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Unusual Signal Peptide Directs Penicillin Amidase from *Escherichia coli* to the Tat Translocation Machinery

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The recently described Tat protein translocation system in *Escherichia coli* recognizes its protein substrates by the consensus twin arginine (SRR*X*FLK) motif in the signal peptide. The signal sequence of *E. coli* pre-pro-penicillin amidase bears two arginine residues separated by one aspargine and does not resemble the Tat-targeting motif but can nevertheless target the precursor to the Tat pathway. Mutational studies have shown that the hydrophobic core region acts in synergism with the positive charged N-terminal part of the signal peptide as a Tat recognition signal and contributes to the efficient Tat targeting of the pre-pro-penicillin amidase. © 2002 Elsevier Science (USA)

Key Words: **bacterial preprotein translocation; Tat-dependent; signal peptide; penicillin amidase.**

Preproteins destined for export or membrane insertion are targeted to the respective transport machinery by their N-terminal signal peptides, which contain three distinct regions: a hydrophilic, basic N-terminal region (n-domain), a hydrophobic core region (h-domain) and a C-terminal region (c-region) with short chain amino acids at −3 and −1 positions (1). The Sec system, which enables proteins to be exported across the bacterial membrane in extended conformation, recognizes signal peptides that lack sequence similarity but share common physical–chemical properties (2, 3). The recent described Tat apparatus has been shown to mediate the translocation of folded proteins which predominantly bind a cofactor molecule in the cytoplasm prior to transport (4–6). An efficient Tat targeting in bacteria requires two consecutive arginine residues embedded within a consensus SRR*X*FLK motif (4). In spite of that it has been reported that the signal peptides with a single mutation of one arginine residue by lysine (7) and even with a deletion of the paired arginines (8) are still recognized by the Tat system. A recent study has described a TtrB subunit of *Salmonella enterica* as a new Tat substrate with a naturally occurring substitution of one arginine for lysine (9). The signal sequence of TtrB lacks one of the invariant arginines in its signal peptide, but the surrounding sequence in the N–H junction is similar to the characteristic Tat targeting motif.

In this study, we present a naturally occurring example of Tat-dependent translocation of a pre-pro-penicillin amidase (ppPA) with a signal peptide without the Tat-targeting motif. Penicillin amidase (PA) is synthesized as a precursor in the cytoplasm and maturates further in an intramolecular autoproteolytic process to an active PA composed of two polypeptide chains: a small one A (MW 23 kDa), and larger one B (MW 62 kDa) (10, 11). An intracellular proteolytic degradation of ppPA in the cytoplasm reduces the yields of active PA and competes with the transport into the periplasm (12). PA is a stable periplasmic enzyme, which allows its export to be studied by intracellular fractionation and conveniently monitored by SDS–PAGE assay of the periplasmic fraction or activity measurements. To determine by which mechanism the membrane translocation of ppPA is driven, we expressed the *pac*-gene under depleted TatA and TatE conditions (13). Furthermore, based on mutational studies the possible recognition signals for the Tat machinery in the ppPA signal peptide were determined.

MATERIALS AND METHODS

Bacterial strains and DNA techniques. *E. coli* strains used in this study were: MC4100 (F⁻ Δ*lacU169 ara139 rpsL150 relA1 ptsF rbs* flbB5301) and JARV15 (MC4100Δ*tatA*Δ*tatE*) (13). The *pac* gene amplified with its RBS and Shine–Dalgarno sequence upstream the start codon using chromosomal DNA from *E. coli* ATCC 11105 as a template was cloned into the polylinker of the low-copy-number vector pMMB207 (14) yielding the plasmid pPAEC. Plasmid pPAOT for *in vivo* expression of OmpT-PA was constructed as follows. The single stranded 5′-phosphorylated oligonucleotides encoding the OmpT-signal peptide sequence (GenBank Accession No. P09169) were converted into double stranded DNA-fragment by heating of stoichiometric amounts of both primers at 75°C for 10 min followed

