In vivo Activation of Tetracycline Repressor by Cre/lox-Mediated Gene Assembly

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Key Words

Tetracycline repressor • Gene regulation • Site-specific recombination • *Bacillus subtilis*

Abstract

Tetracycline repressor (TetR) bears an unstructured loop region between helices $\alpha 8$ and $\alpha 9$, which is moderately permissive to amino acid exchanges and length variations. Recognition sites for the site-specific recombinases FLP (*FRT*) or Cre (*lox*) were inserted in-frame into *tetR*, substituting some of this loop's codons. A number of the deduced TetR variants displayed efficient regulation in vivo, thus allowing the establishment of a new mode of TetR activation on the genetic level. Chromosomally encoded *tetR* in *Bacillus subtilis* was disrupted and inactivated by insertion of a *lox66-aphAIII-lox71* kanamycin resistance cassette. Marker excision by Cre recombinase led to the assembly of a novel *tetR* allele. The encoded regulator, termed TetR^{lox72/1}, is distinguished from wt-TetR by a slightly elongated and altered $\alpha 8$ – $\alpha 9$ loop only, harboring an amino acid stretch encoded by *lox72*. Despite decreased intracellular protein amounts, TetR^{lox72/1} displayed efficient in vivo activity in *B. subtilis* and *E. coli*, indistinguishable from that of wt-TetR. These results underline the sequence flexibility of TetR in the $\alpha 8$ – $\alpha 9$ loop and demonstrate the possible use of the regulator as a read-out tool for the activity of site-specific recombinases.

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Introduction

Tetracycline-dependent gene regulation (*tet* regulation) is a frequently applied means of gaining transcriptional control over genes in prokaryotes and eukaryotes [reviewed in Berens and Hillen, 2003 and in Bertram and Hillen, 2008]. The origin of *tet* regulation can be traced back to the Tc resistance determinants *tet*(B) of Tn10 [Hillen and Schollmeier, 1983] and *tet*(D) of plasmid RA1 [Unger et al., 1984]. Both comprise *tetR*–*tetA* divergences, in which the tc antiporter TetA is transcriptionally controlled by tetracycline repressor (TetR) [reviewed in Hillen and Berens, 1994]. Binding of TetR to its cognate DNA sequence *tetO* can be reversibly suspended by administration of subinhibitory doses of the antibiotic tetracycline (Tc). This results in *tetA* expression with Tc or *tetA* repression without Tc. The genetic background of this system in Tn10 is well understood and involves three partly intertwined promoters and two *tetO* sites in the intergenic *tetR*–*tetA* region [reviewed in Grkovic et al., 2002 and in Hillen and Berens, 1994]. Negative autoregulation of *tetR* results in a balanced amount of the regulator. This

Abbreviations: Ap = Ampicillin; Atc = anhydrotetracycline; Cm = chloramphenicol; Em = erythromycin; FRT = FLP recognition target; Km = kanamycin; lox = locus of cross-over of P1; SSR = site-specific recombination; Tc = tetracycline.

ensures both sensitive induction at low Tc levels and tight regulation in the absence of the drug. For applied *tet* regulation in bacteria, the conventional set-up consists of a promoter containing one or two *tetO* sequences and a downstream target gene, the activity of which can be modulated by Tc and TetR, encoded in *cis* or *trans*. Some of the most efficient settings for applied *tet* regulation in bacteria rely on constitutively high *tetR* expression [Lutz and Bujard, 1997; Skerra, 1994] for two major reasons. First, the tightness of repression correlates to the abundance of TetR. Second, the use of the less antibiotic Tc derivative anhydro-Tc (ATc), which binds TetR with at least 35-fold higher affinity than Tc [Degenkolb et al., 1991], allows for complete induction even at high intracellular TetR amounts. To enable efficient transcription of a *tet*-regulated gene, available TetR is induced by (A)Tc, which is added to the growth medium. Alternatively, target gene expression can also be achieved without inducer, given that no TetR or only marginal amounts of it are present in the cell. To this end, *tetR* can e.g. itself be transcriptionally repressed by a second inducible system [Bertram et al., 2005; Kamionka et al., 2005], such that repression of the target gene requires expression of *tetR*. In the present study, we describe a novel kind of *tetR* activation in vivo that is not based upon transcriptional or translational control, but exploits a particular structural feature of the regulator. TetR's active form consists of two identical monomers which fold to form 10 α -helices connected by turns and loops [Hinrichs et al., 1994; Orth et al., 1998, 2000]. Of these, the loop sequence between helices $\alpha 8$ and $\alpha 9$ is not resolved in published TetR structures. Earlier studies have shown that this stretch in the TetR variants of *tet(B)* and *tet(D)* is permissive for alterations to a certain extent. This pertains to both length and amino acid composition [Berens et al., 1997; Kintrup et al., 2000; Scholz et al., 2001]. The second prerequisite for the described approach is the availability of a site-specific recombination (SSR) system for the organism of choice. The most commonly applied of these in mammalian and bacterial genetics are the yeast derived FLP/*FRT* system [reviewed in Schweizer, 2003] and Cre/*lox*, originating from bacteriophage P1 [reviewed in Sauer, 2002]. Both are very similar in structural and functional terms, requiring 34-bp recognition sequences, which serve as binding sites and substrates for the respective recombinase. Upon FLP or Cre treatment, respectively, a region between two collinear *lox* or *FRT* sequences within a circular or linear DNA molecule is precisely excised [reviewed in Grindley et al., 2006]. A number of functional variant *lox* sites have been identified in the past. Among these are *lox66* and

