

TnTIN and TnTAP: Mini-transposons for site-specific proteolysis *in vivo*

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ABSTRACT Tobacco etch virus (TEV) protease recognizes a 7-aa consensus sequence, Glu-Xaa-Xaa-Tyr-Xaa-Gln-Ser, where Xaa can be almost any amino acyl residue. Cleavage occurs between the conserved Gln and Ser residues. Because of its distinct specificity, TEV protease can be expressed in the cytoplasm without interfering with viability. Polypeptides that are not natural substrates of TEV protease are proteolyzed if they carry the appropriate cleavage site. Thus, this protease can be used to study target proteins in their natural environment *in vivo*, as well as *in vitro*. We describe two Tn5-based mini-transposons that insert TEV protease cleavage sites at random into target proteins. TnTIN introduces TEV cleavage sites into cytoplasmic proteins. TnTAP facilitates the same operation for proteins localized to the bacterial cell envelope. By using two different target proteins, SecA and TolC, we show that such modified proteins can be cleaved *in vivo* and *in vitro* by TEV protease. Possible applications of the site-specific proteolysis approach are topological studies of soluble as well as of inner and outer membrane proteins, protein inactivation, insertion mutagenesis experiments, and protein tagging.

Proteases are important tools in molecular biology. Until recently, however, they have been primarily applied to *in vitro* studies. To extend the use of proteases, we showed in a previous paper that the site-specific tobacco etch virus (TEV) protease can be used to selectively cleave an essential cytoplasmic protein *in vivo* (1).

To facilitate the generation of TEV protease recognition sites into proteins, we have constructed two mini-transposons that can insert into a gene to generate “sandwich” fusions of a short DNA fragment encoding the TEV protease cleavage site and the target gene that remains otherwise intact. These mini-transposons are derivatives of Tn5 that generate stable inserts, because Tn5 transposase is expressed in trans. To detect inserts in which the ORF of target gene and of TEV protease recognition sequence match, the transposons carry the bacterial reporter genes *uidA* or signal sequenceless *phoA*, both lacking a promoter and translation initiation signals. *uidA* encodes cytoplasmic β -glucuronidase (2). *phoA* encodes periplasmic alkaline phosphatase, which must be exported to the periplasm to be active (3). Signal sequenceless *phoA* is the most widely used tool for generating fusions to cytoplasmic membrane proteins for studying membrane protein topology (4). The presence of *uidA* and *phoA* fusions can be easily monitored on agar indicator plates containing specific dyes, i.e. 5-bromo-4-chloro-3-indolyl- β -D-glucuronide (X-GLUC) (5) and 5-bromo-4-chloro-3-indolyl-phosphate, respectively. A related strategy was applied recently by Manoil and Bailey (6) in the construction of a set of transposons that insert 31 codons into target genes. Here, fusions to *lacZ* and *phoA* are generated

to detect insertions into the reading frame of the target gene. These transposons have been shown to be useful for structure-function studies of LacY, LacI, and MalK (6–8).

TnTIN was used to introduce protease recognition sites into SecA, an essential cytoplasmic protein involved in secretion (9). One insert, located after amino acid 830, left SecA function intact. On coexpression of TEV protease, cleavage of this SecA derivative occurred *in vivo*. In addition, the purified SecA derivative was cleaved by TEV protease *in vitro*.

TnTAP was used to insert protease sites into the outer membrane protein TolC. TolC is involved in the uptake of antibiotics and colicins and in the export of the virulence factor hemolysin (10–12). TolC derivatives carrying TEV site insertions after amino acids 79, 157, and 306 were cleaved after adding TEV protease to whole cells.

