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Malfolded recombinant Tat substrates are Tat-independently degraded in *Escherichia coli*

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ABSTRACT

The twin-arginine translocation (Tat) system translocates folded proteins across biological membranes. It has been suggested that the Tat system of *Escherichia coli* can direct Tat substrates to degradation if they are not properly folded [Matos, C.F., Robinson, C. and Di Cola, A. (2008) The Tat system proofreads FeS protein substrates and directly initiates the disposal of rejected molecules. *EMBO J.* 27, 2055–2063; Matos, C.F., Di Cola, A. and Robinson, C. (2009) TatD is a central component of a Tat translocon-initiated quality control system for exported FeS proteins in *Escherichia coli*. EMBO Rep. 10, 474–479]. Contrary to the earlier reports, it is now concluded that reported differences between tested strains were due to variations in expression levels and inclusion body formation. Using the native Tat substrate NrfC and a malfolded variant thereof, we show that the turnover of these proteins is not affected by the absence of all known Tat components. Malfolded NrfC is degraded more quickly than the native protein, indicating that Tat-independent protease systems can recognize malfolded Tat substrates.

1. Introduction

The twin-arginine translocation (Tat) system serves to translocate fully folded proteins across the cytoplasmic membrane of most prokaryotes and the thylakoid membrane of plant plastids [1]. Many of the proteins that are transported via Tat ("Tat substrates") contain cofactors that are assembled prior to transport and often Tat substrates cross the membrane as heterooligomers, underlining the outstanding adaptation of the Tat system to globular proteins [2]. It has been postulated by several groups that a quality control system exists to prevent translocation of malfolded proteins [3–6]. The idea of a folding quality control was mainly based on studies that demonstrated the requirement of a folded state for functional translocation with substrates such as c-type cytochromes or alkaline phosphatase [4,7]. For Tat-dependent protein transport, the term "quality control" describes a recognition and rejection of malfolded proteins and may involve the initiation of proteolysis of malfolded proteins. One possibility would be that such a quality control is an intrinsic ability of the translocation machinery itself [8]. Alternatively, the cytoplasmic chaperone and protease systems suffice to ensure that no malfolded proteins reach the Tat system at natural expression levels [9].

Recently, evidence for a Tat-dependent degradation of malfolded Tat substrates was obtained, which supported the idea of a quality control in which the Tat system recognizes malfolded Tat substrates and directs them to proteolysis [10,11]. Conclusions were based on experiments that monitored the accumulation or degradation of recombinantly produced Tat substrates in *Escherichia coli* strain MC4100 and several *tat* deletion mutants thereof. In order to use an arabinose-inducible expression system in these normally arabinose-sensitive *E. coli* strains, arabinose-resistant clones of each of their strains were selected on arabinose-containing media. We now report that the reason for the published observations were differing recombinant expression levels in the tested strains, which did not depend on the *tat* genotypes. We could obtain wild type and *tat* deficient strains that produced native or malfolded Tat substrates at similar levels, thus allowing a direct comparison of the Tat substrate turnover in wild type and Tat-deficient strains. The data suggest that proteases can recognize and degrade malfolded Tat substrates without an involvement of Tat components. Tat substrates are thus under control of a cytoplasmic quality control system that functions prior to Tat-specific interactions and that can direct malfolded Tat substrates to cytoplasmic proteolysis. As Tat substrates fold prior to translocation, our data suggest that the known cytoplasmic folding quality control systems suffice and

Abbreviation: Tat, twin-arginine translocation

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there may be no need for an extra Tat-dependent degradation pathway.

2. Materials and methods

2.1. Strains and growth conditions

E. coli MC4100 [12], or its *tatABCDE* deficient derivative DADE [13], were used for physiological studies, and *E. coli* XLl-Blue Mrf' Kan (Stratagene) was used for cloning. Cultivation was done as described previously [10,11]. Results were compared with the ones obtained with the original strains of the studies by Matos et al. [10,11].

2.2. Genetic methods and plasmids

The plasmids used to produce the Tat substrates, pBAD-nrfC and pBAD-nrfC-M2, were described previously [10,11]. We also constructed a pBAD-lacZ vector to quantify the level of the pBAD-vector dependent induction in the strains. *E. coli lacZ* was amplified with genomic DNA as template using the primers pBAD-lacZ-MfeI-F (5′-ATA GAT CAA TTG CTA TGA CCG TCG TTT TAC AAC GTC GTG-3′) and pBAD-lacZ-PstI-R (5′-ATA GAT CTG CAG TTA TTT TTG ACA CCA GAC CAA ATG G-3′). The PCR-fragment was digested with MfeI and PstI and cloned into EcoRI and PstI digested pBAD24. All constructs were confirmed by restriction analyses and sequencing. The NrfC variants were also cloned into the IPTG-inducible vector pEXT22 using the NheI/XbaI sites from the pBAD derivatives and the XbaI site of pEXT22 [14].

2.3. Biochemical methods

Quality control assays were carried out as described previously [10,11]. LacZ activity was quantified three hours after L-arabinose induction according to Miller [15] with triplicate samples.

