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Brief report

# Intracellular monitoring of target protein production in *Staphylococcus aureus* by peptide tag-induced reporter fluorescence

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## Summary

**An intracellular approach for monitoring protein production in *Staphylococcus aureus* is described. mCherry, fused to the dodecapeptide Tip, was capable of inducing tetracycline repressor (TetR). Time- and concentration-dependent production of mCherry could be correlated to TetR-controlled GFPmut2 activity. This approach can potentially be extended to native *S. aureus* proteins.**

*Staphylococcus aureus* is a pathogenic bacterium causative of numerous human diseases. A profound understanding of *S. aureus* biology is imperative for finding cures against infections caused by strains recalcitrant to antibiotics. Changes in the transcriptome and proteome patterns have been elucidated for many *S. aureus* strains and mutants (see Nagarajan and Elasri, 2007; François *et al.*, 2010; Hecker *et al.*, 2010 for recent overviews); however, analysing intracellular protein production of *S. aureus*, especially during infection, remains challenging. Translational fusions of genes of interest to easily detectable and quantifiable reporters, such as *lacZ* or *gfp*, result in a considerable increase of the deduced proteins in molecular mass, which may not only render them less abundant in the cell but potentially also less soluble or impeded in localization or activity (Cubitt *et al.*, 1995; Lesley *et al.*, 2002). In contrast, proteins bearing short peptide appendices, exemplified by the hexa-histidine- or the nine-amino-acid encompassing strep-tag (Hochuli *et al.*, 1988; Schmidt and Skerra, 1994), typically used to facilitate purification, generally provide high chances of retained activity. The dodecapeptide Tip (TetR-inducing peptide) has been identified as an alternative inducer of tetracycline (Tc) repressor TetR (Klotzsche *et al.*, 2005), an extensively characterized and widely applied regulator in bacteria (Bertram and Hillen, 2008). Notably, in contrast to TetR's conventional low-molecular-weight effectors Tc or anhydrotetracycline (ATc) (Degenkolb *et al.*, 1991), TetR(B) from transposon Tn*10* is susceptible to Tip, which is not the case for the frequently used TetR(BD) hybrid (Schnappinger *et al.*, 1998; Schubert *et al.*, 2001). This specific interaction and the chemical nature of Tip can be exploited in the context of a genetic read-out system, which monitors the specific production of a target protein tagged with Tip. To date, the expression of at least six full-length or truncated genes translationally fused to Tip could be correlated to an increase of *lacZ* activity subject to TetR control (Klotzsche *et al.*, 2005; Schlicht *et al.*, 2006). Since the Tip system has only been applied in *Escherichia coli* and *Salmonella* so far (C. Berens, pers. comm.), we decided to adapt Tip-dependent TetR induction to *S. aureus*, a Gram-positive bacterium of broad general and clinical interest, in a proof-of-concept study.

To set-up strains capable of responding to Tip, a *tetR(B)* expression cassette was inserted into the chromosomal *lip* locus of *S. aureus* SA113 (wt) and its derivative RAB171 (harbouring a TetR-controlled $P_{xyl/tet}$-*gfpmut2* fusion). To this end, *tetR(BD)* was exchanged against *tetR(B)* in a pRAB2-derived vector, which was subsequently integrated as described (Stary *et al.*, 2010). Oligonucleotides, plasmids and bacterial strains are listed in Tables 1 and 2; further details are available on request. Epifluorescence microscopy (DM 5500 B microscope and DFC

Received 12 July, 2011; revised 11 August, 2011; accepted 15 August, 2011. *For correspondence. E-mail ralph.bertram@uni-tuebingen.de; Tel. (+49) 707129 75934, Fax (+49) 7071 29 5937. We dedicate this paper to the memory of Wolfgang Hillen, pioneer in TetR research and co-author of the inaugural article of *Microbial Biotechnology*.

