Export of *Thermus thermophilus* alkaline phosphatase via the twin-arginine translocation pathway in *Escherichia coli*

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Abstract The bacterial twin-arginine translocation (Tat) pathway is distinct from the Sec system by its remarkable capacity to export folded enzymes. To address the question whether the two systems are capable of translocating homologous enzymes catalyzing the same reaction, we cloned the *tap* gene encoding *Thermus thermophilus* alkaline phosphatase (Tap) and expressed it in *Escherichia coli*. Unlike the alkaline phosphatase of *E. coli*, which is translocated through the Sec system and then activated in the periplasm, Tap was exported exclusively via the Tat pathway and active Tap precursor was observed in the cytoplasm. These results demonstrate that two sequence and functional related enzymes are exported by distinct protein transport systems, which may play an integral role in the bacterial adaptation to their environment during the evolution.

Key words: Sec; Tat pathway; Alkaline phosphatase; Thermophile bacterium; Adaptation; Evolution

1. Introduction

Bacteria export numerous proteins across the cytoplasmic membrane via either the Sec machinery [1,2], or the twin-arginine translocation (Tat) (also called MTT) system [3–7]. The Tat system is different from the Sec pathway by its unusual ability to transport folded enzymes often containing redox cofactors [8,9]. In addition, it is also capable of exporting tightly folded green fluorescent protein that cannot be transported by the Sec system [10,11]. The signal peptides of the proteins exported by the Tat pathway resemble Sec-dependent signal peptides in their overall structures, but possess a twin-arginine motif in the positively charged n-region, a weakly hydrophobic h-region and a positively charged Sec-avoidance signal in the c-region [12]. In *Escherichia coli*, Tat components are encoded by the *tatABCD* operon and the *tatE* gene [6]. TatD is not required for the Tat function [13]. TatC is an integral membrane protein with six predicted transmembrane segments. The deletion of *tatC* leads to mislocation of all the enzymes analyzed [7]. Therefore, TatC is an essential element for the Tat function. TatA, TatB and TatE show a sequence homology at their N-termini, including one transmembrane segment and an adjacent amphipathic domain, whereas their C-termini vary in sequence and in length [14]. A TatABC complex has been recently purified from *E. coli* and a functional Tat system has been reconstituted in vitro from cells overproducing TatABC [15–18]. The composition of a Tat system varies from one organism to another and a minimal Tat system consists of one copy of *tatC*- and one copy of *tatA*-homologous genes [9]. The Tat system is not ubiquitously distributed in all genomes and is unlikely to be among the minimal genes required for life [9]. However, it is essential for some bacteria under certain growth conditions, suggesting that the Tat system may confer the bacterium an advantage in the competition for an ecological niche [9].

Alkaline phosphatase (AP, EC 3.1.3.1) is a proficient phosphomonoesterase that functions through a phosphoseryl intermediate to produce free inorganic phosphate or to transfer the phosphoryl group to other alcohols [19]. This enzyme is ubiquitously distributed and has been highly conserved during evolution. The extensively studied AP from *E. coli* (PhoA) is a homodimeric, metalloenzyme consisting of 449 amino acids, two tightly bound atoms of zinc and one atom of magnesium per monomer [20]. PhoA is synthesized with a classic signal peptide and translocated into the periplasm via the Sec pathway. It is activated in the periplasmic space where it reaches its active conformation upon the formation of two intramolecular disulfide bonds in each monomer [21,22]. This property has been widely used to study protein subcellular location and membrane protein topology [23]. Interestingly, the recently sequenced AP of *Thermus caldophilus* GK24 contains an RRxIxK motif in its potential signal peptide [24], raising the question whether this AP folds in the cytoplasm and is exported via the Tat pathway. In this study, we cloned the *tap* gene from *T. thermophilus* HB27, which encodes an AP (Tap)

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Abbreviations: AP, alkaline phosphatase; ECF, enhanced chemifluorescence; PhoA, alkaline phosphatase of *Escherichia coli*; PNPP, *p*-nitrophenyl phosphate; PVDF, polyvinylidene difluoride; Tap, *Thermus thermophilus* alkaline phosphatase; Tat, twin-arginine translocation

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showing 100% amino acid identity to that of *T. caldophilus*. When expressed in *E. coli*, the AP precursor of Tap is active in the cytoplasm and it is apparently translocated into the periplasm via the Tat system. This result represents a marvelous example of bacterial adaptation to its environment by modifying the mechanism of activation and translocation of one essential enzyme during its evolution.

