Vectors for improved Tet repressor-dependent gradual gene induction or silencing in *Staphylococcus aureus*

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A set of vectors for improved tetracycline-dependent gene regulation in *Staphylococcus aureus* is presented. Plasmid pRAB11 was generated from pRMC2 by adding a second tet operator within the TetR-regulated promoter P\_xyl/tet. Pronounced repression was observed in the absence of anhydrotetracycline (ATc) combined with high induction in the presence of the drug, as demonstrated for pRAB11 bearing staphylococcal nuclease nuc1, lacZ or gfp. Also, in plasmid pCG261, the pRAB11 \(\text{tetR}^{-}\text{P}\_\text{xyl/tet}\) regulatory architecture permitted tight repression and a stepwise increase in transcript amounts of the target gene *rny* (putative RNase) correlated with rising ATc concentrations. Additionally, pRAB11-derived vectors harbouring semi-rationally designed P\_xyl/tet-like fragments, mutated at up to six defined positions, were constructed. Sixteen mutant sequences with single to quadruple exchanges were analysed for transcriptional strength and ATc-dependent inducibility. A set of promoters with gradually decreased activities and improved repression is presented. Finally, the implementation of reverse TetR revtetR-2, which exhibits three amino acid exchanges and binds to tetO in the presence of ATc, yielded an efficiently co-repressible vector within the pRAB11 system. Intriguingly, revtetR was found to contain a fourth mutation only after propagation in *S. aureus*. We predict that the described vectors constitute valuable tools for staphylococcal genetics.

INTRODUCTION

*Staphylococcus aureus* remains a serious health threat in hospitals and in the community (Levy & Marshall, 2004). There are great and justified concerns about the emergence of strains combining multiple antibiotic resistances with high degrees of virulence and physical fitness (DeLeo et al., 2010). The spread of such community-associated methicillin-resistant *S. aureus* strains would render staphylococcal infections even more difficult to treat, and therefore the development of anti-staphylococcal compounds must not be neglected. A wealth of molecular genetics and genomics data from different *S. aureus* strains and a number of studies that revealed essential genes in these bacteria (Bae et al., 2004; Chaudhuri et al., 2009; Forsyth et al., 2002; Ji et al., 2001) have contributed to the revelation of novel potential drug targets. For the validation of respective cellular factors, inducible gene expression systems are very useful by altering the molar drug to target ratio which helps to clarify modes of action. Most of the gene regulation systems available for *S. aureus* act at the level of transcription initiation and exploit repressor proteins which respond to low molecular mass effectors such as carbohydrates or antibiotics. Popular systems to date are based upon the repressors XylR (Wieland et al., 1995), LacI (Jana et al., 2000) or TetR (Ji et al., 1999). The latter binds to tet operator (tetO) and is induced by tetracycline (Tc) or derivatives such as the less antibiotically active anhydrotetracycline (ATc) (Degenkolb et al., 1991), which both cause dissociation of the TetR : tetO complex. (A)Tc-dependent\(^{-}\) gene regulation systems have been applied in dozens of bacterial species (Bertram & Hillen, 2008, http://tinyurl.com/tetreg). TetR’s cognate DNA operator binding site tetO is usually embedded in one or two copies in a suitable target gene promoter. Tet regulation in both eukaryotes and bacteria can be divided into tet-ON and
tet-OFF systems according to the response elicited by addition of the effector compound (Berenis & Hillen, 2003). tet-ON systems then undergo a rapid increase in the transcriptional activity, whereas the hallmark of tet-OFF systems is gene silencing upon drug administration. tet-OFF responses in S. aureus were initially achieved by TetR-dependent inducible expression of a target gene’s antisense RNA fragment (Ji et al., 1999), resulting in post-transcriptional downregulation. More recently, however, a reverse variant of TetR which binds to tetO only in the presence of ATc has been applied for use in S. aureus (Stary et al., 2010). Enabling tet-OFF regulation acting on the level of transcription, this regulator termed revTetR-r2 exhibits a total tetR-encoded diversity of tet regulation architectures has been described for S. aureus, whereby TetR controls target genes encoded in cis or in trans (Bateman et al., 2001; Gründling & Schneewind, 2007; Stary et al., 2010). A convenient and popular system for complementation studies in staphylococci is represented by pALC2073 and vectors derived therefrom (Bateman et al., 2001). Such one-plasmid systems carry both tetR and a target gene located adjacent on one common vector. In pALC2073, tetR is driven by the autoregulated promoter PR*, whereas the target gene is expressed divergently by a hybrid promoter termed Pxyl/tet. It represents a modified Bacillus subtilis PxyA promoter equipped with tetO. During its initial application in both B. subtilis (Geissendorfer & Hillen, 1990) and S. aureus (Zhang et al., 2000) Pxyl/tet had turned out to mediate very strong transcription. An undesired leakiness of target gene expression in the absence of inducer, inherent to this regulatory architecture, was markedly reduced by strengthening the promoter of tetR in plasmid pRMC2 (Corrigan & Foster, 2009). In this study, we describe a number of new vectors enabling an even tighter repression of target genes by exploiting semi-rationally designed Pxyl/tet variants which also confer gradual transcriptional activity in the induced state. These vectors were efficient for the regulation of both reporter genes and native S. aureus factors in different strain backgrounds. Furthermore, we present for the first time to our knowledge, a plasmid that enabled transcriptional tet-OFF control in cis by means of revTetR in S. aureus.

