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NOTES

Chloramphenicol Acetyltransferase, a Cytoplasmic Protein Is Incompatible for Export from *Bacillus subtilis*

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Bacillus subtilis **cells expressing a hybrid protein (Lvs_{ss}-Cat) consisting of the *B. amyloliquefaciens* levansucrase signal peptide fused to *B. pumilus* chloramphenicol acetyltransferase (Cat) are unable to export Cat protein into the growth medium. A series of tripartite protein fusions was constructed by inserting various lengths of the Cat sequences between the levansucrase signal peptide and staphylococcal protein A or *Escherichia coli* alkaline phosphatase. Biochemical characterization of the various Cat protein fusions revealed that multiple regions in the Cat protein were causing the export defect.**

One of the interesting problems related to the mechanism of protein secretion is determining whether there are any intrinsic structural differences between proteins that cross membranes (e.g., secreted proteins) and intracellular proteins that normally do not cross membranes. Attempts to secrete certain intracellular proteins in *Escherichia coli*, including β-galactosidase, chicken triosephosphate isomerase, human superoxide dismutase, and dihydrofolate reductase (3, 7, 16–18, 19), have been reported. We wanted to determine whether *Bacillus pumilus* chloramphenicol acetyltransferase (Cat86) can be secreted from *B. subtilis* when fused to a signal peptide. *B. pumilus* Cat was used as a model primarily because of its small size. In addition, certain structural features of Cat can be inferred from amino acid sequence homology (66%) with *E. coli* Cat, whose structure has been solved at 1.8-Å (0.18-nm) resolution (8). Cat, a trimeric protein, is 220 amino acids in length and requires acetyl coenzyme A as a cofactor for activity. The effect of the Cat sequence on protein export was studied by inserting various lengths of Cat protein between the levansucrase (Lvs) signal peptide and staphylococcal protein A (Spa) or *E. coli* alkaline phosphatase (PhoA). The Lvs signal peptide can efficiently transport Spa and PhoA across the *B. subtilis* membrane (11, 12). In this paper, we report that Cat protein contains multiple sequences that are unable to cross the *B. subtilis* membrane.

B. subtilis BG4103 (Δ*sacB trpC2*), constructed by Dennis Henner, was the host. Plasmids pBE60, pBE504, pBE45, pBE311, and pBE597 have been described elsewhere (11). The various constructs described in Table 1 are all derivatives of shuttle vector pBE60. The *B. pumilus cat* gene was derived from pPL703 (4). Plasmid pBE550 contains the *cat* gene in pBE60 with an *Eco*RV restriction site between codons 2 and 3 of Cat (4). Single-stranded DNA from pBE554 (Lvs_{ss}-Cat) was used as the template for site-directed mutagenesis to introduce an *Msc*I (TGGCCA) restriction site at various positions in the *cat* gene. The *cat-spa* and *cat-phoA* gene fusions were made by replacement of a *cat* fragment (*Msc*I-*Pst*) with a *spa* or *phoA* fragment (*Eco*RV-*Pst*) from pBE45 or pBE597 (11). The fusion junctions were verified by DNA sequencing.

To identify whether a particular region in Cat was responsible for the slow rate of export of Lvs_{ss}-Cat, a series of tripartite gene fusions was constructed. The length of the Cat sequence inserted between the Lvs signal peptide and Spa varied. The ratios of the precursor (Lvs_{ss}-Cat-Spa) to mature Cat-Spa (m-Cat-Spa) of the various tripartite fusions were compared (Fig. 1). *B. subtilis* cells expressing these hybrid fusions (Lvs_{ss}-Cat_{50}-Spa, Lvs_{ss}-Cat_{78}-Spa, Lvs_{ss}-Cat_{99}-Spa, Lvs_{ss}-Cat_{144}-Spa, and Lvs_{ss}-Cat_{220}-Spa) were labeled for 30 s, chased for 0, 1, and 2 min, and immunoprecipitated. In the absence of any Cat sequence between the signal peptide and Spa, a majority of the precursor was converted to m-Spa at min 0 of the chase period, and after 1 min of chase, all of the residual Lvs_{ss}-Spa was converted to m-Spa. However, the rates of conversion of the precursor to the mature form differed for the various Lvs_{ss}-Cat-Spa fusions. The amount of m-Cat-Spa present at chase time zero was dependent on the length of the Cat protein between the signal peptide and Spa. An increase in the length of the Cat sequence resulted in a decrease in the amount of m-Cat-Spa observed at chase time zero. Conversion of Lvs_{ss}-Cat_{50}-Spa to m-Cat_{50}-Spa was faster than for the other Lvs_{ss}-Cat-Spa fusions and was complete by the 1-min chase period. Conversion of the precursor to mature protein was inhibited by carbonyl cyanide *m*-chlorophenylhydrazone, suggesting that it was due to initiation of translocation as opposed to intracellular proteolysis.