2. Materials and methods

2.1. Bacterial strains, plasmids, and media

TTY1 (*ΔpyrE*, *ΔleuB*), a derivative of *T. thermophilus* HB27, arabinose-resistant *E. coli* strains MC4100A (F′ *lacΔU169 araD139 rpsL150 thi flbB5301 deoC7 ptsF25 relA1*) and B1LK0A (as MC4100, Δ*tatC*) are described in [25] and [11], respectively. Other *E. coli* strains, CC118 (*araD139 Δ*(*ara, leu*)7697 *ΔlacX74 phoAΔ20 galE galK thi rpsE rpoB argEam recA1*) and TG1 (Δ(*lac-pro*) *supE thi hsdD5*/F′ *traD36 proA*$^+$*B*$^+$ *lacI*^{q} *lacZ*ΔM15) are laboratory stocks. The pBAD24 is described in [26].

E. coli strains were routinely grown at 37°C in Luria–Bertani (LB) medium or on LB plates. *T. thermophilus* was grown at 75°C in rich medium (TM) composed of 8 g polypeptone, 4 g yeast extracts, 2 g NaCl, 0.35 mM $CaCl_2$ and 0.4 mM $MgCl_2$ per liter of medium. The pH was adjusted to 7.5 prior to autoclaving. As required, glucose (0.2%), arabinose (0.2%) ampicillin (100 μg/ml) and kanamycin (50 μg/ml) were added to the medium. Pre-cultures were grown from single colonies and used at 100-fold dilutions for inoculation of experimental cultures.

2.2. Cloning and expression of the tap gene

T. thermophilus HB27 *tap* gene was amplified from the chromosome of TTY1 by PCR using primers TAP5ECO (5′-gtgaattccatgaagcgaagggacatcctg-3′) and TAP3SAL (5′-acgcgtcgacttaggcccagacgtcctcgg-3′), with the Expand High Fidelity PCR System according to the manufacturer's instructions (Roche). The amplified fragment was digested with *Eco*RI and *Sal*I and cloned into the corresponding sites of the pBAD24. The structure of the resulting plasmid, pTAP, was verified by *Eco*RI, *Sal*I and *Nco*I endonuclease digestion. Expression of *phoA* was under the tight control of the PBAD promoter as described by Guzman et al. [26].

2.3. Enzyme assays

Phosphatase activity was assayed by hydrolysis of *p*-nitrophenyl phosphate (PNPP) and the absorption of the produced *p*-nitrophenol was measured at 410 nm with a Cary 50 spectrophotomer using a control without extract as the reference blank. One unit of enzyme activity is defined as the release of 1 μmol of nitrophenol/min. Alternatively, extracts were, directly or after being separated on a polyacrylamide gel, immobilized on a polyvinylidene difluoride (PVDF) membrane, and AP activity was measured by the enhanced chemifluorescence (ECF) detection reagent according to the manufacturer's instructions (Amersham Pharmacia Biotech). The AP activity was visualized and digitized by using the Kodak Image Station. Fluorescent signal was analyzed by Kodak 1D Image Analysis Software.

2.4. Cellular fractionation, electrophoresis and mass spectrometry

Spheroplasts and cellular fractions were prepared by lysozyme/EDTA/cold osmoshock and ultracentrifugation as described previously [4]. Proteins were separated by polyacrylamide gel electrophoresis in the presence (denaturing) or in the absence (non-denaturing) of SDS on 12.5% acrylamide gels.

For mass spectrometry, protein samples were separated on a SDS-denaturing gel, stained by Coomassie blue and the specific protein band was excised. After crushing and washing of the excised gel, the proteinaceous material was reduced with dithiothreitol and alkylated with iodoacetamide in 100 mM NH_4HCO_3. Proteolytic digestion by trypsin was then performed overnight at 37°C. The supernatant was collected, the salts were removed by flow through a R2 Poros column and the sample was analyzed by mass spectrometry. The protein was identified by the Mascot Search program at http://www.matrixscience.com based on the Mowse Score, $-10*\log(P)$, where P is the probability that the observed match is a random event. Protein scores greater than 65 are significant ($P < 0.05$).