**Table 1.** Oligonucleotides used in this study.

| Primer | Sequence (5′→3′) | Characteristics and comments |
|---|---|---|
| GPMCHE-F | GGATCC**GAATTC**TT*AGGAGGA*TGATTATTTATGGT GAGCAAGGGCGAG | EcoRI, *sod* ribosomal binding site; cloning of *gp-mcherry* into pJL71 |
| GPMCHE-R | GGATCC**GGCGCGCC**TTACTTGTACAGCTCGTCCATGC | AscI, cloning of *gp-mcherry* into pJL71 |
| gpmCh_fw_BamHI | GATC**GGATCC***AGGAGGA*TGATTATTTATGGT | BamHI, cloning of *gp-mcherry* into pRAB32-gp |
| gpmCh_rev_XmaI | CTAG**CCCGGG**TTACTTGTACAGCTCGTC | XmaI, cloning of *gp-mcherry* into pRAB32-gp |

Endonuclease restriction sites are given in boldface, ribosomal binding sites are italicized and start codons are underlined.

**Table 2.** Bacterial strains and plasmids used in this study.

| Strain or plasmid | Relevant characteristic(s) | References and comments |
|---|---|---|
| Strains | | |
| *E. coli* | | |
| DH5α | *F-(φ80d ΔlacZ M15) Δ(lacZYA-argF) U169hsdR17(r-M) recA1 endA1 relA1 deoR)λ-, phoA supE44thi-1, gyrA96* | Hanahan (1983) |
| *S. aureus* | | |
| RAB171 | SA113 *fdh*::$P_{xyl/tet}$-*gfpmut2* | Stary *et al.* (2010) |
| RAB200B | SA113 *lip*::Pt17-*tetR(B), lox66-aphAIII-lox71* | This study |
| RAB201B | SA113 *lip*::Pt17-*tetR(B), lox72* | This study |
| RAB210B | SA113 *lip*::Pt17-*tetR(B), lox66-aphAIII-lox71, fdh*::$P_{xyl/tet}$-*gfpmut2* | This study |
| RAB211B | SA113 *lip*::Pt17-*tetR(B), lox72, fdh*::$P_{xyl/tet}$-*gfpmut2* | This study |
| RN4220 | NCTC8325-4 derivative, acceptor of foreign DNA | Iordanescu and Surdeanu (1976) |
| SA113 (ATCC 35556) | NCTC8325 derivative, *agr*⁻, 11 bp deletion in *rbsU* | Iordanescu and Surdeanu (1976) |
| Plasmids | | |
| pJL71 | pCN54 derivative (Charpentier *et al.*, 2004) bearing *gp-mcherry* | This study |
| pKX15 | *aphAIII*, *xylR*, $P_{xylA}$, pTX15 derivative (Peschel *et al.*, 1996) | This study, *aphAIII* cloned from pDG782 (Guérout-Fleury *et al.*, 1995) via StuI and Acc65I |
| pRAB2-B | *cat*, *bla*, *'lip'*-Pt17-*tetR(B)-lox66-aphAIII-lox71-'lip'* pBT2 derivative (Brückner, 1997) | This study, *tetR(B)* cloned from pWH1925-derivative (Scholz *et al.*, 2004) via XbaI and BstEII |
| pRAB32-gp/-ntgp/-ctgp | pKX15 derivative bearing native, N-terminally or C-terminally *tip*-tagged *gp-mcherry* | This study, *gp-mcherry* cloned from pJL71, attachment of *tip*-tags by commercially purchased DNA fragments (Entelechon, Regensburg) |

Antibiotics were used, where appropriate, at the following final concentrations: ampicillin 100 μg ml$^{-1}$, chloramphenicol 10 μg ml$^{-1}$ and kanamycin 15 μg ml$^{-1}$.

