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Independent Regulation of Two Genes in *Escherichia coli* by Tetracyclines and Tet Repressor Variants

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We report a regulation system in *Escherichia coli* for independent regulation of two distinct reporter genes by application of Tet repressors with different specificities. One Tet repressor variant comprises wild-type *tet* operator (*tetO*) recognition and exclusive induction with the novel inducer 4-dedimethylamino-anhydrotetracycline. The other Tet repressor variant shows *tetO*-4C recognition and induction with tetracycline. We demonstrate that both variants are independently active in vivo and allow selective regulation of two genes in the same cell without any cross talk.

Inducible promoters are powerful tools for studying gene function in prokaryotes. The most widely used regulated expression systems in *Escherichia coli* have been developed from the *lac*, *ara*, and *tet* genes (2, 9, 14), while the *tet*, *spac*, and *xyl* systems have been used mostly with gram-positive bacteria (1, 6, 7, 16, 18). The advantage of *tet* regulation lies in the combination of tight control and sensitive induction by compounds that diffuse passively across biological membranes and do not require the presence of uptake proteins. Since complex cellular processes are often determined by more than one gene, multiple gene regulation systems that can be distinctly addressed are of general interest. We describe here modifications of the *tet* regulatory system that allow differential expression control of two genes in *E. coli*.

Independent *tet* regulation of two genes requires different *tet* operators and TetR variants with unique recognition specificity for them. Two such pairs have been described previously (4, 5). For differential induction, TetR variants with exclusive specificity for distinct inducers are needed. We have recently constructed a TetR variant that is inducible by 4-dedimethylamino-anhydrotetracycline (4-DDMA-ATC) (Fig. 1) but not inducible by tetracycline (TET). This would allow independent induction of two genes. Finally, since native TetR is a homodimer, cross talk between the TetR variants needs to be avoided, which can be accomplished by using *tetR* alleles from different naturally occurring sequence variants, as has been described previously (8, 11). Here, we ask whether the mutations leading to these functionally different TetR proteins can be combined to yield specifically addressable TetR variants and if their phenotypes are stringent enough for distinct regulation in *E. coli*. In addition to the *E. coli lacZ*, we used *xynB* from *Bacillus subtilis* encoding a β-xylosidase (β-Xyl) as a second reporter gene to quantitatively distinguish regulatory efficiencies.

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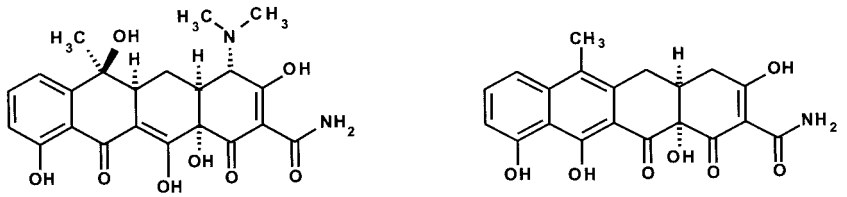
Construction of the dual regulation system. Strains and plasmids used in this study are presented in Table 1. Both reporter genes were expressed in the *E. coli* strain WH207$_{\lambda tet50}$, which carries a chromosomal *tetA-lacZ* transcriptional fusion (17) in which *lacZ* is under wild-type *tetO* control. This construct is regulated by the TetR(BD) mutant H64K L131I S135L, which is inducible with 4-DDMA-ATC but not with TET. The second reporter gene, *xynB*, is regulated by the *tet* operator 4C (*tetO*-4C) mutant, with two palindromic sequence changes from the wild type (4). *xynB* was amplified by PCR from chromosomal DNA of *B. subtilis* strain 168, introducing a downstream BsmBI site. *tetO*-4C was amplified from the plasmid pWH1012-4C (13) containing an upstream BsmBI site and then fused to the *xynB*-carrying fragment in a subsequent PCR without primers. The resulting product was cloned in two steps into a variant of pWH1411 with the *tetR* mutant. The resulting plasmid was termed pWH628 (Fig. 2). TetR-4C, the TetR(B) E37A P39Q Y42M mutant that specifically recognizes *tetO*-4C instead of wild-type *tetO* (4), was expressed from the plasmid pWH1925-*tetR-4C*. Due to different origins of replication (Fig. 2), pWH1925-*tetR-4C* and pWH628 can coexist in WH207$_{\lambda tet50}$. This plasmid compatibility allows the regulation of *xynB* with TetR-4C. In addition, TetR-4C is inducible with TET but not