lox71, each with 5-bp exchanges compared to wt-*loxP*. After Cre-mediated recombination between these two sites, a sequence called *lox72* is obtained, which is mutated at ten positions [Albert et al., 1995]. Although *lox* and *FRT* sites are functionally noncoding, the addition of two basepairs renders them able to encode dodecapeptides. In this study, we analyzed the potential of *tetR* alleles containing internal *lox* or *FRT* sequences for encoding functional TetR variants. Furthermore, the Cre/*lox* system was exploited to assemble a functional gene from an artificially split, bipartite *tetR*-like sequence within the *Bacillus subtilis* chromosome. To this end, a floxed (flanked by *lox*) resistance marker was first used to disrupt wt-*tetR* and was then excised by Cre. This yielded an ORF harboring one in-frame *lox* sequence, whose deduced TetR variant, containing an altered $\alpha 8$ - $\alpha 9$ loop sequence, was functional in vivo with full efficiency.

Results

In vivo Activity of TetR Variants Harboring SSR Recognition Sites

The loop region between helices $\alpha 8$ and $\alpha 9$ (fig. 1a) consists of 15 residues (positions 152 to 167) in TetR and is identical to that of TetR(D). Previous studies have shown that length and sequence variations of this region are tolerated to a certain extent. The first aim was to replace codons 161 to 167 for different SSR recognition sites, thereby maintaining translational read-through. In vivo activity analyses of these new TetR variants should shed further light on the permissivity of this region. *FRT*- or *lox72*-containing sequences were cloned into the *tetR* expression vector pWH1926, yielding pWH1926-F1, pWH1926-L1 and pWH1926-L2 as the desired constructs as well as pWH1926-F1-inv, pWH1926-L1-inv and pWH1926-L2-inv, in which the fragments inserted in opposite orientation. Due to the position of the two *PstI* sites used for cloning within *tetR*, the deduced proteins of the 'inv' constructs bore sequence alterations not only affecting the loop, but also the helix $\alpha 9$ region. In the case of L2-inv, this causes premature termination by an ochre stop codon. Hence, this construct, termed *tetR*^{*lox72/2-inv*}, encodes a truncated TetR-like polypeptide of 170 amino acids only. All other ORFs code for full-length TetR monomers of 214 or 215 residues, which are designated as follows: TetR^{*FRT*} (*FRT* site), TetR^{*lox72/1*} (*lox72*, first reading frame), TetR^{*lox72/2*} (*lox72*, second reading frame), TetR^{*FRT-inv*} and TetR^{*lox72/1-inv*}. The altered sequence stretches of the variants, as well as their in vivo activities in *E. coli*, are depicted in

