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Generating Tetracycline-Inducible Auxotrophy in *Escherichia coli* and *Salmonella enterica* Serovar Typhimurium by Using an Insertion Element and a Hyperactive Transposase

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We report the construction and application of a novel insertion element for transposase-mediated mutagenesis in gram-negative bacteria. Besides Km^r as a selectable marker, the insertion element $InsTet^{G-}1$ carries the anhydrotetracycline (atc)-regulated outward-directed P_A promoter so that atc-dependent conditional gene knockouts or knockdowns are generated. The complex formed between the purified hyperactive transposase and $InsTet^{G-}1$ was electroporated into *Escherichia coli* or *Salmonella enterica* serovar Typhimurium, and mutant pools were collected. We used *E. coli* strains with either TetR or the reverse variant $revTetR^{r2}$, while only TetR was employed in *Salmonella*. Screening of the $InsTet^{G-}1$ insertion mutant pools revealed 15 atc-regulatable auxotrophic mutants for *E. coli* and 4 atc-regulatable auxotrophic mutants for *Salmonella*. We have also screened one *Salmonella* mutant pool in murine macrophage-like J774-A.1 cells using ampicillin enrichment. Two mutants with the $InsTet^{G-}1$ insertion in the gene *pyrE* or *argA* survived this procedure, indicating a reduced intracellular growth rate in J774-A.1 cells. The nature of the mutants and the modes of their regulation are discussed.

In recent years, the application of sophisticated tools has led to a rapidly growing wealth of genomic, transcriptomic, and proteomic information for bacteria. It has been assumed that many new targets for antiinfectives may be identified from these data. However, the function of about 40% of the genes (26) and their encoded proteins turns out to be unknown in most organisms. Therefore, there is now a need to develop new generally applicable methods to analyze gene functions. Responding to this need, several efficient mutagenesis protocols have been developed for a wide range of pathogenic and nonpathogenic bacteria. Many of them contain transposable elements to create insertion mutants because the site of mutation is tagged. The expression of transposase in the target cell is required for transposition but should be eliminated afterwards to ensure the stability of the mutants (16). Therefore, in vitro transposition systems, like the one developed previously by Goryshin et al. in which an insertion element is complexed with purified transposase, which is followed by electroporation of this complex into bacterial cells, are used for mutagenesis in vitro or in vivo (10, 17). While this system is very efficient for creating randomized knockouts of genes, it is incapable of revealing essential genes directly. Insertion elements carrying a regulatable promoter yielding conditional expression of downstream genes may circumvent this limitation. Several such constructs make use of the arabinose-inducible promoter pBAD in combination with transposition (32). This promoter responds to arabinose, which requires an uptake system to enter bacteria. Furthermore, it cannot be used in mammalian pathogenicity models because that inducer is metabolized by the infected animal. The Tn*5tac* system (8) responds to IPTG (isopropyl-β-D-thiogalactopyranoside) and is limited by the strong P_{tac} promoter, causing overexpression effects and a lack of tight repression. We have designed and constructed an insertion element for gram-negative bacteria using a tetracycline-dependent transcriptional control system to regulate genes located downstream from the insertion site. The inducers tetracycline, doxycycline, and anhydrotetracycline (atc) do not require an uptake system to penetrate most cells (33, 34) and function in eukaryotic cell lines (13) and transgenic mammals (38). Thus, the regulation of genes should also be possible in infection models. We describe here the construction and efficiency of an insertion element, based on the Tn*5* in vitro system developed previously by Goryshin et al. (12), to obtain atc-responsive conditional gene knockouts in *Escherichia coli* and *Salmonella enterica* serovar Typhimurium.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. Bacterial strains used in this study are listed in Table 1. *E. coli* and *Salmonella* serovar Typhimurium strains were grown at 37°C in Luria-Bertani (LB) broth or M9 minimal medium containing 0.2% glucose as a carbon source. For antibiotic resistance marker selection, bacterial media were additionally supplemented with 25 μg/ml of chloramphenicol (Cm), 60 μg/ml or 100 μg/ml of kanamycin (Km), or 100 μg/ml of ampicillin (Ap) where needed.