MATERIALS AND METHODS

Bacteria and Plasmids. *Escherichia coli* strains used are: MM52 [$F^- \Delta lacU169 araD136 rbsR relA rpsL thi secA(Ts)$] (13); ME7000 (*rpsL* $\Delta(add-uid-man) recA::cam$); CC118 (*araD139* $\Delta(ara, leu)7697 \Delta lacX74 phoA\Delta 20 galE galK thi rpsE rpoB argE_{am} recA1$) (14); and MM118 (CC118 *tolC recA::cam pcnB80 zid::Tn10*) (15).

pDHB60 is a high copy number pBR322 derivative. It was derived from pDHB32 (16) by replacement of the *malFG'* insert with the polylinker: GAATTCGAGCTCGCCCGGGGATCCTCTAGAGTCGACCTGCAGCCCAAGCTT. pMM70 encodes the wild-type *tolC* gene under its own promoter from pBR322. pMF8 expresses wild-type *secA* under its own promoter from pBR322 (17).

Construction of TnTIN. Oligonucleotides containing a *Bam*HI site at the 5' end, the coding sequence for the TEV protease recognition sequence, internal *Not*I, *Nco*I, *Eco*RI, *Sal*I, and *Not*I restriction sites, and a *Hind*III site at the 3' end were annealed and filled in by using Klenow enzyme. Oligonucleotides used were: CGGATCCTGACTCTTATACACAAGTTTGAAAACCTGTACTTCCAGTCAGCGGCCGCCATGGAATTGAATTCGTC and CCCAAGCTTC-TGACTCTTATACACAAGTATTGCGGCCGCGTCGACGAATTCAATTCCATGGCG. Subsequently, the double-stranded DNA was cut with *Bam*HI and *Hind*III and then ligated into pDHB60 cut with *Bam*HI and *Hind*III to yield pPB201. The sequence of the insert was verified by nucleotide sequencing. The *E. coli uidA* gene was moved as a 1.9-kb *Nco*I-*Eco*RI fragment from pGUS N358- \rightarrow S (CLONTECH) into pPB201 that was cut with *Nco*I and *Eco*RI, yielding pPB202. Finally, the *neo* gene conferring kanamycin resistance

Abbreviations: TEV, tobacco etch virus; X-GLUC, 5-bromo-4-chloro-3-indolyl- β -D-glucuronide; OE, outside end.

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was cloned as a 1.2-kb *SalI* fragment from pUC4K (from Pharmacia) into pPB202 cut with *SalI*, yielding pPB203.

Transposition of TnTIN into a pACYC184 Derivative Containing Tn5 Transposase. To clone Tn5 transposase into pACYC184 (18), pRZ7016MA.EK.LP (19) was cut with *EcoRI* and *NcoI*. The transposase-containing fragment was cloned into pACYC184 cut with *EcoRI* and *NcoI*, yielding pPB101. To allow transposition of TnTIN into pPB101, pPB101 and pPB203 were transformed into strain ME7000. During growth overnight, TnTIN transposed into pPB101. Plasmid DNA was prepared and digested with *DraIII* and *NdeI*. These restriction enzymes cleave pPB203, but not pPB101 or TnTIN. This restriction digest was transformed into strain ME7000. Selection for Tet^r (from pPB101) and Kan^r (from TnTIN) yielded strains containing plasmids where TnTIN transposed into pPB101. This event was verified by restriction mapping. One candidate was chosen and termed pPB301.

Construction of TnTAP. To construct TnTAP, a signal sequenceless *phoA* gene lacking its stop codon was cloned from pSWFII (20) as a 1.2-kb *BamHI*-*BstEII*-digested fragment filled in with Klenow, into pPB203, which was cut with *NcoI* and *EcoRI* and then filled with Klenow, yielding pRL1. Because this *phoA* fragment contained an extra set of 19 bp outside end (OE) of Tn5, the extra OE had to be removed. For this purpose, a fragment lacking the extra 19-bp inverted repeat was generated via PCR by using the following oligonucleotide: TTCGGTACCTTGCCCTGTTCTGGAAA-ACCG. This primer starts just downstream of the extra 19-bp inverted repeat of the *phoA* fragment and has a *KpnI* restriction site at its 5' end. The second primer, CATCCTGCAACTCTGCGGTAGAAACG, is located downstream of the *BssHII* site in *phoA*. The 458-bp PCR product was cut with *KpnI* and *BssHII* and ligated into pRL1 cut with *KpnI* and *BssHII*, yielding pMM100.