Table 1. Bacterial strains or plasmids used in this study

	Relevant characteristics or genotype	Reference
<i>Strains</i>		
<i>E. coli</i> DH5 α	<i>recA1 endA1 gyrA96 thi relA1 hsdR17</i> (r_K^- , m_K^+) <i>supE44</i> Φ 80 <i>dlacZ</i> Δ Δ <i>lacU169</i>	Hanahan, 1983
<i>E. coli</i> WH207 λ <i>tet50</i>	<i>galK rpsL thi</i> Δ <i>lacX74 recA13 Tn10 tetA-lacZ</i> transcriptional fusion <i>bet</i> ⁺ <i>gam</i> ⁺ <i>cIII</i> ⁺ <i>cI</i> ⁺	Smith and Bertrand, 1988; Wissmann et al., 1991
<i>B. subtilis</i> WH557	<i>trpC2 lacA::Pt17-tetR lox66-aphAIII-lox71</i>	Bertram et al., 2005
<i>B. subtilis</i> WH558	<i>trpC2 lacA::Pt17-tetR lox66-aphAIII-lox71 amyE::P_{xyl/tet}-lacZ</i>	Bertram et al., 2005
<i>B. subtilis</i> WH560	<i>trpC2 amyE::P_{xyl/tet}-lacZ</i>	Bertram et al., 2005
<i>B. subtilis</i> RAB100	<i>trpC2 lacA::Pt17-tetR-lox72 amyE::P_{xyl/tet}-lacZ</i>	this study
<i>B. subtilis</i> RAB101	<i>trpC2 lacA::Pt17-tetR'-lox66-aphAIII-lox71-'tetR;lox72 amyE::P_{xyl/tet}-lacZ</i>	this study
<i>B. subtilis</i> RAB102	<i>trpC2 lacA::Pt17-tetR^{lox72/1} lox72 amyE::P_{xyl/tet}-lacZ</i>	this study
<i>Plasmids</i>		
pCrePA	Em ^R , P _{pagA} - <i>cre</i> thermosensitive, expression of <i>cre</i> in <i>Bacillus</i>	Pomerantsev et al., 2006
pWH1925 Δ	Ap ^R , pUC19 derivative, no <i>tetR</i>	Bertram et al., 2004
pWH1926	Ap ^R , pUC19 derivative, constitutive expression of <i>tetR</i>	Kamionka et al., 2006
pWH1926-F	pWH1926 derivative, <i>tetR</i> ^{FRT}	this study
pWH1926-F-inv	pWH1926 derivative, <i>tetR</i> ^{FRT-inv}	this study
pWH1926-L1	pWH1926 derivative, <i>tetR</i> ^{lox72/1}	this study
pWH1926-L1-inv	pWH1926 derivative, <i>tetR</i> ^{lox72/1-inv}	this study
pWH1926-L2	pWH1926 derivative, <i>tetR</i> ^{lox72/2}	this study
pWH1926-L2-inv	pWH1926 derivative, <i>tetR</i> ^{lox72/2-inv}	this study
pWH1926-TLKL	pWH1926 derivative, <i>tetR'-lox66-aphAIII-lox71-'tetR</i>	this study
pWH1411BD	Cm ^R , pACYC177 derivative, <i>tetR</i>	Scholz et al., 2003
pWH1411-TLKL	pWH1411BD derivative, <i>tetR'-lox66-aphAIII-lox71-'tetR</i>	this study
pWH1411-TLKLTextd	pWH1411BD derivative, <i>tetR'-lox66-aphAIII-lox71-'tetR 'lacA'</i>	This study

^R = Resistant.

figure 1b. Intriguingly, all three TetR variants in which only the loop region was altered displayed full inducibility with ATc. Also, the repression capacities of TetR^{FRT} and TetR^{lox72/1} were almost identical to that of wt-TetR, but were diminished in case of TetR^{lox72/2}. This sharply contrasts with the behavior of the '-inv' constructs, none of which has regulatory properties. The truncated TetR^{lox72/2-inv} is inactive, whereas TetR^{FRT} and TetR^{lox72/1} apparently bind to *tetO*, but are induction deficient.

A Vector for Chromosomal Integration of a Split tetR-Like Sequence into B. subtilis

The next aim was to provide a model system through which an artificially interrupted *tetR* variant could be activated upon treatment with a site-specific recombinase. Based upon the results described above, a *lox66*-(marker gene)-*lox71* cassette between two parts of a *tetR* gene was intended to be excised in vivo, to obtain *tetR*^{lox72/1} as the final product. The corresponding manipulations were ac-

complished within the chromosome of *B. subtilis* using the Cre/*lox* system for a number of reasons. First, *B. subtilis* is conveniently accessible to transformation and integration of foreign DNA into the chromosome by double homologous recombination [Fernandez et al., 2000]. Second, strains of this species are available to monitor the in vivo activity of TetR variants [Bertram et al., 2005; Kamionka et al., 2005]. Finally, a Cre/*lox* setting has recently been described for use in *Bacillus* species [Pomerantsev et al., 2006; Yan et al., 2008]. In order to clone an integration vector, an *aphAIII* cassette conferring Km resistance was amplified and modified by PCR. Thereby, it became flanked by *lox66* and seven codons corresponding to positions 168–174 of TetR helix α 9 upstream and by a *lox71* site downstream. This product was inserted between the *PstI* sites of *tetR*, yielding a *tetR'-lox66-aphAIII-lox71-'tetR* sequence termed *tlklt*. After subcloning and minor modifications (see 'Material and Methods'), the final *B. subtilis* integration vector pWH1411-TLKLTextd was obtained.

