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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 Tn*10*, *tetR* regulates the expression of a divergently oriented *tetA* gene that encodes a Tc antiporter. These components of Tn*10* 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 Tn*10* 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 α1 to α3) harbouring a helix–turn–helix motif for binding to *tetO* followed by a protein core (α4 to α10) required for dimerization and inducer binding. The most intensively investigated Tet repressors originate from the Tc-resistant determinants *tet*(B) of Tn*10* (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 α8 and α9 (Bertram *et al*., 2009).

Natively, TetR binds $[Tc\text{-}Mg]^+$ 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

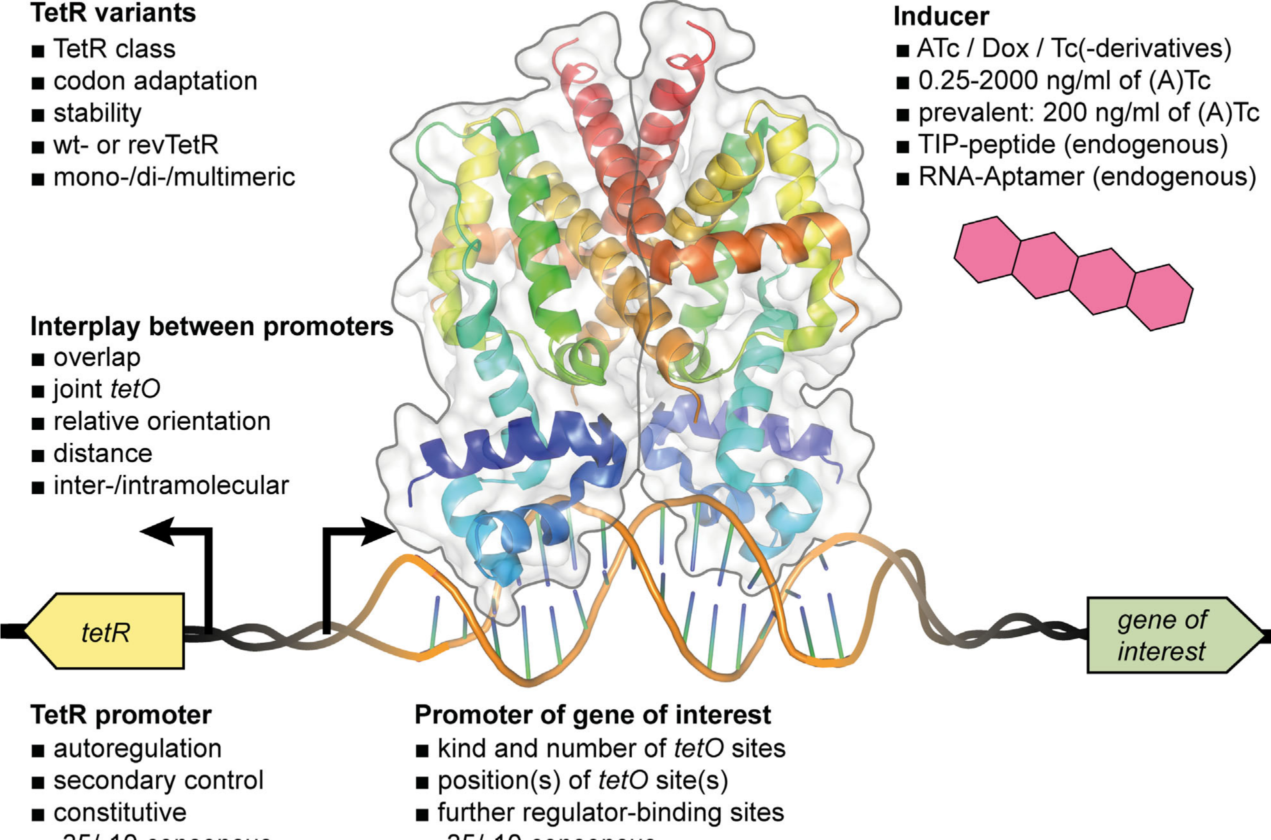

Fig. 1. Variables of bacterial *tet* systems.
Key parameters and variables affecting the outcome and efficiency of *tet regulation*. TetR is shown in the DNA bound form. Bent arrows denote promoters, and double helical part of schematized DNA represents *tetO*.