Subsequently, TnTAP was transposed into pPB101 by following the same procedure as given above for TnTIN, yielding pMM1.

Transposition of TnTIN into *secA* and TnTAP into *tolC*. To isolate in-frame insertions of TnTIN into *secA*, pMF8 containing the wild-type *secA* gene first was transformed into strain ME7000 carrying pPB301. During growth overnight, TnTIN transposed into pMF8. Plasmids were purified and digested with *BstEII* and *XbaI*, which cleave pPB301, but not pMF8 or TnTIN. The restriction digest was transformed into strain ME7000. Selection for Amp^r (from pMF8) and Kan^r (from TnTIN) yielded strains containing plasmids where TnTIN transposed into pMF8. These strains were tested for insertions of TnTIN into the *secA* gene, which resulted in in-frame fusions of *uidA* to *secA* by plating on agar plates containing 40 μ g/ml X-GLUC. Blue colonies (UidA⁺) were purified, and the expression of *secA-tev-uidA* fusions was tested by Western blotting that used polyclonal antibodies against SecA.

To isolate in-frame fusions of TnTAP to *tolC*, pMM70 containing the wild-type *tolC* gene first was transformed into strain MM118 carrying pMM1. After growth overnight, plasmid DNA was isolated and digested with *NheI* to destroy pMM1, but not pMM70 or TnTAP. The restriction digest was transformed into strain MM118. Selection for Amp^r (from pMM70) and Kan^r (from TnTAP) yielded strains containing plasmids where TnTAP transposed into pMM70. These strains were tested for the insertion of TnTAP into the *tolC* gene, which resulted in in-frame fusions of *phoA* to *tolC* by plating on agar plates containing 40 μ g/ml of 5-bromo-4-chloro-3-indolyl-phosphate. Blue colonies (PhoA⁺) were purified, and the expression of *tolC-tev-phoA* fusions was tested by Western blotting that used polyclonal antibodies against TolC.

Deletion of *uidA* (or *phoA*) and *neo* from TnTIN (or TnTAP) Inserts in *secA* (or *tolC*). Plasmids containing TnTIN (TnTAP)

inserts in *secA* (*tolC*) were cut with *NotI* and religated, deleting *uidA* (*phoA*) and *neo*. The remaining insert of 72 bp encodes LTLIHKFENLYFQSAAAAILVYKSQ. TEV protease recognition sequence is ENLYFQS. The resulting plasmids were verified by restriction analysis. Expression of SecA (TolC) proteins containing the desired insert was detected on Western blots, because these derivatives migrated at slightly higher molecular weight on 7% (10%) SDS/PAGE.

Sequencing of Fusion Joints Generated by TnTIN and TnTAP. Fusion joints were sequenced by using primers TTCACGGGTTGGGGTTTCTACAG and GCAGTA-ATATCGCCCTGAGCAGC reading out of the reporter genes *uidA* and *phoA* into the target gene, respectively.

Expression of TEV Protease from a pACYC184 Derivative. We wanted to express TEV protease under *tac* promoter control as a glutathione *S*-transferase (GST) hybrid from a pACYC184 derivative that is compatible with the pBR322-derived *secA* plasmids. For this construct, pGEX2T-27K (21) was digested with *ApaI* and *BspHI* filled in with Klenow. This 3-kb fragment, containing *Ptac* and the GST-TEV protease fusion was ligated into a derivative of pACYC184 (that contained the *lacI^q* gene inserted into the *EcoRI* site) cut with *XmnI* and *ApaI* to yield pMM13.

Proteolysis of SecA Containing TEV Protease Cleavage Site *in Vivo* and *in Vitro*. pME410 was transformed into strain ME7000 containing pMM13. Cells were grown with and without 100 μ M isopropyl β -D-thiogalactoside used to induce expression of TEV protease. Cleavage of SecA was detected by Western blotting.

