

Minireview

Status quo of *tet* regulation in bacteria

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Summary

The tetracycline repressor (TetR) belongs to the most popular, versatile and efficient transcriptional regulators used in bacterial genetics. In the tetracycline (Tc) resistance determinant *tet(B)* of transposon Tn10, *tetR* regulates the expression of a divergently oriented *tetA* gene that encodes a Tc antiporter. These components of Tn10 and of other natural or synthetic origins have been used for tetracycline-dependent gene regulation (*tet* regulation) in at least 40 bacterial genera. *Tet* regulation serves several purposes such as conditional complementation, depletion of essential genes, modulation of artificial genetic networks, protein overexpression or the control of gene expression within cell culture or animal infection models. Adaptations of the promoters employed have increased *tet* regulation efficiency and have made this system accessible to taxonomically distant bacteria. Variations of TetR, different effector molecules and mutated DNA binding sites have enabled new modes of gene expression control. This article provides a current overview of *tet* regulation in bacteria.

Introduction

A key process to control bacterial gene expression is transcription initiation, frequently modulated by

alternative sigma factors or transcriptional regulators. These usually represent activator or repressor proteins that interact with specific DNA sequences. A textbook example is the lactose repressor LacI, natively a regulator of carbon catabolism in Gram-negative bacteria and exploited for inducible gene expression in many bacterial species (Wilson *et al.*, 2007). Numerous of these systems activate gene expression upon administration of a low molecular weight inducer (Terpe, 2006). Among the most frequently used transcriptional regulators for inducible gene expression in bacteria is the tetracycline repressor (TetR). Its original function is the control of tetracycline (Tc) resistance genes found in more than a dozen Tc-resistant determinants (Thaker *et al.*, 2010). These are widespread among the Eubacteria, present in at least 35 genera covering five of 24 phyla (Berens and Hillen, 2004; Agersø and Guardabassi, 2005; Thompson *et al.*, 2007). TetR encoded by transposon Tn10 found in Enterobacteriaceae is a homodimeric transcriptional repressor of the TetR/CamR family (Ramos *et al.*, 2005). It controls its own transcription by negative autoregulation as well as expression of the *tetA* gene, which encodes a proton-dependent antiporter (Hillen and Berens, 1994). Upon interaction with an inducer, usually a Tc or a Tc-derivative, TetR detaches from its cognate DNA site *tetO* and gene expression is initiated. The *tet* regulation system aggregates several characteristics advantageous for inducible gene expression as the specific requirements of Tc-resistant control have shaped *tet* regulation to provide both tight repression and sensitive induction. The inducer is non-metabolizable and can rather freely traverse bacterial membranes, and subinhibitory concentrations are sufficient to trigger a response. In addition, *tet* regulation functions well during infection to enable *in vivo* gene regulation in cell cultures or animal models of infection. Finally, the components of the *tet* system have extensively been engineered to yield Tet repressors with new specificities for inducer- or operator-binding, or a reversed allostery, as well as a plethora of promoters. Besides its broad use in bacteria, TetR-based gene regulation is well established also in eukaryotic cells and organisms (Gossen and Bujard, 1992; Deuschle *et al.*, 1995; Berens and Hillen, 2003; Sprengel and Hasan, 2007; Das *et al.*, 2016) and has been adapted to work

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with archaea (Guss *et al.*, 2008), thus covering all three kingdoms of life. This article provides a comprehensive overview of *tet regulation* in bacteria and focuses on recent developments. We present the components and variables of *tet regulation* (Fig. 1), the multitude of bacterial genera and species that were made accessible to regulation by TetR, and new modes of target gene control.

Wild-type and engineered variants of TetR and their interaction partners

TetR monomers consist of 10 α -helices, with an N-terminal part (helices α 1 to α 3) harbouring a helix–turn–helix motif for binding to *tetO* followed by a protein core (α 4 to α 10) required for dimerization and inducer binding. The most intensively investigated Tet repressors originate from the Tc-resistant determinants *tet(B)* of Tn10 (Hillen and Schollmeier, 1983) and *tet(D)* of the *Salmonella* plasmid RA1 (Unger *et al.*, 1984). A TetR(BD) hybrid that consists of TetR(B) DNA-binding domain and the protein core of TetR(D) was found to provide enhanced stability and regulatory properties compared to

both wild-type variants (Schnappinger *et al.*, 1998). Extensive research and developments have yielded TetR variants with altered specificities for interaction partners, or reversed allostery (Fig. 2). The translational fusion of two *tetR* alleles (differing in codon usage to avoid recombination) gave rise to single-chain TetR (scTetR). Here, the two halves of the functional unit, each resembling one monomer in the TetR wild type, are linked by a polypeptide stretch of 25 amino acids (Kamionka *et al.*, 2006). Zeng *et al.* (2018) constructed repression-proficient and inducible ‘split’ TetR variants which in their active form are not composed of two but of four polypeptide chains held together by short interacting peptides. In a study conducted in *B. subtilis*, *tetR* was activated by intragenic Cre-*lox* recombination resulting in a functional TetR variant with an altered loop sequence between helices α 8 and α 9 (Bertram *et al.*, 2009).

Natively, TetR binds [Tc-Mg]⁺ complexes in a 1:1 stoichiometry relative to the monomer (Hinrichs *et al.*, 1994). Anhydrotetracycline (ATc), which is less toxic to bacteria and a more potent inducer of TetR (Degenkolb *et al.*, 1991), has replaced Tc as the predominant effector of

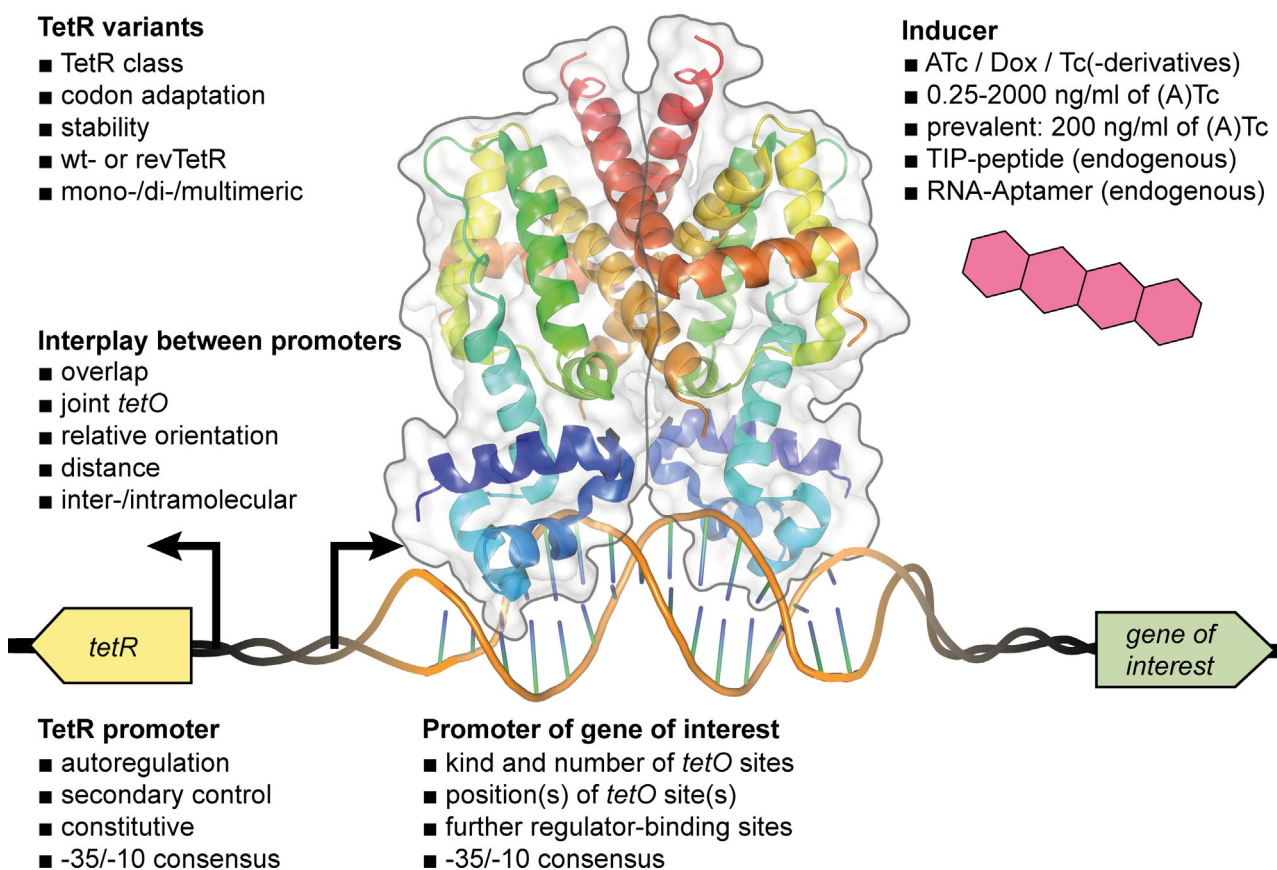


Fig. 1. Variables of bacterial *tet* systems.

Key parameters and variables affecting the outcome and efficiency of *tet regulation*. TetR is shown in the DNA bound form. Bent arrows denote promoters, and double helical part of schematized DNA represents *tetO*.

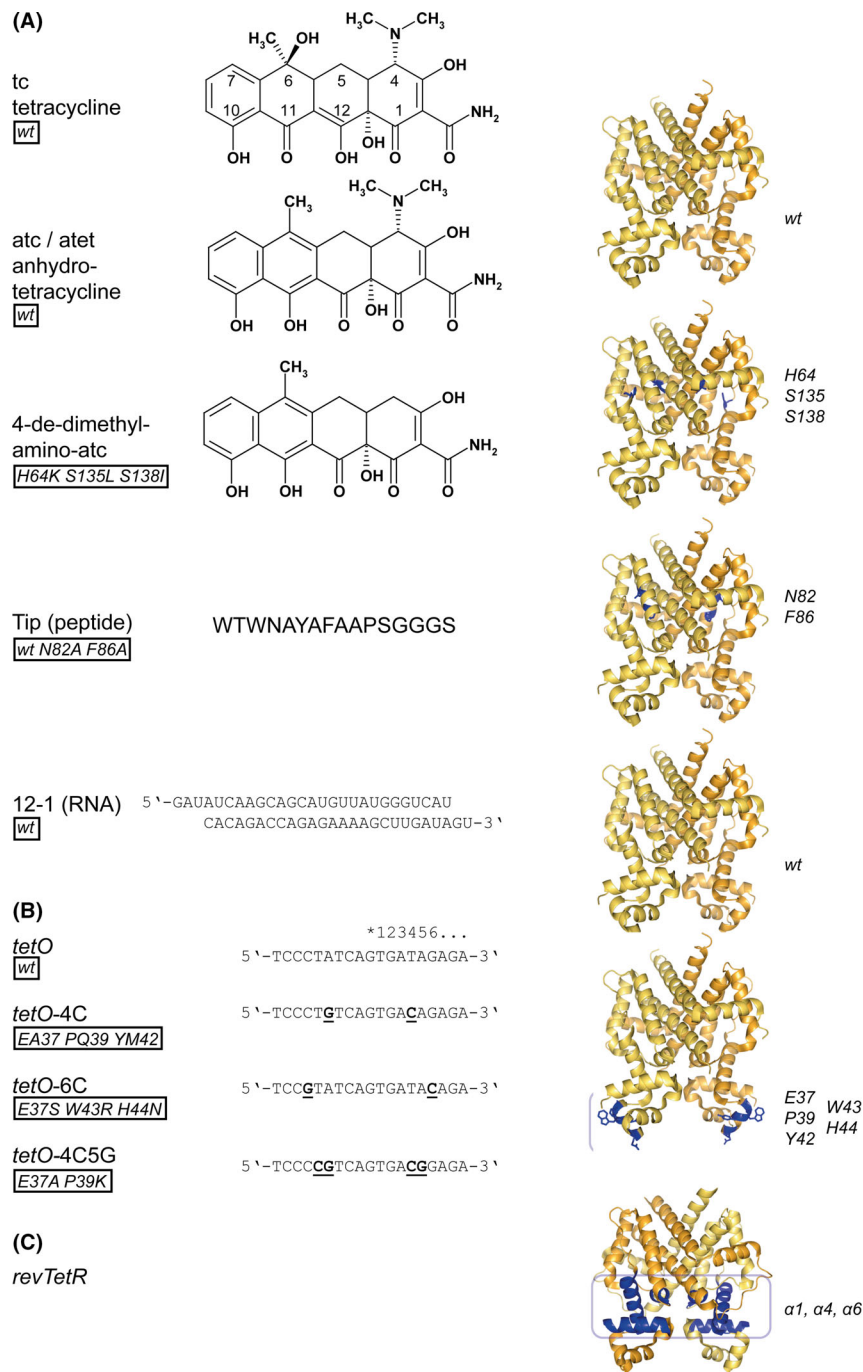


Fig. 2. Different inducers and operators and suitable Tet repressors.

A. Left side: Shown are selected tetracyclines, and the sequences of the Tip peptide and the 12-1 RNA. The boxes indicate which TetR variants are (best) inducible by these compounds. TetR H64K S135L S138I is designated TetR i2. Note that inducibility by Tip in wt-TetR is enhanced by mutations N82A F86A. Right side: TetR with the positions mutated for binding of 4-de-dimethylamino-atc or enhanced interaction with Tip highlighted.

B. Left side: Upper strands of the *tet* operator and selected variants. The grey boxes indicate which TetR variants (best) interact with these sites. Right side: TetR with the positions mutated for binding to *tetO* variants highlighted.