Table 2. Primers used in this study (*FRT* and *lox* sites in boldface)

Oligonucleotide	Sequence (5' → 3')
DP3	GCATTATTGCGCAGAAAT
DP8neu	CAGCGGTCAGTCATTTTA
flp_fw	TTGAAGTTCCTATACTTTCTAGAGAATAGGAACTTCCCGCTATTGCGGGAAGCGCTGCA
flp_rev	GCGCTTCCCGCAATAGCGGGAAGTTCCTATTCTCTAGAAAGTATAGGAACTTCAATGCA
Km_66_inv	GCTACTGCAGCGCTTCCCGCAATAGCGGGATACCGTTTCGTATAATGTATGCTATACGAAGTTATGC-GAACCATTTGAGGTGATAGGT
Km_71_inv	GCTACTGCAGCCTACCGTTTCGTATAGCATACATTATACGAAGTTATGAGTATGGACAGTTGCGGATG
Km1	ATGACGGACAGCCGGTATAAAGG
Km2	AGATGTTGCTGTCTCCAGGTCG
lacA_extd	GTCGCATGCCTATCCGGAAG
lockP	GCCTACCGTTCGTATAGCATACATTATACGAACGGTATCCCGCTATTGCGGGAAGCGCTGCA
lockP_fw	GCTACCGTTCGTATAGCATACATTATACGAACGGTACCGCTATTGCGGGAAGCGCTGCA
lockP_rev	GCGCTTCCCGCAATAGCGGTACCGTTCGTATAATGTATGCTATACGAACGGTAGCTGCA
lox_test_fw	CGGCGCTGTTAATGTGGCAC
lox_test_rev	AGCGCGCAATTAACCTCACTA
Pkcol	GCGCTTCCCGCAATAGCGGGATACCGTTCGTATAATGTATGCTATACGAACGGTAGGCTGCA

Multiple Modifications of the *B. subtilis* Genome and Cre-Mediated Activation of *tetR*^{lox72/1}

B. subtilis WH558 (fig. 2a) was chosen as the initial host strain [Bertram et al., 2005]. It carries wt-*tetR*, driven by a constitutive promoter, integrated in the *lacA* locus. As a reporter gene, *lacZ* downstream of a P_{xyl/tet} promoter [Geissendörfer and Hillen, 1990] with two *tet* operators is located within *amyE*. First, an *aphAIII* gene in the WH558 genome had to be removed. Reinstating this strain's Km sensitivity was mandatory for later selection of the *tlklt* cassette. In anticipation of the future development of a Cre/*lox* system for *Bacillus*, the *aphAIII* resistance cassette used to select for WH558 had been flanked by *lox66* and *lox71* in an earlier study [Bertram et al., 2005]. Upon treatment with plasmid pCrePA, generated for expression of Cre recombinase in *B. anthracis* [Pomerantsev et al., 2006], a Km-sensitive descendant of WH558, designated RAB100, was obtained. The observed size reduction of the region downstream of *tetR* in the chromosome corresponded to the length of the floxed marker gene, strongly indicating Cre mediated eviction of *aphAIII*. RAB100 was transformed with pWH1411BD-TLKLTextd. The integration of the *tlklt* fragment into *tetR* of RAB100 yielded strain RAB101, which was confirmed by PCR. In order to assemble and activate *tetR*^{lox72/1}, competent RAB101 cells were treated with pCrePA (see above). PCR analyses of Km-sensitive

candidates corroborated the absence of the floxed resistance cassette. One of the positive candidates, the *tetR* region of which was confirmed by sequencing, was termed RAB102. In accordance with the expectations, Cre treatment had exactly assembled the two halves of the *tetR*-like sequence to *tetR*^{lox72/1}, leaving one *lox72* scar within the α8 to α9 loop codons behind (see above). The described strain manipulation procedure is schematically summarized in figure 2b, with the corresponding PCR analyses shown in figure 2c. The regulatory capacities of the newly constructed *B. subtilis* WH558 derivatives were assayed by β-gal measurements, the results of which are shown in the upper part of figure 3. No differences in β-gal activity were observed between WH558 and RAB100, which differ in the *aphAIII* selection marker downstream of *tetR* only. RAB101 shows no *lacZ* repression, which is explained by a stop-codon immediately downstream of *lox71* in *tlklt* (not shown). As in the case of *tetR*^{lox72/2-inv}, this construct thus does not form a functional repressor. For RAB102 (*tetR*^{lox72/1}), an almost identical activity profile to that of WH558 or RAB100 was observed. This is in accordance with the results in *E. coli* (fig. 1b). To assay the amounts of TetR (or the shorter *tlklt*-encoded product) of the respective *Bacillus* strains, Western Blot analyses were conducted. Figure 3 (lower part) depicts the results obtained with the monoclonal antibody TOP19, whose epitope maps to the helix-turn-