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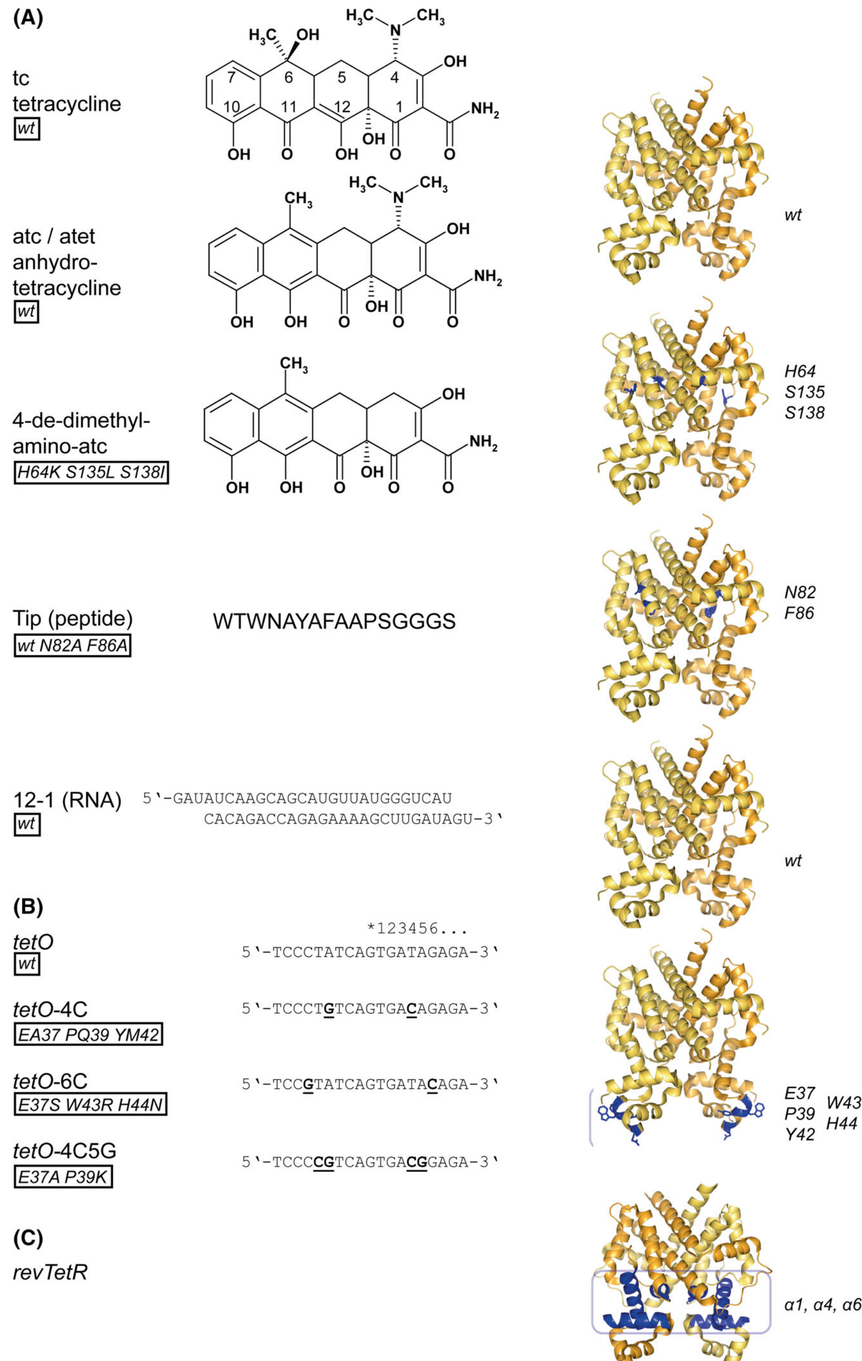

Fig. 2. Different inducers and operators and suitable Tet repressors.
A. Left side: Shown are selected tetracyclines, and the sequences of the Tip peptide and the 12-1 RNA. The boxes indicate which TetR variants are (best) inducible by these compounds. TetR H64K S135L S138 is designated TetR i2. Note 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 α1, α4 and α6 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

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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 μM) 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 α3 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 α1, α4 or α6 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).