S. aureus strains by electroperoration or transduction using phage Φ11. Strains and plasmids used in this study are listed in Table 1.

### Isolation, manipulation and detection of nucleic acids.

Plasmid DNA was prepared from E. coli or staphylococci using commercially available kit systems from Qiagen or Macherey-Nagel. For cell disruption, staphylococci were generally treated with lysostaphin at a final concentration of 12.5 μg ml⁻¹ for 30 min at 37 °C unless stated otherwise. Chromosomal DNA of S. aureus was prepared by phenol/chloroform extraction or by using the InstaGene system (Bio-Rad). DNA sequencing was carried out at GATC or 4base lab. Detection of tet-controlled rry transcript amounts by Northern-blotting was achieved as follows. Overnight cultures of S. aureus Newman HG-217 bearing plasmid pCG260 or pCG261 were grown in TSB medium and were diluted with fresh medium to an initial OD₆₀₀ of 0.05. Cultures were shaken at 37 °C to OD₆₀₀ 0.5, when ATc was added at different concentrations. After another 30 min the cells were harvested and treated as described previously (Goerke et al., 2000). Briefly, bacteria were lysed in 1 ml Trizol reagent from Invitrogen/Life Technologies with 0.5 ml zirconia-silica beads (0.1 mm diameter) in a high-speed homogenizer (Savant Instruments). RNA was then isolated as described in the instructions provided by the Trizol manufacturer. The digoxigenin (DIG)-labelled probe for the detection of rry transcript was generated with the DIG-labelling PCR from Roche Applied Science using oligonucleotides rry-dig-for and rry-dig-rev and DNA from strain RN6390 (Peng et al., 1988) as a template. Primers used in this study are summarized in Table 2.

### Reporter gene and enzyme assays.

β-Galactosidase activity of S. aureus SA113 cells bearing different plasmids was determined as described by Geiger et al. (2008). For flow cytometry analyses of cells expressing GFP, SA113 (pRMC2-gfp) or SA113 (pRAB11-gfp) were cultivated overnight in TSB, inoculated into fresh TSB to OD₆₀₀ 0.05 and cultivated to 0.5 (each with or without ATc, respectively). Prior to measurements, cultures were diluted 1:5 in 0.9% (w/v) NaCl. Using a BD FACS Calibur system, cells were excited with a blue argon laser (488 nm) and fluorescence emission was detected using an FITC detector channel (530 ± 15 nm). To test for exonuclease activity, S. aureus strains were grown on DNA agar CM0321 (Oxoid). Plates containing freshly grown colonies were flooded with 1M HCl and zones of clearing around the colonies indicated positive exonuclease results.

### Construction of pRMC2 derivatives, pRAB11 with and without target genes, and control vectors.

Plasmids used in this study were propagated and cloned in E. coli DH5α, XL1-Blue or One Shot TOP10 chemically competent cells (Invitrogen/Life Technologies). Final constructs were verified by analytical restriction and sequencing. A fragment of 768 bp composed of S. aureus SA113 nucleotide sequence was amplified from chromosomal DNA using primers Nucl-BglII_ fw and Nucl-EcoRI_ rev. It was digested with BglII and EcoRI and was ligated with the similarly restricted vector pRMC2, yielding pRMC2-nuc1. A 103 bp fragment containing the PxyA variant with a second tetO sequence originating from plasmid pWH354 (Geissendorfer & Hillen, 1990) was used to replace the one-tetO PxyA promoter in pRMC2-nuc1 via XhoI and KpnI yielding pRAB11-nuc1. An 868 bp KpnI/PstI sequence containing tetR, its promoter and the divergent two-tetO PxyA version was then ligated with the 5.6 kb KpnI/PstI vector backbone of pRMC2 to obtain pRAB11. Gene gfpmut2 (Cormack et al., 1996) was amplified from plasmid pPRAB4 (Stary et al., 2010) using primers pPRAB4-gfpmut2-fw and -rev, and a 3.3 kb translational spoVG-lacZ fusion was obtained using primers pRAB11-lacZ-fw and -rev and pWH102 as a template (Kamionka et al., 2005). Each of the fragments was inserted into pRAB11 and pRMC2 via BglII and EcoRI to yield pRAB11-lacZ, pRAB11-gfp and the correspondingly designated pRM2C2 derivatives. Of note, a mutation within gfpmut2 in both reporter plasmids was identified, resulting in an L421S exchange of the protein. To provide a