C. TetR with helices $\alpha 1$, $\alpha 4$ and $\alpha 6$ highlighted. Mutations resulting in the reverse phenotype are mostly found in these regions.

applied bacterial *tet* regulation. One drawback of ATc is its instability upon longer exposure to light (Baumschlager *et al.*, 2020), which can result in undesired

changes in inducer concentrations during an experiment. Doxycycline (Dox) is the typical effector of *tet* systems in eukaryotes but Dox antibiotic activity is disadvantageous

for use in many bacteria. Concentrations of tetracyclines applied for bacterial *tet* systems range from ~ 0.25 to ~ 2.000 ng ml⁻¹. 200 ng ml⁻¹ (equalling ~ 0.5 μ M) of ATc is sufficient to fully induce most bacterial *tet* systems. Numerous examples have demonstrated that *tet* regulation permits tuning target gene expression dependent on inducer concentration (see examples below). Various tetracyclines are synthesized by Streptomycetes (reviewed by Chopra and Roberts (2001)) or in a (semi) synthetic fashion (reviewed by Liu and Myers (2016)). Engineered TetR variants respond to Tc derivatives unsuitable for wt-TetR. In particular, TetR i2 is exclusively induced by 4-de-dimethylamino-ATc, but not by ATc or Dox (Henssler *et al.*, 2004; Klieber *et al.*, 2009) (Fig. 2A). Notably, also specific RNA or peptide molecules are capable of inducing TetR. A dodecameric peptide termed Tip (transcription inducing peptide) can trigger an allosteric conformational change in TetR leading to dissociation from *tetO* (Klotzsche *et al.*, 2005; Luckner *et al.*, 2007). Also, the RNA aptamer 12-1 with a minimal length of 49 nt represents an alternative inducer of TetR (Hunsicker *et al.*, 2009). According to the structure of the TetR-12-1 complex, this interaction resembles the binding of TetR to *tetO* (Grau *et al.*, 2020). Mutations in helix $\alpha 3$ of TetR can shift operator recognition from wild-type *tetO* to variants such as *tetO*-4C (Helbl and Hillen, 1998), *tetO*-6C (Helbl *et al.*, 1998) or *tetO*-4C5G (Krueger *et al.*, 2007) (Fig. 2B). Reverse TetR or revTetR variants are characterized by interaction with *tetO* in the presence of ATc, in this case called 'effector' instead of 'inducer' (Kamionka *et al.*, 2004a; Scholz *et al.*, 2004; Resch *et al.*, 2008). One or few single amino acid exchanges found predominantly in helices $\alpha 1$, $\alpha 4$ or $\alpha 6$ are sufficient for the reverse phenotype (Fig. 2C). revTetR variants such as *revtetR* r1.7 (*revtetR-r2*) show regulation efficiencies comparable to wt-TetR and function with the same promoters. Of note, relaxed or shifted specificities of Tet repressors to inducers or operators can be combined and even be transferred to revTetR (Bertram *et al.*, 2004; Henssler *et al.*, 2005; Krueger *et al.*, 2007).

Tet-ON and Tet-OFF control

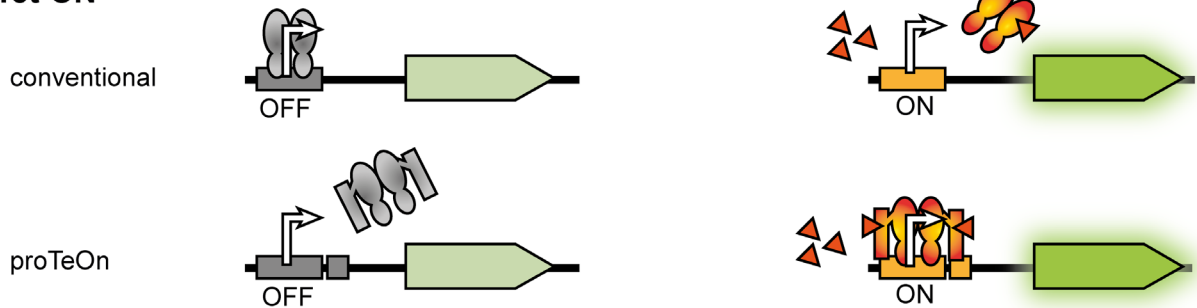
Tet regulation entails two different general outcomes, Tet-ON or Tet-OFF (Fig. 3). If the addition of an effector initiates gene expression, the system obeys Tet-ON logic. In the simplest, and by far most abundant form of Tet-ON, TetR binds to one or more *tetO* site(s) in a *tet*-sensitive promoter, which activates a downstream gene upon addition of ATc. In contrast, gene silencing in the presence of an effector defines Tet-OFF control. Tet-OFF can be achieved at the level of transcription initiation, when revTetR is used, or by wt-TetR that controls

antisense-RNA (AS-RNA) expression. More recent and sophisticated modes of Tet-ON and Tet-OFF control are discussed later. Generic Tet-ON regulation achieves rapid gene expression with observable phenotypic changes in as fast as 15 min, as shown in *Listeria monocytogenes* (Schmitter *et al.*, 2017). A return to the OFF state requires removal or dilution of the inducer. According to a study in *Chlamydia trachomatis*, the transcriptional response of a reporter gene was reduced by 50% after 20 min and by 90% after 120 min after removal of the inducer (Wickstrum *et al.*, 2013). Phenotypes attributed to gene silencing by revTetR were observed in *Mycobacterium smegmatis* after about 4 h by Western blotting against the target proteins, with only faint signals visible after 12 h (Guo *et al.*, 2007). Comparably, shutting off conditional complementation of the *dap* gene by revTetR in *Helicobacter pylori* resulted in growth retardation beginning 10 h after addition of ATc (Debowski *et al.*, 2015). Combined rapid ON and OFF kinetics can be realized by toggle switches. In *E. coli*, a respective synthetic, bistable gene-regulatory network was established with TetR and LacI mutually controlling each other's expression and a LacI-controlled promoter for target gene regulation (Gardner *et al.*, 2000). Recently, a comparable TetR/LacI toggle switch was developed for *Streptococcus pneumoniae* (Sorg *et al.*, 2020). Regarding the dynamic ranges of *tet* regulation, the induction factor (IF) provides a simple metric by dividing signal strength in the ON by the OFF state. Among the highest IF was reported for the P_{LtetO-1}-based *tet* system with a value of about 5000 using luciferase as a reporter (Lutz and Bujard, 1997). Reverse TetR achieved an IF up to 102 in a *lacZ*-based assay (Scholz *et al.*, 2004). Notably, the choice of the reporter gene or the mode of quantification of another signal (such as Western blotting, or RNA detection) strongly influence the quantification of the IF, which is why we mostly refrain from stating or comparing dynamic ranges of different *tet* systems.

Fundamentals of *tet* regulation architectures

Some systems rely on the native architecture with the *tetR* gene located adjacent to and divergent from *tetA*, whereas others separate *tetR* and the gene under *tet* control. The *tetR* gene and the *tet*-sensitive promoter may be located in *cis* on plasmids or the chromosome, or on different genomic entities, as described below. *Tet*-sensitive promoters have different requirements to function efficiently in different bacteria. This includes the number and position of *tetO* sites as well as specificities of promoters, such as those of low G + C Gram-positive bacteria (Voskuil *et al.*, 1995) or specific Gram-negative bacteria (Bayley *et al.*, 2000; Mastropaolo *et al.*, 2009).

(A) Tet-ON



(B) Tet-OFF

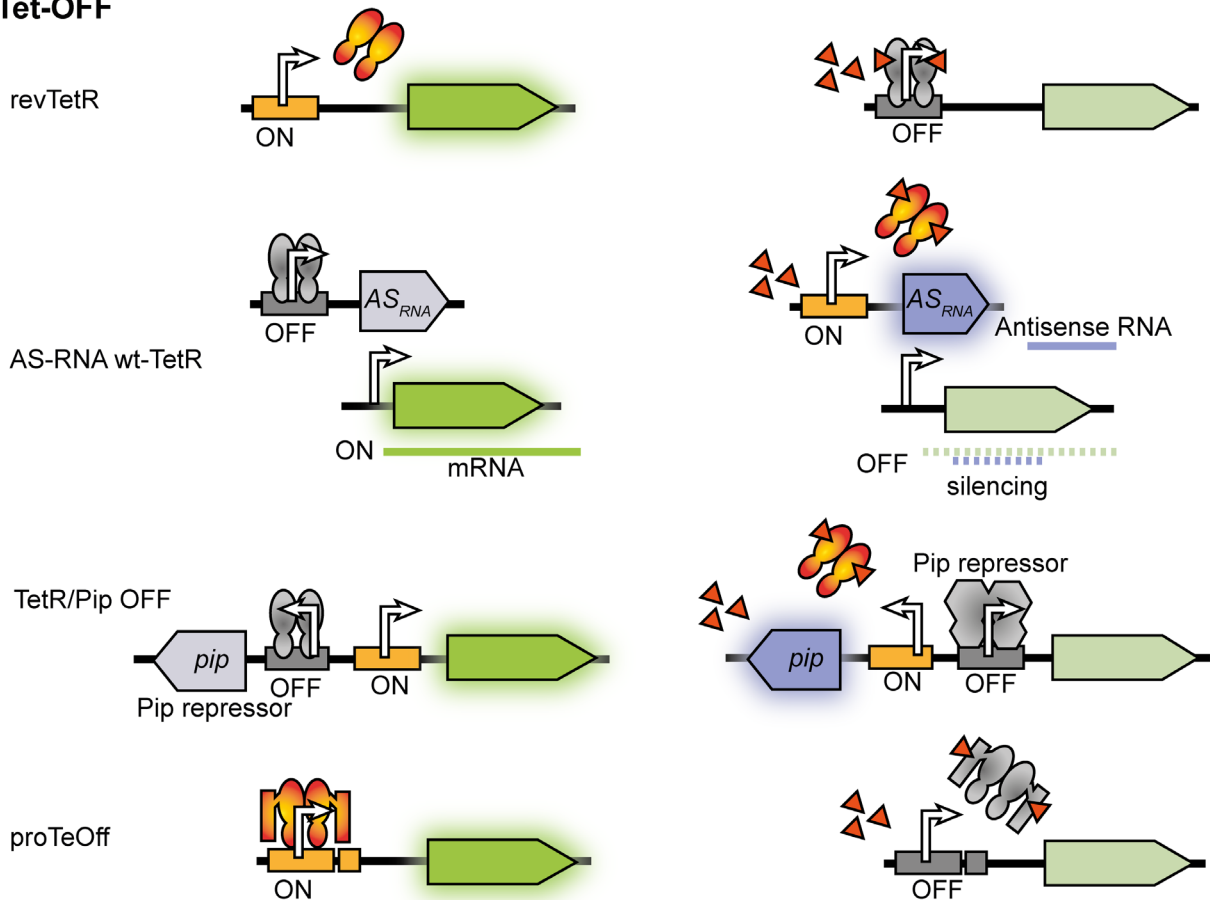


Fig. 3. Types of Tet-ON and Tet-OFF control.

A. upper panel: conventional Tet-ON control with wt-TetR; lower panel: Control by proTeOn (Volzing *et al.*, 2011).

B. 1st panel: Tet-OFF control by revTetR; 2nd panel: Expression of AS-RNA by wt-TetR; 3rd panel: The TetR/Pip OFF system (Boldrin *et al.*, 2010). A similar mode is represented by *tet*-regulated expression of dCas9 (Mariscal *et al.*, 2018) (not shown); 4th panel: Control by proTeOff. Bent arrows denote promoters. Boxes below the promoters symbolize *tetO*, or binding sites of Pip (TetR/Pip OFF) or LuxR (ProTeOn or ProTeOff). Effector is depicted as triangles. Light green arrows symbolize the inactive state; bright green arrows denote actively transcribed gene.

Expression of *tetR* can be constitutive, or autoregulated, or controlled by a second regulation system. Strong constitutive expression of *tetR* results in tight repression (Kamionka *et al.*, 2005), while decreased TetR

abundance can raise sensitivity of induction (Georgi *et al.*, 2012). Autoregulation of *tetR* may decrease transcriptional noise and thereby improve the response uniformity of a population (Hensel, 2017).

Fields of application

The versatility of the *tet* system is reflected by various fields of application. Soon after the publication of the first *tet* regulation system, which was based upon transposon Tn10 (de la Torre *et al.*, 1984), related mobile genetic elements permitted *tet*-dependent phenotypes in Enterobacteriaceae (Way *et al.*, 1984; Takiff *et al.*, 1992; Rappleye and Roth, 1997). Using transposon Tn5-derived integrative elements equipped with *tet*-responsive promoters, strains of *E. coli*, *Salmonella enterica* serovar Typhimurium or *Bacillus subtilis* with conditional lethal phenotypes were obtained (Bertram *et al.*, 2005; Köstner *et al.*, 2006). Conditional expression of a *tet*-controlled random pool of antisense-RNA identified *S. aureus* genes for infection and strains was characterized in infected mice (Ji *et al.*, 2001). The usefulness of the *tet* system in bacteria that inhabit cell cultures, lower- and vertebrate animals or plants is of particular appeal. This was shown for *Salmonella enterica* Serovar Typhi, *Staphylococcus aureus*, *Yersinia pestis*, *Helicobacter pylori*, Mycobacteria and Bacteroides (Ji *et al.*, 1999, 2001; Bateman *et al.*, 2001; Qian and Pan, 2002; Blokpoel *et al.*, 2005; Lathem *et al.*, 2007; Debowski *et al.*, 2017; Lim *et al.*, 2017). TetR is also a popular tool to control overexpression of genes of interest as demonstrated for antibody fragments (Schiweck *et al.*, 1997), anticalins (Beste *et al.*, 1999) and many other proteins expressed by plasmids derived from the pASK vector series (Skerra, 1994). The *tet* system served to construct biosensor strains to detect minute amounts of Tc or derivatives in the rat intestine (Bahl *et al.*, 2004), milk or pork serum (Kurittu *et al.*, 2000), or in soil (Hansen *et al.*, 2001). TetR and *tet*-sensitive promoters have emerged as popular parts and devices in synthetic biology to establish toggle switches, circuits or logic gates, frequently using *E. coli* as chassis (reviewed by Cameron *et al.* (2014)). Synthetic biology also pursues of generating bacteria with novel functionalities. Camacho *et al.* (2016) have engineered *Salmonella enterica* serovar Typhimurium to release a cytotoxic peptide upon addition of ATc when the bacteria proliferate inside tumour cells. The finding that specific peptides and RNA molecules can function as inducers of TetR (Klotzsche *et al.*, 2005; Hunsicker *et al.*, 2009) has rendered the regulator also suitable as a signal processing unit for translational or transcriptional activity within bacterial cells. Finally, TetR has also been applied in bacterial genetics apart from transcriptional regulation. To this end, TetR fused to a yellow-fluorescent protein bound to *tetO* sites inserted into selected chromosomal locations and the addition of inducer relieved a block of replication (Possoz *et al.*, 2006).