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Reference or source
Strains		
E. coli DH5α	*recA1 endA1 gyrA96 thi relA1 hsdR17*(r_K^- m_K^+) *supE44* ϕ80d*lacZ*ΔM15 Δ*lacU169*	3
B. subtilis 168	*trpC2*	Laboratory stock
E. coli WH207	*galK rpsL thi* Δ*lacX74 recA13*	17
E. coli WH207$_{\lambda tet50}$	Tn*10 tetA-lacZ* transcriptional fusion, *bet*$^+$ *gam*$^+$ *c*III$^+$ *c*I$^+$ *lacZ*$^+$	15
Plasmids		
pWH1925-*tetR*-4C	Ap^r, pMB1 *tetR*-4C	This study
pWH1925-Δ	pWH1925-*tetR-4C* without *tetR* gene	12a
pWH1411-i1.9	Cm^r *tetR*-H64K L131I S135L	12
pWH628	Cm^r, pWH1411-i1.9 with *tetO*-4C–*xynB* region	This study
pWH627	pWH628 without *tetR* gene	This study



tetracycline (tc) 4-dedimethylamino anhydrotetracycline (4-ddma-atc)

FIG. 1. Chemical structures and designations of tetracyclines used in this study. The chemical structures of tetracycline and the antibiotically inactive 4-ddma-atc are shown.

with 4-DDMA-ATC, and the class B and BD sequence variants of TetR do not heterodimerize (11).

In vivo evaluation of enzyme activities. β-Galactosidase (β-Gal) activity was determined as described previously (10) by using *ortho*-nitrophenyl-β-D-galactopyranoside as a substrate. β-Xyl activity was determined in the same way except with *para*-nitrophenyl-xylanopyranoside as a substrate. We tested for potential cross-reaction of the enzymes by adding *ortho*-nitrophenyl-β-D-galactopyranoside to a culture expressing only β-Xyl. No absorption was detected at 420 nm, and the same result was obtained with β-Gal and *para*-nitrophenyl-xylanopyranoside (data not shown). Measurements of dual regulation were performed in parallel, where one part of the culture was assayed for β-Gal and the other for β-Xyl activity. Both effector concentrations were adjusted to achieve optimal induction within the tolerance range of *E. coli* (0.2 μM for TET and 1.6 μM for 4-DDMA-ATC) (data not shown). The regulation of β-Gal expression is shown in Fig. 3A. Independent of the presence or absence of any inducer, TetR-4C does not repress β-Gal expression and therefore does not recognize *tetO*, as is shown on the left side of Fig. 3A. The right side of Fig. 3A shows that in the absence of inducer, the 4-DDMA-ATC-specific TetR binds to *tetO*, as indicated by the tight repression of *lacZ*, while TET does not induce this TetR variant at all. In contrast, *lacZ* is highly expressed when 4-DDMA-ATC is added, indicating that this TetR variant is inducible only with 4-DDMA-ATC and not with TET. β-Gal activity remains high in the presence of both inducers, suggesting that TET does not interfere with the induction of TetR H64K L131I S135L by 4-DDMA-ATC. Figure 3B shows the results of β-Xyl activity determinations of the same cultures. TetR H64K L131I S135L does not bind to *tetO*-4C (Fig. 3B, left). No XynB expression was detectable in the absence of an inducer, indicating that TetR-4C binds tightly to its cognate sequence *tetO*-4C. Upon addition of TET, β-Xyl was induced to 65% activity. 4-DDMA-ATC does not induce TetR-4C, as shown by the repression of *xynB*, and it does not interfere with induction by TET (Fig. 3B, right).