Fig. 1. a Structure of TetR(D). The two monomers are colored in blue and grey with the α -helices illustrated as barrels or as the backbone trace, respectively. Tc is depicted in yellow. In the blue-colored monomer, the termini and the helices $\alpha 8$ and $\alpha 9$ are marked. The connecting loop, which is modeled in this representation, is highlighted in red. The region between the two arrows was targeted in the described approach. **b** Amino acid sequence alignment of different $\alpha 8$ – $\alpha 9$ loop regions. The amino acid sequences from the five C-terminal residues of helix $\alpha 8$ to the end of helix $\alpha 9$ of TetR variants or TetR-like polypeptides are shown. Identical positions are shaded black or grey. The C-terminal end of TetR^{lox72/2-inv} is followed by a diamond. The positions of two *Pst*I sites used for cloning within the respective *tetR* alleles are indicated by arrows. β -gal activities of the respective constructs as assayed in *E. coli* (in %) are given on the right.

	$\alpha 8$		$\alpha 9$	- / + ATc
TetR	QQEHTAALTDRPAA	PDENLP-----	PLLREALQIMD	0.7 / 92
TetR ^{FRT}	QQEHTAALTDRPAL	KFLYFLENRNF-	PLLREALQIMD	0.7 / 87
TetR ^{FRT-inv}	QQEHTAALTDRPAA	LPATAGSSYSLES	SIGTSM-QIMD	4.7 / 1.6
TetR ^{lox72/1}	QQEHTAALTDRPAA	YRSYSIHYTNGI	PLLREALQIMD	1.0 / 106
TetR ^{lox72/1-inv}	QQEHTAALTDRPAA	LPATAGYRSYNVC	YTNGRLQIMD	2.8 / 1.9
TetR ^{lox72/2}	QQEHTAALTDRPAA	TVRIAYIIRTV-	PLLREALQIMD	7.7 / 104
TetR ^{lox72/2-inv}	QQEHTAALTDRPAA	LPATAVPFV♦		100 / 113

helix motif of TetR(B) [Pook et al., 1998], which is identical to that of TetR. The approximately equally strong signals obtained with soluble protein extracts of WH557, WH558 and RAB100 contrast to the weaker band in case of RAB102. No signal was observed with RAB101, which encodes the prematurely terminated TLKLT product corresponding to residues 1–161 of TetR with an additional eleven amino acids at the C-terminus. Additional Western analyses using polyclonal antibodies raised against TetR revealed an even stronger reduction of signal intensity in RAB102, when compared to WH558 and RAB100 (not shown). This may be explained by fewer antibody recognition epitopes in the altered loop region of TetR^{lox72/1} compared to wt-TetR. Nevertheless, the re-

duced regulator amounts of RAB102 are sufficient to achieve high-capacity *tet* regulation in the *B. subtilis* model system.

Discussion

Exerting transcriptional control is the most prevalent technique for artificial gene regulation in bacteria to date. In most cases, transcription initiation is modulated by a regulator protein which conditionally binds to its cognate DNA sequence, dependent on the interaction with a suitable small ligand. For TetR we here describe an additional stage of control of higher hierarchy. The described kind