General methods. For measurements of β-galactosidase (β-gal) activity, *E. coli* cells were cotransformed with pWH1867 and pWH1411BD, encoding wild-type (wt) TetR, or $pWH1411^{r2}$, encoding $revTetR^{r2}$, respectively (Table 1). Three independent clones of each strain were taken from log-phase cultures at 37°C, and β-gal activities were determined as described previously (25, 30).

For the preparation of electrocompetent cells, 1 liter of LB broth containing 25 mg chloramphenicol was inoculated with *E. coli* MG1655/pWH1411BD or $pWH1411^{r2}$ or *Salmonella* serovar Typhimurium 12023/pWH1411BD. After the optical density at 600 nm (OD_{600}) had reached 0.4, the culture was chilled on ice for 30 min and then harvested by centrifugation at 4,000 × *g* for 10 min. The supernatant was discarded, and the pellet was washed four times in 10 ml of 10% ice-cold glycerol and finally resuspended in 1 ml of 10% ice-cold glycerol. This cell suspension was stored in 100-μl aliquots at −70°C.

Electroporation of $InsTet^{G-}1$–transposase complexes into *E. coli* and *Salmo-*

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TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Genotype	Reference or source
Strains		
E. coli K-12		
DH5α	*recA1 endA1 gyrA96 thi relA1 hsdR17* (r_K^- m_K^+) *supE44* ϕ80d*lacZ*Δ Δ*lacU169*	4
MG1655	*rph-1*	5
ER2566	*fhuA2 lacZ*::T7 gene *1* [*lon*] *ompT gal sulA11 R*(*mcr-73*::mini-Tn*10*-Tet^s)*2* [*dcm*] *R*(*zgb-210*::Tn*10*-Tet^s) *endA1*Δ (*mcrC-mrr*) *114*::IS*10*	7
Salmonella enterica serovar Typhimurium NCTC12023	Wild type	ATCC 14028
Plasmids		
pCB302b	Ap^r, pBR322 derivative, divergent promoterless *lacZ* and *galK*	29
pDG972	Km^r, pMTL23 derivative	14
pTYB2	Cm^r, pACYC derivative, C-terminal intein-chitin binding domain fusion	7
pUC18	Ap^r *lacZ*α	37
pWH1411BD	Cm^r, pACYC177 derivative, *tetR*(BD)51-208	31
pWH1411^{r2}	Cm^r, pACYC177 derivative, *tetR*^{r2}(BD)E15A, L17G, L25V; XbaI/NcoI from pWH1925-TetR^{r2}	This study
pWH1865	Ap^r/Km^r, pUC18 derivative, InsTet^{G-}1 in XbaI/NcoI pWH1865 ΔKm	This study
pWH1865ΔKm^r	Ap^r, pUC18 derivative, InsTet^{G-}1 Δ*aphA*III XbaI/NcoI	This study
pWH1866	Ap^r/Km^r, pUC18 derivative, InsTet^{G-}1 flanked by PvuII sites	This study
pWH1867	Ap^r/Km^r, pCB302b derivative, InsTet^{G-}1 upstream of a promoterless *lacZ*	This study
pWH1891	Ap^r, pTYB2 derivative, Tn*5* transposase E54K, M56A, L372P; C-terminal intein-chitin binding domain fusion	This study
pWH1925^{r2}	Ap^r, pUC19 derivative, *tetR*(BD)E15A, L17G, L25V	30