3. Results

3.1. Cloning and expression of the tap gene of T. thermophilus HB27

Sequence analysis revealed that three homologous APs with more than 90% sequence identity from *Thermus* sp FD3041, *Thermus aquaticus* subs. *caldophilus* GK24 and *T. thermophilus* HB8 contain a potential twin-arginine signal peptide, suggesting that they might be exported by the Tat pathway. To assess this hypothesis, we cloned the *tap* gene encoding the Tap HB27 into the pBAD24. The resulting pTAP and the vector pBAD24 were introduced into *E. coli* strain CC118 (Δ*phoA*). The crude extract of CC118/pTAP grown with arabinose exhibited a specific activity of 59.2 units/mg of protein, whereas that of CC118/pBAD24 was less than 1 unit/mg of protein. The periplasmic fractions prepared from these cells were thermo-denatured at 65°C for 20 min. After eliminating the denatured polypeptides by centrifugation, the supernatants were resolved on a 12.5% polyacrylamide denaturing gel and visualized by Coomassie blue staining. A polypeptide with an apparent molecular mass of about 55 kDa was present in the periplasmic fraction of CC118/pTAP, but was absent from that of CC118/pBAD24 (Fig. 1, lane 2 versus lane 1). To establish its authenticity, the 55-kDa band was excised from the gel, digested with trypsin and the peptides analyzed by MALDI-TOF mass spectrometry. The peptide distributions corresponded to only two proteins in the data base: the APs of *T. aquaticus* subs. *caldophilus* GK24 and *Thermus* sp. FD3041 with significant Mowse scores of 143 and 95, respectively. Furthermore, the cloned *tap* gene showed 97% nucleotide and 100% amino acid sequence identities to the *tap* gene and the AP of the *T. aquaticus* subs. *caldophilus* GK24, respectively (S. Kuramitsu, personal communication). Taken together, the peptide mapping and activity assay confirm that the *T. thermophilus tap* gene has been successfully cloned and expressed in *E. coli*.

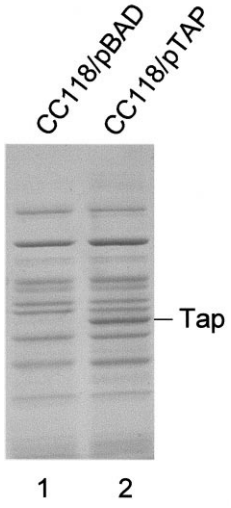

Fig. 1. Expression and identification of Tap. The periplasmic fractions (20 μg of protein each) obtained from CC118/pBAD (lane 1) and CC118/pTap (lane 2) were treated at 60°C for 20 min and the supernatants were separated on a 12.5% SDS-denaturing gel. The band of about 55 kDa (Tap) present only in CC118/pTap was identified by mass spectrometry as described in Section 2.

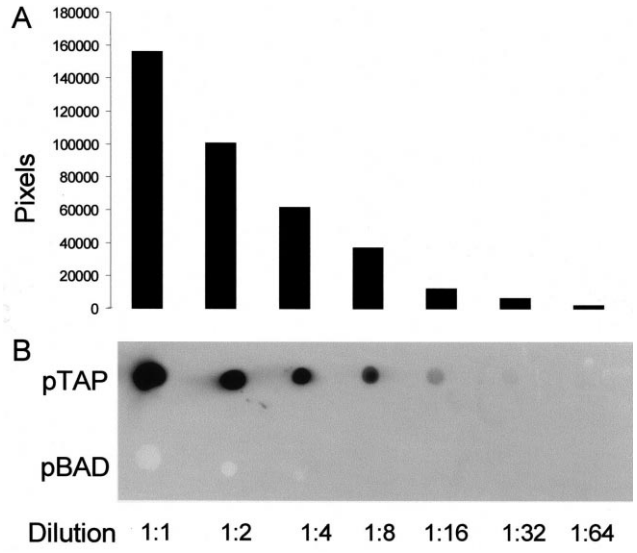

Fig. 2. Detection of Tap activity by using the ECF reagent. The crude extracts of CC118/pBAD (pBAD) and CC118/pTap (pTAP) were diluted as indicated in panel B. Four microliters of the dilutions were immobilized on a PVDF membrane, which was then developed by the ECF detection reagent. The AP activity was visualized and digitized by using the Kodak Image Station and the values in pixels obtained with the CC118/pTap strain are presented in panel A.