That the length of the Cat protein rather than any particular amino acid sequence had a role in the efficiency of targeting Cat into the membrane was shown by using a series of internal deletions in Lvs_{ss}-Cat_{99}-Spa. The length of the Cat sequence varied (21, 50, and 78 residues). A strain containing 21 Cat residues (Lvs_{ss}-$Cat_{99(\Delta 3-78)}$-Spa) was processed faster than the other Cat-Spa fusions. Strains containing 50 [Lvs_{ss}-$Cat_{99(\Delta 3-50)}$-Spa] and 78 [Lvs_{ss}-$Cat_{99(\Delta 51-78)}$-Spa] residues had signal peptide processing comparable to that of strains Lvs_{ss}-Cat_{50}-Spa and Lvs_{ss}-Cat_{78}-Spa, respectively. Thus, the amount of mature processed protein at

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TABLE 1. Plasmids constructed for this study

Plasmid	Genes[a]	Proteins
pBE554	*lvs*$_{ss}$-*cat*	Lvs$_{ss}$-Cat
pBE333	*lvs*$_{ss}$-*spa*	Lvs$_{ss}$-Spa
pBE585M	*lvs*$_{ss}$-*cat*$_{50}$-*spa*	Lvs$_{ss}$-Cat$_{50}$-Spa
pBE825	*lvs*$_{ss}$-*cat*$_{78}$-*spa*	Lvs$_{ss}$-Cat$_{78}$-Spa
pBE586	*lvs*$_{ss}$-*cat*$_{99}$-*spa*	Lvs$_{ss}$-Cat$_{99}$-Spa
pBE587	*lvs*$_{ss}$-*cat*$_{144}$-*spa*	Lvs$_{ss}$-Cat$_{144}$-Spa
pBE588	*lvs*$_{ss}$-*cat*$_{204}$-*spa*	Lvs$_{ss}$-Cat$_{204}$-Spa
pBE589	*lvs*$_{ss}$-*cat*$_{220}$-*spa*	Lvs$_{ss}$-Cat$_{220}$-Spa
pBE577	*lvs*$_{ss}$-*cat*$_{99(\Delta3-50)}$-*spa*	Lvs$_{ss}$-Cat$_{99(\Delta3-50)}$-Spa
pBE827	*lvs*$_{ss}$-*cat*$_{99(\Delta-78)}$-*spa*	Lvs$_{ss}$-Cat$_{99(\Delta3-78)}$-Spa
pBE826	*lvs*$_{ss}$-*cat*$_{99(\Delta51-78)}$-*spa*	Lvs$_{ss}$-Cat$_{99(\Delta51-78)}$-Spa
pBE597	*lvs*$_{ss}$-*phoA*	Lvs$_{ss}$-PhoA
pBE598	*lvs*$_{ss}$-*cat*$_{220}$-*phoA*	Lvs$_{ss}$-Cat$_{220}$-PhoA
pBE885	*lvs*$_{ss}$-*cat*$_{50}$-*phoA*	Lvs$_{ss}$-Cat$_{50}$-PhoA
pBE886	*lvs*$_{ss}$-*cat*$_{99}$-*phoA*	Lvs$_{ss}$-Cat$_{99}$-PhoA
pBE887	*lvs*$_{ss}$-*cat*$_{144}$-*phoA*	Lvs$_{ss}$-Cat$_{144}$-PhoA
pBE888	*lvs*$_{ss}$-*cat*$_{204}$-*phoA*	Lvs$_{ss}$-Cat$_{204}$-PhoA
pBE890	*lvs*$_{ss}$-*cat*$_{220(\Delta3-50)}$-*phoA*	Lvs$_{ss}$-Cat$_{220(\Delta3-50)}$-PhoA
pBE891	*lvs*$_{ss}$-*cat*$_{220(\Delta3-79)}$-*phoA*	Lvs$_{ss}$-Cat$_{220(\Delta3-79)}$-PhoA
pBE892	*lvs*$_{ss}$-*cat*$_{220(\Delta3-99)}$-*phoA*	Lvs$_{ss}$-Cat$_{220(\Delta3-99)}$-PhoA
pBE893	*lvs*$_{ss}$-*cat*$_{220(\Delta3-144)}$-*phoA*	Lvs$_{ss}$-Cat$_{220(\Delta3-144)}$-PhoA
pBE894	*lvs*$_{ss}$-*cat*$_{220(\Delta3-204)}$-*phoA*	Lvs$_{ss}$-Cat$_{220(\Delta3-204)}$-PhoA