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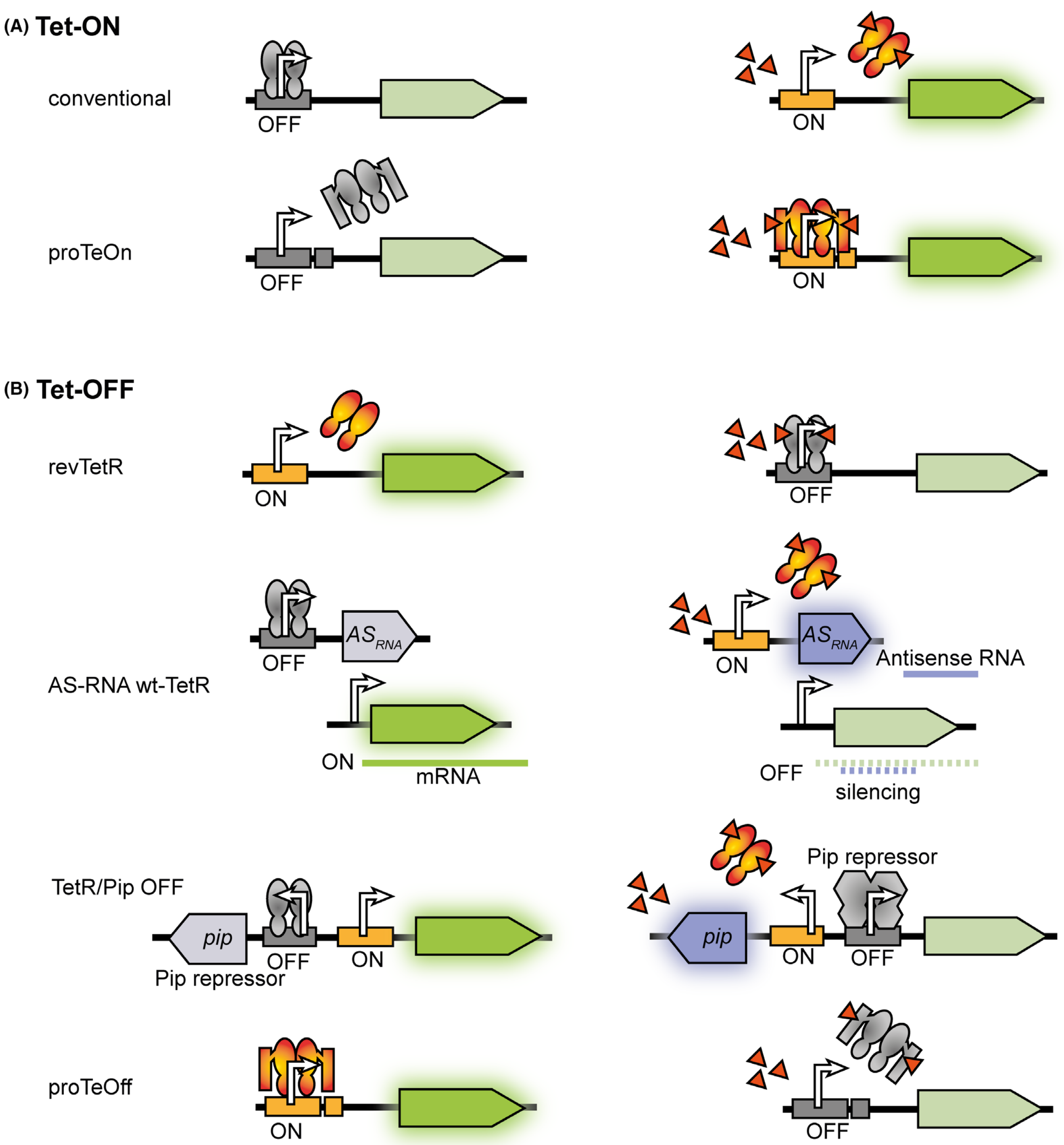