### METHODS

**Bacterial strains, growth conditions and manipulations.** Bacteria were grown in liquid or on solid BM (Bera et al., 2005), BOG (BM without glucose) or TSB (Sigma). Antibiotics were used, where appropriate, at the following final concentrations: 100 μg ampicillin ml⁻¹, 10 μg chloramphenicol ml⁻¹ and 2.5 or 10 μg erythromycin ml⁻¹. As effector for (rev)TetR, ATc (purchased as a dry chemical from Acros, prepared as a 10 mM stock solution in 70% ethanol) was added to bacterial cultures at a final concentration of 0.4 μM unless stated otherwise. Escherichia coli was made competent and transformed using standard techniques (Hanahan, 1983). S. aureus cells were subjected to electroporation according to Augustin & Götz (1990). Plasmids cloned in E. coli were used to transform the restriction-deficient S. aureus RN4220 and were introduced into other...
basal expression control for \( \beta \)-galactosidase measurements, plasmid pRAB11-lacZ \( \Delta \)pXyl/tet was constructed by eliminating the \( \text{pXyl/tet} \) promoter of pRAB11-lacZ via XhoI and KpnI digestion, followed by sticky end polishing with T4 polymerase and religation of the vector.

**Construction of plasmid pCG246 and its derivatives bearing rny**. Plasmid pCG246 was obtained by exchanging the \( \text{ermC} \) cassette in the pCN47 backbone for the cat194 selection marker of pCG246 via PstI and SalI yielding plasmids pCG247 and pCG248, respectively. A fragment of 1675 bp consisting of \( \text{rny} \) (encoding a putative RNase), including its putative ribosome-binding site, was amplified from strain RN6390 (Peng et al., 1988) using primers \( \text{BglII-RNaseY-for} \) and \( \text{BglII-RNaseY-rev} \) the \( \text{tetO} \) primer was then digested with \( \text{BglII} \) and ligated with the similarly restricted vectors pCG247 and pCG248, yielding pCG260 and pCG261, respectively.

**Design of \( \text{pXyl/tet} \) variants and cloning of pRAB11 derivatives with different promoters and \( \text{tetR} \) alleles**. DNA with sequences comprising the original \( \text{pXyl/tet} \) promoter with two \( \text{tetO} \) elements were amplified and cloned into Restriction enzymes were used for cloning of vectors and plasmids.