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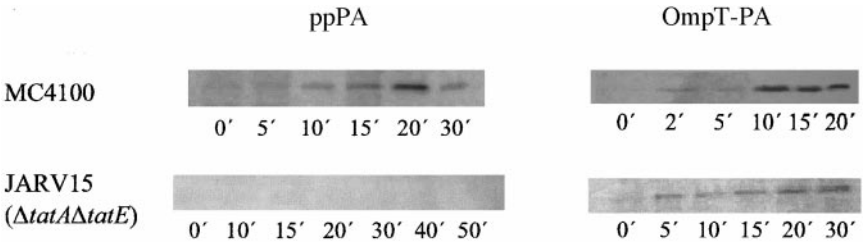

FIG. 1. Pulse–chase analysis of the ppPA and OmpT-PA transport into the periplasm. The *E. coli* strains MC4100 and JARV15 (Δ*tatA*Δ*tatE*) were grown at 28°C in the chase medium. 5 min after induction of the synthesis of PA precursors cells were pulse labeled with [^{35}S]methionine for 2 min and then chased with nonradioactive methionine. The quantitatively isolated PA by immunoaffinity chromatography (for details see Materials and Methods) was assessed by SDS–PAGE followed by autoradiography.

by slowly cooling down to room temperature. The 5′-blunt end *pac*-amplificate without signal sequence was digested with *Kpn*I and together with double stranded OmpT-signal sequence carrying an 5′-*Eco*RI-end was cloned into the corresponding sites of the plasmid pMMB207 (14). pPAEC was used as the template for the introduction of site-specific mutations into the n-region of the signal peptide coding sequence of ppPA. PA-precursor mutants with signal peptides, which are hybrids between original ppPA signal sequence and the OmpT signal sequence, were engineered by oligo-directed mutagenesis using pPAEC or pPAOT as templates. All mutations were verified by DNA sequencing (SeqLab, Germany).

Pulse-chase experiments. Bacteria were grown at 28°C in the following chase medium: M9 salt medium supplemented with glucose (0.3%) and methionine-free amino acid mix (0.02% each), pH 7.4. As selection marker chloramphenicol 25 μg/ml was added. The cells were grown to the mid-logarithmic phase and the expression of the ppPA and mutant precursors was induced with 0.5 mM IPTG. 5 min after induction, cells were pulse labeled with [^{35}S]methionine for 2 min at 28°C, followed by an addition of an excess of unlabeled methionine as described elsewhere (15). The mature PA was quantitatively isolated by immunoaffinity chromatography using Bio-Rad columns containing 75 μl suspension of immobilized monoclonal antibodies with an epitope against the B-chain of PA as described elsewhere (16). The fractions eluted with 50 μl phosphate buffer, pH 3.5, were assessed by SDS–PAGE followed by autoradiography.

RESULTS AND DISCUSSION

The export of ppPA in the *E. coli* strain MC4100 was completed within 20 min (Fig. 1). Parallel, we performed a control experiment with an OmpT-PA precursor constructed by a fusion of the typical Sec-targeting signal peptide (OmpT) to PA. The OmpT-PA precursor was exported efficiently into the periplasm when expressed in *E. coli* MC4100, verified also by PA activity measurements (data not shown). A strain bearing the combined deletion in the *tatA* and *tatE* genes (JARV15) has been described previously to be completely defective in the export of proteins with Tat signal peptides (13). In the presence of *tatA* and *tatE* mutation the transport of ppPA was completely blocked and no mature PA was detected over a 50-min chase (Fig. 1). In contrast, the translocation of OmpT-PA was not influenced under TatATatE impaired functions and was completed with about the same half-life time as in the control *E. coli* strain MC4100 (Fig. 1). These experiments show that the transport of ppPA through the membrane is exclusively Tat-dependent.