The current taxonomic spectrum of applied *tet* regulation in bacteria

So far, *tet* regulation has been applied for conditional gene expression in at least 40 bacterial genera of seven phyla (Table 1 and Fig. 4). Among the Gram-negative bacteria, *tet* systems have been established in Alpha-, Beta-, Gamma-, Delta- and Epsilonproteobacteria, Cyanobacteria, Spirochaetes and Chlamydiae. In Gram-positive bacteria, *tet* regulation was developed for use in Firmicutes, Actinobacteria and Tenericutes.

The next of kin: *tet* in Gram-negative bacteria

Systems based upon the Tn10 *tet* sequence

In Tn10, TetR binds to two palindromic tetracycline operator (*tetO*) sites embedded in bidirectional intertwined promoters (P_{R1} , P_{R2} and P_A) (Fig. 5A). The promoters P_{R1} and P_{R2} face towards *tetR* and are autoregulated. P_A controls expression of *tetA*, encoding a proton-dependent Tc antiporter. The two *tet* operators *tetO*₁ and *tetO*₂ of Tn10 share a core dyad symmetry of 19 bp but differ at four positions. TetR interaction with *tetO*₁ inhibits transcription of both genes, while the occupation of *tetO*₂ represses only *tetA*. The affinity of TetR is higher to *tetO*₂ than to *tetO*₁ (Kleinschmidt *et al.*, 1988).

De la Torre *et al.* (1984) showed that a *tetA-lacZ* translational fusion encoded on a plasmid could be controlled upon addition of Tc. Also, the first *tet* system applied in *Salmonella* was based upon a Tn10 derivative termed T-POP (Rappleye and Roth, 1997). Recently, Tn10 *tet* regulation cassettes were developed for ectopic expression of fimbriae on a low copy plasmid in *Salmonella enterica* serovar Typhimurium (Hansmeier *et al.*, 2017) and for promoter replacement in the chromosomes of *Yersinia enterocolitica* (Schulte *et al.*, 2019). In the zoonotic pathogen *Coxiella burnetii*, the type IVB secretion system (T4BSS) was controlled by Tn10 *tet* regulation (Beare *et al.*, 2011). An allelic exchange vector harbouring the *tetR-P_{tetA}* sequence found use in the nosocomial pathogen *Providencia stuartii* (Armbruster *et al.*, 2017) and in *Sodalis glossinidius*, which infects tsetse flies (Kendra *et al.*, 2020). Yin *et al.* (2015) employed *tetR-tetA* based *tet* regulation to induce putative secondary metabolite gene clusters in the insect pathogens *Photobacterium luminescens* and *Xenorhabdus stockiae*. To analyse the transcriptional activity of *Brucella abortus* in endosomal vacuoles of macrophages, the *tetR-tetA* sequence of Tn10 served to regulate a *gfp* reporter gene (Starr *et al.*, 2012). In the facultative pathogen *Burkholderia thailandensis*, the *tet*-controlled twin arginine translocation (Tat) secretion system was found essential for aerobic growth (Wagley *et al.*, 2014).

Table 1. Current list of bacterial organisms in which *tet* control is available.

Bacterium	Phylum (class)	Reference of initial <i>tet</i> regulation
<i>Acetobacterium woodii</i>	Firmicutes	Beck <i>et al.</i> (2020)
<i>Acinetobacter oleivorans</i>	Proteobacteria (gamma)	Hong and Park (2014)
<i>Agrobacterium tumefaciens</i>	Proteobacteria (alpha)	Hu <i>et al.</i> (2014)
<i>Anabaena spec.</i>	Cyanobacteria	Higo <i>et al.</i> (2016)
<i>Bacillus subtilis</i>	Firmicutes	Geissendörfer and Hillen (1990)
<i>Bacteroides thetaiotaomicron</i> , <i>B. fragilis</i> , <i>B. ovatus</i> , <i>B. uniformis</i> , <i>B. xylanisolvens</i> , <i>B. intestinalis</i> , <i>B. dorei</i> , <i>B. vulgatus</i> , <i>B. cellulosilyticus</i> , <i>B. eggerthii</i>	Bacteroidetes	Lim <i>et al.</i> (2007)
<i>Borrelia burgdorferi</i>	Spirochaetes	Whetstine <i>et al.</i> (2009)
<i>Brucella abortus</i>	Proteobacteria (alpha)	Starr <i>et al.</i> (2012)
<i>Burkholderia thailandensis</i>	Proteobacteria (beta)	Wagley <i>et al.</i> (2014)
<i>Campylobacter jejuni</i>	Proteobacteria (epsilon)	Cohen <i>et al.</i> (2019)
<i>Chlamydia trachomatis</i>	Chlamydiae	Wickstrum <i>et al.</i> (2013)
<i>Citrobacter freundii</i>	Proteobacteria (gamma)	Nyerges <i>et al.</i> (2016)
<i>Clostridium acetobutylicum</i> , <i>C. difficile</i>	Firmicutes	Fagan and Fairweather (2011), Dong <i>et al.</i> (2012)
<i>Corynebacterium glutamicum</i>	Actinobacteria	Radmacher <i>et al.</i> (2005)
<i>Coxiella burnetii</i>	Proteobacteria (gamma)	Beare <i>et al.</i> (2011)
<i>Edwardsiella tarda</i>	Proteobacteria (gamma)	Nyerges <i>et al.</i> (2016)
<i>Escherichia coli</i> , <i>E. hermannii</i>	Proteobacteria (gamma)	de la Torre <i>et al.</i> (1984), Nyerges <i>et al.</i> (2016)
<i>Francisella novicida</i> , <i>F. tularensis</i>	Proteobacteria (gamma)	LoVullo <i>et al.</i> (2012), Brodmann <i>et al.</i> (2018)
<i>Geobacter sulfurreducens</i>	Proteobacteria (delta)	Ueki <i>et al.</i> (2016)
<i>Helicobacter pylori</i>	Proteobacteria (epsilon)	Debowski <i>et al.</i> (2013), McClain <i>et al.</i> (2013)
<i>Klebsiella oxytoca</i>	Proteobacteria (gamma)	Temme <i>et al.</i> (2012)
<i>Laribacter hongkongensis</i>	Proteobacteria (beta)	Woo <i>et al.</i> (2005)
<i>Listeria monocytogenes</i>	Firmicutes	Schmitter <i>et al.</i> (2017)
<i>Magnetospirillum gryphiswaldense</i> , <i>M. magneticum</i>	Proteobacteria (alpha)	Yoshino <i>et al.</i> (2010), Borg <i>et al.</i> (2014)
<i>Methylobacterium extorquens</i>	Proteobacteria (alpha)	Chubiz <i>et al.</i> (2013)
<i>Mycobacterium abscessus</i> , <i>M. bovis BCG</i> , <i>M. smegmatis</i> , <i>M. tuberculosis</i>	Actinobacteria	Blokpoel <i>et al.</i> (2005), Ehrh <i>et al.</i> (2005), Cortes <i>et al.</i> (2011)
<i>Mycoplasma agalactiae</i> , <i>M. genitalium</i> , <i>M. mycoides</i> (JCVI-syn 1.0), <i>M. pneumoniae</i>	Tenericutes (Mollicutes)	Breton <i>et al.</i> (2010), Mariscal <i>et al.</i> (2016), Mariscal <i>et al.</i> (2018)
<i>Photorhabdus luminescens</i>	Proteobacteria (gamma)	Yin <i>et al.</i> (2015)
<i>Providencia stuartii</i>	Proteobacteria (gamma)	Armbruster <i>et al.</i> (2017)
<i>Pseudomonas putida</i>	Proteobacteria (gamma)	Gauttam <i>et al.</i> (2020)
<i>Ralstonia eutropha</i>	Proteobacteria (beta)	Li and Liao (2015)
<i>Salmonella enterica</i> serovar Thyphi, -Typhimurium	Proteobacteria (gamma)	Rappleye and Roth (1997), Qian and Pan (2002)
<i>Sodalis glossinidius</i>	Proteobacteria (gamma)	Kendra <i>et al.</i> (2020)
<i>Spiroplasma citri</i>	Tenericutes (Mollicutes)	Breton <i>et al.</i> (2010)
<i>Staphylococcus aureus</i> , <i>S. carnosus</i> , <i>S. epidermidis</i>	Firmicutes	Ji <i>et al.</i> (1999), Giese <i>et al.</i> (2009), Christner <i>et al.</i> (2010)
<i>Streptococcus agalactiae</i> , <i>S. mutans</i> , <i>S. pneumoniae</i> , <i>S. pyogenes</i>	Firmicutes	Bugrysheva and Scott (2010), Lartigue and Boulloc (2014), Stieger <i>et al.</i> (1999), Wang and Kuramitsu (2005)
<i>Streptomyces ambofaciens</i> , <i>S. avermitilis</i> , <i>S. coelicolor</i> , <i>S. griseus</i> , <i>S. lividans</i> , <i>S. rimosus</i> , <i>S. roseosporus</i> , <i>S. venezuelae</i>	Actinobacteria	Hansen <i>et al.</i> (2001), Rodriguez-Garcia <i>et al.</i> (2005)
<i>Synechococcus</i> sp. strain PCC7002	Cyanobacteria	Zess <i>et al.</i> (2016)
<i>Synechocystis</i> sp. strain PCC 6803	Cyanobacteria	Huang and Lindblad (2013)
<i>Vibrio cholerae</i>	Proteobacteria (gamma)	Hsiao <i>et al.</i> (2006)
<i>Xenorhabdus stockiae</i>	Proteobacteria (gamma)	Yin <i>et al.</i> (2015)
<i>Yersinia enterocolitica</i> , <i>Y. pestis</i>	Proteobacteria (gamma)	Lathem <i>et al.</i> (2007), Obrist and Miller (2012)

Taxonomic spectrum of *tet* regulation in bacteria. See Fig. 4 for the phylogenetic distribution.

In the anaerobic soil bacterium *Geobacter sulfurreducens*, expression of *gltA* (encoding citrate synthase) from a plasmid bearing the Tn10 *tet*-control sequence rendered growth on acetate dependent on the presence of ATc. This *tet* system was also used in concert with *lacI*/IPTG-dependent induction, to constitute an AND gate device (Ueki *et al.*, 2016). A *tet* system applied in *Magnetospirillum gryphiswaldense* relied on chromosomal expression of *tetR* driven by the neomycin promoter

P_{neo} and the native Tn10 P_A promoter for control of target genes (Borg *et al.*, 2014). Bina *et al.* (2014) described a *tet*-inducible vector system for *Vibrio cholerae* using the Tn10 *tet* regulation sequence. In another study on *V. cholerae*, Cakar *et al.* (2018) cloned a promoterless *tetR* gene and a resolvase gene driven by Tn10 P_A to investigate gene expression profiles. *TetR* was used for promoter probing upon random insertion into the chromosome.

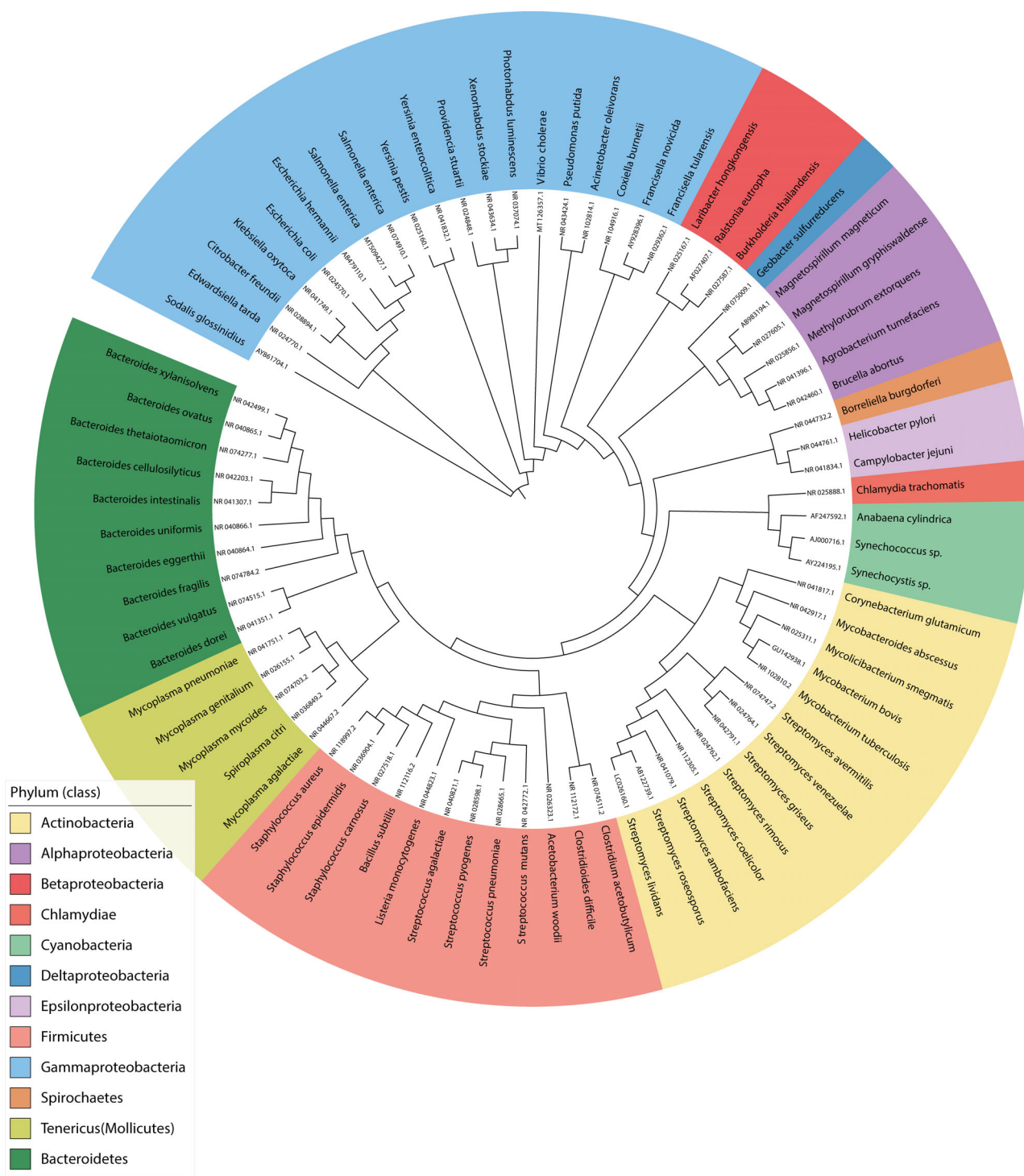
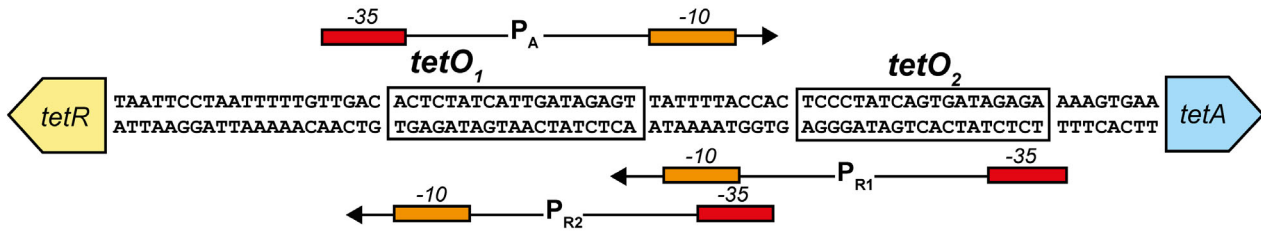
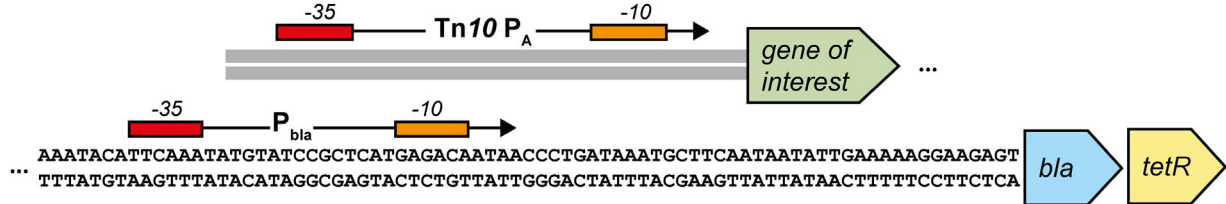