### Table 1. Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristic(s)</th>
<th>Reference</th>
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<tbody>
<tr>
<td><strong>Strains</strong></td>
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<td><strong>E. coli</strong></td>
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<td>DH5a</td>
<td>F(^-) ((\Delta)lacZ M15) (\Delta)lacZYA-argF) U169 hsdR17(r(^-)M) recA1 endA1 relA1 deoR (\lambda) phoA supE44 thi-1, gyrA96</td>
<td>Hanahan (1983)</td>
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<td>One Shot TOP10</td>
<td>F(^-) mcrA (\Delta)mrr- (\Delta)hsdRMS-mrrBC) (\Delta)lacX74 recA1 araD139 ((\Delta)ara leu)7697 galU galK rpsL (Str(^B)) endA1 supG</td>
<td>Invitrogen/Life Technologies</td>
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<td>XL1-Blue</td>
<td>hsdR17((K, m_{\text{F}}) (\gamma)) recA1 endA1 gyrA96 thi-1 supE44 relA1 lacI(^F) proA1 lacF(^I) ZAM15 Tra10 (Tet(^R))</td>
<td>Stratagene/Agilent</td>
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<td><strong>S. aureus</strong></td>
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<td>HG001</td>
<td>NCTC8325 derivative, (\text{rbsU} ) repaired, source of gene ( \text{rny} )</td>
<td>Herbert et al. (2010)</td>
</tr>
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<td>HG003 (\Delta)nuc1 (\Delta)nuc2</td>
<td>NCTC8325 derivative, (\text{rbsU} ) and ( \text{tcar} ) repaired, (\text{nuc1::aadB}, \text{nuc2::ermB} )</td>
<td>This study</td>
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<td>NewmanHG</td>
<td>Newman derivative, with ( \text{sacSL} ) from strain HG001</td>
<td>Mainiero et al. (2010)</td>
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<td>NewmanHG-217</td>
<td>NewmanHG, (\Delta) (\text{rny}::\text{ermC} )</td>
<td>This study</td>
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<td>RN4220</td>
<td>NCTC8325-4 derivative, acceptor of foreign DNA</td>
<td>Iordanescu &amp; Surdeanu (1976)</td>
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<td>RN6390</td>
<td>Derivative of NCTC8325</td>
<td>Peng et al. (1988)</td>
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<td>SA113 (ATCC 35556)</td>
<td>NCTC8325 derivative, ( \text{agr} ), 11 bp deletion in ( \text{rbsU} )</td>
<td>Iordanescu &amp; Surdeanu (1976)</td>
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<td><strong>Plasmids</strong></td>
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<td>pCG246</td>
<td>( \text{cat} , \text{bla} ), E. coli/\text{Staphylococcus} shuttle vector, pCN47 derivative</td>
<td>This study</td>
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<td>pCG247</td>
<td>pCG246 with ( \text{pXyl/tet} ) ((1 \times \text{tetO})) of pRM2C</td>
<td>This study</td>
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<td>pCG248</td>
<td>pCG246 with ( \text{pXyl/tet} ) ((2 \times \text{tetO})) of pRAB11</td>
<td>This study</td>
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<td>pCG260</td>
<td>pCG247 with ( \text{pXyl/tet} )-( \text{rny} ) fusion</td>
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<td>pCG261</td>
<td>pCG248 with ( \text{pXyl/tet} )-( \text{rny} ) fusion</td>
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<td>pRAB11 with ( \text{pXyl/tet-spoVG-lacZ} ) fusion</td>
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<td>pRAB11BD-lacZ</td>
<td>pRAB11-lacZ with ( \text{tetR(BD)} )</td>
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<td>pRAB11-lacZ without ( \text{pXyl/tet} )</td>
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<td>pRAB11 with ( \text{pXyl/tet} )-( \text{nuc1} ) fusion</td>
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<td>pRAB11-gfp</td>
<td>pRAB11 with ( \text{pXyl/tet} )-( \text{gfpmut2} ) fusion, ( \text{gfpmut2} ) L241S mutation</td>
<td>This study</td>
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<td>pRAB12-lacZ to pRAB17-lacZ</td>
<td>pRAB12-lacZ derivatives with mutant ( \text{pXyl/tet} ) sequences</td>
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<td>pRAB13-(r2^*)-lacZ</td>
<td>pRAB11-lacZ derivative with promoter ( \text{pXyl/tet} ) no. 40 and ( \text{revtetR-r2}^* ) ((\text{E15V} , \text{L17G} , \text{L25V} , \text{R49Q}))</td>
<td>This study</td>
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<td>pRM2C</td>
<td>( \text{cat} , \text{bla} ), ( \text{pXyl/tet} ) ((1 \times \text{tetO})), E. coli/\text{Staphylococcus} shuttle vector, pAL2073 derivative</td>
<td>Corrigan &amp; Foster (2009)</td>
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<td>pRM2C-gfp</td>
<td>pRM2C with ( \text{pXyl/tet} )-( \text{gfpmut2} ) fusion, ( \text{gfpmut2} ) L241S mutation</td>
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<td>pRM2C-lacZ</td>
<td>pRM2C with ( \text{pXyl/tet-spoVG-lacZ} ) fusion</td>
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<td>pRM2C-nuc1</td>
<td>pRM2C with ( \text{pXyl/tet} )-( \text{nuc1} ) fusion</td>
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<td>pWH134</td>
<td>( \text{knt} , \text{bla} ), improved ( \text{Pr}^* ) promoter upstream of ( \text{tetR(B)} ), E. coli/Bacillus subtilis shuttle vector, pUB110 derivative</td>
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<td>pWH1926</td>
<td>( \text{bla} ) ( \text{tetR(BD)} ), pUC19 derivative</td>
<td>Kamionka et al. (2006)</td>
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<td>pWH1926</td>
<td>( \text{cat} , \text{bla} ), ( \text{pXyl/tet-spoVG-lacZ} ) fusion</td>
<td>Kamionka et al. (2005)</td>
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<td>pCN50</td>
<td>cat194 ( \text{bla} ), E. coli/\text{Staphylococcus} shuttle vector</td>
<td>Charpentier et al. (2004)</td>
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positions within the −35 or the −10 promoter regions were variable during synthesis. The degree of variability was adjusted to a probability of 70% for the original consensus base and 10% each for one of the three remaining bases. According to these settings, exchanges at the designated positions would be expected to occur with the following frequencies: no exchange, 11.8%; one exchange, 30.3%; two exchanges, 32.4%; three exchanges, 18.5%; four exchanges, 5.95%; five or more mutations, ≤1.1%. These values reflect a binomial (Bernoulli) distribution calculated by the following equations.