Nevertheless, these observations are insufficient to resolve how the Tat system recognizes the ppPA signal peptide since it does not bear the Tat targeting motif. To address this question we replaced a range of site-specific amino acids in the n-domain of the signal peptide (Fig. 2A) and studied the translocation of these engineered variants by pulse–chase experiments (Fig. 2B) or by measurement of the transported amount of mature PA over a period of 3 h (Fig. 2C). The exchange of the positions of the fifth aspargine and fourth arginine resulted in a mutant precursor RR-PA with consecutive arginines in the n-domain which served as a positive control. The export of RR-PA was completely blocked in *tatA* and *tatE* deletion strain, whereas the replacement of the second arginine with lysine had no discernible effect on the translocation compared to ppPA and still permitted export by the Tat pathway (Figs. 2B and 2C). This is in contrast to the effect of equivalent mutation in *Zymomonas mobilis* GFOR causing an inhibition of the Tat-dependent transport (15). However, it was not contradictory as other studies describing the transport of SufI (7) and TtrB (9) showed that the Tat translocation system recognizes either two arginines, or one arginine and one lysine residue.

An individual arginine to serine mutation (R6S-PA) retarded the translocation of the mutant precursor in *tatA* and *tatE* deleted background and transport efficiency decreased (Fig. 2C) compared to the control strain *E. coli* 4100. That mutation seemed to reroute the mutant precursor (R6S-PA) onto the Sec export pathway. Nevertheless, it should be remembered that there is no evidence that the transport of R6S-PA observed in the control *E. coli* strain MC4100 was mediated by the Sec pathway. The half-life time of the translocation of R6S-PA was equal to that of the ppPA but the achieved final intensity was lower (Fig. 2B) and in the *tatA* and *tatE* deletion strain the transport efficiency decreased. Taken together, these observations cannot exclude the possibility that the export of

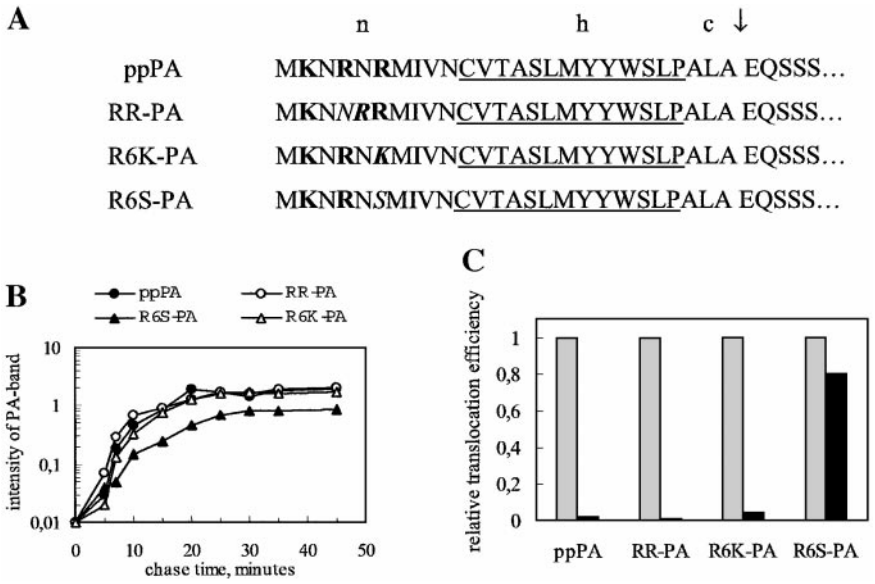

FIG. 2. Analysis of the export of ppPA signal peptide mutants. (A) Amino acid sequence of the signal peptide of ppPA and the engineered signal peptides. The hydrophobic core segments are underlined; positive charged residues are in bold letters; the residues introduced by mutagenesis are italicized. The n-, h-, and c-regions are indicated. The cleavage site by signal peptidases is marked by an arrow. (B) Comparative *in vivo* pulse–chase analysis of the export of PA-precursors with alterations in the n-region in the *E. coli* MC4100 strain. Each point on the graph represents the scanned intensity of the mature periplasmic PA from the autoradiograms. The transport of ppPA with its original signal sequence in *E. coli* MC4100 served as a control. Cells were cultivated at 28°C in chase medium. (C) Relative translocation efficiency of ppPA signal peptide mutants was estimated from the scanned intensity of the B-chain of periplasmic PA from the Western blot. ppPA precursor mutants expressed in *E. coli* strains MC4100 (gray bars) and JARV15 (Δ*tatA*Δ*tatE*) (black bars) were cultured at 28°C in chase medium containing Met (0.02%) as well. Cells were harvested 3 h after induction with IPTG (0.5 mM) and PA was detected by immunoblot using monoclonal antibodies with an epitope against the B-chain of mature PA [16]. Expression of ppPA signal peptide mutants *in E. coli* MC4100 served as controls and the values of the intensity of their B-chains were taken as 1.