3.2. Export of thermophilus AP via the Tat system in E. coli

To characterize the translocation of Tap, we analyzed the cellular distribution of Tap activity in the wild type MC4100A and in its *tatC* derivative B1LK0A. In contrast to CC118, MC4100A contains an intact chromosomal copy of the *phoA* gene and thus exhibited a weak endogenous AP activity (Table 1). The introduction of the pTAP in MC4100A increased the AP activity measured at 37°C about six-fold. Interestingly, only the basic level of AP activity was observed in the *tatC* mutant in either the presence or absence of the pTAP (Table 1). These results strongly suggest that the deletion of the *tatC* gene would reduce the Tap activity without affecting the PhoA activity. Since Tap has a thermophile origin, we also preformed the assays at 65°C. The PNPP hydrolysis by PhoA or Tap was increased about two-fold, suggesting that these enzymes function better at higher temperatures. Henceforth, the PNPP-hydrolysis assays in this study was carried out at 65°C.

It was not possible to determine the distribution of Tap in the periplasmic and cytoplasmic fractions as the activities measured by PNPP-hydrolysis assays were too low to provide reliable results. In addition, it was not possible to utilize antisera against PhoA for analyzing the distribution by immunoblot detection as it did not significantly cross-react with the Tap protein. Therefore, we sought an alternative sensitive and quantitative method to measure the AP activities. A series of dilutions of the CC118/pTAP and CC118/pBAD24 crude extracts were immobilized on a PVDF membrane, which was then developed by the ECF method. The AP activity generates a fluorescent product that was visualized and digitized by using the Kodak Image Station. A fluorescent signal was detected in the extract of CC118/pTAP at up to 1/64-fold dilution, but it was absent from all dilutions of the extract of CC118/pBAD24 (Fig. 2), reflecting the high specificity and sensitivity of this method. In addition, the increase in signal was linear in the region from 1/2 to 1/16 dilutions.

When the ECF method was used to study the cellular distribution of APs, a single fluorescent band was found in the periplasm of the Δ*phoA*/*tap*$^+$ strain CC118/pTAP, whereas it was replaced by a slower migrating band in the cytoplasm of this strain (Fig. 3, lane 1 versus lane 10). Since both bands were absent from the extracts of CC118 (data not shown) or MC4100A carrying the pBAD24 (Fig. 3, lanes 8 and 9), we assumed that these bands correspond to the Tap synthesized from the pTAP with the slower migrating form (Tap-P) corresponding to the precursor of this enzyme. Similarly, Tap-P and Tap were observed in the cytoplasm and the periplasm, respectively, of the *phoA*$^+$/*tap*$^+$ strain MC4100A/pTAP (Fig. 3, lane 6). Using Image-analysis software, we calculated that 83% of total Tap was located in the periplasm of MC4100A/pTAP. Significantly, in addition to Tap, a third active band was also detected in the periplasm of the *phoA*$^+$/*tap*$^+$ strain MC4100A/pTAP (Fig. 3, lane 6). Two observations support the conclusion that this active band is PhoA. First, this band was also found in the periplasm of the *phoA*$^+$ strain MC4100A/pBAD24 (Fig. 3, lane 8), but absent from the periplasm of the Δ*phoA*/*tap*$^+$ strain CC118/pTAP (Fig. 3, lane 1). Second, anti-PhoA antiserum could specifically recognize this band in immunoblot (data not shown).

Table 1
AP activities of the wild type MC4100A and the *tatC* mutant B1LK0A

Strains	Specific activity (unit/mg protein)	
	37°C	65°C
MC4100A/pBAD24	3.2	7.9
MC4100A/pTAP	17.9	36.4
B1LK0A/pBAD24	2.0	4.4
B1LK0A/pTAP	2.2	5.4

Strains were grown at 37°C in LB-ampicillin medium plus 0.2% arabinose. AP activity was assayed at 37 or 65°C by PNPP hydrolysis.

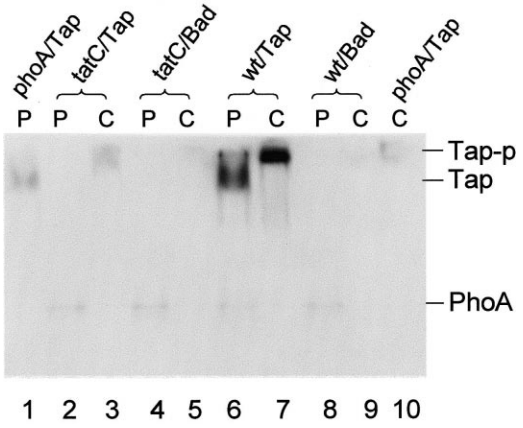

Fig. 3. Cellular distributions of Tap and PhoA in different strains. The periplasmic (P, 15 μg of protein each) and cytoplasmic (C, 60 μg of protein each) fractions from CC118 (*phoA*), MC4100A (wt) and B1LK0A (*tatC*) carrying pBAD24 (Bad) or pTAP (Tap) were separated by electrophoresis on a non-denaturing 12.5% polyacrylamide gel, electro-transferred onto a PVDF membrane and developed by ECF-detection reagent. The AP activity was visualized and digitized by using the Kodak Image Station, and analyzed by Kodak 1D Image Analysis software.

