amounts of free Cat (Figure 4, see also Figure 2C). Relatively high levels of free Cat (indicated by an arrow in Figure 4) are seen with hybrids Q100Cat and S194Cat (Figure 4, lanes 1 and 7). The deleted hybrids Q100CatΔ(37−71) and S194CatΔ(144−163) release only small amounts of free Cat (Figure 4, lanes 4 and 10). Of the full-length products, only the putative periplasmic hybrids Q100Cat and S194CatΔ(144−163) are significantly degraded by proteinase K in the absence of Triton X100 (Figure 4, lanes 2 and 11). The cytoplasmically oriented hybrids Q100CatΔ(37−71) and S194Cat are significantly more resistant to proteinase K under these conditions (Figure 4, lanes 5 and 8) but undergo complete cleavage in the presence of Triton X100 (Figure 4, lanes 6 and 9). These results are in good agreement with previous topological studies of LacY (Calamia & Manoil, 1990) and demonstrate that Cat is translocated to the periplasm when fused to periplasmic loops of the permease.

DISCUSSION

Chloramphenicol acetyltransferase derived from transposon Tn9 represents a large family of prokaryotic proteins responsible for chloramphenicol resistance. The intracellular enzyme appears as a noncovalent homotrimer, and the protein folds rapidly to form the stable functional species in the cytoplasm (Kim & Kang, 1991). Interestingly, the enzyme also folds correctly and rapidly in many heterologous expression systems, including plant and mammalian cells. This property makes Cat a useful reporter in studies of gene expression in prokaryotic and in eukaryotic systems. In this study, we demonstrate that Cat can be useful also as a sensor for membrane protein topology in *E. coli*. The major advantage of Cat over β-galactosidase, which has been used as a cytoplasmic reporter, is that, like its periplasmic counterpart, β-lactamase, the orientation of Cat can be determined by its ability to confer antibiotic resistance *in vivo*. We show that, in contrast to the outwardly oriented β-galactosidase which undergoes only partial membrane integration and might alter the integrity of the plasma membrane, externally oriented Cat is fully translocated into the periplasm. Of importance for its use in gene fusion studies is the finding that Cat is active on both sides of the membrane but confers resistance to chloramphenicol only in its cytosolic form, while alkaline phosphatase and β-galactosidase are active only on one side of the membrane.

In previous attempts to translocate Cat across the membrane, it was suggested that Cat cannot be secreted. For example, when fused to bacterial signal sequences, Cat does not cross the cytoplasmic membrane of *Bacillus subtilis* (Chen & Nagarajan, 1993). It was proposed that its rapid folding into a stable, translocation-incompetent state prevents successful interaction of Cat with the translocation apparatus. In this study, however, it is demonstrated that, when fused to putative periplasmic loops of LacY, the enzyme is translocated across the membrane and exhibits acetyltransferase activity and that in the periplasmic orientation Cat is unable to confer chloramphenicol resistance. We suggest that, when Cat is fused to a signal sequence of a secreted protein, it is posttranslationally targeted to the membrane, but unlike secretory proteins, Cat is unable to retain a translocation-competent conformation and remains in the cytoplasm. In contrast, when Cat is fused to an integral membrane protein which might utilize a cotranslational pathway for membrane insertion, it is then translocated to the periplasm [see also Xie and Morimoto (1995)]. Thus, membrane insertion of N-terminal LacY polypeptides and the subsequent translocation of the Cat moiety may be coupled so that the initiation of the translocation of Cat is cotranslational. In this manner, the Cat polypeptide is prevented from folding prior to translocation across the membrane. In addition to its use in topology studies, Cat fusions may serve as antibiotic selection markers in screening for *E. coli* mutants defective in membrane protein biogenesis. For this purpose, screening for resistant clones among chloramphenicol sensitive *E. coli* cells harboring any of the outwardly directed LacY−Cat fusions may lead to identification of assembly defective mutants (temperature sensitive or not). Using a similar approach but with another reporter, the secretory protein alkaline phosphatase, Akiyama et al. (1994) identified mutants carrying a defective membrane protease, FtsH. The use of an antibiotic resistance marker may improve the screening efficiency in order to reveal relevant mutations in cellular components which take part in the biogenesis of membrane proteins.

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