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...
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 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 α8 and α9 (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.

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applied bacterial tet regulation. One drawback of ATc is its instability upon longer exposure to light (Baum- schlager 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 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 Streptomyces (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 2i is exclusively induced by 4-de-dimethylamino-ATc, but not by ATc or Dox (Henssler et al., 2004; Klieber et al., 2007) (Fig. 2A). Notably, also specific RNA or peptide molecules are capable of inducing TetR. A dodericamer 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). Notably, the choice of the reporter gene or the mode of quantification of the IF, which is why we mostly refrain from stating or comparing dynamic ranges of Tet regulation (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).
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; Latham 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 the generation of 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 tetO1 and tetO2 of Tn10 share a core dyad symmetry of 19 bp but differ at four positions. TetR interaction with tetO2 inhibits transcription of both genes, while the occupation of tetO1 represses only tetA. The affinity of TetR is higher to tetO2 than to tetO1 (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 (Bear et al., 2011). An allelic exchange vector harbouring the tetR-P_tetA sequence found use in the nosocomial pathogen Providencia stuartii (Armaruster 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 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 tet regulation |
|------------------------------------------------|----------------|-------------------------------------|
| Acetobacterium woodii                         | Firmicutes     | Beck et al. (2020)                  |
| Acinetobacter olivovarans                    | 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. cellulosolyticus, B. eggertii |                  |                                     |
| Borrelia burgdorferi                          |                  |                                     |
| Bruccella abortus                             |                  |                                     |
| Burkholderia thailandensis                   |                  |                                     |
| Campylobacter jejuni                          |                  |                                     |
| Chlamydia trachomatis                         |                  |                                     |
| Citrobacter freundii                          |                  |                                     |
| Clostridium acetobutylicum, C. difficile      |                  |                                     |
| Corynebacterium glutamicum                    |                  |                                     |
| Coxella burnetii                              |                  |                                     |
| Edwardsiella tarda                            |                  |                                     |
| Escherichia coli, E. hermannii                |                  |                                     |
| Francisella novicida, F. tularensis           |                  |                                     |
| Geobacter sulfurreducens                      |                  |                                     |
| Helicobacter pylori                           |                  |                                     |
| Klebsiella oxytoca                            |                  |                                     |
| Lanibacter hongkongensis                      |                  |                                     |
| Listeria monocytogenes                        |                  |                                     |
| Magnetospirillum gryphiswaldense, M. magnetcum|                  |                                     |
| Methylobacterium extorquens                   |                  |                                     |
| Mycobacterium abscessus, M. bovis BCG, M. smegmatis, M. tuberculosis |                  |                                     |
| Mycoplasma agalactiae, M. genitalium, M. mycoides (JCVI-syn 1.0), M. pneumoniae |                  |                                     |
| Photobabesia luminescens                      |                  |                                     |
| Providencia stuartii                          |                  |                                     |
| Pseudomonas putida                            |                  |                                     |
| Ralstonia eutropha                            |                  |                                     |
| Salmonella enterica serovar Thyphi, -Typhimurium|              |                                     |
| Sodalis glossinisid                        |                  |                                     |
| Spiroplasma citri                             |                  |                                     |
| Staphylococcus aureus, S. carnosus, S. epidermidis |              |                                     |
| Streptococcus agalactiae, S. mutans, S. pneumoniae, S. pyogenes | Firmicutes | Bugysheva and Scott (2010), Lartigue and Bouloc (2014), Stieger et al. (1998), Wang and Kuramitsu (2005) |
| Streptomycyes ambofaciens, S. avermilitia, 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               |                  |                                     |
| Synechocystis sp. strain PCC 6803             |                  |                                     |
| Vibrio cholerae                               |                  |                                     |
| Xenorhabdus stockiae                          |                  |                                     |
| Yersinia enterocolitica, Y. pestis            |                  |                                     |

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 Pneo 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, Čakar 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.

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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).
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 PA 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 k-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).

**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 PA 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 cl repressor binding sequences that flank the −35 hexamer have been replaced by two tetO 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 Typhimurium and also the first tet...
system in *V. cholerae* was based upon the \(P_{\text{LtetO-1}}\) promoter (Hsiao et al., 2008). \(P_{\text{LtetO-1}}\) 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 \(P_{\text{N25}}\) 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 insufficient, 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 \(P_{\text{refA}}\) which is active in the presence of nitrate or \(P_{\text{petE}}\), 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. Conjugal 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 \(P_{\text{bla}}\) 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 \(P_{\text{tetH}}\) promoter (similar to \(P_{X}\) of *Tn10*) could be detected at nanomolar concentrations of Doc (Hong and Park, 2014). In a tet system for *M. magneticum*, the tetR gene was expressed constitutively by \(P_{\text{msp3}}\) 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 cumamate-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 \(P_{\text{phaC}}\) 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. Debowska 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 \(uP_{\text{tetO1}}\) (one tetO) and \(uP_{\text{tetO2}}\) (two tetO). TetR was expressed from one of three different *H. pylori* promoters in the chromosome. Compared to \(uP_{\text{tetO1}}\), expression by \(uP_{\text{tetO2}}\) 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 \(-35\) (Debowska 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 (Debowska et al., 2017). A tet regulation system in *Campylobacter jejuni*, also causing