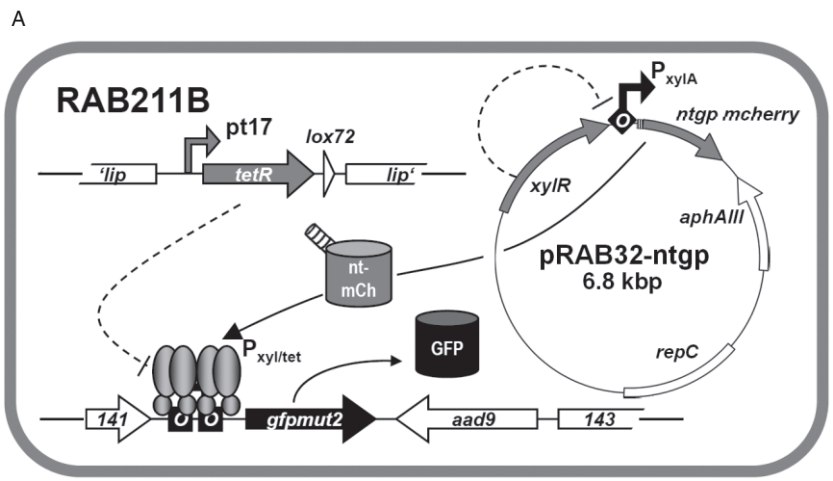


**Fig. 1.** A. Schematic representation of RAB211B (pRAB32-ntgp). Tip or *tip* are symbolized by hatched tubes or boxes respectively. Repression is symbolized by dashed lines. Squares or diamonds marked 'O' denote TetR- or XylR-binding sites respectively. Promoters are represented by bent arrows (with TetR bound to $P_{xyl/tet}$). nt-mCh: N-terminally Tip-tagged mCherry; GFP: GFPmut2.
B. Representation of Tip-tagged or native mCherry proteins' primary structure termini. The Tip dodecapeptide is given in bold and is marked by a hatched box.