\[ C = \frac{n!}{k!(n-k)!} \]

\((n=\text{amount of variable positions, here, 6; } k=\text{total number of actually mutated positions per fragment; } C=\text{number of possible arrangements of the } k \text{ exchanges}).\)

\[ P_0 = (1-p)^{(n-k)} \cdot p^k \]

\((p=\text{probability for exchange at each position, here 0.3}).\)

\[ P_1 = C \cdot P_0 \]

The pool of fragments synthesized obeying these settings was cloned via XhoI and KpnI into the vector backbone of the similarly digested vectors pRAB11-nuc1 or pRAB11-lacZ. In order to construct pRAB11BD-lacZ, pRAB11-lacZ was digested with XhoI and BstElI and a similarly restricted 646 bp fragment released from pWH1926 (Kamionka et al., 2006) was ligated. Thereby, tetR class (B) of transposon Tn10 was replaced with tetR class (BD) (Scholz et al., 2004) into pRAB11-lacZ and derived P\text{y syn} variant vectors pRAB12-lacZ, pRAB13-lacZ and pRAB14-lacZ.

### RESULTS AND DISCUSSION

**Complementation of an S. aureus nuc1 deletion strain using two similar tet vectors**

*S. aureus* harbours two genes encoding extracellular nucleases, designated Nuc1 and Nuc2, and it was found previously that *S. aureus* RN4220 Δnuc1 exhibits only low exonuclease activity (Tang et al., 2008). This is in agreement with results obtained with an SA113 Δnuc1 mutant previously generated in our laboratory (Thumm & Götz, unpublished data), as judged by nuclease plate assays. We aimed to complement SA113 Δnuc1 by using the recently developed ATc-inducible plasmid pRMC2 (Corrigan & Foster, 2009), into which nuc1 had been cloned downstream of the TetR addressable P\text{y syn} promoter. Unexpectedly, SA113 Δnuc1 (pRMC2-nuc1) exhibited discernible nuclease activity even in the absence of ATc, as deduced from clearly visible zones of clearing around colonies on HCl-treated DNase agar. In the original study describing this vector system, Corrigan & Foster (2009) placed sasG under tet control which resulted in undetectable levels of the encoded surface protein in the absence of ATc. Presumably, whole cell dot immunoblotting is less sensitive, reflected by higher threshold levels of detection than the nuc1-directed nuclease plate assay used in the present study. As pRMC2 contains only one copy of tetO within P\text{y syn}, the addition of a second TetR binding site seemed to be a promising approach to enhance target gene repression in the absence of an inducer, as indicated by previous studies in *B. subtilis* (Geissendorfer & Hillen, 1990; Kamionka et al., 2005) and *S. aureus* (Xu et al., 2010). Hence, a double-stranded DNA fragment resembling the P\text{y syn} promoter of pWH354 (Geissendorfer & Hillen, 1990), identical to that of pRMC2, albeit with a second tetO site 15 bp downstream of the first operator, was synthesized. This two-tetO promoter version
was used to replace P<sub>xyl/tet</sub> in pRMC2-nuc1 resulting in plasmid pRAB11-nuc1. Semiquantitative analysis of SA113 Δnuc1 (pRAB11-nuc1) gave reason to assume a tighter repression of nuc1 under non-induced conditions in this vector compared with the pRMC2 background, as judged by smaller halo diameters of colonies subjected to nuclease activity testing (data not shown). Subsequently, an S. aureus HG003 Δnuc1Δnuc2 strain (our unpublished results) was transformed with pRAB11-nuc1. SDS-PAGE analyses of respective crude soluble protein extracts of cells grown either with or without ATc were conducted. Compared with the pattern of the non-induced control, a prominent band clearly indicated highly elevated amounts of a ~25 kDa protein, corresponding to the molecular mass of Nuc1 (Fig. 1a). Since the pRAB11 system appeared useful for the control of other target genes, pRAB11-nuc1 was sequenced completely on both strands. The sequence of the deduced vector pRAB11 without nuc1 (Fig. 1b) has been deposited in GenBank under accession no. JN635500.