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gastroenteritis in humans, was composed of \( \text{P}_{\text{psb}-\text{tet}R} \) and target genes cloned downstream of \( \text{pOs} \)-based promoters carrying one or two \( \text{tetO} \) sites. This study not only established Tet-ON control, but also used rev\( \text{tetR} \) r6.2 (V99E) in \( \text{C. jejuni} \) (Cohen et al., 2019). For \( \text{Borellia burgdorferi} \), the aetiological agent of Lyme disease, one copy of \( \text{tetO} \) was integrated into the \( \text{P}_{\text{ospA}} \) promoter at the unusual position +1. The \( \text{tetR} \) gene was constitutively expressed by \( \text{P}_{\text{psb}} \) 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 \( \text{Synechococcus} \) sp. strain PC7002. To this end, the −35 region of a truncated cyanobacterial promoter \( \text{P}_{\text{psb}} \) was flanked by two \( \text{tetO} \) sites and minor further mutations yielded five potentially tet-sensitive promoters. Both the tet-regulated \( \text{gfp} \) reporter and \( \text{tetR} \) were integrated in the chromosome and expression of \( \text{tetR} \) was tested with three different constitutive promoters. Not only administration of ATc but also the concentration of \( \text{CO}_2 \) 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 \( \text{O}^{70} \)-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 \( \text{Laribacter hongkongensis} \) (Woo et al., 2005) was based upon the \( \text{P}_{\text{psyl/tet}} \) promoter that had been developed for Gram-positive bacteria, as detailed in the following.

**Gram-positive solutions of tet regulation**

*The \( \text{P}_{\text{psyl/tet}} \) promoter system*

The first tet system of Gram-positive bacteria was established in \( \text{Bacillus subtilis} \) (Geissendörfer and Hillen, 1990). The tet regulation sequences of Tn10 were found to be unsuitable, and hence, a modified autoregulated promoter termed \( \text{P}^+ \) was constructed to drive \( \text{tetR} \), while the \( \text{B. subtilis} \) \( \text{P}_{\text{psly}} \) promoter was vested with one or two \( \text{tetO} \) sequences to yield two versions of \( \text{P}_{\text{psyl/tet}} \) for tet regulation of genes of interest (Fig. 6). All required components were cloned in one plasmid, termed pWH353 (one \( \text{tetO} \) in \( \text{P}_{\text{psyl/tet}} \)) or pWH354 (two copies of \( \text{tetO} \)). 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 \( \text{P}_{\text{psyl/tet}} \) in pathogenic Gram-positive bacteria is marked by Ji et al. (1999), when the virulence gene \( \text{hla} \) 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 \( \text{S. camosus} \), the \( \text{hla} \) gene was cloned into pALC2084 to analyse the effect regarding phagolysosomal escape (Giese et al., 2009). In \( \text{S. epidermidis} \), \( \text{P}^+\text{tetR}-\text{P}_{\text{psyl/tet}} \) (one \( \text{tetO} \)) was used to control expression of the extracellular matrix-binding protein-gene \( \text{emb} \) after promoter exchange in the chromosome (Christner et al., 2010). Inducible antisense-RNA expression was accomplished by the \( \text{P}_{\text{psyl/tet}} \) sequence of pALC2073 in \( \text{Streptococcus mutants} \) (Wang and Kuramitsu, 2005). Bugrysheva and Scott (2010) have applied the \( \text{P}^+\text{tetR}-\text{P}_{\text{psyl/tet}} \) (two \( \text{tetO} \)) sequence in \( \text{Streptococcus pyogenes} \) to replace the native promoters of two putative essential RNase encoding genes, thereby rendering growth of strains \( \text{ATc} \)-dependent. The use of a target gene promoter with three vs. two \( \text{tet} \) operators permitted tighter control but at the cost of reduced induction. The facultative intracellular food spoilage bacterium \( \text{Listeria monocytogenes} \) 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 \( \text{P}_{\text{psyl/tet}} \) (two \( \text{tetO} \)) from the chromosome, while \( \text{tetR} \) was episomally encoded and driven by a strong synthetic promoter termed pt17 (Bertram et al., 2005). The resulting strain facilitated \( \text{ATc} \)-dependent spatio-temporal control of ActA and consequently actin recruitment within epithelial human cells.

Corrigan and Foster (2009) modified the \( \text{P}^+ \) promoter of the \( \text{P}^+\text{tetR}-\text{P}_{\text{psyl/tet}} \) sequence, rendering expression of \( \text{tetR} \) constitutive (Fig. 6). The resulting plasmid pRM22 was used in \( \text{S. aureus} \), and its tet regulation architecture was cloned also into a shuttle-vector applied in \( \text{Streptococcus agalactiae} \) (Lartigue and Bouloc, 2014). Helle et al. (2011) added a second \( \text{tetO} \) site to \( \text{P}_{\text{psyl/tet}} \) of pRM22 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 \( \text{tetR} \) 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 \( \text{fabI} \), 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 \( \text{S. aureus} \), a one-plasmid tet system resembling pWH354 (Geissendörfer and Hillen, 1990), as well as \( \text{ATc} \)-dependent regulation of chromosomal target genes by \( \text{TetR} \) or rev\( \text{TetR} \) or inducible AS-RNA expression. Apparently, tet regulation of AS-
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 Pxyl/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 Pxyl/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 CRISPR 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. 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 Pxyl/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.

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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 Tn10 tetA promoter. TetR was expressed from a plasmid and driven by Pgap. Repression of emb led to growth retardation and an elevated glutamate efflux. In an alternative, episomal setup, the Pgap-tetR fragment and the Pxy/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 Pnat 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 Pnat 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 Gautam et al. (2019) described a dual expression plasmid of C. glutamicum exploiting the PnatO1 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, but not with tetO2. 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 (Voizling 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

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