Fig. 4. Phylogenetic distribution of applied *tet* regulation in bacteria. The presented phylogeny is based on 16S DNA sequences of respective species, assessed from the NCBI Nucleotide database. Sequences were aligned and the phylogeny was calculated using the EMBL-EBI web services (https://www.ebi.ac.uk/Tools/phylogeny/simple_phylogeny/) using default parameters. The visualization was done using the iTOL (version 6.3) web tool (Letunic and Bork, 2021).

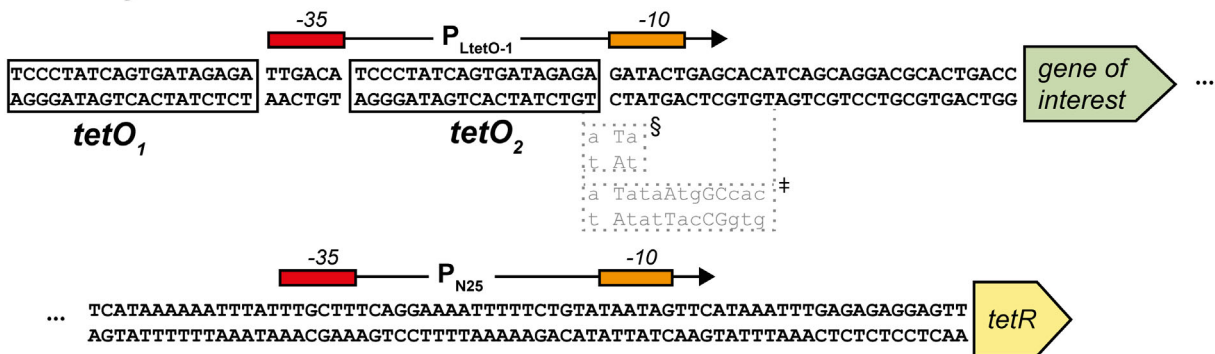
Tn10



pASK75



pZ vector system / E. coli DH5αZ1



§ Lee *et al.* (2016); † Huang and Lindblad (2013);

Fig. 5. Selected promoters of popular bacterial *tet* regulation systems for Gram-negative bacteria.

- A. P_A , P_{R1} and P_{R2} of transposon Tn10.
 B. P_A of Tn10 and P_{bla} of pBR322 in the pASK75 system. The P_A promoter is identical to that in A). *bla*: beta-lactamase.
 C. $P_{LtetO-1}$ and P_{N25} in the pZ vector system. Sequence deviations of promoter variants are given in grey dotted boxes.

The pASK75 vector system

Another popular *tet* regulation vector system in Gram-negative bacteria was initially described as the expression plasmid pASK75 (Skerra, 1994) (Fig. 5B). It contains the Tn10 P_A promoter, whereas *tetR* is expressed constitutively as a bicistronic transcript downstream of the beta-lactamase gene *bla* derived from the plasmid pBR322. In the phytopathogen and transgenesis vector *Agrobacterium tumefaciens* this *tet* system was applied to control expression of λ -Red recombination (Hu *et al.*, 2014). For use in *Chlamydia trachomatis*, a sexually transmitted obligate intracellular bacterium, the pASK75 *tet* system was

cloned into a suitable shuttle-vector and doseable expression was observed (Wickstrum *et al.*, 2013).

 $P_{LtetO-1}$ -based set-ups

$P_{LtetO-1}$ represents a phage λ P_L promoter in which two λ *ci* repressor binding sequences that flank the -35 hexamer have been replaced by two *tetO*₂ sites (Lutz and Bujard, 1997) (Fig. 5C). In the original set-up, *tetR* is driven by the P_{N25} promoter of phage T5 and integrated into the λ phage attachment of the *E. coli* chromosome. Qian and Pan (2002) employed the $P_{LtetO-1}$ promoter in *Salmonella enterica* serovar Thyphi and also the first *tet*

system in *V. cholerae* was based upon the $P_{\text{LtetO-1}}$ promoter (Hsiao *et al.*, 2006). $P_{\text{LtetO-1}}$ was applied to establish *tet* regulation in *Escherichia hermannii*, *Citrobacter freundii* and *Edwardsiella tarda* to control multiplex automated genome engineering (Nyerges *et al.*, 2016). In *Klebsiella oxytoca*, a nitrogen fixation cluster was decoupled from native control instances and redesigned genes controlled by a $P_{\text{LtetO-1}}$ sequence, with two nucleotide exchanges (Temme *et al.*, 2012). The $P_{\text{LtetO-1}}$ *tet* system was used together with IPTG induction in *Pseudomonas putida* to exert independent dual control of two genes or operons (Gauttam *et al.*, 2020). The first published *tet* system in *Yersinia pestis* was employed for regulated expression of the plasminogen activator Pla in a mouse infection model. *TetR* was integrated into the chromosome and driven by the P_{N25} promoter (Lathem *et al.*, 2007). In a set of low copy plasmids for *tet* regulation in *Y. enterocolitica*, the *tetR* expression module is separated from the *tet*-sensitive promoter (Obrist and Miller, 2012). Lee *et al.* (2016) altered $P_{\text{LtetO-1}}$ at selected positions to increase transcription in *E. coli*. For use in the cyanobacterium *Synechocystis* sp. the $P_{\text{LtetO-1}}$ promoter proved inefficient, but four exchanges around the -10 region yielded promoter L03 (Fig. 5C) that permitted a wide dynamic range (Huang and Lindblad, 2013). Induction was dependent not only on ATc but also on the light conditions. The L03 promoter was used also in the multicellular filamentous cyanobacterium *Anabaena* sp. PCC 7120 (Higo *et al.*, 2016). Expression of *tetR* was driven by either P_{nirA} , which is active in the presence of nitrate or P_{petE} , which is unresponsive to nitrogen source. Stability of TetR was decreased by addition of the protein degradation tag LVA to the C-terminus. Of note, an increase in target gene activity could be achieved merely by modulating nitrate concentrations. In a follow-up study, a positive feedback loop was constructed by means of a *tetR* directed and *tet*-controlled small antisense-RNA (Higo *et al.*, 2017). This resulted in elevated levels and extended duration of induction in *Anabaena*.

Specific promoters for *tet* regulation in Gram-negative bacteria

For the zoopathogenic *Francisella tularensis*, a *groESL* promoter was equipped with *tetO* downstream of the -10 consensus sequence and *tetR* was expressed by a constitutive promoter (LoVullo *et al.*, 2012). This one-plasmid system enabled *in vivo* regulation of *ripA*, which is required for *F. tularensis* replication in macrophages and also established Tet-OFF control by employing *revtetR* r1.7. Conjugative plasmids for *F. novicida* were constructed by the same *tet*-regulatory sequence (Brodmann *et al.*, 2018). In another system for use in *F. novicida*, *tetR* was transcribed from a P_{bla} promoter inserted

into a transposon attachment site of the chromosome (McWhinnie and Nano, 2014). Of several synthetic promoters tested for target gene expression, ten were found to be regulatable by TetR and ATc. Conditional expression of the virulence factor *vgrG* permitted inducer-dependent growth of a mutant strain within a macrophage cell line. Unusually, a TetR(H) variant (Hansen *et al.*, 1993; Chopra and Roberts, 2001) was chosen to construct a Tc biosensor strain of *Acinetobacter oleivorans*, a soil bacterium able to degrade diesel oil. Reporter gene expression driven by the P_{tetH} promoter (similar to P_{A} of Tn10) could be detected at nanomolar concentrations of Dox (Hong and Park, 2014). In a *tet* system for *M. magneticum*, the *tetR* gene was expressed constitutively by P_{msp3} and the promoter for *tet*-control contained *tetO* sites integrated upstream of the -35 and -10 regions of P_{msp1} (Yoshino *et al.*, 2010). A *tet* system applicable to *Methylobacterium extorquens* was generated with a *tetO* sequence placed downstream of the -10 region of a rhizobial phage promoter and *tetR* expressed from a *lac* promoter. Compared to a cumate-dependent induction system, *tet*-control provided tighter repression (Chubiz *et al.*, 2013). To establish *tet* regulation in *Ralstonia eutropha*, a producer of sustainable, biodegradable materials or biofuels, one or two *tet* operators were integrated into the *rrsC* promoter (Li and Liao, 2015). A library of 300 mutant promoters based upon P_{phaC1} was tested for suitable expression of *tetR*. Using the most favourable combination, *tet*-controlled expression of a toxic gene from *B. subtilis* hampered growth of *R. eutropha*.

In 2013, two groups reported *tet* system set-ups in the gastric ulcer bacterium *Helicobacter pylori*. McClain *et al.* (2013) chose an unusual number of three *tetO* sites chromosomally inserted into the *cagUT* promoter, natively driving transcription of type IV secretion system (T4SS) genes. Codon-optimized *tetR* was expressed from the *ureA* locus. Debowski *et al.* (2013) employed a *ureA* promoter to insert one *tetO* site downstream of -10 and alternatively an additional one between -35 and -10 , to yield promoters termed uP_{tetO1} (one *tetO*) and uP_{tetO2} (two *tetO*). TetR was expressed from one of three different *H. pylori* promoters in the chromosome. Compared to uP_{tetO1} , expression by uP_{tetO2} yielded lower activities. A *tet*-controlled reporter gene was also inducible in a mouse model of infection. Second-generation *tet* promoters for *H. pylori* are characterized by a *tetO* site in between -35 and -10 and the addition of a second *tet* operator upstream of -35 (Debowski *et al.*, 2015). This study also introduced regulation by *revTetR*-r1.7 in *H. pylori*. One *tet*-sensitive promoter with three *tetO* sites was applied to generate conditional urease mutants, which were investigated in a mouse model of infection (Debowski *et al.*, 2017). A *tet* regulation system in *Campylobacter jejuni*, also causing

gastroenteritis in humans, was composed of $P_{\text{rpsL}}\text{-tetR}$ and target genes cloned downstream of $rpsO$ -based promoters carrying one or two $tetO$ sites. This study not only established Tet-ON control, but also used revTetR r6.2 (V99E) in *C. jejuni* (Cohen *et al.*, 2019). For *Borellia burgdorferi*, the aetiological agent of Lyme disease, one copy of $tetO$ was integrated into the P_{ospA} promoter at the unusual position +1. The $tetR$ gene was constitutively expressed by P_{flaB} in cis, located downstream of the tet -controlled reporter gene, providing a one-plasmid set-up (Whetstone *et al.*, 2009).

Zess *et al.* (2016) constructed a tet -inducible promoter to act in concert with an inducible sRNA for posttranscriptional regulation in *Synechococcus* sp. strain PC7002. To this end, the -35 region of a truncated cyanobacterial promoter P_{cpcB} was flanked by two $tetO$ sites and minor further mutations yielded five potentially tet -sensitive promoters. Both the tet -regulated gfp reporter and $tetR$ were integrated in the chromosome and expression of $tetR$ was tested with three different constitutive promoters. Not only administration of ATc but also the concentration of CO_2 affected tet regulation efficiency. In addition, this tet system was used for posttranscriptional control by regulated expression of an sRNA.

The gut-colonizing Bacteroides have evolved rather unusual σ^{70} -dependent promoters, with conserved -33 and -7 elements (Bayley *et al.*, 2000; Mastropaolo *et al.*, 2009). Tet operators were placed at different sites of the P1 promoter of 16S rRNA and alternative ribosome binding sites were tested. This system permitted inducible gene regulation in the respective bacteria in the gut of mice (Lim *et al.*, 2017). A tet system developed for use in *Laribacter hongkongensis* (Woo *et al.*, 2005) was based upon the $P_{\text{xyl/tet}}$ promoter that had been developed for Gram-positive bacteria, as detailed in the following.