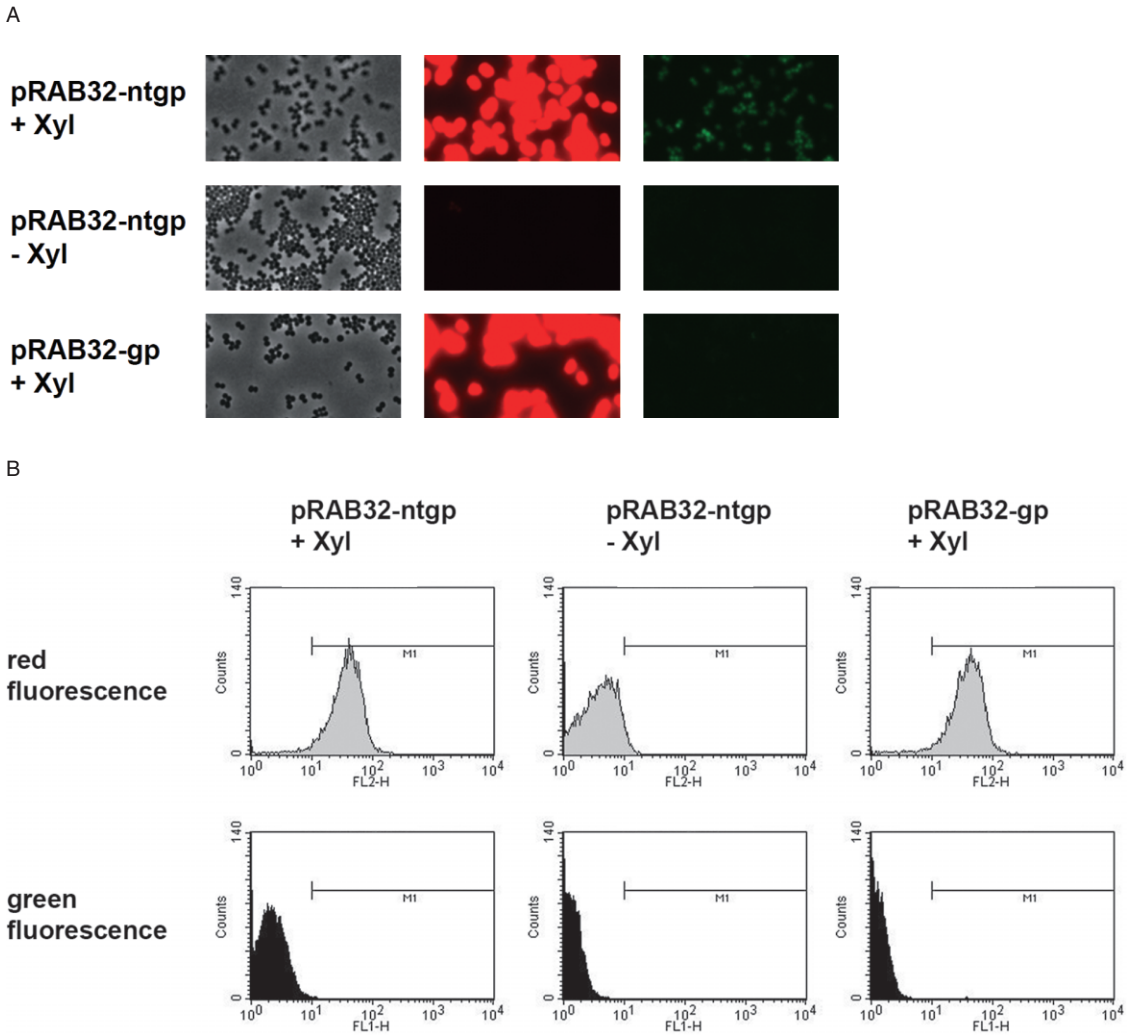


**Fig. 2.** RAB211B cells harbouring vectors and grown under conditions as indicated.
A. Microscopic observation of cells 330 min after xylose induction in phase-contrast (left), using filters for red- (middle) or green-fluorescence (right).
B. Flow cytometric analysis of cells carrying pRAB32-gp or -ntgp grown in medium with or without xylose. Upper panels show populations' red fluorescence, lower panels indicate green fluorescence. The M1 threshold was arbitrarily set to 10. It should be noted that GFPmut2 fluorescence is also partially detected in the FL2-H channel.

360 FX camera, Leica) confirmed that cells of the RAB171-derived strain RAB201B ($P_{xyl/tet}$-*gfpmut2*, *tetR*(*B*)), but not those of RAB200B (*tetR*(*B*)), devoid of a TetR controlled reporter gene, exhibited bright green fluorescence when cultured with a final concentration of 0.4 μM ATc (data not shown). Thus, TetR(B) (henceforth referred to as TetR for convenience) can functionally regulate gene expression in this genomic architecture, as shown for TetR(BD) before (Stary *et al*., 2010). Subsequent excision of the *aphAIII* resistance markers of RAB200B and RAB201B by Cre recombinase (Leibig *et al*., 2008) yielded kanamycin-sensitive strains RAB210B and RAB211B respectively (Fig. 1A). As a prototype carrier for Tip, the monomeric protein mCherry was chosen (Shaner *et al*., 2004), which is an established red fluorescent reporter in *S. aureus* (Malone *et al*., 2009; Pereira *et al*., 2010). Intracellular detection and quantification does not require substrates or co-factors and its structural properties, including solvent exposed N- and C-termini (Shu *et al*., 2006), make it very suitable for protein fusions. In analogy to comparable fluorescent protein encoding genes, successfully adapted to the codon usage of *S. aureus* (Sastalla *et al*., 2009; Paprotka *et al*., 2010), a new *mcherry* allele termed *gp-mcherry* (Gram-positive adapted mCherry) was designed (DNA 2.0, Menlo Park, CA, USA). Furnished with the ribosomal binding site of *sod* (Franke *et al*., 2007), it was cloned into the xylose inducible vector pKX15. Cloning of further synthetic DNA fragments (Entelechon, Regensburg) into the obtained vector pRAB32-gp gave rise to plasmids pRAB32-ntgp and pRAB32-ctgp, encoding