**Quantification of regulatory capacities of pRMC2 and pRAB11**

In order to quantify the regulatory capacities of pRAB11 in relation to its antecessor pRMC2, transcriptional fusions of gfp<sub>mut2</sub> (Cormack et al., 1996) or lac<sub>Z</sub>, respectively, were cloned into each vector downstream of the P<sub>xyl/tet</sub> promoters. Quantification of green fluorescence of SA113 (pRMC2-gfp) and SA113 (pRAB11-gfp) cells resulted in very similar activity profiles, in both the repressed and induced state (Fig. 2a). Flow cytometry confirmed an expected shift of the majority of the populations to an increased fluorescence level in both reporter strains when ATc was present (Fig. 2b). Following measurement of β-galactosidase, lac<sub>Z</sub> appeared to be slightly less active in pRAB11 compared with the pRMC2 context under both induced and non-induced conditions (Fig. 2c). Although the difference was not statistically significant in this reporter gene assay, it might indicate a reduced basal level of transcription, as also observed with nuc1 as the target gene (see above), at the cost of reduced inducibility in pRAB11.

**tet control of the rny gene in a related vector system**

A second tet vector system was established, based upon components of the pCN series of plasmids, which is characterized by three different origins of replication to choose from and a modular structure for simplified subcloning and modification (Charpentier et al., 2004). Exchange of cat in pCN47 for the ermC marker of pCN50 gave rise to pCG246, which bears the low copy number origin of replication of pT181 wild type. The newly constructed

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**Fig. 1.** (a) SDS-PAGE separation of 20 μg crude soluble protein extracts of HG003 Δnuc1Δnuc2 (pRAB11-nuc1) cultivated with or without ATc as indicated. A prominent band of ~25 kDa (corresponding to the molecular mass of Nuc1), observed in extracts of cells grown with ATc (6 h post-induction), is indicated by an arrow. Selected bands of a molecular mass marker are indicated on the left (in kDa). (b) Representation of pRAB11. Relevant features are given as boxes (origins of replication in grey, tetO in black), arrows [selection markers and ‘lacZα in grey, tetR(B) in black] and bent arrows (promoters). The sequence of P<sub>xyl/tet</sub> used in this study is given above. Lower-case letters in the −35 or −10 regions (underlined) indicate variable positions in the related plasmids pRAB11-lacZ to pRAB17-lacZ (see below). Single restriction sites downstream of P<sub>xyl/tet</sub> are listed.
plasmids pCG247 and pCG248 derived from pCG246 additionally contained the tet regulatory regions of pRMC2 or pRAB11, respectively (Fig. 3a). Here, the gene rny, encoding a putative RNase (Commichau et al., 2009), was cloned into both vectors, giving rise to pCG260 and pCG261. Cells of an rny deletion strain NewmanHG-217 (data not shown) carrying either pCG260 or pCG261 were analysed for ATc-dependent episomal rny transcription. As depicted in Fig. 3(b), an increase in rny mRNA correlated with rising ATc concentrations in the strain containing the two-tetO promoter construct pCG261 (right part). In contrast, specific signals in the case of the single tetO version pCG260 were generally stronger compared with pCG261, and little ATc dose-dependency could be observed (left part). Qualitatively, these observations are in line with nuclease assays obtained with the parental plasmids pRMC2-nuc1 or pRAB11-nuc1, respectively, indicating an enhanced transcriptional tightness when a P_xyl/tet promoter is equipped with two instead of

Fig. 3. (a) Map of pCG248. Representations are as described in Fig. 1(b). (b) Northern blot analysis of rny expressed in strain NewmanHG-217 carrying pCG260 (left) or pCG261 (right), cultivated with 0–100 ng ATc ml^{-1} (0 to ~0.22 μM), as indicated. Signals in the lower panel indicate 16S rRNA as loading control.

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one tetO site. However, the level of inducibility as well as the dose-dependency seems to be dependent on not only the tetO-bearing promoter but also the gene analysed and the plasmid system used.