Gram-positive solutions of tet regulation

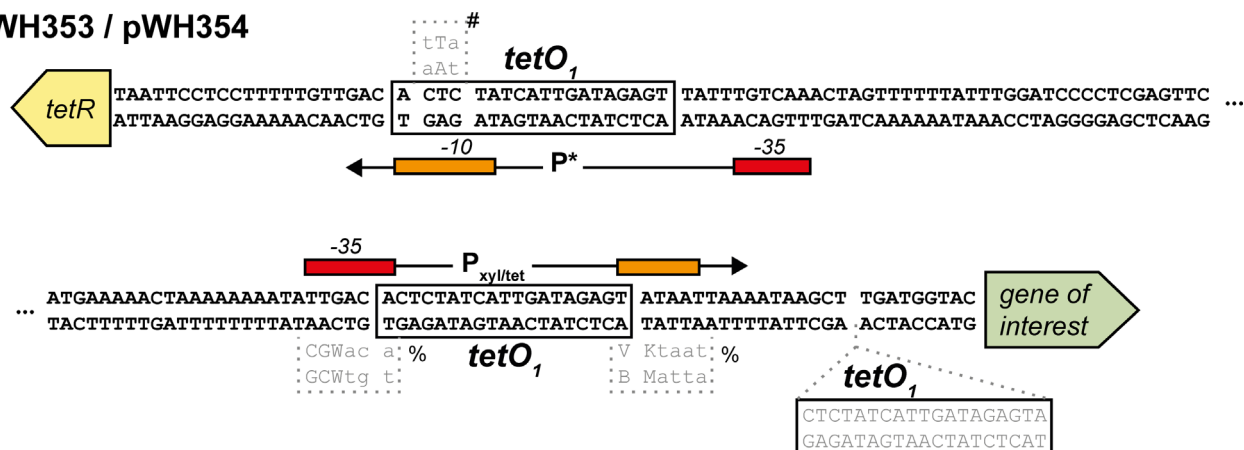
The $P_{\text{xyl/tet}}$ promoter system

The first tet system of Gram-positive bacteria was established in *Bacillus subtilis* (Geissendörfer and Hillen, 1990). The tet regulation sequences of Tn10 were found to be unsuitable, and hence, a modified autoregulated promoter termed P^* was constructed to drive $tetR$, while the *B. subtilis* P_{xylA} promoter was vested with one or two $tetO$ sequences to yield two versions of $P_{\text{xyl/tet}}$ for tet regulation of genes of interest (Fig. 6). All required components were cloned in one plasmid, termed pWH353 (one $tetO$ in $P_{\text{xyl/tet}}$) or pWH354 (two copies of $tetO$). Expression by pWH353 is strongly inducible with some basal expression in the absence of the inducer. In turn, pWH354 produces no detectable basal expression at the expense of reduced inducibility. The first use of $P_{\text{xyl/tet}}$ in pathogenic Gram-positive bacteria is marked by Ji *et al.*

(1999), when the virulence gene *hla* encoding alpha-toxin was regulated by tet -controlled antisense-RNA. The tet regulation cassette of pWH353 can be found in the popular plasmids pALC2073 and pALC2084 used in staphylococci (Bateman *et al.*, 2001). In the non-pathogenic *S. carnosus*, the *hla* gene was cloned into pALC2084 to analyse the effect regarding phagolysosomal escape (Giese *et al.*, 2009). In *S. epidermidis*, $P^*\text{-tetR-P}_{\text{xyl/tet}}$ (one $tetO$) was used to control expression of the extracellular matrix-binding protein-gene *embp* after promoter exchange in the chromosome (Christner *et al.*, 2010). Inducible antisense-RNA expression was accomplished by the $P_{\text{xyl/tet}}$ sequence of pALC2073 in *Streptococcus mutants* (Wang and Kuramitsu, 2005). Bugrysheva and Scott (2010) have applied the $P^*\text{-tetR-P}_{\text{xyl/tet}}$ (two $tetO$) sequence in *Streptococcus pyogenes* to replace the native promoters of two putative essential RNase encoding genes, thereby rendering growth of strains ATc-dependent. The use of a target gene promoter with three vs. two tet operators permitted tighter control but at the cost of reduced induction. The facultative intracellular food spoilage bacterium *Listeria monocytogenes* spreads from one host cell to another by means of the ActA protein. In a study by Schmitter *et al.* (2017), the *actA* gene was expressed by $P_{\text{xyl/tet}}$ (two $tetO$) from the chromosome, while $tetR$ was episomally encoded and driven by a strong synthetic promoter termed pt17 (Bertram *et al.*, 2005). The resulting strain facilitated ATc-dependent spatio-temporal control of ActA and consequently actin recruitment within epithelial human cells.

Corrigan and Foster (2009) modified the P^* promoter of the $P^*\text{-tetR-P}_{\text{xyl/tet}}$ sequence, rendering expression of $tetR$ constitutive (Fig. 6). The resulting plasmid pRMC2 was used in *S. aureus*, and its tet regulation architecture was cloned also into a shuttle-vector applied in *Streptococcus agalactiae* (Lartigue and Bouloc, 2014). Helle *et al.* (2011) added a second $tetO$ site to $P_{\text{xyl/tet}}$ of pRMC2 to yield plasmid pRAB11 and also exchanged selected positions in the target gene promoter in a semi-randomized fashion (Fig. 6). Six of the promoter variants were analysed and all led to lower expression levels in the induced state and tighter repression in the absence of an inducer. Schleimer *et al.* (2019) reported that a restoration of $tetR$ autoregulation of pRAB11 reduced growth retardation, which was attributed to a decreased abundance of the repressor. The resulting plasmid was employed to induce AS-RNA directed to the essential gene *fabI*, a gene investigated in similar studies before (Ji *et al.*, 2004; Stary *et al.*, 2010). Stary *et al.* (2010) presented four different tet architectures in *S. aureus*, a one-plasmid tet system resembling pWH354 (Geissendörfer and Hillen, 1990), as well as ATc-dependent regulation of chromosomal target genes by TetR or revTetR or inducible AS-RNA expression. Apparently, tet regulation of AS-

pWH353 / pWH354



Corrigan and Foster (2009); % Helle *et al.* (2011)

Fig. 6. The $P_{xyl/tet}$ promoter.

P^* and $P_{xyl/tet}$ in pWH353 (one *tetO*) and pWH354 two *tetO*). Note that the sequence is continued from the upper to the lower part. Sequence deviations of promoter variants are given in grey dotted boxes.

RNA expression remains a popular choice in staphylococcal genetics (Yan *et al.*, 2011; Xu *et al.*, 2017).

Meiers *et al.* (2017) used the *tet*-control module of pRAB11 (Helle *et al.*, 2011) for chromosomal integration in *S. pneumoniae*. Hyperactive two-component histidine kinases, usually recalcitrant to cloning due to genetic instability, could thereby be tightly repressed and regulated. The $P_{xyl/tet}$ promoter of plasmid pRMC2 (Corrigan and Foster, 2009) was applied for *tet* regulation in the nosocomial pathogen *Clostridium difficile* (Fagan and Fairweather, 2011). An accessory secretory (*sec*) system was analysed by conventional Tet-ON regulation, as well as by inducible AS-RNA expression targeted to the 5' ends of *secA1*, or *secA2* mRNA, respectively. The *tet* system described by Fagan and Fairweather (2011) also found use in the acetone-producing *Acetobacterium woodii* (Beck *et al.*, 2020). A comparison with three other inducible systems revealed that only a theophylline riboswitch (Topp *et al.*, 2010) and *tet* regulation yielded tight repression of a reporter in the non-induced state. Breton *et al.* (2010) described the implementation of *tet*-control in Mollicutes by a one-plasmid set-up in the plant pathogen *Spiroplasma citri* and the animal pathogen *Mycoplasma agalactiae*. Target gene expression was controlled by $P_{xyl/tet}$ ($2 \times tetO$) and *tetR* was driven by the *Spiroplasma* spiralin promoter. Unusually, *tetR* and the target gene were positioned in adjacent, yet convergent orientation. Gene expression in *S. citri* could be induced when dwelling in either the insect vector leafhopper or in periwinkle plants. A similar architecture with a slightly truncated version of the *tet*-controlled promoter was used to express Cre recombinase in *M. genitalium* (Mariscal *et al.*, 2016). The excision frequency of a *lox*-flanked

resistance marker served as a readout for *tet* regulation efficiency. For use in *M. mycoides*, including the synthetic cell JCVI-syn1.0 (Gibson *et al.*, 2010), this *tet* system required higher amounts of Tc, which was tolerated by the cells possibly by concomitant expression of the Tc-resistant factor *tetM*. Tet-OFF regulation was achieved by combining the regular *tet* system and CRISPRi (clustered regularly interspaced short palindromic repeat-mediated interference). A nuclease defective Cas9 protein (dCas9) and a target gene-directed single-guide RNA were controlled by a *tet* promoter. This inducible CRISPRi system yielded a decrease of around 75% of mCherry reporter activity (Mariscal *et al.*, 2018).

Tet systems adapted to Actinobacteria

Hansen *et al.* (2001) developed a *Streptomyces rimosus* strain to function as an oxytetracycline biosensor in soil by a *gfp* gene controlled by the Tn10 *tet* sequence. *Tet*-sensitive promoters based upon the strong *ermEp1* promoter were constructed for use in *Streptomyces coelicolor* (Rodriguez-Garcia *et al.*, 2005). The establishment of *tet* regulation in Mycobacteria was achieved by four independent approaches, each of which relies on different genetic components. Ehrhart *et al.* (2005) had chosen to modify mycobacterial promoters, Blokpoel *et al.* (2005) made use of the *tet(Z)* resistance determinant of Corynebacteria, Carroll *et al.* (2005) relied on the $P_{xyl/tet}$ promoter, and Hernandez-Abanto *et al.* (2006) modified a *tet*-sensitive promoter previously applied in Streptomyces. In the latter study, *tetR* was expressed from an acetamide inducible promoter and expression of a *gfp* reporter in *Mycobacterium smegmatis* in a mouse model

of infection was modulated by acetamide and ATc. Williams *et al.* (2010) improved the *tet(Z)*-based system by changing the vector background, providing an episomal or chromosomal architecture, and by exploiting the TetR T40G mutation to increase affinity to *tetO* (Baumeister *et al.*, 1992). A Tet-OFF mode of control was enabled by placing the pristinamycin repressor Pip under *tet*-control. Thereby, a gene of interest downstream of a Pip-controlled promoter is indirectly repressible by addition of ATc (Boldrin *et al.*, 2010) (Fig. 3). This TetR/Pip OFF system was used to regulate genes *ftsZ* in *M. smegmatis* and *fadD32* in *M. tuberculosis* and *M. abscessus* (Cortes *et al.*, 2011). Guo *et al.* (2007) established gene regulation in *Mycobacterium smegmatis* via revTetR-r1.7, as proven by conditional expression of *secA1*, essential for *in vitro* growth. Improved revTetR regulation in Mycobacteria was achieved by two adaptations. First, the codon usage of *revtetR* r1.7 was altered from *E. coli* to Mycobacteria. Second, residues at selected positions were exchanged for amino acids stemming from a different TetR class (Klotzsche *et al.*, 2009). The use and the versatility of *tet* systems in Mycobacteria have been summarized by Schnappinger and Ehrh (2014), as well as Evans and Mizrahi (2015).

To use *tet regulation* in *Corynebacterium glutamicum*, Radmacher *et al.* (2005) replaced the native *emb* promoter in the chromosome for the Tn10 *tetA* promoter. TetR was expressed from a plasmid and driven by P_{gap}. Repression of *emb* led to growth retardation and an elevated glutamate efflux. In an alternative, episomal setup, the P_{gap}-*tetR* fragment and the P_{xyI/tet} promoter were cloned in divergent orientation into an expression vector. In comparison with an established IPTG inducible promoter, this *tet system* achieved 30% of reporter activity in the induced state, but much tighter repression, close to the detection limit (Lausberg *et al.*, 2012).

Other promoters for *tet regulation* in Gram-positive bacteria

In a study in *S. aureus*, Xu *et al.* (2010) inserted one or two *tetO* sites into the P_{N25} promoter of bacteriophage T5 and two other chimeric promoters to obtain six different promoters with different basal and induced expression strengths. Also, the first *tet regulation* system applied in streptococci was based upon a P_{N25} promoter with one *tetO* site between -10 and -35 regions (Stieger *et al.*, 1999). The most recently published *tet system* for *S. pneumoniae* exploits synthetic promoters carrying one or two *tetO* sites (upstream, downstream or flanking the -10 consensus) and codon-adapted *tetR* expressed from a strong constitutive promoter. The combined use of the LacI-system permitted the construction of synthetic regulatory networks with different outputs to implement a genetic

inverter, amplifier and toggle switch. Different sophisticated modes of regulation were shown to also function in mice (Sorg *et al.*, 2020). A study by Gauttam *et al.* (2019) described a dual expression plasmid of *C. glutamicum* exploiting the P_{LtetO-1} architecture, which later was used by the same group in *Pseudomonas* (see previous chapter). The P_{cm} promoter, originally part of a chloramphenicol resistance cassette, was modified to include one or two *tetO* sites at different positions to establish *tet regulation* in *Clostridium acetobutylicum* (Dong *et al.*, 2012). *TetR* was expressed from a constitutive thiolase gene promoter (P_{thl}). Notably, promoters were functional only with *tetO*₁ but not with *tetO*₂. The addition of a second *tetO* site upstream of -35 improved the dynamic range. Another *tet regulation* system for use in *B. subtilis* exploited the *tet(A)* determinant of transposon Tn1721 (Heravi *et al.*, 2015).