Fig. 2. a Schematic representation of the initial *B. subtilis* measurement strain WH558. The locations of *tetR* and the $P_{xyl/tet}$ -*lacZ* fusion are schematically depicted. Open bent arrows denote the promoters Pt17 (for *tetR* expression) and $P_{xyl/tet}$ (*tet*-regulatable, upstream of *lacZ*). Black trapezoids depict various *lox* sites as denoted, flanking the kanamycin resistance cassette (*aphAIII*), also indicating their direction. Boxes marked 'O' represent *tet* operators. A transcriptional terminator is drawn as a hairpin. Three successive stop codons are symbolized by a capital 'S'. The figure is not drawn to scale. **b** Multiple alterations of the *tetR* region. As a first step, the *aphAIII* gene was excised by Cre recombinase, leading to the marker-free strain RAB100. Secondly, the *tetR* gene was replaced by double homologous recombination for a *tetR'*-*lox66*-*aphAIII*-*lox71*-*tetR* (*tlklt*) sequence, resulting in *B. subtilis* RAB101. Third, the *aphAIII* gene was once again eliminated by Cre, giving rise to the final construct RAB102 harboring *tetR*^{*lox72/1*}. Primers for analytic PCR reactions (see fig. 2c), indicated by small arrows and latin numbering, are: (I) *lox_test_fw*, (II) DP3, (III) DP8neu, (IV) Km1, (V) Km2, (VI) *lox_test_rev*. The figure is not drawn to scale. **c** Results of analytic PCRs PCR products of the four indicated *B. subtilis* strains obtained with the following primer combinations (see fig. 2b for approximate primer locations): lane 1: I + VI, lane 2: III + VI, lane 3: I + II and lane 4: IV + V (from left to right). Sizes of selected reference bands (in bp) are indicated.

of activation abrogated undesired leaky repression of the target gene (in this study *tetR*), which is occasionally encountered in transcriptional regulation systems. Instead, activation of TetR was achieved at the genomic level by exploiting site-specific recombination (SSR), in this case by Cre/lox technology. A plasmid for Cre expression in *B. anthracis* [Pomerantsev et al., 2006] turned out to be very effective also in *B. subtilis* and was used to consecutively eliminate a *lox66-aphAIII-lox71* cassette twice out of two adjacent genomic loci (fig. 2b). The presence of a *lox72* scar after the first round of Cre treatment did not impede the second recombination between *lox66* and *lox71*, corroborating previous findings that *lox72* is a poor substrate for Cre [Albert et al., 1995; Lambert et al., 2007]. In the past, SSR has already been exploited for gene activation in bacteria. Genes in *E. coli* were re-oriented using FLP/FRT, so that they became attached to a promoter and thus expressed [Sektas et al., 1999; Sektas and Szybalski, 1998]. Unlike our approach and earlier studies, this method did not incorporate SSR binding sites into an ORF. The group of Boccard fused the 5'-part of *lacZ* to *attL* and the 3'-part to *attR* of phage λ . A *lacZ* allele harboring *attB* could thus reversibly be assembled by the recombinases Int and Xis in *E. coli*. [Esnault et al., 2007; Valens et al., 2004]. The encoded β -gal variant served the purpose of a readout for spatial proximity of distant chromosomal sites, but showed only about 50% of the wild type enzyme's activity. Kaczmarczyk and Green [2001] also described SSR-mediated β -gal activation by excision of a floxed *egfp* gene in order to monitor Cre activity. In the resulting protein the *lox* encoded residues were within a fused N-terminus, which is not part of the native enzyme. By contrast, our approach implies that a target protein must tolerate primary structure alteration. In the case of TetR, the $\alpha 8$ – $\alpha 9$ loop seemed the most promising region to this end. Among the 15 different naturally occurring TetR orthologues known to date [Agersø and Guardabassi, 2005; Berens and Hillen, 2004; Brown et al., 2008; Thompson et al., 2007], little primary sequence conservation is observed within the loop, which is 12 to 21 amino acids long. This also holds true for the unique loops of the TetR variants described here. Scholz et al. [2001] have constructed a 'loop-less' TetR(D) variant and have also substituted this region for stretches of two to 42 mostly alanine residues. It proved to be the case that the loop per se, but not its sequence is critical for TetR(D) induction by ATc. Variants with loop lengths of about 17–29 were most efficient in that study and the 21-residue loop of TetR^{lox72/1} thus fits into this range. The fact that an [ED]R motif (corresponding to positions 156 and 157 of TetR),

Fig. 3. Regulatory capacities and regulator amounts of different *B. subtilis* strains. Results of β -gal measurements are depicted in the upper panel, with Miller units given on the y-axis. Control strains are WH557, encoding *tetR* but not *lacZ* and WH560, which harbors *P_{xyl/tet}-lacZ* but no *tetR*. Open bars depict values obtained without ATc, whereas closed bars represent 0.4 μ M ATc conditions. Western blot results for the corresponding strains, obtained with monoclonal antibody, are shown below. 80 ng of purified TetR was used as a control.