Fig. 3. Types of Tet-ON and Tet-OFF control.
A. upper panel: conventional Tet-ON control with wt-TetR; lower panel: Control by proTeOn (Volzing *et al.*, 2011).
B. 1st panel: Tet-OFF control by revTetR; 2nd panel: Expression of AS-RNA by wt-TetR; 3rd panel: The TetR/Pip OFF system (Boldrin *et al.*, 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 ProTe-Off). 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 Tn*10* (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 Tn*5*-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 pursuits 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 Tn*10 tet *sequence*

In Tn*10*, 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 Tn*10* 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 Tn*10* derivative termed T-POP (Rappleye and Roth, 1997). Recently, Tn*10 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 burnetiid,* the type IVB secretion system (T4BSS) was controlled by Tn*10 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 *Photorhabdus luminescens* and *Xenorhabdus stockiae*. To analyse the transcriptional activity of *Brucella abortus* in endosomal vacuoles of macrophages, the *tetR-tetA* sequence of Tn*10* 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 *tet regulation*
Acetobacterium woodii	Firmicutes	Beck *et al.* (2020)
Acinetobacter oleivorans	Proteobacteria (gamma)	Hong and Park (2014)
Agrobacterium tumefaciens	Proteobacteria (alpha)	Hu *et al.* (2014)
Anabaena spec.	Cyanobacteria	Higo *et al.* (2016)
Bacillus subtilis	Firmicutes	Geissendörfer and Hillen (1990)
Bacteroides thetaiotaomicron, B. fragilis, B. ovatus, B. uniformis, B. xylanisolvens, B. intestinalis, B. dorei, B. vulgatus, B. cellulosilyticus, B. eggerthii	Bacteroidetes	Lim *et al.* (2007)
Borrelia burgdorferi	Spirochaetes	Whetstine *et al.* (2009)
Brucella abortus	Proteobacteria (alpha)	Starr *et al.* (2012)
Burkholderia thailandensis	Proteobacteria (beta)	Wagley *et al.* (2014)
Campylobacter jejuni	Proteobacteria (epsilon)	Cohen *et al.* (2019)
Chlamydia trachomatis	Chlamydiae	Wickstrum *et al.* (2013)
Citrobacter freundii	Proteobacteria (gamma)	Nyerges *et al.* (2016)
Clostridium acetobutylicum, C. difficile	Firmicutes	Fagan and Fairweather (2011), Dong *et al.* (2012)
Corynebacterium glutamicum	Actinobacteria	Radmacher *et al.* (2005)
Coxiella burnetii	Proteobacteria (gamma)	Beare *et al.* (2011)
Edwardsiella tarda	Proteobacteria (gamma)	Nyerges *et al.* (2016)
Escherichia coli, E. hermannii	Proteobacteria (gamma)	de la Torre *et al.* (1984), Nyerges *et al.* (2016)
Francisella novicida, F. tularensis	Proteobacteria (gamma)	LoVullo *et al.* (2012), Brodmann *et al.* (2018)
Geobacter sulfurreducens	Proteobacteria (delta)	Ueki *et al.* (2016)
Helicobacter pylori	Proteobacteria (epsilon)	Debowski *et al.* (2013), McClain *et al.* (2013)
Klebsiella oxytoca	Proteobacteria (gamma)	Temme *et al.* (2012)
Laribacter hongkongensis	Proteobacteria (beta)	Woo *et al.* (2005)
Listeria monocytogenes	Firmicutes	Schmitter *et al.* (2017)
Magnetospirillum gryphiswaldense, M. magneticum	Proteobacteria (alpha)	Yoshino *et al.* (2010), Borg *et al.* (2014)
Methylobacterium extorquens	Proteobacteria (alpha)	Chubiz *et al.* (2013)
Mycobacterium abscessus, M. bovis BCG, M. smegmatis, M. tuberculosis	Actinobacteria	Blokpoel *et al.* (2005), Ehrt *et al.* (2005), Cortes *et al.* (2011)
Mycoplasma agalactiae, M. genitalium, M. mycoides (JCVI-syn 1.0), M. pneumoniae	Tenericutes (Mollicutes)	Breton *et al.* (2010), Mariscal *et al.* (2016), Mariscal *et al.* (2018)
Photorhabdus luminescens	Proteobacteria (gamma)	Yin *et al.* (2015)
Providencia stuartii	Proteobacteria (gamma)	Armbruster *et al.* (2017)
Pseudomonas putida	Proteobacteria (gamma)	Gauttam *et al.* (2020)
Ralstonia eutropha	Proteobacteria (beta)	Li and Liao (2015)
Salmonella enterica serovar Thyphi, -Typhimurium	Proteobacteria (gamma)	Rappleye and Roth (1997), Qian and Pan (2002)
Sodalis glossinidius	Proteobacteria (gamma)	Kendra *et al.* (2020)
Spiroplasma citri	Tenericutes (Mollicutes)	Breton *et al.* (2010)
Staphylococcus aureus, S. carnosus, S. epidermidis	Firmicutes	Ji *et al.* (1999), Giese *et al.* (2009), Christner *et al.* (2010)
Streptococcus agalactiae, S. mutans, S. pneumoniae, S. pyogenes	Firmicutes	Bugrysheva and Scott (2010), Lartigue and Bouloc (2014), Stieger *et al.* (1999), Wang and Kuramitsu (2005)
Streptomyces ambofaciens, S. avermitilis, S. coelicolor, S. griseus, S. lividans, S. rimosus, S. roseosporus, S. venezuelae	Actinobacteria	Hansen *et al.* (2001), Rodriguez-Garcia *et al.* (2005)
Synechococcus sp. strain PCC7002	Cyanobacteria	Zess *et al.* (2016)
Synechocystis sp. strain PCC 6803	Cyanobacteria	Huang and Lindblad (2013)
Vibrio cholerae	Proteobacteria (gamma)	Hsiao *et al.* (2006)
Xenorhabdus stockiae	Proteobacteria (gamma)	Yin *et al.* (2015)
Yersinia enterocolitica, Y. pestis	Proteobacteria (gamma)	Lathem *et al.* (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 Tn*10 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 Tn*10* 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 Tn*10 tet regulation* sequence. In another study on *V. cholerae*, Cakar *et al.* (2018) cloned a promoterless *tetR* gene and a resolvase gene driven by Tn*10* P_A to investigate gene expression profiles. *TetR* was used for promoter probing upon random insertion into the chromosome.