SecA410 containing a TEV protease cleavage site after amino acid 830 was purified by a method to be described elsewhere. One microgram of purified SecA in proteolysis buffer (50 mM Tris-HCl, pH 8.0/0.5 mM EDTA pH 8.0) was incubated in the presence of 5 mM DTT (final concentration) and 1 μ l TEV protease (1 mg/ml stock solution from GIBCO/BRL). After 3 hr at 30°C, proteolysis was stopped by addition of 100 mM iodoacetamide (final concentration). Proteolysis of SecA was detected on SDS/PAGE followed by staining with Coomassie blue (22).

Proteolysis of Whole Cells. Cells expressing TolC containing TEV protease sites were grown in Luria-Bertani medium (23) to an OD₆₀₀ of 0.3. Cells were washed once in proteolysis buffer (see above). For proteolysis, 5 mM DTT and 2.5 μ l TEV protease (70 μ g/ml final concentration) was added to 42.5 μ l cells. After 4 hr at 30°C, 100 mM iodoacetamide was added to inactivate TEV protease. After precipitation with trichloroacetic acid, samples were subjected to SDS/PAGE and Western blotting by using polyclonal antibodies against TolC (24, 25).

RESULTS

Construction of TnTIN and TnTAP. We have constructed a set of mini-transposons that introduce TEV protease cleavage sites into target proteins (Fig. 1). The basic architecture of TnTIN is: the 19-bp OE of Tn5 required for transposition, a 7-codon TEV protease cleavage site, *uidA*, *neo*, and another 19-bp OE of Tn5. TnTAP is basically the same except that *uidA* was replaced by signal sequenceless *phoA*. The Tn5 transposase, which acts on the flanking 19-bp OE sequences, is supplied in trans, generating stable insertions. When inserted in the correct orientation and reading frame, the transposons form translational fusions with the target gene. This protein fusion includes a 5' portion of the target gene, TEV protease cleavage site, and either *uidA* or *phoA*. These translational fusions can be detected on indicator plates containing specific dyes, i.e., X-GLUC for *uidA* fusions and 5-bromo-4-chloro-3-indolyl-phosphate for *phoA* fusions. TnTIN is used for cytoplasmically localized target proteins, TnTAP for cell envelope proteins. The transposons carry the *neo* gene, conferring to