which is conserved in most of the original TetR variants' loops, was maintained may have improved inducibility by ATc [Scholz et al., 2001]. Poly-alanine TetR(D) variants furnished with loops of more than 20 residues had previously shown decreased intracellular protein levels. This is in accordance with our observations of TetR^{lox72/1} (fig. 2c), which, nonetheless, displayed tight repression. The weaker in vivo repression capacities of TetR^{lox72/2} may be rationalized by instability. The induction defects of the full-length 'inv' constructs underline the participation of helix $\alpha 9$ in forming TetR's inducer binding pocket. Interestingly, however, these constructs are stable enough to mediate repression. It can therefore be inferred that formation of the four-helix bundle of the helices $\alpha 8$ and $\alpha 10$ of both monomers, which build a rigid scaffold

of TetR [Hinrichs et al., 1994; Kisker et al., 1995] is not impaired. Taken together, the variants TetR^{lox72/1} and TetR^{FRT}, and also TetR^{lox72/2} to a lesser extent (fig. 1b) retained the functions of both DNA-binding and ATc inducibility. Thus, to our knowledge, the present study for the first time reports Cre/lox-mediated gene assembly resulting in the activation of a fully efficient allosteric protein, which is only slightly altered in a carefully selected permissive region. Further manipulations of *tetR* within cells are also conceivable through the described approach. These include reversible Cre-mediated alteration or (in)activation of TetR function by inserting or excising stop codons or transcription factor encoding DNA into *tetR* in vivo. This may enable both the generation of novel regulators with altered functions and switching TetR activity in an inducer-independent manner. Furthermore, the described mode of TetR activation can be viewed as an attractive read-out tool for monitoring the activity of SSR systems. Based upon our observations that TetR^{lox} and TetR^{FRT} variants displayed full efficiency, it can be assumed that TetR also tolerates further similar-sized SSR recognition sites within its ORF. Thus, TetR's sequence flexibility might prove useful for analyzing activities of both the known and new SSR systems, which are as yet untapped for molecular genetics purposes.

Experimental Procedures

Bacterial Strains and Growth Conditions

Cloning was carried out in *Escherichia coli* DH5 α [Hanahan, 1983]. Genetic manipulations of *tetR* in vivo were conducted in *B. subtilis* WH558 [Bertram et al., 2005] and derivatives thereof, as outlined in table 1. Cells were generally grown either in liquid (shaking) or on solid LB or BM [Bera et al., 2005]. When appropriate, media were supplemented with ampicillin (Ap; 100 mg/l for *E. coli*), kanamycin (Km; 30 mg/l for *E. coli*, or 15 mg/l for *B. subtilis*), chloramphenicol (Cm; 25 mg/l for *E. coli*, or 5 or 10 mg/l for *B. subtilis*) or erythromycin (Em; 2.5 mg/l for *B. subtilis*). *E. coli* strains for cloning were made competent and transformed using standard techniques [Hanahan, 1983]. Naturally competent *B. subtilis* cells were obtained using an established protocol [Kraus et al., 1994]. All strains used in this study are summarized in table 1.

DNA Isolation and Modification

Plasmid DNA was prepared from *E. coli* according to the manufacturers' protocols using the E.Z.N.A. Plasmid Miniprep Kit (Qiagen, Hilden, Germany), NucleoSpin Plasmid (Macherey-Nagel, Düren, Germany), or the Plasmid Midi Kit (Qiagen, Hilden, Germany). For amplification of *B. subtilis* chromosomal DNA, colonies from solid medium were directly inoculated into a PCR reaction mix using illustra PuReTaq Ready-To-Go PCR beads (GE Healthcare, Munich, Germany). Sequencing of plasmids or PCR

products was accomplished using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Weiterstadt, Germany) or carried out at GATC (Constance, Germany). Primers were purchased from Biomers (Ulm, Germany) or MWG-Biotech (Ebersberg, Germany), longer oligonucleotides were obtained from TIB-MOLBIOL (Berlin, Germany). The sequences of the primers used for cloning or sequencing in this study are listed in table 2.