 

mCherry N- or C-terminally linked to Tip respectively (Fig. 1B). RAB210B and RAB211B cells carrying either one of the three pRAB32 plasmids (Fig. 1A) were cultivated in rich basic medium (BM) (Bera *et al*., 2005) without glucose and analysed for xylose-dependent red fluorescence using three different methods. In all strains induced by adding xylose to a final concentration of 0.5% (w/v), cells glowed strongly red, indicating high levels of functional mCherry irrespective of any Tip-appendix. In contrast, weak but clearly discernible green fluorescence was observed only in induced RAB211B (pRAB32-ntgp) cells, as depicted in Fig. 2A. The same reporter strains were subjected to flow cytometry (FACSCalibur, BD) and accordingly, a shift towards red fluorescence by about one order of magnitude was observed in all populations of induced cultures. Again, *gfpmut2* expression appeared to be solely triggered in the strain disposing of N-terminally Tip-tagged mCherry (Fig. 2B). In order to quantify red and green fluorescence, cells harvested at different time-points during exponential growth phase were analysed using a microplate reader (Infinite M200 Pro, Tecan). Activity profiles of mCherry and GFPmut2 expressed from the reporter strains were subsequently monitored in a time-resolved and xylose-dependent manner. As depicted in Fig. 3A, red fluorescence increased steadily over time in xylose-induced cells, albeit somewhat less pronounced in case of C-Tip mCherry. This might be due to improper folding or lower protein amounts, one or both of which might also be causative for the inefficiency of C-Tip mCherry to induce GFPmut2 production. This also agrees with previous results, in which C-terminally Tip-tagged TrxA had proven to be a less effective inducer of TetR than N-tip TrxA in *E. coli* (Klotzsche *et al*., 2005). The observed GFPmut2 activity was also consistent with observations from microscopy and flow cytometry, because only cells expressing N-terminally tagged mCherry, fluoresced both red and green (Fig. 3B). To provide a control for full, rapid and direct induction of TetR, RAB211B was induced by 0.4 μM ATc. In comparison, green fluorescence due to Tip-mediated induction in RAB211B (pRAB32-ntgp) was delayed by ~ 200 min. This might in part be attributed to 15 min maturation time for mCherry (Shaner *et al*., 2004), but most likely primarily reflects the time span between xylose induction and the production of sufficient amounts of N-Tip mCherry to efficiently induce TetR. Induced RAB211B (pRAB32-ntgp) cells reached ~ 30% of the fluorescence intensity determined for RAB211B with ATc. Indeed, binding constants of Tip to TetR had been established to be about 2–3 orders of magnitude lower than for Tc effectors (Degenkolb *et al*., 1991; Klotzsche *et al*., 2005). As indicated by Figs 2 and 3A, the induced reporter strain appears to produce high amounts of mCherry when cultured with 0.5% xylose. According to a study by Zhang and colleagues (2000), a rough estimation indicates the presence of mCherry in a range of ~ $10^4$ proteins per cell in this state. Reducing the inducer concentrations for RAB211B (pRAB32-ntgp) resulted in stepwise decreased red and green fluorescence (Fig. 4). Thus, the system appears to be capable of graded responses to different amounts of the Tip-tagged target protein. Strains with enhanced sensitivity might be required to detect the presence of native *S. aureus* proteins that are only weakly or moderately produced. Increasing the inducer to TetR ratio is one promising way, since strains with less TetR, controlling the $P_{xyl/tet}$ promoter upstream of *gfpmut2*, putatively respond better to lower levels of Tip-tagged proteins (Klotzsche *et al*., 2005). To this end, *S. aureus* strains bearing autoregulated *tetR*, which generally ensures a balanced level of repressor molecules, might be exploited (Gründling and Schneewind, 2007), optionally combined with improved TetR/Tip pairs (Klotzsche *et al*., 2005; Klotzsche *et al*.,