**Variants of P<sub>xyl/tet</sub> promoters provide a wide dynamic range of tet regulation**

Many previous studies have shown that the P<sub>xyl/tet</sub> promoter is highly active when induced in Bacillales such as <i>B. subtilis</i>, <i>S. aureus</i> or <i>Streptococcus mutans</i> (Geissendoerfer & Hillen, 1990; Wang & Kuramitsu, 2005; Zhang et al., 2000). In fact, the activity of P<sub>xyl/tet</sub> in the TetR unbound state exceeds that of native <i>S. aureus</i> promoters of low and intermediate strength, such as P<sub>folA</sub>, P<sub>awf</sub> or P<sub>abl</sub>, and rather reflects the transcriptional level of the highly active rpsF promoter (Stary et al., 2010; Xu et al., 2010). Although tet regulation enables inducer dose-dependent responses of a target gene (reviewed by Bertram & Hillen, 2008), the determination and maintenance of a defined inducer concentration resulting in stable and reproducible intermediate expression is cumbersome. Therefore, we aimed to establish further pRAB11-derived vectors with tet-controlled promoters of gradually weaker activity in the fully induced state. Thereby the expression of <i>S. aureus</i> genes, which are otherwise moderately or weakly active in their native chromosomal environment, could be artificially adjusted to meet the genes' physiological expression profiles. To this end, defined positions of a DNA fragment containing the two-tetO version of P<sub>xyl/tet</sub> were chosen to incorporate variable deoxynucleotides during chemical synthesis. Three of the most conserved positions of each of the −35 and −10 regions of σ<sup>A</sup>-dependent <i>B. subtilis</i> promoters (Helmann, 1995; Jarmer et al., 2001), which perfectly match the consensus in P<sub>xyl/tet</sub>, were chosen for biased randomization (see Methods for details). Cloning of the obtained fragment pool into pRAB11-nuc1 and sequencing of 30 different clones revealed 10 different P<sub>xyl/tet</sub>-derived sequences. These exhibited between one and three exchanges at the intended positions in all except two cases, in which either an additional mutation was identified in the poly-A stretch upstream of the −35 site, or a 1 bp deletion had occurred between the −35 and −10 site (see Supplementary Table S1, available with the online version of this paper). Three of the resulting pRAB11-nuc1-derived plasmids with one, two or three exchanges in P<sub>xyl/tet</sub> respectively, were analysed in <i>S. aureus</i> SA113 Δnuc1 for nuclease activity in the absence or presence of ATc. According to halo diameters on indicative media plates (data not shown), these variants were estimated to be weaker than the original P<sub>xyl/tet</sub> version. To quantify the transcriptional strengths of these ten promoter versions, they were cloned into pRAB11-lacZ to replace wild type P<sub>xyl/tet</sub>. Together with further mutant sequences, yielded from cloning of the fragment pool directly into pRAB11-lacZ, a total of 16 reporter plasmids with different P<sub>xyl/tet</sub> promoter variants were obtained. Fig. 4 shows the results of β-galactosidase quantifications of selected plasmids designated pRAB12-lacZ to pRAB17-lacZ, bearing six P<sub>xyl/tet</sub> variants, compared with pRAB11-lacZ. A wide dynamic range of tet regulation was observed. Compared with wild type P<sub>xyl/tet</sub>, all of the 16 tested variants conferred less β-galactosidase activity in the induced state. Notably, this was also associated with improved repression capabilities, indistinguishable from values obtained with pRAB11-lacZ ΔP<sub>xyl/tet</sub> lacking a promoter upstream of lacZ. In the presence of ATc, the reporter gene activities ranged from ~300 (pRAB12) to ~4 relative units (pRAB17), compared with values of ~780 obtained with pRAB11-lacZ. While two further promoters approximately resembled the values of pRAB15-lacZ, eight of the tested sequences resulted in activities below 2.5 and

![Fig. 4. Regulation capacities of plasmids pRAB11-lacZ to pRAB17-lacZ. Representations are as described in Fig. 2(a). The sequences of the −35 sites (above) and −10 sites (below) of the P<sub>xyl/tet</sub> promoter variants corresponding to the respective plasmids are given. Dots indicate consensus bases. Error bars, SD.](chart.png)
were considered to be inactive promoters. The sequences of all assayed promoter fragments and the obtained β-galactosidase values are given in Supplementary Table S1. The most active variants after the original $P_{xyl/tet}$ version were found to contain single point mutations only, whereas exchanges at two or more positions resulted in activities below 5 relative units. Strikingly, promoters with different exchanges at identical positions showed drastically variable activities, as observed for the third position in the $-35$ and the first position in the $-10$ hexamer. Xu et al. (2010) had previously described a total of six promoters for tet regulation in S. aureus, where three highly similar core promoters were equipped with one or two tetO sequences. The authors had found that construct $P_{T00X}$, which is identical to $P_{xyl/tet}$ from positions $-60$ to $-8$ and merely differs with regard to the position and the sequence of the second downstream tetO site, resulted in the highest activity of the two-tetO promoters tested. Also in line with our observations, two $P_{T00X}$-derived promoters, containing either two exchanges in $-35$ ($P_{TOT0X}$) or one exchange each in $-35$ and in $-10$ ($P_{T00W}$), displayed markedly weaker transcriptional activities.