Construction of Plasmids for Expression of *tetR* Alleles Carrying SSR Sites

Throughout this study, the TetR(BD) chimera which comprises the TetR(B) sequence from position 1 to 50 and residues 51 to 208 of TetR(D) was used [Schnappinger et al., 1998; Schubert et al., 2001]. For convenience, TetR(BD) is hereafter referred to as TetR. Complementary oligonucleotides containing *FRT* or *lox* sites for in-frame insertion into *tetR* were 5' phosphorylated by T4 polynucleotide kinase (New England Biolabs, Frankfurt/Main, Germany). Subsequently, two complementary oligonucleotides were hybridized by mixing 37.5 pmol of each in aqueous solution, heating to 90°C and cooling down to RT within approximately 1 h. The hybridized oligonucleotides carried 3' overhangs, compatible or identical to those of *Pst*I cut DNA and contained the following SSR recognition sites: *FRT* within flp_fw/flp_rev, and *lox72* in two different reading frames within lockP/Pkcol and lockP_fw/lockP_rev. Upon insertion into *tetR* of *Pst*I cut pWH1926 [Kamionka et al., 2006], the following desired constructs were obtained: pWH1926-F with *FRT* in its 5'→3' reading frame 2, pWH1926-L1 (*lox72* in 3'→5' frame 1) and pWH1926-L2 (*lox72* 3'→5' frame 2). In all constructs the SSR recognition sites are embedded in a *tetR* ORF. Plasmids in which the fragments were inserted in opposite orientation are designated correspondingly and are suffixed '-inv'.

Cloning of a *B. subtilis* Integration Vector to Allow for Cre-Mediated *tetR* Activation

For disruption of *tetR* with a floxed kanamycin resistance marker, an *aphAIII* cassette was amplified from plasmid pDG792 [Guérout-Fleury et al., 1995] by PCR using primers Km_66_inv and Km_71_inv. The PCR product was inserted into pWH1926 via *Pst*I. Of the two possible resulting plasmids, that with *aphAIII* in opposite orientation to the disrupted *tetR* was used and designated pWH1926-TLKLT (for *tetR'*-*lox66*-*aphAIII*-*lox71*-*tetR*). The target strain *B. subtilis* WH558 carries DNA of pUC19 chromosomally, which is the ancestor of pWH1926 derivatives. To circumvent possible undesired ectopic insertion upon transformation with pWH1926-TLKLT, the *tetR'*-*lox66*-*aphAIII*-*lox71*-*tetR* sequence was subcloned into pWH1411BD [Scholz et al., 2003] via *Bgl*II and *Nco*I, yielding pWH1411-TLKLT. To provide a larger region of sequence identity for genomic integration, a part of the chromosomal sequence within and downstream of *tetR* was amplified from *B. subtilis* RAB100 DNA (table 1) using primers DP8neu and lacA_extd (table 2). This fragment was inserted into pWH1411-TLKLT via *Nco*I and *Pae*I to obtain the integration vector pWH1411-TLKLTxd, which was linearized by *Nhe*I prior to transformation of *B. subtilis*.

Applied Techniques for Genome Modifications of *B. subtilis* Strains

Elimination of floxed *aphAIII* cassettes from *B. subtilis* chromosomes was achieved by treatment of the respective cells with

pCrePA, a plasmid which was constructed for Cre expression in *B. anthracis* [Pomerantsev et al., 2006]. In general, 1 µg of this plasmid was used to transform *B. subtilis*. Transformands were first cultivated at 30°C on media containing 2.5 µg/ml Em but lacking Km, as the resistance marker *aphAIII* was intended to be eliminated. On average, at least 10% of the analyzed clones displayed Km resistance after pCrePA treatment. Em-resistant colonies were re-streaked on plates without Em and incubated at 37°C, a temperature which is not permissive for pCrePA. Candidate clones were streaked out again and incubated overnight at 37°C a second time. Clones which displayed both Em and Km sensitivity after this treatment, indicating marker eviction and pCrePA loss, were checked by PCR analyses.

β-Galactosidase Activity Quantifications

In vivo repression and induction capacities of TetR variants were determined by β-galactosidase (β-gal) assays of mid-log cultures of *E. coli* WH207 λtet50 [Smith and Bertrand, 1988; Wissmann et al., 1991] or various *B. subtilis* strains as described [Kamionka et al., 2005; Scholz et al., 2004]. Cells were grown in LB alone (repressive conditions), or supplemented with 0.4 µM ATc (for induction of TetR). Three independent cultures were assayed, with the measurements conducted at least twice. Values obtained for *E. coli* cells carrying the *tetR*-less plasmid

pWH1925Δ [Bertram et al., 2004], mimicking full induction, were set to 100%. Standard deviations of these measurements were below 10%.

Western Blot Analyses

Immunodetection of TetR in soluble *B. subtilis* extracts was performed using 75 µg of total protein, and either serum of TOP19 monoclonal antibody raised against TetR(B) [Pook et al., 1998] or a 1:20,000 dilution of rabbit polyclonal antibodies recognizing TetR in conjunction with ECL Plus Western Blotting Detection Reagents (GE Healthcare, Munich, Germany) as described in detail previously [Kamionka et al., 2005].

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