[a] *lvs*$_{ss}$ denotes the promoter, regulatory elements, and signal peptide coding region of the *B. amyloliquefaciens* levansucrase gene (20). *spa* and *phoA* were derived from pBE45 and pBE597, respectively (11).

time zero of the chase period of the various Cat-Spa fusions correlated with the length of the Cat sequence between the signal peptide and Spa.

N-terminal fusions to *E. coli* PhoA have been used to determine the topology of membrane proteins and also to identify secretion-incompatible sequences (10). The PhoA activity is indicative of export in both *E. coli* and *B. subtilis* (10, 13). The hydropathy plot of the Cat protein is shown in Fig. 2. We constructed a series of Lvs$_{ss}$-Cat-PhoA fusions in which Cat sequences were sandwiched between the Lvs signal peptide and PhoA (Fig. 2). Bacteria containing the various fusions were labeled for 5 min with [^{35}S]methionine and immunoprecipitated with anti-PhoA serum. Similar amounts of the fusion protein were synthesized in all cases, and the ratio of the precursor to mature protein for the various Cat-PhoA fusions was comparable to that of the corresponding Cat-Spa fusion (data not shown).

The extracellular PhoA activities of the various strains containing the Lvs$_{ss}$-Cat-PhoA fusions were compared in cells grown in a complex medium. The presence of Cat sequences between the Lvs signal peptide and PhoA resulted in a decrease in PhoA activity (6 to 33% residual activity). A strain containing Cat residues 205 to 220 had the maximum PhoA activity (33%) among the Lvs$_{ss}$-Cat-PhoA fusions. Thus, any particular linear sequence in the Cat protein was not responsible for the export defect. Rather, secretion-incompatible sequences were present throughout the Cat protein.

Our results identify blocks in at least two different steps in the export of the Cat protein from *B. subtilis*. The first defect is a slow entry of Lvs$_{ss}$-Cat into the export pathway, as revealed by a high percentage of the precursor protein. The second defect is the presence of sequences that are unable to be translocated across the membrane. We have used a variety of Cat protein fusions to determine the sequences that contribute to both the slow rate of export and the block of export.

The ratio of the mature to precursor protein can be used to arbitrarily determine the efficiency of export. The slow secretion efficiency of Cat can be improved by an insertion of 162 residues of mature Lvs between the signal peptide and Cat (data not shown). The secretion efficiency of the various Cat-Spa fusions revealed that the amount of m-Cat-Spa observed at time zero of the chase period decreased with the