Episomal Tet-ON and Tet-OFF regulation by different tetR alleles

Like the vast majority of tet regulation components used in S. aureus to date, pRAB11 and its derivatives are also based upon the tetR of class (B) originating from transposon Tn10. However, in earlier studies, a chimera of Tet repressors from class (B) and (D), termed TetR(BD) (Schnappinger et al., 1998; Schubert et al., 2001), was found to combine the advantageous features of high tetO binding affinity [by employing the DNA-binding domain of TetR(B)] and higher protein stability [attributed to the dimerization and inducer-binding domain of TetR(D)]. In order to obtain a possible gain of efficacy when exploiting TetR(BD) in our one-plasmid system, pRAB11BD–lacZ was constructed by replacing tetR(B) with tetR(BD). Furthermore, we aimed to demonstrate one-plasmid-based regulation by revTetR, stemming from systematic mutagenesis of TetR(BD) (Scholz et al., 2004). Resulting pRAB11-derived vectors exploiting revTetR were intended to enable tet-OFF control by rapid silencing of a target gene upon administration of ATc in a transcriptional fashion. This contrasts with post-transcriptional tet-inducible antisense RNA-mediated gene knockdown, as described by Ji et al. (1999). To this end, the reverse tetR variant allele revtetR-r2 (deduced mutations E15V L17G L25V) was chosen due to its efficient regulatory capacities in the Gram-positive species B. subtilis (Kamionka et al., 2005), S. aureus (Stary et al., 2010) or Mycobacterium smegmatis (Guo et al., 2007). Surprisingly, revtetR-r2 turned out to be recalcitrant to cloning into pRAB11-, pRAB12-, pRAB13- and pRAB14–lacZ; cells putatively bearing the respective recombinant plasmids repeatedly grew very badly or plasmids prepared exhibited severe deletions. Finally, however, pRAB13-r2 could be isolated from E. coli and its sequence of the tet-regulatory regions was confirmed. Surprisingly, after propagation in S. aureus, revtetR-r2 contained a point mutation in codon 49 (arginine to glutamine). The resulting plasmid, dubbed pRAB13-r2*-lacZ, as well as pRAB11BD-lacZ were subjected to β-galactosidase quantifications (Fig. 5). pRAB11BD-lacZ exhibited slightly higher values than its tetR(BD) counterpart pRAB11-lacZ when induced by ATc, whereas the repression capabilities were virtually identical for both vectors (compare Fig. 5 to Fig. 2c). An efficient ATcépendent downregulation of β-galactosidase activity was observed with pRAB13-r2*-lacZ, whereas lacZ appeared highly expressed in the absence of the drug. Thus, the activity values of pRAB13-r2*-lacZ inversely resembled the ones of pRAB11-lacZ (compare to Fig. 2c). As judged by data from Scholz et al. (2004), codon 49, which was altered in addition to codons 15, 17 and 25 in revtetR-r2*, had generally not appeared to be critical for the reverse phenotype of TetR mutants. Notably, however, TetR(B) R49Q is massively impaired in induction (Wissmann et al., 1991). In mycobacteria, reverse TetR-r2 chimera vested with additional exchanges next to E15V L17G L25V were gain-of-efficiency mutants (Klotzsche et al., 2009), which makes it tempting to speculate that selective pressure had resulted in the manifestation of the additional mutation in revTetR-r2* (E15V L17G L25V R49Q) in S. aureus.

Conclusions

We here present a set of novel vectors for improved tet regulation in S. aureus. The one-plasmid architectures

![Fig. 5. Regulation capacities of plasmids pRAB11BD–lacZ and pRAB13-r2*-lacZ. Representations are as described in Fig. 2(a). Error bars, SD.](http://mic.sgmjournals.org/3321)
described in this study are the result of stepwise modifications along the line of the closely related vectors pALC2073 (Bateman et al., 2001) and pRMC2 (Corrigan & Foster, 2009). A strong and constitutive promoter for expression of tetR in pRMC2 was combined with a PXYL/tet version containing two tetO sites in pRAB11 yielding a very tight repression, combined with a high degree of inducibility. The stepwise abrogation of target gene repression in an ATC-concentration-dependent fashion could be demonstrated in plasmid pCG261, based upon the tet-regulation architecture of pRAB11. PXYL/tet-derived promoters of lower transcriptional activity described in this study cover a comparable regulatory range to the tet-sensitive promoters described by Xu et al. (2010) and have the potential for a very precise fine adjustment of target gene expression. Next to these tet-ON systems, a one-plasmid system for a reverse TetR-based tet-OFF mode of control was also established for the first time. This adds to other architectures of reverse TetR-dependent gene silencing that had previously been adopted for S. aureus in our group (Stary et al., 2010). We assume that the vectors described in this study can be of great help for various purposes such as for complementation, where gene-dosage is critical, or for overexpression of target proteins (Fig. 1a). According to previous experiences (Christner et al., 2010; Giese et al., 2009), these vectors can be assumed to also function efficiently in other staphylococci.

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Tet regulation vectors for S. aureus


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