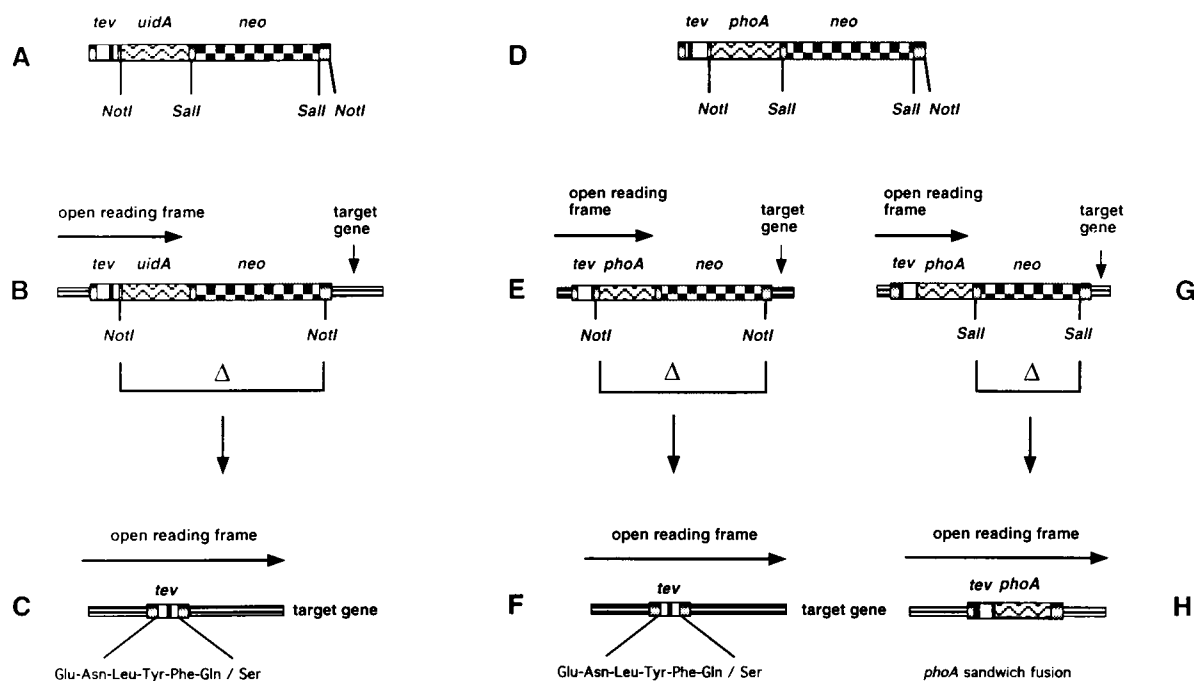


FIG. 1. Structure and use of TnTIN and TnTAP. *tev* represents TEV protease cleavage site, *uidA* encodes β -glucuronidase, *phoA* encodes signal sequenceless alkaline phosphatase, and *neo* confers to kanamycin resistance. (A) Architecture of TnTIN. (B) TnTIN after insertion into a target gene. When inserted in the correct orientation and reading frame, the reading frame of the target gene extends into the *tev* site and *uidA*, resulting in a hybrid gene that encodes a hybrid protein composed of an N-terminal part of the target protein, TEV protease cleavage site, and β -glucuronidase. (C) Digestion by *NotI* removes the *uidA* and *neo* genes, generating a sandwich fusion of the 72-bp TEV protease cleavage site within the target gene. (D) Architecture of TnTAP. (E and G) TnTAP after insertion into a target gene. When inserted in the correct orientation and reading frame, the reading frame of the target gene extends into the *tev* site and *phoA*, resulting in a hybrid gene that encodes a hybrid protein composed of an N-terminal part of the target protein, TEV protease cleavage site, and signal sequenceless alkaline phosphatase. (F) Digestion by *NotI* removes the *uidA* and *neo* genes, generating a sandwich fusion of the 72-bp TEV protease cleavage site within the target gene. (H) Digestion by *SalI* removes the *neo* gene, generating a sandwich fusion between the target gene and *phoA*. The TEV protease site is present at the N terminus of signal sequenceless alkaline phosphatase.

kanamycin resistance, which can be used to select for cells carrying transposon insertions. Both transposons carry *NotI* restriction sites after the codons for TEV protease site and after *neo*. *NotI* deletions remove *uidA* or *phoA* and *neo*, leaving a 72-bp insert in an otherwise intact target gene. We chose the 8-bp cutter *NotI*, because this restriction site occurs very rarely in target genes. The insertion generated by both transposons is identical: LTLIHKFENLYFQSAAAILVYKSQ. The TEV protease recognition sequence is ENLYFQS.

An additional feature of TnTAP is that after a *SalI* deletion, the *neo* gene is removed, leaving a sandwich fusion of *phoA* and the target gene. This fusion occurs because the *phoA* gene lacks a stop codon so that the reading frame at the 3' end of *phoA* remains open and extends into the 3' end of the target gene. Thus, TnTAP is the first transposon that generates *phoA* sandwich fusions at random. To date, *phoA* sandwich fusions could be constructed only by using a cloning vector described earlier (20). The use of TnTAP for construction of *phoA* sandwich fusions will be described separately.

TnTIN Insertions into the *secA* Gene. To test the function of TnTIN, we obtained derivatives of *secA* plasmid pMF8 into which the transposon had inserted. Initially, pMF8 and pPB301 were transformed into strain ME7000 ($\Delta uid recA$). Twenty-four Kan^r colonies, which were blue on agar plates containing X-GLUC, were analyzed. Blue color on X-GLUC plates indicated the presence of a translational fusion of *uidA* to an ORF of pMF8. Western blots of these colonies with antibodies against SecA indicated that nine candidates had insertions within the *secA* gene (data not shown). Nucleotide sequencing indicated that we obtained fusions after codons 351 and 830 of SecA, respectively. Fusions to codon 830 expressed a large *secA*-TEV site-*uidA* fusion migrating at about 150 kDa on SDS/PAGE (Fig. 2). One of these candidates, termed *secAT-*

nTIN410, was chosen for further characterization.