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Sodalis glossinidius
Edwardsiella tarda
Citrobacter freundii
Klebsiella oxytoca
Escherichia coli
Escherichia hermannii
Salmonella enterica
Salmonella enterica
Yersinia pestis
Yersinia enterocolitica
Providencia stuartii
Xenorhabdus stockiae
Photorhabdus luminescens
Vibrio cholerae
Pseudomonas putida
Acinetobacter oleivorans
Coxiella burnetii
Francisella novicida
Francisella tularensis
Laribacter hongkongensis
Ralstonia eutropha
Burkholderia thailandensis
Geobacter sulfurreducens
Magnetospirillum magneticum
Magnetospirillum gryphiswaldense
Methylorubrum extorquens
Agrobacterium tumefaciens
Brucella abortus
Borreliella burgdorferi
Helicobacter pylori
Campylobacter jejuni
Chlamydia trachomatis
Anabaena cylindrica
Synechococcus sp.
Synechocystis sp.
Corynebacterium glutamicum
Mycobacteroides abscessus
Mycolicibacterium smegmatis
Mycobacterium bovis
Mycobacterium tuberculosis
Streptomyces avermitilis
Streptomyces venezuelae
Streptomyces griseus
Streptomyces rimosus
Streptomyces coelicolor
Streptomyces ambofaciens
Streptomyces roseosporus
Streptomyces lividans
Clostridium acetobutylicum
Clostridioides difficile
Acetobacterium woodii
Streptococcus mutans
Streptococcus pneumoniae
Streptococcus pyogenes
Streptococcus agalactiae
Listeria monocytogenes
Bacillus subtilis
Staphylococcus carnosus
Staphylococcus epidermidis
Staphylococcus aureus
Mycoplasma agalactiae
Spiroplasma citri
Mycoplasma mycoides
Mycoplasma genitalium
Mycoplasma pneumoniae
Bacteroides dorei
Bacteroides vulgatus
Bacteroides fragilis
Bacteroides eggerthii
Bacteroides uniformis
Bacteroides intestinalis
Bacteroides cellulosilyticus
Bacteroides thetaiotaomicron
Bacteroides ovatus
Bacteroides xylanisolvens

Phylum (class)
- Actinobacteria
- Alphaproteobacteria
- Betaproteobacteria
- Chlamydiae
- Cyanobacteria
- Deltaproteobacteria
- Epsilonproteobacteria
- Firmicutes
- Gammaproteobacteria
- Spirochaetes
- Tenericus(Mollicutes)
- Bacteroidetes

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).