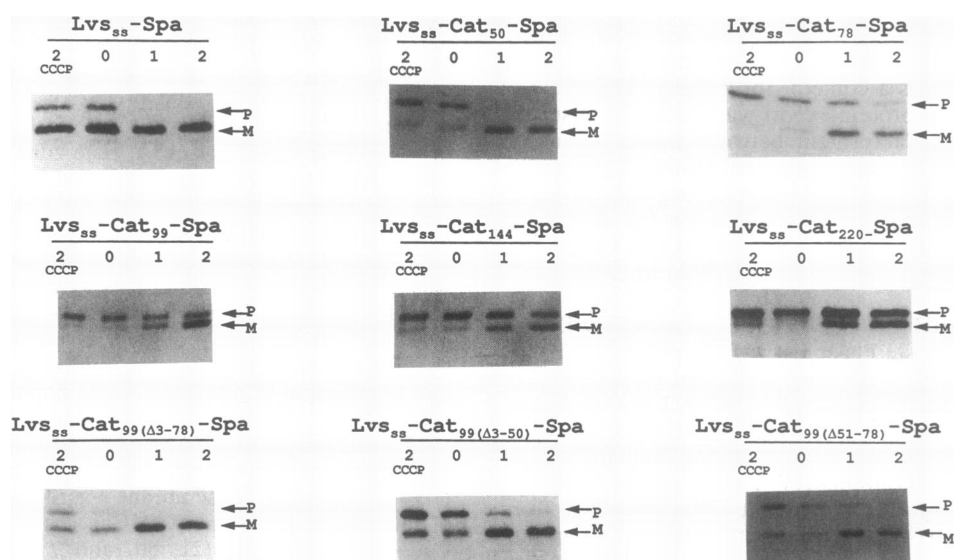

FIG. 1. Comparison of kinetics of signal peptide removal in various Lvs$_{ss}$-Cat-Spa tripartite fusions. *B. subtilis* BG4103 containing various fusions was grown in synthetic medium, induced with sucrose (20 mM) for 30 min, and radiolabeled for 30 s with [^{35}S]methionine; at time zero, a chase solution (methionine) was added (1). An aliquot (500 μl) of each sample was withdrawn and incubated in the presence of carbonyl cyanide *m*-chlorophenylhydrazine (50 μM). Samples (500 μl) were withdrawn at the indicated times, transferred to a tube containing 500 μl of 10% trichloroacetic acid, processed for immunoprecipitation with immunoglobulin G-Sepharose beads, and separated on a sodium dodecyl sulfate-polyacrylamide gel (6). 0, 1, and 2 refer to chase times in minutes. P, precursor; M, mature form.

