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Single particle tracking of dynamically localizing TatA complexes in *Streptomyces coelicolor*

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ABSTRACT

The Tat (twin-arginine translocation) pathway transports folded proteins across the bacterial cytoplasmic membrane and is a major route of protein export in the mycelial soil-dwelling bacterium *Streptomyces*. We recently examined the localization of Tat components (TatABC) in time-lapse imaging and demonstrated that all three components colocalize dynamically with a preference for apical sites. Here we apply an in-house single particle tracking package to quantitatively analyze the movement of the TatA subunit, the most abundant of the Tat components. Segmentation and analysis of trajectories revealed that TatA transitions from free to confined movement and then to fixed localization. The sequence starts with a mixed punctate and dispersed localization of TatA oligomers, which then develop into a few larger still foci, and finally colocalize with TatBC to form a functional translocation system. It takes 15–30 min for the Tat export complex to assemble and most likely become active. With this study we provide the first example of quantitative analysis of dynamic protein localization in *Streptomyces*, which is applicable to the study of many other dynamically localizing proteins identified in these complex bacteria.

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1. Introduction

In recent years, high resolution cell-biology techniques have revealed that, despite their small size, bacteria have a complex internal organization [1–3]. Molecular crowding within cells results in a dynamic equilibrium of components [4], leading to their non-uniform spatial distribution [5]. This necessitates diffusion or active transport to areas where proteins can function, such as in nuclear organization, cell division or differentiation [6,7]. *In vivo* multidimensional fluorescence microscopy allows scientists to visualize these processes, which often display rapid anddirected spatial relocation of proteins in three-dimensional time lapse movies [8–10]. Taking cell division proteins as an example, these have been seen oscillating from one end of the cell to the other, forming rings across its midsection, or focal complexes at specific intracellular sites [11–14]. To better understand these modes of action, and the lifespan of cellular proteins, quantitative measurements of protein abundance and dynamics are necessary.

A challenging subject for the study of dynamic protein localization is the Gram-positive filamentous soil bacterium *Streptomyces*. These mycelial organisms are used as natural producers of a large number of commercially important secondary metabolites, enzymes, and other secreted protein products [15,16,20]. In most bacteria the Sec pathway is the predominant route for protein export, but the streptomycetes encode an unusually large numbers of Tat (twin-arginine translocation) substrates [26–28]. During fermentation, growth of hyphae is a balance of tip extension, branching frequency and fragmentation [17,18], all of which affect the efficiency of production and secretion. Fragmentation of hyphae strongly enhances protein secretion [24], which correlates well with our observation that the components of the Tat pathway in *Streptomyces* localize at the apical sites of hyphae [25]. A better understanding of where and when secretion takes place in the hyphae can lead to important insights for strain-improvement and rational process design.

The *Streptomyces* Tat machinery consists of TatA, TatB and TatC proteins [28,29], functional homologs of proteins found in Gram-negative bacteria such as *Escherichia coli* [30]. The tetrameric TatA is the most abundant component of the Tat complex [31] and many copies of this monotopic membrane protein are believed to cluster around a substrate-bound TatBC complex to bring about the transport of a folded protein across the membrane, with an average of ~25 TatA subunits per complex [32–34].

Analysis of the Tat complex using live imaging revealed that the proteins localize surprisingly dynamically throughout the life cycle [25]. The dynamic localization of the separate components, however, creates difficulty in understanding their biological implication. Quantitative description and modeling of these movements by single particle tracking is therefore necessary to better understand complex assembly in time and space. Here we provide a detailed analysis of the dynamics of the TatA component of the

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Tat protein export pathway, using an in-house foci tracking package which was developed in the frame of the Particle Tracking Challenge of the International Symposium on Biomedical Imaging (ISBI) in 2012 (Chenouard et al., *submitted for publication*). Specific questions we wished to address were (1) whether TatA undergoes different stages of motion prior to co-localization with TatB and TatC; (2) if so, what are the durations of these transitional stages; and (3) what is the time it takes for the complex toassemble.

This is the first mathematical tracking analysis of diffusion-limited foci in vegetative and aerial hyphae of *Streptomyces*, which can be applied to study the movement of many other dynamic proteins in these complex microorganisms.

2. Material and methods

2.1. Strains and culturing conditions

Strain BRO3 is a derivative of *S. coelicolor* FM145, a variant of *S. coelicolor* M145 with reduced autofluorescence [35]. BRO3 produces TatA-eGFP from the native chromosomal location (FM145, TatA::TatA-eGFP). Construction of the strain is described elsewhere [25]. Initial examination of all strains was done by stereo fluorescence and light microscopy with a Zeiss Lumar V12 as described previously [38]. To culture samples for live imaging, uncoated μ-dishes (Ibidi GmbH) were perforated at the side while closed tightly, and subsequently were semi-filled with SFM medium [36]. These dishes were inoculated with 1 μL of spores at a concentration of 10^9 spores/mL, turning the lid so that it was supported on the vents, allowing for gas exchange, and were sealed off by two layers of Parafilm to prevent drying of the medium. Samples were incubated at 30 °C for 48 h before being transferred to the live imaging microscope. Temperature was controlled with a p-insert heating block and kept at 30 °C, and allowed to calibratefor 1 h before commencing imaging.