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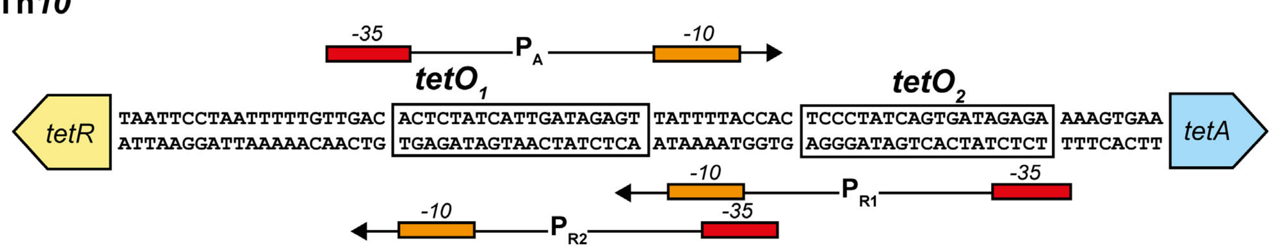

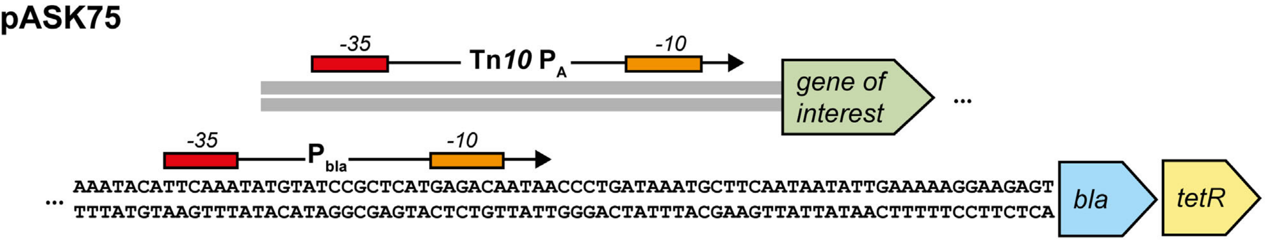

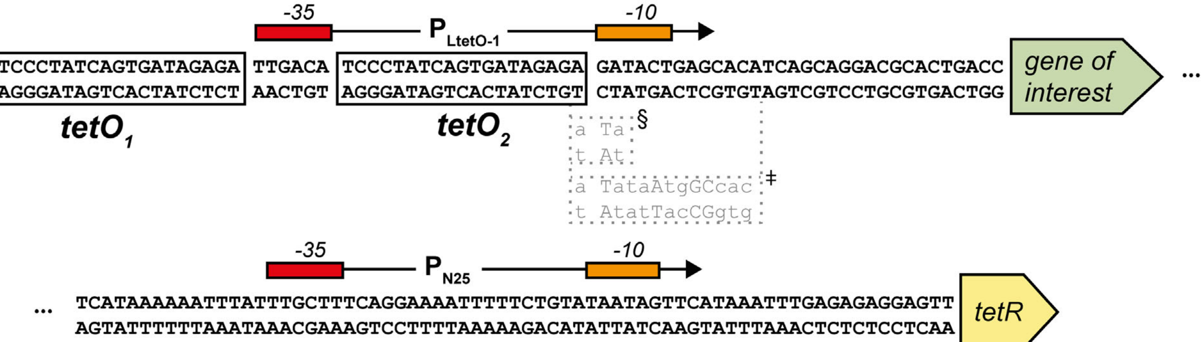

§ Lee *et al.* (2016); ‡ Huang and Lindblad (2013);