nella serovar Typhimurium was carried out in 2-mm-gap electroporation cuvettes with 40 μl of cell suspension and 1 μl of the reaction mixtures, as specified in Results, at 2,500 V (τ = 5 ms) using a Bio-Rad (Munich, Germany) Gene Pulser. For *E. coli*, the suspension was diluted to 2 ml with LB medium, transferred into 2.2-ml microcentrifuge tubes, and incubated for 60 min at 37°C before plating onto agar as specified in Results. In the case of *Salmonella* serovar Typhimurium, SOC medium (100 μl 1 M $MgCl_2$, 100 μl 1 M $MgSO_4$, 200 μl 20% glucose diluted in 10 ml SOB medium [10 g/liter tryptone, 2.5 g/liter yeast extract, 0.85 ml 5 M NaCl, 1 ml 1 M KCl]) was used for dilution after electroporation.

For ampicillin enrichment of the *Salmonella* serovar Typhimurium InsTet^{G-}1 insertion mutant library, a final ampicillin concentration of 100, 200, or 500 μg/ml was used.

Chromosomal DNA was isolated from kanamycin-resistant clones of *E. coli* MG1655 containing either pWH1411BD or pWH1411BD^{r2} and *Salmonella* serovar Typhimurium 12023/pWH1411BD according to the QIAamp DNA Mini kit protocol (QIAGEN, Hilden, Germany). The DNA was digested with EcoRI, which was followed by 20 min heat inactivation at 70°C. A total of 1.5 μg of the DNA was sequenced using 6 μl Big Dye Terminator mix (Applied Biosystems, Weiterstadt, Germany) and 2 μl of the sequencing primer Tn-out_neu (5′-GG TCCTAATTTTTGTTGACACTC-3′) (20 pmol) and adjusted with deionized water to a total volume of 20 μl. The reaction products were ethanol precipitated, washed once with 70% ethanol, and resuspended in 10 μl formamide, and the sequence was determined using an ABI 310 genetic analyzer (PE Biosystems, Weiterstadt, Germany).

Construction of the insertion element InsTet^{G-}1. The oligonucleotides IE1-1 (5′-GGGAATTCTCTAGACTGTCTCTTATACACATCTTAAGTAGGTAAAAA TATAATGACCCTCTTG-3′), IE1-2 (5′-TATATTTTTACCTACTTAAGATGTGT ATAAGAGACAGTCTAGAGAATTCCC-3′), IE2-1 (5′-TACTGGGAGAACTA TTGGGTTCTCCCGTAAAAAATGGTCCCAGGATTAAAAACAACTGTGA GA-3′), IE2-2 (5′-TTTTAATCCTGGGACCATTTTTTACGGGAGAACCCAAT A-3′), IE3-1 (5′-TGTTGACACTCTATCATTGATAGAGTTATTTTACCTCTCCC TATCAGTGATAGAGAAAAAGAT-3′), IE3-2 (5′-ATCACTGATAGGGAGAGG TAAAATAACTCTATCAATGAT-3′), IE-4-1 (5′-TCTCTTTTTCTACACATATTC TCTGTCTTCGAATACATACTCACTACACC-3′), and IE4-2 (5′-GGTGTAGTGA GTATGTATTCGAAGACAGAGAATATGTG-3′) comprise the sequence of the insertion element InsTet^{G-}1 with an NcoI restriction site for insertion of the kanamycin resistance gene. They were phosphorylated with T4 polynucleotide kinase (NEB, Frankfurt/Main, Germany). Equimolar amounts of complementary oligonucleotides were hybridized by heating to 96°C and slow cooling to ambient temperature. The hybridized DNA fragments were ligated for 16 h at 4°C, amplified by PCR using the outside primers IE1-1 and IE4-2, digested with BamHI and XbaI, and ligated with pUC18 restricted with the same nucleases. The resulting plasmid, pWH1865ΔKm, was digested with NcoI and ligated with the kanamycin resistance cassette obtained by PCR from plasmid pDG792 (14) with the oligonucleotides Km1-neu (5′-GAAGAGGATGCCATGGCAGATTGCCTTG-3′) and Km2 (5′-G TAGTTAAAGCTCCATGGACATCTAAATCTAGGTAC-3′) to obtain plasmid pWH1865. A PCR with the oligonucleotides PvuII-hind (5′-GCCAGTGCCAAGC AGCTGTCTCTTATAC-3′) and PvuII-xba (5′-CGGGGATCCTCTCAGCTGTC TCTTATACAC-3′) introduced PvuII sites at each end of InsTet^{G-}1. The PCR fragment and pUC18 were digested with PvuII, and the vector was additionally dephosphorylated and ligated. The resulting plasmid, pWH1866, was cut with PvuII, and InsTet^{G-}1 was purified from an agarose gel using the Nucleo Spin Extract kit (Macherey & Nagel, Düren, Germany). The DNA concentration was determined from the absorption at 260 nm. The reporter plasmid pWH1867 contains InsTet^{G-}1 transcriptionally fused to a promoterless *lacZ*. InsTet^{G-}1 was obtained from pWH1865 by XbaI restriction, blunt ending using T4-DNA polymerase (NEB, Frankfurt/Main, Germany), and then restriction with HindIII. pCB302b (29) was digested with AgeI and HindIII and ligated with the InsTet^{G-}1 fragment to obtain pWH1867.