Characterization of *secA*-TEV Site-*uidA* Fusions. Plasmids containing *secA*-TEV site-*uidA* fusions to codon 830 were digested with *NotI* and religated, yielding pME410. Deletion of *uidA* and *neo* generated a 24-codon insertion containing the TEV protease cleavage site. The presence of the insertion was detectable on SDS/PAGE because this SecA derivative, termed SecA410, migrated at slightly larger molecular weight than wild-type SecA. To test if TEV protease could cleave this

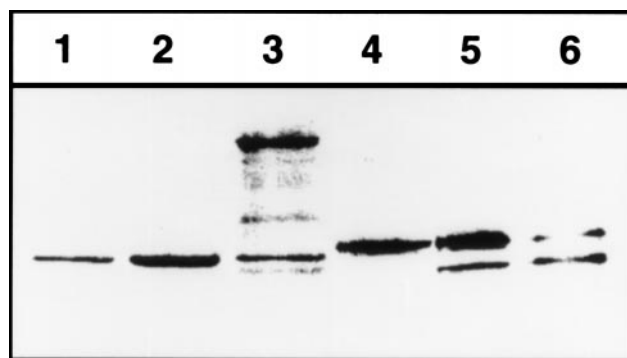


FIG. 2. Proteolysis of SecA410 *in vivo*. To monitor cleavage of SecA410 by TEV protease, a Western blot was prepared by using cells of ME7000 expressing either wild-type SecA (lane 1), wild-type SecA expressed from pMF8 (lane 2), *secATnTIN410* (lane 3), SecA410 resulting from a cutback of TnTIN with *NotI* (lane 4), or SecA410 plus TEV protease expressed from pMM13 (lanes 5 and 6) after overnight growth in Luria-Bertani. The growth medium was without (lane 1-5) or supplemented with 100 μ M isopropyl β -D-thiogalactoside (lane 6) to induce TEV protease. To detect SecA, polyclonal antibodies against SecA were used.

SecA construct, pMM13 expressing TEV protease under *ptac* control was transformed into strain ME7000 containing pME410, expressing the *secA*-TEV cleavage site-*secA* sandwich fusion. Coexpression of TEV protease led to cleavage of SecA, indicating that the TEV protease recognition sequence was surface-exposed in the functional SecA dimer (see below) (Fig. 2). This finding agrees with the model that the C-terminal 70-aa residues of SecA are involved in lipid binding as well as SecB binding and therefore should be surface accessible (26). The efficiency of cleavage was $12 \pm 15\%$ when expression of TEV protease from the *tac* promoter was uninduced and $80 \pm 10\%$ when expression of TEV protease was induced by addition of isopropyl β -D-thiogalactoside to the growth medium (Fig. 2). Because in pMF8 expression of SecA is under the control of the wild-type promoter, incomplete cleavage of SecA might be because of its autoregulation: SecA expression is derepressed when protein secretion is not fully functional (9). This explanation is supported by the finding that the purified SecA410 was cleaved more efficiently by TEV protease *in vitro* (Fig. 3).

To initially test to see if the insertion of the TEV protease site before and after cleavage by TEV protease would interfere with SecA function, we transformed pME410 and pMM13 into strain MM52 (*secATs*). MM52 does not grow at 42°C because of its conditional SecA defect. MM52 expressing the plasmid-encoded SecA410 derivative complemented the *secA*(Ts) allele and grew well at 42°C. Thus, SecA function was not compromised significantly by the insertion containing the TEV protease cleavage site. However, when TEV protease was coexpressed, the *secATs* mutation was no longer complemented as was indicated by the inability of the strain to grow on Luria-Bertani agar plates at 42°C.