Future directions of *tetcontrol* – from tool to toolbox

The discovery that a dodecameric peptide termed Tip can induce TetR (Klotzsche *et al.*, 2005) was followed by fusing various proteins with Tip in *E. coli* (Schlicht *et al.*, 2006) and *Salmonella enterica* serovar Typhimurium (Georgi *et al.*, 2012). A *tet*-controlled reporter system can then serve as a quantitative readout of the carrier-protein production. As shown in *S. aureus*, Tip-tagged mCherry retained red fluorescence and was able to induce expression of *tet*-controlled *gfpmut2* (Gauger *et al.*, 2012). Induction of TetR by Tip was improved by introducing mutations N82A and F86A into the regulator, while selected Tip variants were active as corepressors or anti-inducers of TetR (Klotzsche *et al.*, 2007; Goeke *et al.*, 2012). Another unusual inducer of TetR is the RNA aptamer 12-1. Meitert *et al.* (2013) generated transcriptional fusions resulting in the insertion of the aptamer into untranslated regions of mRNAs as well as into small non-coding RNAs. This served to monitor expression levels of natural transcripts in *E. coli*. In a synthetic biology approach, Higo *et al.* (2017) applied both Tip and 12-1-RNA as inducers of TetR in cyanobacteria to establish a positive feedback loop. New kinds of bacterial Tet-ON and Tet-OFF control became feasible when TetR- and revTetR were modified to function as activators in bacteria (Volzing *et al.*, 2011). The C-terminal part of the quorum-sensing LuxR regulator (encompassing a transactivation domain) was fused to the C-termini of TetR or revTetR. The resulting regulators were termed proTeOn (based upon revTetR) and proTeOff (wt-TetR derived) (Fig. 3). These require promoters with both *tetO* and a binding site for LuxR for target gene regulation. When proTeOn binds to the promoter in the presence of ATc, a gene of interest is activated via RNA polymerase recruitment. proTeOff in contrast deactivates a target gene in the presence of ATc. Another type of Tet-OFF

regulation shown in Mycobacteria made use of targeted degradation of proteins of interest. For this, the *sspB* gene was put under *tet* control to allow for inducible depletion of SsrA-tagged proteins relying on intracellular proteases (Kim *et al.*, 2011). While a conventional Tet-ON system can function in concert as one of a dozen gene regulation systems in a highly engineered *E. coli* chassis (Meyer *et al.*, 2019), different variants of TetR with altered or relaxed inducer or operator specificities have been applied to construct mutually orthogonal *tet* systems (Kamionka *et al.*, 2004b) or to combine Tet-ON and Tet-OFF control in one cell (Kim *et al.*, 2013). Single-chain TetR (Kamionka *et al.*, 2006) or heterodimeric TetR variants (Stiebritz *et al.*, 2010) permit convenient changes in only one half of the protein to yield repressors that require two different inducers or that bind asymmetric *tetO*-like sequences (Krueger *et al.*, 2007). With this toolbox at hand, we can expect to see further *tet* systems being established in as yet untapped bacteria, as well as further refinements and enhanced functionalities in potentially any bacterial species of choice.

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Conflict of Interest

None declared.

References

Agersø, Y., and Guardabassi, L. (2005) Identification of Tet 39, a novel class of tetracycline resistance determinant in *Acinetobacter* spp. of environmental and clinical origin. *J Antimicrob Chemother* **55**: 566–569.

Armbruster, C.E., Forsyth-DeOrnellas, V., Johnson, A.O., Smith, S.N., Zhao, L., Wu, W., and Moble, H.L.T. (2017) Genome-wide transposon mutagenesis of *Proteus mirabilis*: essential genes, fitness factors for catheter-associated urinary tract infection, and the impact of polymicrobial infection on fitness requirements. *PLoS Pathog* **13**: e1006434.

Bahl, M.I., Hansen, L.H., Licht, T.R., and Sorensen, S.J. (2004) In vivo detection and quantification of tetracycline by use of a whole-cell biosensor in the rat intestine. *Antimicrob Agents Chemother* **48**: 1112–1117.

Bateman, B.T., Donegan, N.P., Jarry, T.M., Palma, M., and Cheung, A.L. (2001) Evaluation of a tetracycline-inducible promoter in *Staphylococcus aureus* *in vitro* and *in vivo* and its application in demonstrating the role of *sigB* in microcolony formation. *Infect Immun* **69**: 7851–7857.

Baumeister, R., Helbl, V., and Hillen, W. (1992) Contacts between Tet repressor and *tet* operator revealed by new recognition specificities of single amino acid replacement mutants. *J Mol Biol* **226**: 1257–1270.

Baumschlager, A., Rullan, M., and Khammash, M. (2020) Exploiting natural chemical photosensitivity of anhydrotetracycline and tetracycline for dynamic and setpoint chemo-optogenetic control. *Nat Commun* **11**: 3834.

Bayley, D.P., Rocha, E.R., and Smith, C.J. (2000) Analysis of *cepA* and other *Bacteroides fragilis* genes reveals a unique promoter structure. *FEMS Microbiol Lett* **193**: 149–154.

Beare, P.A., Gilk, S.D., Larson, C.L., Hill, J., Stead, C.M., Omsland, A., *et al.* (2011) Dot/Icm Type IVB secretion system requirements for *Coxiella burnetii* growth in human macrophages. *MBio* **2**: e00175–e1111.

Beck, M.H., Flaiz, M., Bengelsdorf, F.R., and Durre, P. (2020) Induced heterologous expression of the arginine deiminase pathway promotes growth advantages in the strict anaerobe *Acetobacterium woodii*. *Appl Microbiol Biotechnol* **104**: 687–699.

Berens, C., and Hillen, W. (2003) Gene regulation by tetracyclines. Constraints of resistance regulation in bacteria shape TetR for application in eukaryotes. *Eur J Biochem* **270**: 3109–3121.

Berens, C., and Hillen, W. (2004) Gene Regulation By Tetracyclines. In *Genetic Engineering: Principles and Methods. Genetic Engineering: Principles and Methods*, Vol. 26. Setlow, J.K. (ed.). Boston, MA: Springer. https://doi.org/10.1007/978-0-306-48573-2_13

Bertram, R., Kolb, M., and Hillen, W. (2009) In vivo activation of tetracycline repressor by *Cre/lox*-mediated gene assembly. *J Mol Microbiol Biotechnol* **17**: 136–145.

Bertram, R., Köstner, M., Müller, J., Vazquez Ramos, J., and Hillen, W. (2005) Integrative elements for *Bacillus subtilis* yielding tetracycline-dependent growth phenotypes. *Nucleic Acids Res* **33**: e153.

Bertram, R., Kraft, C., Wisshak, S., Mueller, J., Scholz, O., and Hillen, W. (2004) Phenotypes of combined tet repressor mutants for effector and operator recognition and allosteric. *J Mol Microbiol Biotechnol* **8**: 104–110.

Beste, G., Schmidt, F.S., Stibora, T., and Skerra, A. (1999) Small antibody-like proteins with prescribed ligand specificities derived from the lipocalin fold. *Proc Natl Acad Sci USA* **96**: 1898–1903.

Bina, X.R., Wong, E.A., Bina, T.F., and Bina, J.E. (2014) Construction of a tetracycline inducible expression vector and characterization of its use in *Vibrio cholerae*. *Plasmid* **76**: 87–94.

Blokpoel, M.C., Murphy, H.N., O'Toole, R., Wiles, S., Runn, E.S., Stewart, G.R., *et al.* (2005) Tetracycline-inducible gene regulation in mycobacteria. *Nucleic Acids Res* **33**: e22.

- Boldrin, F., Casonato, S., Dainese, E., Sala, C., Dhar, N., Palù, G., *et al.* (2010) Development of a repressible mycobacterial promoter system based on two transcriptional repressors. *Nucleic Acids Res* **38**: e134.
- Borg, S., Hofmann, J., Pollithy, A., Lang, C., and Schuler, D. (2014) New vectors for chromosomal integration enable high-level constitutive or inducible magnetosome expression of fusion proteins in *Magnetospirillum gryphiswaldense*. *Appl Environ Microbiol* **80**: 2609–2616.
- Breton, M., Sagne, E., Duret, S., Beven, L., Citti, C., and Renaudin, J. (2010) First report of a tetracycline-inducible gene expression system for mollicutes. *Microbiology* **156**: 198–205.
- Brodmann, M., Heilig, R., Broz, P., and Basler, M. (2018) Mobilizable plasmids for tunable gene expression in *Francisella novicida*. *Front Cell Infect Microbiol* **8**: 284.
- Bugrysheva, J.V., and Scott, J.R. (2010) The ribonucleases J1 and J2 are essential for growth and have independent roles in mRNA decay in *Streptococcus pyogenes*. *Mol Microbiol* **75**: 731–743.
- Cakar, F., Zingl, F.G., Moisi, M., Reidl, J., and Schild, S. (2018) In vivo repressed genes of *Vibrio cholerae* reveal inverse requirements of an H(+)/Cl(-) transporter along the gastrointestinal passage. *Proc Natl Acad Sci USA* **115**: E2376–E2385.
- Camacho, E.M., Mesa-Pereira, B., Medina, C., Flores, A., and Santero, E. (2016) Engineering *Salmonella* as intracellular factory for effective killing of tumour cells. *Sci Rep* **6**: 30591.
- Cameron, D.E., Bashor, C.J., and Collins, J.J. (2014) A brief history of synthetic biology. *Nat Rev Microbiol* **12**: 381–390.
- Carroll, P., Mutucumaru, D.G., and Parish, T. (2005) Use of a Tetracycline-Inducible System for Conditional Expression in *Mycobacterium tuberculosis* and *Mycobacterium smegmatis*. *Appl Environ Microbiol* **71**: 3077–3084.
- Chopra, I., and Roberts, M. (2001) Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance. *Microbiol Mol Biol Rev* **65**: 232–260.
- Christner, M., Franke, G.C., Schommer, N.N., Wendt, U., Wegert, K., Pehle, P., *et al.* (2010) The giant extracellular matrix-binding protein of *Staphylococcus epidermidis* mediates biofilm accumulation and attachment to fibronectin. *Mol Microbiol* **75**: 187–207.
- Chubiz, L.M., Purswani, J., Carroll, S.M., and Marx, C.J. (2013) A novel pair of inducible expression vectors for use in *Methylobacterium extorquens*. *BMC Res Notes* **6**: 183.
- Cohen, E.J., Quek, R.T., and Beeby, M. (2019) A *tetRA*-based promoter system for the generation of conditional knockouts in *Campylobacter jejuni*. *bioRxiv*, <https://doi.org/10.1101/616649>
- Corrigan, R.M., and Foster, T.J. (2009) An improved tetracycline-inducible expression vector for *Staphylococcus aureus*. *Plasmid* **61**: 126–129.
- Cortes, M., Singh, A.K., Reytrat, J.M., Gaillard, J.L., Nassif, X., and Herrmann, J.L. (2011) Conditional gene expression in *Mycobacterium abscessus*. *PLoS One* **6**: e29306.
- Das, A.T., Tenenbaum, L., and Berkhout, B. (2016) Tet-on systems for doxycycline-inducible gene expression. *Curr Gene Ther* **16**: 156–167.
- de la Torre, J.C., Ortín, J., Domingo, E., Delamarter, J., Allet, B., Davies, J., *et al.* (1984) Plasmid vectors based on Tn10 DNA: gene expression regulated by tetracycline. *Plasmid* **12**: 103–110.
- Debowski, A.W., Sehna, M., Liao, T., Stubbs, K.A., Marshall, B.J., and Benghezal, M. (2015) Expansion of the tetracycline-dependent regulation toolbox for *Helicobacter pylori*. *Appl Environ Microbiol* **81**: 7969–7980.
- Debowski, A.W., Verbrugghe, P., Sehna, M., Marshall, B.J., and Benghezal, M. (2013) Development of a tetracycline-inducible gene expression system for the study of *Helicobacter pylori* pathogenesis. *Appl Environ Microbiol* **79**: 7351–7359.
- Debowski, A.W., Walton, S.M., Chua, E.-G., Tay, A.-Y., Liao, T., Lamichhane, B., *et al.* (2017) *Helicobacter pylori* gene silencing in vivo demonstrates urease is essential for chronic infection. *PLoS Pathog* **13**: e1006464.
- Degenkolb, J., Takahashi, M., Ellestad, G.A., and Hillen, W. (1991) Structural requirements of tetracycline-Tet repressor interaction: determination of equilibrium binding constants for tetracycline analogs with the Tet repressor. *Antimicrob Agents Chemother* **35**: 1591–1595.
- Deuschle, U., Meyer, W.K., and Thiesen, H.J. (1995) Tetracycline-reversible silencing of eukaryotic promoters. *Mol Cell Biol* **15**: 1907–1914.
- Dong, H., Tao, W., Zhang, Y., and Li, Y. (2012) Development of an anhydrotetracycline-inducible gene expression system for solvent-producing *Clostridium acetobutylicum*: a useful tool for strain engineering. *Metab Eng* **14**: 59–67.
- Ehrt, S., Guo, X.V., Hickey, C.M., Ryou, M., Monteleone, M., Riley, L.W., and Schnappinger, D. (2005) Controlling gene expression in mycobacteria with anhydrotetracycline and Tet repressor. *Nucleic Acids Res* **33**: e21.
- Evans, J.C., and Mizrahi, V. (2015) The application of tetracycline-regulated gene expression systems in the validation of novel drug targets in *Mycobacterium tuberculosis*. *Front Microbiol* **6**: 812.
- Fagan, R.P., and Fairweather, N.F. (2011) *Clostridium difficile* has two parallel and essential Sec secretion systems. *J Biol Chem* **286**: 27483–27493.
- Gardner, T.S., Cantor, C.R., and Collins, J.J. (2000) Construction of a genetic toggle switch in *Escherichia coli*. *Nature* **403**: 339–342.
- Gauger, T., Weihs, F., Mayer, S., Krismer, B., Liese, J., Kull, M., and Bertram, R. (2012) Intracellular monitoring of target protein production in *Staphylococcus aureus* by peptide tag-induced reporter fluorescence. *Microb Biotechnol* **5**: 129–134.
- Gauttam, R., Desiderato, C., Jung, L., Shah, A., and Eikmanns, B.J. (2019) A step forward: compatible and dual-inducible expression vectors for gene co-expression in *Corynebacterium glutamicum*. *Plasmid* **101**: 20–27.
- Gauttam, R., Mukhopadhyay, A., and Singer, S.W. (2020) Construction of a novel dual-inducible duet-expression system for gene (over)expression in *Pseudomonas putida*. *Plasmid* **110**: 102514.
- Geissendörfer, M., and Hillen, W. (1990) Regulated expression of heterologous genes in *Bacillus subtilis* using the Tn10 encoded tet regulatory elements. *Appl Microbiol Biotechnol* **33**: 657–663.