Cloning and purification of the hyperactive Tn*5* transposase. The hyperactive transposase mutant (12) was generated from transposon Tn*5* by PCR mutagenesis and cloned into the overexpression vector pTYB2 to yield pWH1891. This plasmid contains a self-cleaving intein tag fused to a chitin binding domain originating from the IMPACT-CN kit (NEB, Frankfurt/Main, Germany) and a T7 promoter for overexpression. *E. coli* ER2566 transformed with pWH1891 was grown in 3 liters of LB medium at 23°C until an OD_{600} of 0.5 was reached, 0.5 mM of IPTG was then added, and the culture was incubated for another 5 h. The cells were harvested, washed once with 80 ml of TEGX (20 mM Tris-HCl, pH 7.5, 0.7 M NaCl, 1 mM EDTA, 10% [vol/vol] glycerol, 0.1% [vol/vol] Triton X-100), resuspended in 20 ml of TEGX containing protease inhibitors (Complete Mini EDTA Free; Roche, Penzberg, Germany), sonified, and centrifuged at 45,000 rpm (L7-55 ultracentrifuge, 60 TI rotor; Beckmann, Munich, Germany) for 1 h at 4°C. A chitin affinity column was loaded with the supernatant, washed with 10 column volumes of TEGX, flushed with 1.5 volumes of TEGX containing 50 mM dithiothreitol, incubated overnight, and eluted the next day with TEGX. Fractions containing transposase were collected, aliquoted, and stored at −20°C in storage buffer (TEGX with 50% glycerol).

Transposition reactions in vitro and in vivo. In vitro insertion of InsTet^{G-}1 into pUC19 was done according to the protocol provided by Epicenter (Madison, Wis.). One hundred nanograms of InsTet^{G-}1 was mixed with 100 ng of pUC19 and different molar ratios of the hyperactive transposase in a total volume of 10 μl in reaction buffer (0.5 mM Tris-acetate, pH 7.5, 1.5 M potassium acetate, 100