Proteolysis of Purified SecA by TEV Protease *in Vitro*. Because proteolysis of SecA occurred *in vivo*, we wanted to examine if similar results could be obtained when using purified SecA and TEV protease. SecA containing the TEV protease site after amino acid 830 was purified. Addition of purified TEV protease resulted in efficient cleavage of SecA (Fig. 3).

TnTAP Insertions into the *tolC* Gene. To test the function of TnTAP, we used the outer membrane protein TolC as a target. As with TnTIN insertions into *secA*, we obtained derivatives of *tolC* plasmid pMM70 into which the transposon had inserted. After analyzing 180 Kan^r colonies, which were blue on agar plates containing 5-bromo-4-chloro-3-indolyl-phosphate, we isolated 115 fusions in which the TEV protease cleavage site was in the *tolC* reading frame. Sequencing of the fusion joints indicated that TnTAP inserted at 50 different positions in *tolC*. Five candidates were chosen for further characterization. Deletion of *phoA* and *neo* yielded *tolC*-TEV site-*tolC* sandwich fusions. Adding TEV protease to whole cells led to cleavage of TolC derivatives containing TEV protease sites after amino acids 79, 157, and 306. No cleavage occurred when the protease sites were inserted after amino acids 148 and 332 (Fig. 4). The TolC derivatives were expressed

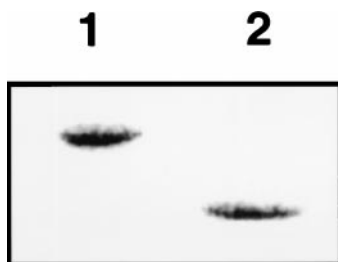


FIG. 3. Proteolysis of SecA410 *in vitro*. Cleavage of SecA by TEV protease *in vitro* was monitored by 7% SDS/PAGE. Lane 1, 1 μ g of purified SecA410. Lane 2, 1 μ g of purified SecA410 after incubation with 1 μ g of TEV protease for 3 hr at 30°C.

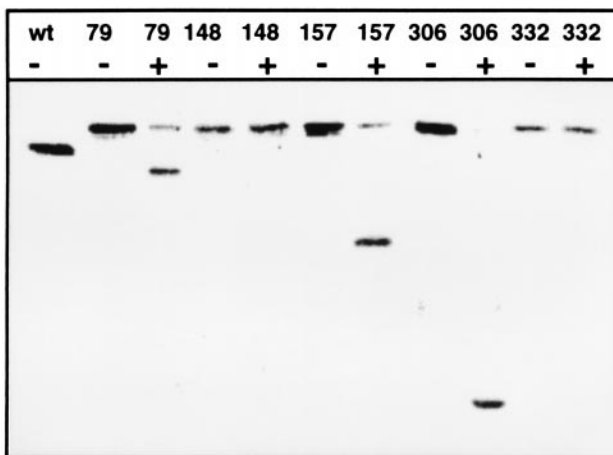


FIG. 4. Proteolysis of TolC derivatives on the extracellular surface of whole cells. Cleavage of TolC carrying TEV protease sites was monitored by Western blotting. TEV protease was added to intact cells for 3 hr at 30°C. After inactivation of TEV protease, cells were suspended in sample buffer and subjected to SDS/PAGE. wt, wild-type TolC. Numbers indicate the amino acid of TolC after which a TEV protease site was inserted. -, no TEV protease added to whole cells. +, TEV protease (70 μ g/ml final concentration) added to whole cells.