R6S-PA remains partly Tat-dependent. The range of site-specific substitutions into the n-region of the ppPA signal peptide confirm that both arginines, although they are not consecutive in the natural ppPA signal peptide, seem to function as a recognition signal for the Tat translocase. Thus the *E. coli* ppPA signal peptide mediates Tat-dependent transport efficiently even though the N-terminal region of its signal peptide does not match the described consensus Tat targeting motif.

Another intriguing question is: Does the h-domain of the *E. coli* ppPA signal peptide also contribute to target the precursor to the Tat system? For this purpose we constructed two hybrid signal peptides, in which either n- or hc-domains of the original ppPA targeting signal were substituted with the corresponding regions from the OmpT signal sequence (Fig. 3). The short designations used for the hybrid constructs are made up of the one-letter codes for the corresponding signal peptide region followed by a capital letter identifying the origin of this region (P for ppPA and O for OmpT). Pulse–chase experiments verified that both precursors with hybrid signal peptides are exported into the periplasm with about the same half-life time as ppPA in the control *E. coli* MC4100 strain (Fig. 3). In the strain JARV15 compared with that control experiment, the export of the engineered precursors was retarded and the end intensity of processed PA was lower (Fig. 3). In the *tatA* and *tatE* deletion strain the nOhcP-PA construct was exported more efficiently than nPhcO-PA suggesting that nOhcP-PA was able to use Tat-dependent pathway but with lower efficiency than nPhcO-PA (Fig. 3). Despite of the lowered export rates these hybrid precursors were translocated to higher or lower extent under depleted TatA and TatE conditions. Both

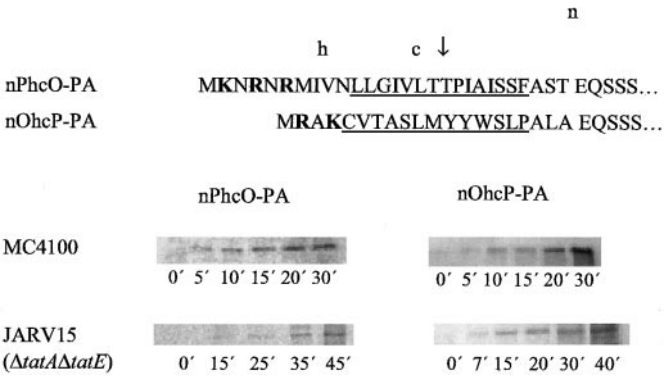

FIG. 3. Pulse–chase analysis of the translocation rates of PA-precursors with hybrid signal peptides. The primary structure of these hybrid signal peptides is shown on the top of the figure and the same description scheme was used as for the sequences in Fig. 2. The experiments are performed under conditions as described for ppPA in Fig. 1. The same amount of cells was loaded per lane.

precursors could function as substrates for Tat pathway. Based on these results a conclusion can be drawn that the n-domain of the ppPA original signal peptide alone is not sufficient to support efficient Tat targeting.

Analysis of the amino acid composition of the h-domain of the signal peptide of ppPA shows relatively low proportion of hydrophobic Leu/Val (27%) residues compared to the Sec-dependent signal peptides. Experimental and statistical data on the hydrophobicity of the h-regions reveals that Tat-targeting signals have less hydrophobic h-segments than Sec-dependent signal peptides (17). Thus, beyond the positively charged n-domain of ppPA signal peptide, the hydrophobicity of h-segment has an important contribution to the Tat targeting. Taken together, these observations demonstrate that both structural determinants within the overall context of the signal peptide are required for a selective targeting of ppPA to the Tat translocation system. The example for such unusual signal peptide, which still efficiently mediates the Tat-dependent translocation of ppPA, expands the group of naturally occurring Tat-dependent signals in bacteria with some "mismatches" to the established Tat consensus motif.

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