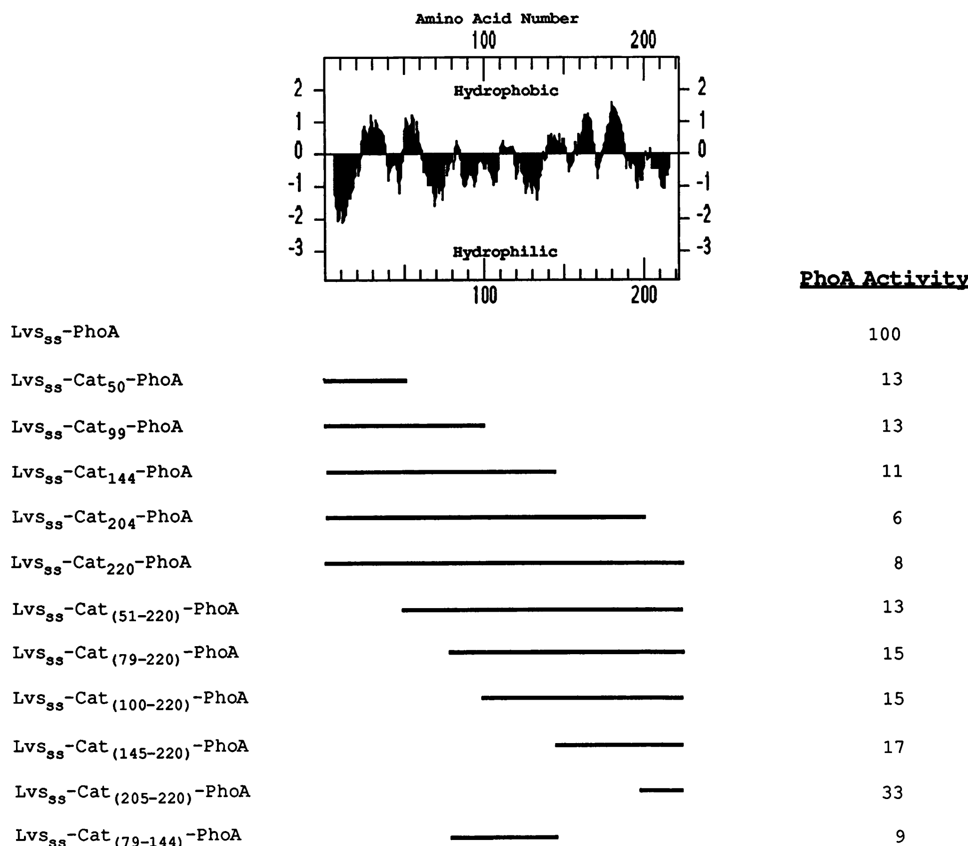

FIG. 2. Comparison of PhoA activities of Lvs_{ss}-Cat-PhoA fusions. The hydropathy profile of Cat, determined by using the Kyte-Doolittle hydropathy plot function in DNA Strider 1.1, is shown at the top. The various Lvs_{ss}-Cat-PhoA fusions are schematically represented; the Cat residues that are inserted between the Lvs signal peptide and PhoA are underlined. PhoA activity is represented as a percentage of the control activity, which was 6.0 mU. Enzyme activities are averages of duplicate cultures.

increase in the length of the Cat protein. This phenomenon appears to be due to size rather than to any particular sequence. A kinetic partitioning between efficient export and folding of the precursor has been proposed (14). The differences in the amounts of the various Lvs_{ss}-Cat-Spa precursor proteins probably reflect in vivo differences in the rates of folding of these precursors. The probability of secondary structure is likely to be greater with an increase in the number of Cat residues. Thus, the slow rate of export of Cat is likely due to a rapid folding of the precursor protein in the cytoplasm into a translocation-incompetent state.

The inability to detect PhoA activity in the various Lvs_{ss}-Cat-PhoA fusions suggests that the Cat-PhoA fusion junction is probably in the cytoplasm. The presence of a long stretch of hydrophobic amino acids is known to block export (2). Similarly, the presence of positively charged residues following a stretch of hydrophobic residues is postulated to block export (9). The hydrophobicity plot and the distribution of charged residues in the Cat protein do not identify any particular sequence as secretion incompatible, given the current knowledge of protein export in bacteria.

Overproduction of β-galactosidase in *B. subtilis* by fusion to the alkaline protease signal peptide or the *B. subtilis* Lvs signal peptide resulted in a lethal phenotype presumably due to interference with the secretion of other proteins (5, 21). We tested the growth of *B. subtilis* strains synthesizing Lvs_{ss}-Cat in a variety of media, and induction of Lvs_{ss}-Cat by sucrose did not result in alteration of the growth rate. The *B. amyloliquefaciens lvs* gene is expressed less efficiently than its *B. subtilis* homolog. The expression of Lvs_{ss}-Cat even in the presence of sucrose may not reach a level high enough to cause interference.

The secretion incompatibility of β-galactosidase from *E. coli* appears to be due to several reasons. One of the factors could be the large size of the β-galactosidase protein (116 kDa). However, a small portion of β-galactosidase when fused to PhoA can be exported to the *E. coli* periplasm (7). In contrast to β-galactosidase, *B. pumilus* Cat is a small cytoplasmic protein (22 kDa) that cannot be exported. However, Cat seems to share several features with β-galactosidase in terms of secretion incompatibility. Both Cat and β-galactosidase can be targeted more efficiently into the secretory pathway by providing some of the mature sequence in addition to the signal peptide (15). The degree of export block may be directly related to the length of Cat present. A similar observation has been reported by Lee et al. for β-galactosidase (7). Both Cat and β-galactosidase contain multiple regions that are incompatible with export.

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