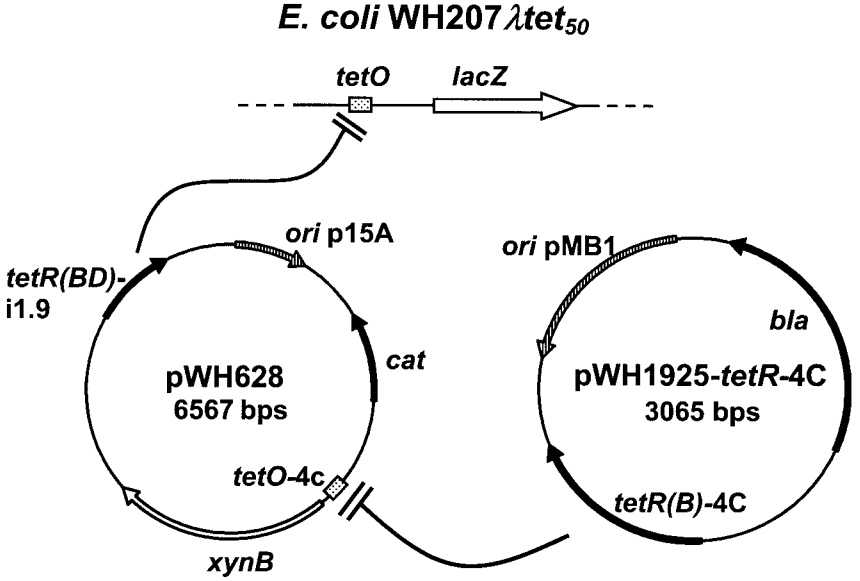


FIG. 2. Components of the dual regulation system. The host strain is WH207$_{\lambda tet50}$. *tetR*(BD)-*i1.9* [encoding the 4-DDMA-ATC-specific mutant TetR(BD) H64K L131I S135L] is constitutively expressed from plasmid pWH628 and binds *tetO* located upstream of *lacZ* on the chromosome. XynB, expressed from pWH628, is regulated via *tetO*-4C that is bound by TetR(B)-4C. TetR(B)-4C is constitutively expressed from pWH1925-*tetR-4C*. *ori* codes for origin of replication, *cat* codes for chloramphenicol acetyltransferase, *bla* codes for β-lactamase, *xynB* codes for β-Xyl, and *lacZ* codes for β-Gal.

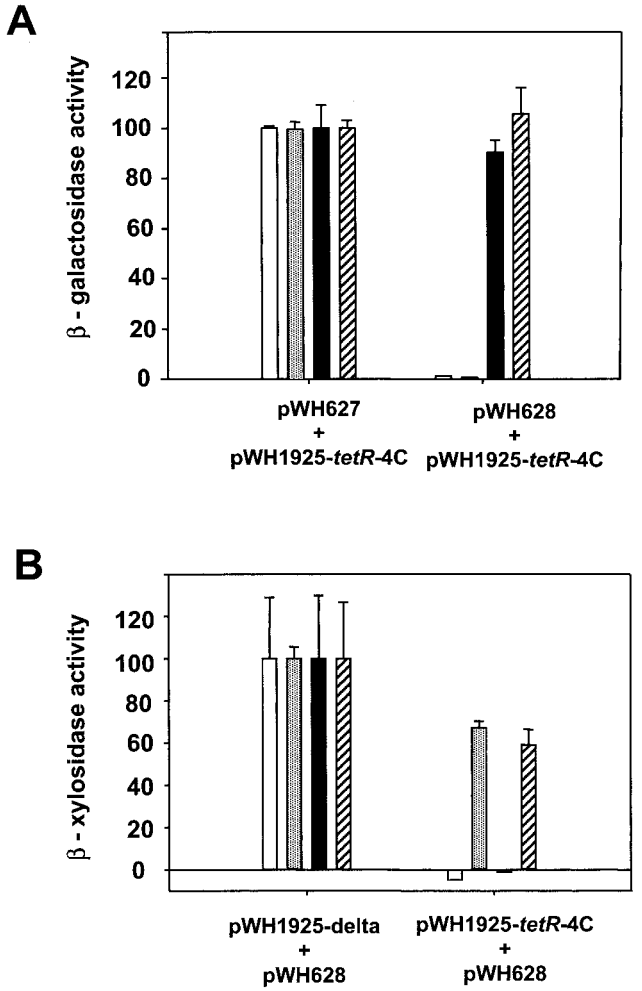


FIG. 3. Induction analysis of the dual regulation system. (A) Induction of β-Gal expression is shown in the presence of the respective inducers. β-Gal activity in the absence of *tetR*(BD)-i1.9 (pWH627) was set to 100% and represents about 2,500 Miller units. Open columns, no inducer; gray columns, 0.2 μM TET; black columns, 1.2 μM 4-DDMA-ATC; striped columns, 0.2 μM TET plus 2 μM 4-DDMA-ATC. (B) Induction of XynB expression in the presence of the inducers. XynB activity obtained in the absence of *tetR*(B)-4C (pWH1925-Δ) was set to 100% and represents about 10 Miller units.

Conclusions. We present for the first time a dual regulation system that combines different TetR inducer specificities with different TetR operator recognition mutations for application in prokaryotes, allowing independent and reversible in vivo regulation of two different genes by two distinct effectors. Apparently, 4-DDMA-ATC resembles TET as a well-permeating effector showing fast and efficient induction in *E. coli*. In addition, this effector exhibits no antibiotic activity and may thus overcome limitations in efficiency of expression that are sometimes observed with induction by TET in sensitive strains. The use of these two independently functioning repressors should be a powerful tool for the analysis of prokaryotic phenotypes that depend on the expression of two genes. It should also have merits in target validation or inhibitor screening assays for heterooligomeric proteins.

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