2.2. Data collection

2.2.1. Fluorescence microscopy

Fluorescence and corresponding light micrographs were obtained with a Zeiss Observer inverted fluorescence microscope (with an Hamamatsu CCD camera at a resolution of 78 nm/pixel) as described [35,37]. The green fluorescent images were created using 470/40-nm bandpass excitation and 525/50 bandpass detection. All images were background-corrected, setting the signal outside the hyphae to 0 to obtain a sufficiently dark background. These corrections were made using Axiovision software 4.8.

2.2.2. Time-lapsed (live) imaging

Imaging was performed with a Zeiss Observer A1 microscope with a Hamamatsu EM-CCD C9100–02 camera as described [39]. Images were taken at 2 min intervals with an exposure time of 100 ms. In order to minimize focal drift, the microscope stage and imaging chamber were allowed to equilibrate for 60 min before imaging. Initially z-stacks of five images with a focal depth of 0.5 μm were taken to prevent out-of-focus movement of the hyphae. These image sequences were then z-projected using the average z-stack projection method implemented in ImageJ.

2.2.3. Computer programs

The particle tracking algorithm was submitted to the Particle Tracking Challenge (http://bioimageanalysis.org/track/) of the 2012 International Symposium on Biomedical Imaging (http://www.biomedicalimaging.org/2012/), in which the performance of existing and newly developed particle tracking algorithms was tested against synthetic image sets with ground truth data. The methods and results of the challenge have been described (Chenouard et al., *submitted for publication*). For details on the algorithm see the Supplementary Methods. The algorithm was implemented in the Java programming language (Sun Microsystems Inc., Santa Clara, CA) in the form of a plug-in for ImageJ (National Institutes of Health, Bethesda, MD), the computer-platform independent public domain image analysis program inspired by NIH-Image. ImageJ version 1.46o was used with the Java 1.7 compiler.

3. Results and discussion

We focused on TatA complex assembly as a whole, which occurs at a timescale several orders of magnitude greater than the fast movement of individual proteins. To capture an entire localization sequence (with an initial estimated duration of roughly 30–60 min) prior to bleaching of the samples, we took images for up to 3.5 h at 2 min intervals. Since mobility data suggests that cytosolic diffusion occurs at a time scale two orders of magnitude faster than membrane diffusion, we observed the dynamics of slow-moving membrane-bound macrocomplexes, and not the cytosolic fraction of TatA. *S. coelicolor* FM145, a derivative from the wild-type strain withreduced autofluorescence [35], was used for the localization studies, allowing imaging of the fluorescently labeled proteins at significantly higher signal to noise ratio. In 18 time lapse imaging movies of diffusion-limited TatA-eGFP, roughly 1500 foci were tracked from the onset of imaging to final localization. Foci tracked/detected in only two consecutive frames were not taken into account. The distribution of tracks (Fig. 1) demonstrates that short tracks predominate, with roughly half of all tracks corresponding to 10 or less consecutive frames.

Our previous study revealed increased amounts of localization in aerial hyphae, and imaging was therefore performed on early aerial hyphae after 48 h of growth. At this point, many hyphae had already stopped growing due to crowding in the sample, butoccasionally extending tips were still observed. In the growing hyphae, the Tat complex was seen following the tips.

The broad range of stoichiometries exhibited by TatA suggests that dynamic polymerization and depolymerization occurs during assembly [33]. This is evident in our time lapse sequences, where protein complexes display different types or stages of movement prior to fixed localization (ex. Fig. 2 and Supplementary Video 1). At the start of imaging, mainly dispersed fluorescence is seen, with gradually many faint foci appearing to 'race' along the hyphae. During this fast movement, several strong fluorescent foci become visible localizing (mainly) near the tips at roughly a 2 μm distance. Over the course of the time lapse, the faint focieither fade or merge with larger stable fluorescent foci. These faint foci have short tracks lasting only 3–4 frames or 6–8 min. Particle tracking highlights these tracks as brief directed bursts of motion. The larger foci remain stationary, or wobble before stabilizing.

A large variation in fluorescence intensity is seen over the course of imaging. Initial faint foci assemble over the course of the time lapse imaging to form complexes with increased fluorescent intensity, likely reflecting natural protein oligomerization. Although formation of protein complexes can be enhanced by an eGFP fusion, which may affect protein functionality [40], we previously demonstrated that the TatA fusion is fully functional, restoring secretion of Tat substrates to wild-type levels. For full functional analysis of the TatA fusion product and Tat complex formation we refer to our previous work [25].

Supplementary Video 2 shows another representative example of dynamic localization. The final image from the time lapse is presented (Fig. 3A), with the detected TatA-eGFP tracks projected onto the image. Despite the fading of foci due to the imaging, three tracks could be followed for the entire duration (1 h) of the movie. The trajectories of these tracks were plotted (Fig. 3B), demonstrating representative trajectories of a still focus, a rapidly traveling focus (which becomes confined after 10 min) and a wobbling focus.

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