- Georgi, C., Buerger, J., Hillen, W., and Berens, C. (2012) Promoter strength driving TetR determines the regulatory properties of Tet-controlled expression systems. *PLoS One* **7**: e41620.
- Gibson, D.G., Glass, J.I., Lartigue, C., Noskov, V.N., Chuang, R.-Y., Algire, M.A., *et al.* (2010) Creation of a bacterial cell controlled by a chemically synthesized genome. *Science* **329**: 52–56.
- Giese, B., Dittmann, S., Paprotka, K., Levin, K., Weltrowski, A., Biehler, D., *et al.* (2009) Staphylococcal alpha-toxin is not sufficient to mediate escape from phagolysosomes in upper-airway epithelial cells. *Infect Immun* **77**: 3611–3625.
- Goeke, D., Kaspar, D., Stoeckle, C., Grubmuller, S., Berens, C., Klotzsche, M., and Hillen, W. (2012) Short peptides act as inducers, anti-inducers and corepressors of Tet repressor. *J Mol Biol* **416**: 33–45.
- Gossen, M., and Bujard, H. (1992) Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. *Proc Natl Acad Sci USA* **89**: 5547–5551.
- Grau, F.C., Jaeger, J., Groher, F., Suess, B., and Muller, Y.A. (2020) The complex formed between a synthetic RNA aptamer and the transcription repressor TetR is a structural and functional twin of the operator DNA-TetR regulator complex. *Nucleic Acids Res* **48**: 3366–3378.
- Guo, X.V., Monteleone, M., Klotzsche, M., Kamionka, A., Hillen, W., Braunstein, M., *et al.* (2007) Silencing *Mycobacterium smegmatis* by using tetracycline repressors. *J Bacteriol* **189**: 4614–4623.
- Guss, A.M., Rother, M., Zhang, J.K., Kulkarni, G., and Metcalf, W.W. (2008) New methods for tightly regulated gene expression and highly efficient chromosomal integration of cloned genes for *Methanosarcina* species. *Archaea* **2**: 193–203.
- Hansen, L.H., Ferrari, B., Sorensen, A.H., Veal, D., and Sorensen, S.J. (2001) Detection of oxytetracycline production by *Streptomyces rimosus* in soil microcosms by combining whole-cell biosensors and flow cytometry. *Appl Environ Microbiol* **67**: 239–244.
- Hansen, L.M., McMurry, L.M., Levy, S.B., and Hirsh, D.C. (1993) A new tetracycline resistance determinant, Tet H, from *Pasteurella multocida* specifying active efflux of tetracycline. *Antimicrob Agents Chemother* **37**: 2699–2705.
- Hansmeier, N., Miskiewicz, K., Elpers, L., Liss, V., Hensel, M., and Sterzenbach, T. (2017) Functional expression of the entire adhesiome of *Salmonella enterica* serotype Typhimurium. *Sci Rep* **7**: 10326.
- Helbl, V., and Hillen, W. (1998) Stepwise selection of TetR variants recognizing *tet* operator 4C with high affinity and specificity. *J Mol Biol* **276**: 313–318.
- Helbl, V., Tiebel, B., and Hillen, W. (1998) Stepwise selection of TetR variants recognizing *tet* operator 6C with high affinity and specificity. *J Mol Biol* **276**: 319–324.
- Helle, L., Kull, M., Mayer, S., Marincola, G., Zelder, M.-E., Goerke, C., *et al.* (2011) Vectors for improved Tet repressor-dependent gradual gene induction or silencing in *Staphylococcus aureus*. *Microbiology* **157**: 3314–3323.
- Hensel, Z. (2017) A plasmid-based *Escherichia coli* gene expression system with cell-to-cell variation below the extrinsic noise limit. *PLoS One* **12**: e0187259.
- Henssler, E.M., Bertram, R., Wisshak, S., and Hillen, W. (2005) Tet repressor mutants with altered effector binding and allostery. *FEBS J* **272**: 4487–4496.
- Henssler, E.M., Scholz, O., Lochner, S., Gmeiner, P., and Hillen, W. (2004) Structure-based design of Tet repressor to optimize a new inducer specificity. *Biochemistry* **43**: 9512–9518.
- Heravi, K.M., Watzlawick, H., and Altenbuchner, J. (2015) Development of an anhydrotetracycline-inducible expression system for expression of a neopullulanase in *B. subtilis*. *Plasmid* **82**: 35–42.
- Hernandez-Abanto, S.M., Woolwine, S.C., Jain, S.K., and Bishai, W.R. (2006) Tetracycline-inducible gene expression in mycobacteria within an animal host using modified *Streptomyces tcp830* regulatory elements. *Arch Microbiol* **186**: 459–464.
- Higo, A., Isu, A., Fukaya, Y., and Hisabori, T. (2016) Efficient gene induction and endogenous gene repression systems for the filamentous cyanobacterium *Anabaena* sp. PCC 7120. *Plant Cell Physiol* **57**: 387–396.
- Higo, A., Isu, A., Fukaya, Y., and Hisabori, T. (2017) Designing synthetic flexible gene regulation networks using RNA devices in cyanobacteria. *ACS Synth Biol* **6**: 55–61.
- Hillen, W., and Berens, C. (1994) Mechanisms underlying expression of Tn10 encoded tetracycline resistance. *Annu Rev Microbiol* **48**: 345–369.
- Hillen, W., and Schollmeier, K. (1983) Nucleotide sequence of the Tn10 encoded tetracycline resistance gene. *Nucleic Acids Res* **11**: 525–539.
- Hinrichs, W., Kisker, C., Düvel, M., Müller, A., Tovar, K., Hillen, W., and Saenger, W. (1994) Structure of the Tet repressor-tetracycline complex and regulation of antibiotic resistance. *Science* **264**: 418–420.
- Hong, H., and Park, W. (2014) TetR repressor-based bioreporters for the detection of doxycycline using *Escherichia coli* and *Acinetobacter oleivorans*. *Appl Microbiol Biotechnol* **98**: 5039–5050.
- Hsiao, A., Liu, Z., Joelsson, A., and Zhu, J. (2006) *Vibrio cholerae* virulence regulator-coordinated evasion of host immunity. *Proc Natl Acad Sci USA* **103**: 14542–14547.
- Hu, S., Fu, J., Huang, F., Ding, X., Stewart, A.F., Xia, L., and Zhang, Y. (2014) Genome engineering of *Agrobacterium tumefaciens* using the lambda Red recombination system. *Appl Microbiol Biotechnol* **98**: 2165–2172.
- Huang, H.H., and Lindblad, P. (2013) Wide-dynamic-range promoters engineered for cyanobacteria. *J Biol Eng* **7**: 10.
- Hunsicker, A., Steber, M., Mayer, G., Meitert, J., Klotzsche, M., Blind, M., *et al.* (2009) An RNA aptamer that induces transcription. *Chem Biol* **16**: 173–180.
- Ji, Y., Marra, A., Rosenberg, M., and Woodnutt, G. (1999) Regulated antisense RNA eliminates alpha-toxin virulence in *Staphylococcus aureus* infection. *J Bacteriol* **181**: 6585–6590.
- Ji, Y., Yin, D., Fox, B., Holmes, D.J., Payne, D., and Rosenberg, M. (2004) Validation of antibacterial mechanism of action using regulated antisense RNA expression in *Staphylococcus aureus*. *FEMS Microbiol Lett* **231**: 177–184.
- Ji, Y., Zhang, B., Van Horn, S.F., Warren, P., Woodnutt, G., *et al.* (2001) Identification of critical staphylococcal genes using conditional phenotypes generated by antisense RNA. *Science* **293**: 2266–2269.

- Kamionka, A., Bertram, R., and Hillen, W. (2005) Tetracycline-dependent conditional gene Knockout in *Bacillus subtilis*. *Appl Environ Microbiol* **71**: 728–733.
- Kamionka, A., Bogdanska-Urbaniak, J., Scholz, O., and Hillen, W. (2004a) Two mutations in the tetracycline repressor change the inducer anhydrotetracycline to a corepressor. *Nucleic Acids Res* **32**: 842–847.
- Kamionka, A., Majewski, M., Roth, K., Bertram, R., Kraft, C., and Hillen, W. (2006) Induction of single chain tetracycline repressor requires the binding of two inducers. *Nucleic Acids Res* **34**: 3834–3841.
- Kamionka, A., Sehnal, M., Scholz, O., and Hillen, W. (2004b) Independent regulation of two genes in *Escherichia coli* by tetracyclines and Tet repressor variants. *J Bacteriol* **186**: 4399–4401.
- Kendra, C.G., Keller, C.M., Bruna, R.E., and Pontes, M.H. (2020) Conjugal DNA transfer in *sodalis glossinidius*, a maternally inherited symbiont of tsetse flies. *mSphere* **5**: e00864-20. <https://doi.org/10.1128/mSphere.00864-20>
- Kim, J.-H., O'Brien, K.M., Sharma, R., Boshoff, H.I.M., Rehren, G., Chakraborty, S., *et al.* (2013) A genetic strategy to identify targets for the development of drugs that prevent bacterial persistence. *Proc Natl Acad Sci USA* **110**: 19095–19100.
- Kim, J.H., Wei, J.R., Wallach, J.B., Robbins, R.S., Rubin, E.J., and Schnappinger, D. (2011) Protein inactivation in mycobacteria by controlled proteolysis and its application to deplete the beta subunit of RNA polymerase. *Nucleic Acids Res* **39**: 2210–2220.
- Kleinschmidt, C., Tovar, K., Hillen, W., and Porschke, D. (1988) Dynamics of repressor-operator recognition: the Tn10-encoded tetracycline resistance control. *Biochemistry* **27**: 1094–1104.
- Klieber, M.A., Scholz, O., Lochner, S., Gmeiner, P., Hillen, W., and Muller, Y.A. (2009) Structural origins for selectivity and specificity in an engineered bacterial repressor-inducer pair. *FEBS J* **276**: 5610–5621.
- Klotzsche, M., Berens, C., and Hillen, W. (2005) A peptide triggers allostery in tet repressor by binding to a unique site. *J Biol Chem* **280**: 24591–24599.
- Klotzsche, M., Ehrh, S., and Schnappinger, D. (2009) Improved tetracycline repressors for gene silencing in mycobacteria. *Nucleic Acids Res* **37**: 1778–1788.
- Klotzsche, M., Goeke, D., Berens, C., and Hillen, W. (2007) Efficient and exclusive induction of Tet repressor by the oligopeptide Tip results from co-variation of their interaction site. *Nucleic Acids Res* **35**: 3945–3952.
- Köstner, M., Schmidt, B., Bertram, R., and Hillen, W. (2006) Generating tetracycline-inducible auxotrophy in *Escherichia coli* and *Salmonella enterica* serovar Typhimurium by using an insertion element and a hyperactive transposase. *Appl Environ Microbiol* **72**: 4717–4725.
- Krueger, M., Scholz, O., Wisshak, S., and Hillen, W. (2007) Engineered Tet repressors with recognition specificity for the *tetO*-4C5G operator variant. *Gene* **404**: 93–100.
- Kurittu, J., Karp, M., and Korpela, M. (2000) Detection of tetracyclines with luminescent bacterial strains. *Luminescence* **15**: 291–297.
- Lartigue, M.F., and Boulouc, P. (2014) A tetracycline-inducible expression vector for *Streptococcus agalactiae* allowing controllable gene expression. *J Microbiol Methods* **96**: 16–18.
- Lathem, W.W., Price, P.A., Miller, V.L., and Goldman, W.E. (2007) A plasminogen-activating protease specifically controls the development of primary pneumonic plague. *Science* **315**: 509–513.
- Lausberg, F., Chattopadhyay, A.R., Heyer, A., Eggeling, L., and Freudl, R. (2012) A tetracycline inducible expression vector for *Corynebacterium glutamicum* allowing tightly regulable gene expression. *Plasmid* **68**: 142–147.
- Lee, J.W., Gyorgy, A., Cameron, D.E., Pyenson, N., Choi, K.R., Way, J.C., *et al.* (2016) Creating single-copy genetic circuits. *Mol Cell* **63**: 329–336.
- Letunic, I., and Bork, P. (2021) Interactive Tree Of Life (iTOL) v5: an online tool for phylogenetic tree display and annotation. *Nucleic Acids Res* **49**: W293–W296.
- Li, H., and Liao, J.C. (2015) A synthetic anhydrotetracycline-controllable gene expression system in *Ralstonia eutropha* H16. *ACS Synth Biol* **4**: 101–106.
- Lim, B., Zimmermann, M., Barry, N.A., and Goodman, A.L. (2017) Engineered regulatory systems modulate gene expression of human commensals in the gut. *Cell* **169**: 547–558.e15.
- Liu, F., and Myers, A.G. (2016) Development of a platform for the discovery and practical synthesis of new tetracycline antibiotics. *Curr Opin Chem Biol* **32**: 48–57.
- LoVullo, E.D., Miller, C.N., Pavelka, M.S. Jr, and Kawula, T.H. (2012) TetR-based gene regulation systems for *Francisella tularensis*. *Appl Environ Microbiol* **78**: 6883–6889.
- Luckner, S.R., Klotzsche, M., Berens, C., Hillen, W., and Muller, Y.A. (2007) How an agonist peptide mimics the antibiotic tetracycline to induce Tet-repressor. *J Mol Biol* **368**: 780–790.
- Lutz, R., and Bujard, H. (1997) Independent and tight regulation of transcriptional units in *Escherichia coli* via the LacR/O, the TetR/O and AraC/11-12 regulatory elements. *Nucleic Acids Res* **25**: 1203–1210.
- Mariscal, A.M., Gonzalez-Gonzalez, L., Querol, E., and Pinol, J. (2016) All-in-one construct for genome engineering using Cre-lox technology. *DNA Res* **23**: 263–270.
- Mariscal, A.M., Kakizawa, S., Hsu, J.Y., Tanaka, K., González-González, L., Broto, A., *et al.* (2018) Tuning gene activity by inducible and targeted regulation of gene expression in minimal bacterial cells. *ACS Synth Biol* **7**: 1538–1552.
- Mastroiolo, M.D., Thorson, M.L., and Stevens, A.M. (2009) Comparison of *Bacteroides thetaiotaomicron* and *Escherichia coli* 16S rRNA gene expression signals. *Microbiology (Reading)* **155**: 2683–2693.
- McClain, M.S., Duncan, S.S., Gaddy, J.A., and Cover, T.L. (2013) Control of gene expression in *Helicobacter pylori* using the Tet repressor. *J Microbiol Methods* **95**: 336–341.
- McWhinnie, R.L., and Nano, F.E. (2014) Synthetic promoters functional in *Francisella novicida* and *Escherichia coli*. *Appl Environ Microbiol* **80**: 226–234.
- Meiers, M., Laux, A., Eichinger, D., Sexauer, A., Marx, P., Bertram, R., and Brückner, R. (2017) A tetracycline inducible integrative expression system for *Streptococcus pneumoniae*. *FEMS Microbiol Lett*.
- Meitert, J., Aram, R., Wiesemann, K., Weigand, J.E., and Suess, B. (2013) Monitoring the expression level of