in *pcnB* mutant strain MM118. *pcnB* decreases the plasmid copy number of ColE1 ori plasmids (15), thus reducing the problem of overexpression. To obtain evidence for the functionality of the constructed TolC derivatives, we tested growth on MacConkey plates. TolC is known to confer to resistance against hydrophobic agents such as bile salts (27). Growth on MacConkey plates containing 0.15% bile salts is a convenient monitor of TolC function. Cells expressing TolC derivatives containing TEV protease sites after amino acid 79 and 306 grew as well as the wild type. Inserts after amino acid 157 and 332 conferred to slightly reduced growth, whereas the insert after amino acid 148 was apparently nonfunctional because no growth on MacConkey plates could be detected. In addition, we wanted to test to see if the expression of these TolC derivatives had a negative effect on the integrity of the outer membrane. Thus, we investigated hypersensitivity against the antibiotics rifampicin and chloramphenicol on Luria-Bertani agar plates containing 1, 5, and 50 μ g/ml rifampicin and 0.1 and 1 μ g/ml chloramphenicol. Because growth of cells expressing the TolC derivatives was essentially the same as that of wild-type cells, we concluded that the outer membrane was not hyperpermeabilized by the TolC constructs.

DISCUSSION

We describe two mini-transposons, TnTIN and TnTAP, that fuse a short insert of 24 codons into target genes. The insert encodes the cleavage site of TEV protease that can proteolyze target proteins *in vivo*, *in vitro*, or on the extracellular side of whole cells when the recognition sequence is surface accessible. We showed that this site-specific proteolysis approach can be applied to soluble cytoplasmic as well as to integral outer membrane proteins. Because only surface-exposed TEV protease cleavage sites are proteolyzed, this approach can be used in topological studies. The insertion and cleavage of TEV protease sites may represent a novel tool for studying the topology of outer membrane proteins. Experiments currently are being carried out to test this idea.

The 24-codon insertions can be used to detect regions in proteins tolerating additional sequence, which may provide helpful information for protein engineering and structure-function studies. Sites that can accommodate the 24-aa inser-

tion without loss of activity can be used to inactivate essential proteins *in vivo* by site-specific proteolysis via coexpressed TEV protease. This approach may serve as an alternative or addition to existing methods, e.g., conditional lethal or suppressible nonsense mutations and dilution experiments. It remains to be seen, however, what fraction of all possible inserts in permissive sites will yield proteins that are effectively inactivated, either as a primary or secondary consequence of cleavage. Examples for secondary consequences of cleavage are dissociation of proteolytic fragments and/or further degradation of these fragments by cellular proteases. For inactivation of essential proteins, the experimental system can be optimized such that TEV protease is under control of the tight arabinose promoter (28) to minimize cleavage of the target protein when expression of TEV protease is not induced. When, as in the case of SecA, the expression of the target protein is autoregulated by using a foreign promoter, e.g., the *lac* promoter, will exclude increased expression after inactivation of the target protein.

The transposons provide a technically simple and rapid alternative to inserting protease cleavage sites via linker or oligonucleotide mutagenesis. Another advantage is that transposition occurs rather randomly, as was detected when sequencing 115 insertions into the *tolC* gene. Random insertions may be desired for searching permissive sites in target proteins for which detailed structural information is not available.

Because TEV protease can be actively expressed in yeast cells without affecting viability (29), site-specific proteolysis may be applied in eukaryotes and cell cultures as well. Also, because the use of Tn5 is not restricted to *E. coli*, and *uidA* is commonly used as a reporter gene in plant molecular biology, we anticipate general applicability.

We thank Jon Beckwith for challenging us, many years ago, to devise an *in vivo* selection for *E. coli* mutants defective in membrane protein insertion. Our efforts have directly resulted in this method. We thank J. Barondess for discussions, R. Benz and I. Gentschev for antibodies to TolC and bacterial strains, Kelly Hughes for reading the manuscript, D. Oliver for a *secA* plasmid, K.-L. Schimz for antibodies to SecA, W. Reznikoff for transposase plasmids, Kate Wilson for strains and discussions, and C. Manoil and B. Traxler for sharing unpublished results. We are grateful to W. Boos in whose laboratory part of this work was carried out and to D. Pette and SFB156 for support. This work was supported by a grant from the Deutsche Forschungsgemeinschaft. D.B. was supported by National Institutes of Health Grants GM54160 and GM41883, and M.M. was supported by a fellowship from the Studienstiftung des deutschen Volkes.

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