Finally, we analyzed the cellular location of the APs in B1LK0A (*ΔtatC*). The only band observed in the periplasm of this strain carrying either pTAP or pBAD corresponds to the fast migration PhoA bands (Fig. 3, lanes 2 and 4). These results confirm that PhoA is translocated into the periplasm via the Sec pathway, independently of the Tat system. In marked contrast, Tap was absent from the periplasm and was detected only as the precursor form (Tap-P) in the cytoplasm of B1LK0A/pTAP (Fig. 3, lanes 2 and 3). Unlike PhoA, the *T. thermophilus* counterpart is exported into the periplasm exclusively through the Tat pathway in *E. coli*, and as detected by the ECF method, it accumulates in an active form in the cytoplasm of the *tatC* deletion mutant.

4. Discussion

APs are ubiquitously distributed and highly conserved in bacteria, Archaea, yeast and mammalian cells [27]. A survey of the exported APs reveals that most of them are synthesized with a typical Sec-dependent signal sequence, but that the APs of the three *Thermus* species carry an obvious twin-arginine signal peptide. PhoA, the prototype for studying structure, catalytic mechanisms and biosynthesis of APs, is synthesized as an inactive precursor which is activated only after it is exported through the Sec pathway into the periplasmic space ([23]; this study). The inactivity of cytoplasmic AP appears to be due to, at least in part, the absence of essential intramolecular disulfide bonds [28]. In addition, it has been speculated that PhoA remains inactive in the cytoplasm to avoid non-specific phosphate-bond hydrolysis [29]. Strikingly, the four cysteines that form two intramolecular disulfide bonds are not conserved among the APs, which thus challenges the generality of this activation mechanism. Indeed, in this report, we have observed an active precursor of Tap in the cytoplasm of *E. coli*. Most importantly, in marked contrast to the PhoA, the Tap is exported to the periplasm exclusively via the Tat pathway in *E. coli*. The Tat system is thus involved in the phosphate metabolism in *T. thermophilus*. In addition, we provide the first evidence for the export of orthologous enzymes via distinct pathways. Sequence analysis has revealed that other homologous proteins, such as the copper-containing nitrite reductases from mesophilic bacteria, can be synthesized with either Sec- or Tat-targeting sequences [8]. It would be interesting to assess if our observation can be generalized to these enzymes.

Intriguingly, the cytoplasmic level of active Tap precursor is reduced in the *ΔtatC* mutant compared to the wild type strain. Since the *tap* gene is expressed from the same plasmid in both strains, the reduction of Tap must occur post-translationally in *ΔtatC*. This observation would suggest a housekeeping mechanism that cleans up Tap in the absence of the functional Tat system. Indeed, the depletion of the *tat* genes often results in inactivation and degradation of enzymes exported by this pathway [11,14].

Why should homologous enzymes follow distinct pathways for their export? A current working hypothesis accounting for protein thermostability is that hyperthermophilic enzymes are more rigid than their mesophilic homologs and that rigidity is a prerequisite for high protein thermostability [30]. High rigidity would be more suitable for a Tat-pathway-dependent translocation. However, the rigidity is not the only explanation for the use of the Tat pathway by Tap since the *tat* genes are absent from the genomes of two thermophile organisms, *Pyrococcus abyssi* and *Thermotoga maritima* [9], and their APs are synthesized with the Sec-dependent signal peptides. The AP of *Thermotoga neapolitana*, which is closely related to that of *T. maritima*, exhibits much higher thermostability than those of *Thermus* species [31]. The intrinsically higher thermostability would permit these APs to remain stable in either the unfolded or partially folded conformation and thus be suitable for export by the Sec pathway. In contrast, to become thermostable, the Tap may have to fold as soon as it is synthesized. Since the Sec system cannot export folded proteins, evolution would route the folded AP to the Tat pathway. The unusual capacity of the Tat system to translocate folded proteins would confer the microorganisms an advantage in competition for an ecological niche.

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