- coding and non-coding RNAs using a TetR inducing aptamer tag. *Bioorg Med Chem* **21**: 6233–6238.
- Meyer, A.J., Segall-Shapiro, T.H., Glassey, E., Zhang, J., and Voigt, C.A. (2019) *Escherichia coli* "Marionette" strains with 12 highly optimized small-molecule sensors. *Nat Chem Biol* **15**: 196–204.
- Nyerges, Á., Csörgő, B., Nagy, I., Bálint, B., Bihari, P., Lázár, V., *et al.* (2016) A highly precise and portable genome engineering method allows comparison of mutational effects across bacterial species. *Proc Natl Acad Sci USA* **113**: 2502–2507.
- Obrist, M.W., and Miller, V.L. (2012) Low copy expression vectors for use in *Yersinia* sp. and related organisms. *Plasmid* **68**: 33–42.
- Possoz, C., Filipe, S.R., Grainge, I., and Sherratt, D.J. (2006) Tracking of controlled *Escherichia coli* replication fork stalling and restart at repressor-bound DNA in vivo. *EMBO J* **25**: 2596–2604.
- Qian, F., and Pan, W. (2002) Construction of a *tetR*-integrated *Salmonella enterica* serovar Typhi CVD908 strain that tightly controls expression of the major merozoite surface protein of *Plasmodium falciparum* for applications in human Vaccine production. *Infect Immun* **70**: 2029–2038.
- Radmacher, E., Stansen, K.C., Besra, G.S., Alderwick, L.J., Maughan, W.N., Hollweg, G., *et al.* (2005) Ethambutol, a cell wall inhibitor of *Mycobacterium tuberculosis*, elicits L-glutamate efflux of *Corynebacterium glutamicum*. *Microbiology* **151**: 1359–1368.
- Ramos, J.L., Martínez-Bueno, M., Molina-Henares, A.J., Terán, W., Watanabe, K., Zhang, X., *et al.* (2005) The TetR family of transcriptional repressors. *Microbiol Mol Biol Rev* **69**: 326–356.
- Rappleye, C.A., and Roth, J.R. (1997) A Tn10 derivative (T-POP) for isolation of insertions with conditional (tetracycline-dependent) phenotypes. *J Bacteriol* **179**: 5827–5834.
- Resch, M., Striegl, H., Henssler, E.M., Sevana, M., Egerer-Sieber, C., Schiltz, E., *et al.* (2008) A protein functional leap: how a single mutation reverses the function of the transcription regulator TetR. *Nucleic Acids Res* **36**: 4390–4401.
- Rodriguez-Garcia, A., Combes, P., Perez-Redondo, R., and Smith, M.C. (2005) Natural and synthetic tetracycline-inducible promoters for use in the antibiotic-producing bacteria *Streptomyces*. *Nucleic Acids Res* **33**: e87.
- Schiweck, W., Buxbaum, B., Schatzlein, C., Neiss, H.G., and Skerra, A. (1997) Sequence analysis and bacterial production of the anti-c-myc antibody 9E10: the V(H) domain has an extended CDR-H3 and exhibits unusual solubility. *FEBS Lett* **414**: 33–38.
- Schleimer, N., Kaspar, U., Ballhausen, B., Fotiadis, S.A., Streu, J.M., Kriegeskorte, A., *et al.* (2019) Adaption of an episomal antisense silencing approach for investigation of the phenotype switch of *Staphylococcus aureus* small-colony variants. *Front Microbiol* **10**: 2044.
- Schlicht, M., Berens, C., Daam, J., and Hillen, W. (2006) Random insertion of a TetR-inducing peptide tag into *Escherichia coli* proteins allows analysis of protein levels by induction of reporter gene expression. *Appl Environ Microbiol* **72**: 5637–5642.
- Schmitter, S., Fieseler, L., Klumpp, J., Bertram, R., and Loessner, M.J. (2017) TetR-dependent gene regulation in intracellular *Listeria monocytogenes* demonstrates the spatiotemporal surface distribution of ActA. *Mol Microbiol* **105**: 413–425.
- Schnappinger, D., and Ehrh, S. (2014) Regulated expression systems for mycobacteria and their applications. *Microbiol Spectr* **2**: MGM2-0018-2013. https://doi.org/10.1128/microbiolspec.MGM2-0018-2013_3
- Schnappinger, D., Schubert, P., Pfeleiderer, K., and Hillen, W. (1998) Determinants of protein-protein recognition by four helix bundles: changing the dimerization specificity of Tet repressor. *Embo J* **17**: 535–543.
- Scholz, O., Henssler, E.M., Bail, J., Schubert, P., Bogdanska-Urbaniak, J., Sopp, S., *et al.* (2004) Activity reversal of Tet repressor caused by single amino acid exchanges. *Mol Microbiol* **53**: 777–789.
- Schulte, M., Sterzenbach, T., Miskiewicz, K., Elpers, L., Hensel, M., and Hansmeier, N. (2019) A versatile remote control system for functional expression of bacterial virulence genes based on the *tetA* promoter. *Int J Med Microbiol* **309**: 54–65.
- Skerra, A. (1994) Use of the tetracycline promoter for the tightly regulated production of a murine antibody fragment in *Escherichia coli*. *Gene* **151**: 131–135.
- Sorg, R.A., Gally, C., Van Maele, L., Sirard, J.C., and Veening, J.W. (2020) Synthetic gene-regulatory networks in the opportunistic human pathogen *Streptococcus pneumoniae*. *Proc Natl Acad Sci USA* **117**: 27608–27619.
- Sprengel, R., and Hasan, M.T. (2007) Tetracycline-controlled genetic switches. *Handb Exp Pharmacol* **178**: 49–72. https://doi.org/10.1007/978-3-540-35109-2_3
- Starr, T., Child, R., Wehrly, T.D., Hansen, B., Hwang, S., López-Otin, C., *et al.* (2012) Selective subversion of autophagy complexes facilitates completion of the *Bruceella* intracellular cycle. *Cell Host Microbe* **11**: 33–45.
- Stary, E., Gaupp, R., Lechner, S., Leibig, M., Tichy, E., Kolb, M., and Bertram, R. (2010) New architectures for Tet-on and Tet-off regulation in *Staphylococcus aureus*. *Appl Environ Microbiol* **76**: 680–687.
- Stiebritz, M.T., Wengrzik, S., Klein, D.L., Richter, J.P., Srebrzynski, A., Weiler, S., and Muller, Y.A. (2010) Computational design of a chain-specific tetracycline repressor heterodimer. *J Mol Biol* **403**: 371–385.
- Stieger, M., Wohlgensinger, B., Kamber, M., Lutz, R., and Keck, W. (1999) Integrational plasmids for the tetracycline-regulated expression of genes in *Streptococcus pneumoniae*. *Gene* **226**: 243–251.
- Takiff, H.E., Baker, T., Copeland, T., Chen, S.M., and Court, D.L. (1992) Locating essential *Escherichia coli* genes by using mini-Tn10 transposons: the *pdxJ* operon. *J Bacteriol* **174**: 1544–1553.
- Temme, K., Zhao, D., and Voigt, C.A. (2012) Refactoring the nitrogen fixation gene cluster from *Klebsiella oxytoca*. *Proc Natl Acad Sci USA* **109**: 7085–7090.
- Terpe, K. (2006) Overview of bacterial expression systems for heterologous protein production: from molecular and biochemical fundamentals to commercial systems. *Appl Microbiol Biotechnol* **72**: 211–222.
- Thaker, M., Spanogiannopoulos, P., and Wright, G.D. (2010) The tetracycline resistome. *Cell Mol Life Sci* **67**: 419–431.

- Thompson, S.A., Maani, E.V., Lindell, A.H., King, C.J., and McArthur, J.V. (2007) Novel tetracycline resistance determinant isolated from an environmental strain of *Serratia marcescens*. *Appl Environ Microbiol* **73**: 2199–2206.
- Topp, S., Reynoso, C.M.K., Seeliger, J.C., Goldlust, I.S., Desai, S.K., Murat, D., *et al.* (2010) Synthetic riboswitches that induce gene expression in diverse bacterial species. *Appl Environ Microbiol* **76**: 7881–7884.
- Ueki, T., Nevin, K.P., Woodard, T.L., and Lovley, D.R. (2016) Genetic switches and related tools for controlling gene expression and electrical outputs of *Geobacter sulfurreducens*. *J Ind Microbiol Biotechnol* **43**: 1561–1575.
- Unger, B., Klock, G., Hillen, W., and Wells, R.D. (1984) Nucleotide sequence of the repressor gene of the RA1 tetracycline resistance determinant: structural and functional comparison with three related Tet repressor genes. *Nucleic Acids Res* **12**: 7693–7703.
- Volzing, K., Biliouris, K., and Kaznessis, Y.N. (2011) proTeOn and proTeOff, new protein devices that inducibly activate bacterial gene expression. *ACS Chem Biol* **6**: 1107–1116.
- Voskuil, M.I., Voepel, K., and Chambliss, G.H. (1995) The -16 region, a vital sequence for the utilization of a promoter in *Bacillus subtilis* and *Escherichia coli*. *Mol Microbiol* **17**: 271–279.
- Wagley, S., Hemsley, C., Thomas, R., Moule, M.G., Vanaporn, M., Andreae, C., *et al.* (2014) The twin arginine translocation system is essential for aerobic growth and full virulence of *Burkholderia thailandensis*. *J Bacteriol* **196**: 407–416.
- Wang, B., and Kuramitsu, H.K. (2005) Inducible antisense RNA expression in the characterization of gene functions in *Streptococcus mutans*. *Infect Immun* **73**: 3568–3576.
- Way, J.C., Davis, M.A., Morisato, D., Roberts, D.E., and Kleckner, N. (1984) New Tn10 derivatives for transposon mutagenesis and for construction of *lacZ* operon fusions by transposition. *Gene* **32**: 369–379.
- Whetstone, C.R., Slusser, J.G., and Zuckert, W.R. (2009) Development of a single-plasmid-based regulatable gene expression system for *Borrelia burgdorferi*. *Appl Environ Microbiol* **75**: 6553–6558.
- Wickstrum, J., Sammons, L.R., Restivo, K.N., and Hefty, P.S. (2013) Conditional gene expression in *Chlamydia trachomatis* using the tet system. *PLoS One* **8**: e76743.
- Williams, K.J., Joyce, G., and Robertson, B.D. (2010) Improved mycobacterial tetracycline inducible vectors. *Plasmid* **64**: 69–73.
- Wilson, C.J., Zhan, H., Swint-Kruse, L., and Matthews, K.S. (2007) The lactose repressor system: paradigms for regulation, allosteric behavior and protein folding. *Cell Mol Life Sci* **64**: 3–16.
- Woo, P.C., Ma, S.S., Teng, J.L., Li, M.W., Kao, R.Y., Lau, S.K., and Yuen, K.Y. (2005) Construction of an inducible expression shuttle vector for *Laribacter hongkongensis*, a novel bacterium associated with gastroenteritis. *FEMS Microbiol Lett* **252**: 57–65.
- Xu, H.H., Trawick, J.D., Haselbeck, R.J., Forsyth, R.A., Yamamoto, R.T., Archer, R., *et al.* (2010) *Staphylococcus aureus* TargetArray: comprehensive differential essential gene expression as a mechanistic tool to profile antibacterials. *Antimicrob Agents Chemother* **54**: 3659–3670.
- Xu, T., Wu, Y., Lin, Z., Bertram, R., Götz, F., Zhang, Y., and Qu, D. (2017) Identification of genes controlled by the essential YycFG two-component system reveals a role for biofilm modulation in *Staphylococcus epidermidis*. *Front Microbiol* **8**: 724.
- Yan, M., Yu, C., Yang, J., and Ji, Y. (2011) The essential two-component system YhcSR is involved in regulation of the nitrate respiratory pathway of *Staphylococcus aureus*. *J Bacteriol* **193**: 1799–1805.
- Yin, J., Zhu, H., Xia, L., Ding, X., Hoffmann, T., Hoffmann, M., *et al.* (2015) A new recombineering system for *Photobacterium* and *Xenorhabdus*. *Nucleic Acids Res* **43**: e36.
- Yoshino, T., Shimojo, A., Maeda, Y., and Matsunaga, T. (2010) Inducible expression of transmembrane proteins on bacterial magnetic particles in *Magnetospirillum magneticum* AMB-1. *Appl Environ Microbiol* **76**: 1152–1157.
- Zeng, Y., Jones, A.M., Thomas, E.E., Nassif, B., Silberg, J.J., and Segatori, L. (2018) A split transcriptional repressor that links protein solubility to an orthogonal genetic circuit. *ACS Synth Biol* **7**: 2126–2138.
- Zess, E.K., Begemann, M.B., and Pfleger, B.F. (2016) Construction of new synthetic biology tools for the control of gene expression in the cyanobacterium *Synechococcus* sp. strain PCC 7002. *Biotechnol Bioeng* **113**: 424–432.