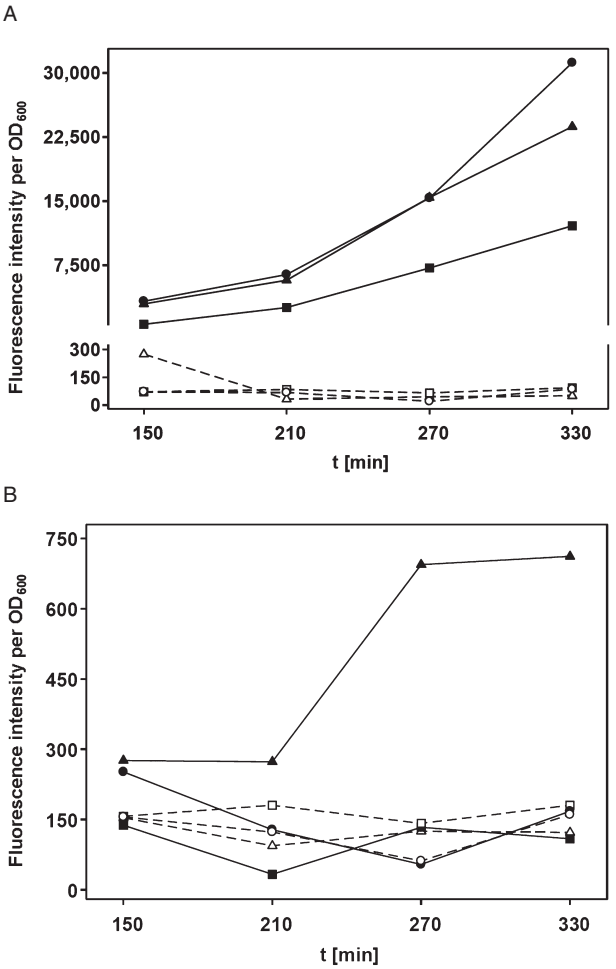


**Fig. 3.** Fluorescence intensities of RAB211B cells bearing pRAB32-gp (circles), pRAB32-ntgp (triangles) or pRAB32-ctgp (squares), (A) red-, (B) green-fluorescence. Open symbols (connected by dashed lines) represent growth under non-induced conditions and filled symbols denote cells induced with xylose. Fluorescence values were correlated to cell densities ($OD_{600}$).

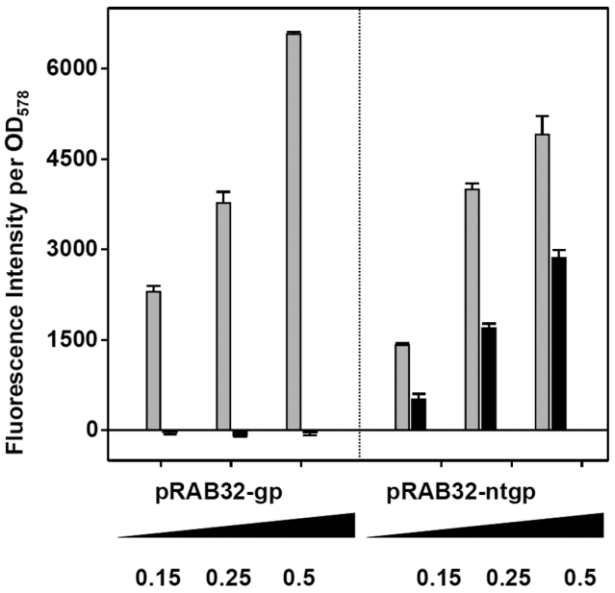


**Fig. 4.** Influence of different xylose concentrations on red- (grey bars) and green-fluorescence (black bars) of RAB211B cells bearing vectors as indicated. Fluorescence values were correlated to cell densities ($OD_{578}$) and basal fluorescence from non-induced cells was subtracted from values obtained with 0.15%, 0.25% and 0.5% xylose.

2007; Daam *et al*., 2008). Taken together, proof-of-concept for a functional Tip-tagging architecture in *S. aureus* was achieved using mCherry as a carrier protein. Based upon our observations that N-tip mCherry both glowed red and moonlighted as an inducer for TetR, it appears feasible to apply this approach to expression profiling of *S. aureus* encoded proteins. To this end, *tip* could be either fused to genes of interest on plasmids for chromosomal integration (Brückner, 1997; Arnaud *et al*., 2004; Bae and Schneewind, 2006), or it could be randomly attached to chromosomal genes via an integrative element in a transposon-like fashion, as demonstrated for *E. coli* before (Schlicht *et al*., 2006). The Tip-tagging technique might be particularly useful for *in vivo* grown *S. aureus* cells, e.g. in infection models, in which standard proteomic techniques might not be applicable.

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