3. Results

3.1. Individual L-arabinose-resistant clones of E. coli MC4100 and its derivatives vary in L-arabinose-inducible expression levels

In 2008, an assay has been developed to study the turnover of Tat substrates during growth [10,11]. In this assay, Tat substrates are produced from pBAD vectors after induction by L-arabinose and culture aliquots from different time points during growth are analyzed by SDS–PAGE and detection of the Tat substrates after Western blotting. When Tat substrates were mutated in a way that affects folding, these substrates were degraded in the *tat* wild type strain MC4100 and in derivatives deleted in *tatA*, *tatB*, *tatC* and *tatE*. The mutated substrates apparently were stabilized in the strains deleted in *tatAE*, *tatD*, and *tatABCDE*. At that time it was concluded that one of the components TatA or TatE, and the component TatD are essential for a degradation of malfolded Tat substrates in a complementary way. This seemed to be clear evidence for a Tat-machinery dependent quality control mechanism, furthermore implicating that TatA, TatE, and TatD play important roles in this pathway [10,11].

In later studies we made the puzzling observation that differing results were obtained with apparently identical strains from the two laboratories involved in this report. The authors noted quantitative differences in Western blot signal intensities that depended on the source of the individual strains, although these strains ought to be identical (see below).

As the clones of the strains used in the two laboratories were independently selected for L-arabinose-resistance, it was possible that differences in expression levels were caused by these independent selection processes. To test this, we generated 16 independent L-arabinose-resistant clones of the wild type strain MC4100 and analyzed the L-arabinose-induced expression level by the use of a pBAD-lacZ vector. The LacZ activity differed in a clone-specific manner and varied between below 1000 and about 10000 Miller Units (Fig. 1A). Having confirmed that expression levels vary between isolated L-arabinose-resistant clones, we likewise measured the expression levels in the clones that had been used in the previously published studies [10,11]. Strikingly, the Δ*tatABCDE* strain, the Δ*tatAE* strain, and the Δ*tatD* strain showed a very high LacZ activity, whereas the other five strains showed intermediate LacZ activities (Fig. 1B). Importantly, the strains that exhibited high expression levels were those that had been thought to be defect in Tat-dependent degradation. These results pointed to the testable possibility that only the higher expression level caused the accumulation that had been interpreted as being a quality control defect in the Δ*tatABCDE,* Δ*tatAE,* and Δ*tatD* strains.

3.2. Malfolded Tat substrates are Tat-independently degraded

To analyze whether the clone-specific expression levels had caused the accumulations or transient appearances of native or malfolded recombinant substrates, we carried out the quality control assay with arabinose-resistant clones of previously determined low, intermediate and high expression levels. We compared L-arabinose-resistant clones of the *tat* wild type strain (MC4100) and the *tatABCDE* deficient strain (DADE) and used the original pBAD-nrfC and pBAD-nrfC-M2 vectors of Matos et al. We found that degradation kinetics and accumulations solely depended on the expression level (Fig. 2). Clones with lowest expression levels (1000–2000 Miller Units) showed no accumulation of NrfC or malfolded NrfC (NrfC-M2) precursor. Intermediate expression levels (4000–7000 Miller Units) resulted in longer but still transient presence of detectable NrfC and NrfC-M2 precursor, and high expression levels (8000–10000 Miller Units) resulted in a strong accumulation of native and malfolded NrfC (Fig. 2A). We also tested the possible formation of inclusion bodies and found that high expression levels resulted in large amounts of inclusion bodies. The amount of soluble protein was variable. Most importantly, there was no stabilization of NrfC-M2 in the *tatABCDE* deficient strain when clones with low or intermediate expression levels were examined. The turnover of NrfC-M2 was the same in the *tatABCDE* deficient strain as it was in the *tat* wild type parental strain. As expected for a malfolded protein, NrfC-M2 was always more rapidly degraded than native NrfC, and this did not depend on the presence of Tat components. The lower stability of NrfC-M2 must therefore be a result of Tat- independent proteolytic degradation within the cytoplasm, which is expected since malfolded proteins should be more susceptible to degradation. At high expression levels, NrfC-M2 almost exclusively formed inclusion bodies and thus escaped proteolytic degradation. Also native NrfC formed significant amounts of inclusion bodies under these conditions. The threshold above which inclusion body formation was observed with these substrates was near 8000 Miller Units (Fig. 2C). Apparently, the high expression levels in the Δ*tatABCDE*, Δ*tatAE*, and Δ*tatD* L-arabinose-resistant clones that were used previously were due to the coincidental selection of high expression level arabinose-resistant clones of these strains, which had caused the substrate accumulations that were interpreted as being due to *tat* mutations and thus incorrectly taken as evidence for a TatAE- and TatD-dependent quality control [10,11].

3.3. The RR > KK mutation in the signal peptide of NrfC and NrfC-M2 triggers inclusion body formation

Having clarified how the differences in accumulation of NrfC and NrfC-M2 were caused, there remained the puzzling