Fig. 5. Selected promoters of popular bacterial *tet regulation* systems for Gram-negative bacteria.
A. P_A, P_{R1} and P_{R2} of transposon Tn*10*.
B. P_A of Tn*10* 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 PN25 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 Tn*10* 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 *tetO₂* 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 $P_{LtetO-1}$ promoter (Hsiao *et al.*, 2006). $P_{LtetO-1}$ 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 $P_{LtetO-1}$ sequence, with two nucleotide exchanges (Temme *et al.*, 2012). The $P_{LtetO-1}$ *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 $P_{LtetO-1}$ at selected positions to increase transcription in *E. coli*. For use in the cyanobacterium *Synechocystis* sp. the $P_{LtetO-1}$, 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 Tn*10*) 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 Pmsp1 (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_{rpsL}-*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 (Whetstine *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 $P_{xyl/tet}$ promoter that had been developed for Gram-positive bacteria, as detailed in the following.

Gram-positive solutions of *tet regulation*

The $P_{xyl/tet}$ promoter system

The first *tet system* of Gram-positive bacteria was established in *Bacillus subtilis* (Geissendörfer and Hillen, 1990). The *tet regulation* sequences of Tn*10* 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 $P_{xyl/tet}$ for *tet regulation* of genes of interest (Fig. 6). All required components were cloned in one plasmid, termed pWH353 (one *tetO* in $P_{xyl/tet}$) 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 $P_{xyl/tet}$ 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*-*tetR*–$P_{xyl/tet}$ (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 $P_{xyl/tet}$ sequence of pALC2073 in *Streptococcus mutants* (Wang and Kuramitsu, 2005). Bugrysheva and Scott (2010) have applied the P*-*tetR*–$P_{xyl/tet}$ (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 $P_{xyl/tet}$ (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*-*tetR*–$P_{xyl/tet}$ 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 $P_{xyl/tet}$ 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-

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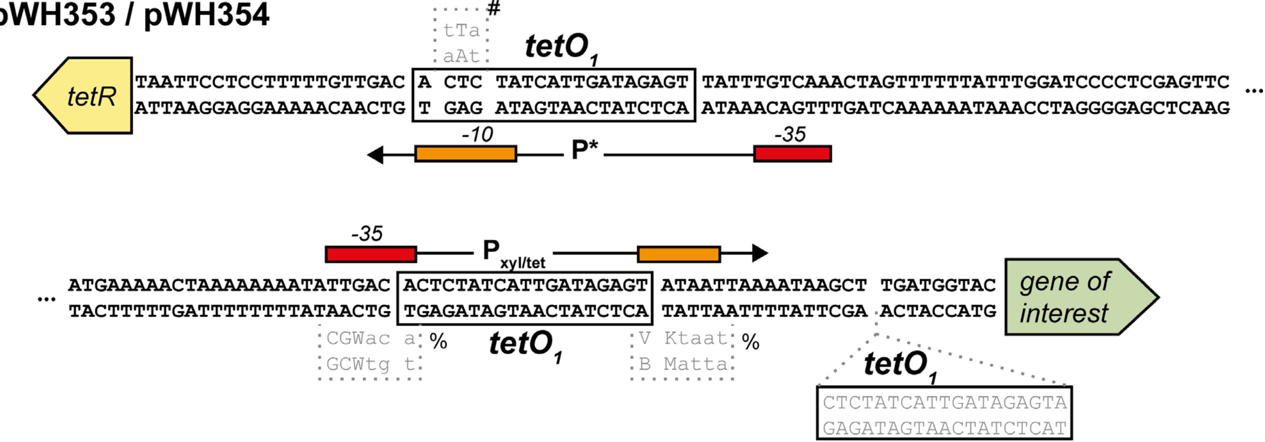

Fig. 6. The $P_{xyl/tet}$ promoter.
P* and $P_{xyl/tet}$ in pWH353 (one *tetO*) and pWH354 two *tetO*). Note that the sequence is continued from the upper to the lower part. Sequence deviations of promoter variants are given in grey dotted boxes.

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×*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 Tn*10 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. Ehrt *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 Streptomycetes. 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 Ehrt (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 Tn*10 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 set-up, the P_{gap}-*tetR* fragment and the $P_{xyl/tet}$ 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 Pcm 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 (Pthl). 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 Tn*1721* (Heravi *et al*., 2015).

Future directions of *tet*control – 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

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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.

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