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Flow cytometric monitoring of protein secretion via the Tat-pathway in *E.coli*

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Introduction

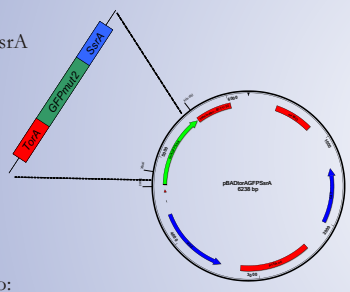
Optimization of the secretion pathway by *Escherichia coli* is of high interest for biotechnological purposes. The export of proteins into the culture medium or into the periplasm of Gram-negative bacteria facilitates downstream-processing, and thereby reduces the costs of the production process.
A secretion pathway of special interest in Gram-negative bacteria is the the twin-arginine translocation Tat-pathway, which mediates the export of folded proteins across the cytoplasmic membrane in a ATP-independent manner. However, only a relative low amount of proteins is naturally secreted via this pathway.

Different attempts are made to enhance the level of secreted proteins. The use of transcriptional analysis via DNA-microarray-technology for instance should help identifying some key elements of the protein export, thus offering chances for optimization of the Tat protein export by cell engineering.
To evaluate the success of such strategies a quantitative method for examinations of the protein-secretion is needed. In this work we present the construction of a reporter strain based on the system of DeLisa et al. (2002) in order to investigate the Tat specific protein export in the strain *E.coli* Bl21.

Reportersystem

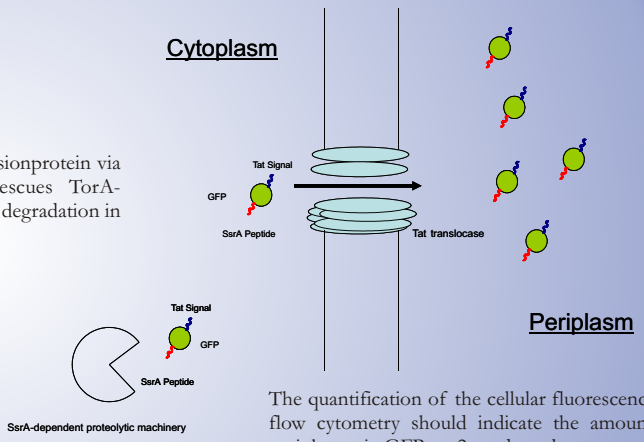
Strain: *E.coli* Bl21

Plasmid:
pBADTorAGFPmut2SsrA



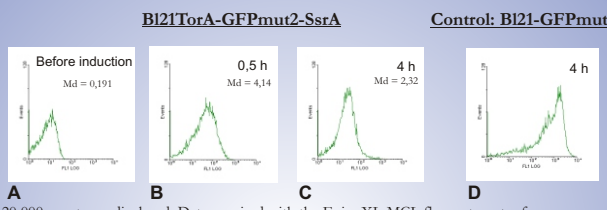
GFPmut2 is fused to:
- a substrate of the Tat-pathway, the leaderpeptide of trimethylamine N-oxide reductase (TorA),
- a SsrA peptide which targets the protein for degradation by the cytoplasmic proteolytic ClpXP machinery.

The export of the fusionprotein via the Tat pathway rescues TorA-GFPmut2-SsrA from degradation in the cytoplasma.



The quantification of the cellular fluorescence via flow cytometry should indicate the amount of periplasmatic GFPmut2 and can be consequently used as a measure for secreted proteins.

Results I



20 000 events are displayed. Data acquired with the Epics XL-MCL flow cytometer from Beckman Coulter. Green fluorescence is measured using a band-pass filter transmitting light between 505 nm and 545 nm.

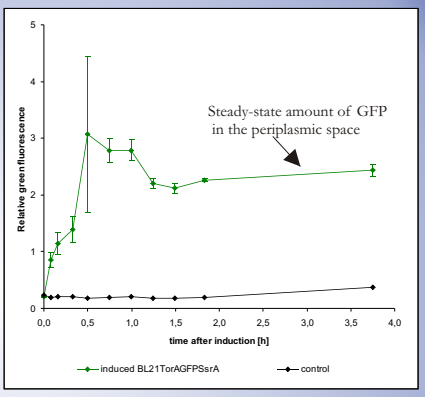
Figures A, B, C present the green fluorescence distribution of Bl21TorA-GFPmut2-SsrA cell populations before/after induction with 0,2 % L-Arabinose.
Figure D presents the fluorescence distribution of a control population BL21eGFP approx. 4 hours after induction. In this control strain the reporterprotein is expressed without the TorA and SsrA peptides. Thus the accumulation in the cytoplasm of the expressed GFPmut2 can be observed.

The experiment clearly indicates that only a small amount of the expressed GFPmut2 is exported via the Tat-pathway in the strain Bl21.

Results II

Kinetic Measurements

The progression of the cellular fluorescense after induction can be seen in figure E. A maximum of the GFPmut2 fluorescence was measured after 0,5 h. A subsequent decrease is observed. Approximately after 2 hours a steady state of cellular fluorescence is reached. The kinetic measurement of the GFPmut2 fluorescence suggests that the intracellular TorA-GFPmut2-SsrA first saturates the SsrA-dependent proteolytic machinery: over the first hour the measured fluorescence results from cytoplasmic and periplamsic GFP. The observed steady state fluorescence should correspond to the amount of periplasmic GFP.
Consequently, the quantitative measurement of exported GFP via the Tat-pathway in the periplasm should first be assayed at least 2 hours after induction.



E

GFPmut2 fluorescence after induction with 0,2 % L-Arabinose. The relative green fluorescence of the *E.coli* Bl21TorA-GFPmut2-SsrA populations is expressed as the median of the fluorescence distribution. Standard deviation results from the relative green fluorescence of three different cultures (n=3). The fluorescence of a non-induced cell population was monitored over the time as a negative control.

Discussion and Outlook

The presented reporter system allows the flow cytometric monitoring of the Tat-specific protein export in the expression strain BL21. However, one should note that the reporter system is not sensitive enough over the first 2 hours after induction with L-Arabinose: over this time the cytoplasmic proteolytic machinery is overwhelmed by the expression of the Tora-GFPmut2-SsrA. Only after 2 hours the measured signals should be considered as a measure of the periplasmatic reporterprotein. Using this approach, we could show that only a small amount of the expressed GFPmut2 is exported via the Tat-pathway in the strain Bl21.
The transcriptom of this *E.coli*-secretion strain after induction is presently being analyzed by our group using whole genome arrays and secretion specific low-density microarrays. This screening may allow to identify some key elements of the Tat-pathway, which may be up- or down-regulated in order to enhance the protein flux to the periplasm. Flow cytometric monitoring of exported proteins will provide a useful tool to evaluate the success of such optimization strategy: one aim would be the maximization of the steady state periplasmic level of the reporter fusionprotein.