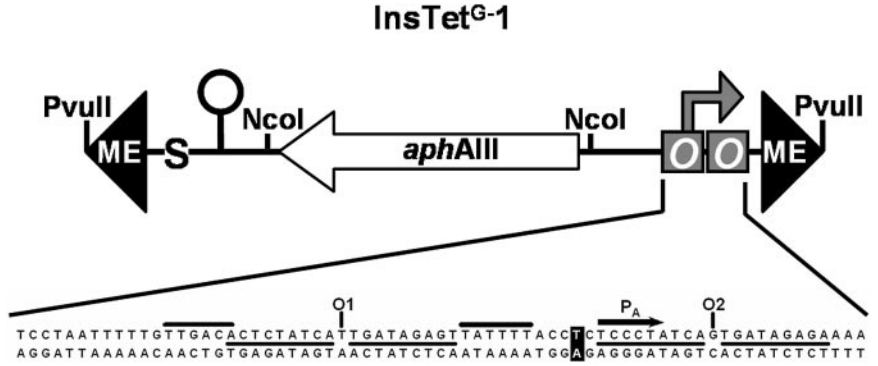

FIG. 1. Architecture of the insertion element InsTet^{G-}1. The entire element is shown schematically. It is 1,376 bp long and flanked by 19-bp recognition sites for hyperactive Tn*5* transposase (ME). The modified *tet* P_A promoter (right-directed gray arrow) on the one side containing two *tet* operators (gray boxes) can drive transcription of downstream genes. The kanamycin resistance gene (*aph*AIII) allows the selection of candidates. A bidirectional transcription terminator from Tn*10* (stem-loop) upstream of the Km^r cassette blocks ingoing and outgoing transcription, and stop codons in all three forward reading frames ("S") terminate possible translation from upstream. Restriction sites for NcoI at both ends of the kanamycin cassette facilitate the exchange of the selection marker, and restriction sites for PvuII at both ends of the insertion element enable blunt cutting for in vitro transposome formation. The sequence of the modified tetracycline-dependent control region of transposon Tn*10* is displayed underneath. One base pair mutation (black boxed letters) shuts down the activity of both P_R promoters without influencing the P_A promoter. O_1/O_2 and the underlined sequences indicate the operator sequences, and the −35 and −10 regions of the P_A promoter are indicated by black boxes. The arrow depicts the transcription start site of the promoter.

mM magnesium acetate, 40 mM spermidine). After incubation at 37°C for 2 h, 1 μl of a 1% sodium dodecyl sulfate solution was added, and the incubation continued for 10 min at 70°C. One microliter of the reaction mixture was transformed into *E. coli* DH5α, and the transformants were plated onto LB agar with kanamycin (60 mg/liter).

Creation of the InsTet^{G-}1–transposase complex (transposomes) in vitro was performed by mixing 110 fmol (*E. coli*) or 545 fmol (*Salmonella* serovar Typhimurium) InsTet^{G-}1 with a fivefold molar excess of the hyperactive Tn*5* transposase. Glycerol was then added to a final concentration of 25% in a total volume of 10 μl, the mixture was incubated at 37°C for 30 min, and 1 μl of the mixture was used for electroporation.

Ampicillin enrichment of intracellular InsTet^{G-}1-derived *Salmonella* serovar Typhimurium mutants. J774-A.1 cells were maintained in a 100-mm petri dish in a humidified atmosphere with 5% CO_2 at 37°C in Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal bovine serum and 2 mM L-glutamine. About 0.5×10^6 J774-A.1 cells between passages 6 and 30 were seeded into each well of a six-well plate and incubated for 24 h. A culture of the InsTet^{G-}1 insertion library of *Salmonella* serovar Typhimurium was grown overnight at 37°C in LB medium and used to inoculate two cultures. One culture was grown in M9 medium to an OD_{600} of about 0.3, and the other culture was first grown in LB medium to an OD_{600} of about 0.3 and was then washed twice with M9 medium, resuspended in M9 medium, and incubated for 1.5 h at 37°C. The J774-A.1 cells were washed with 1 ml 1× phosphate-buffered saline (PBS) and infected with about 1×10^7 *Salmonella* serovar Typhimurium cells in 1 ml 1× PBS. After a 30-min incubation at 37°C, the macrophage cells were washed three times with 1× PBS followed by another incubation for 1 h with DMEM and 100 μg/ml gentamicin. The monolayers were then washed three times with 1× PBS and incubated for 20 h in DMEM with 500 μg/ml of ampicillin and 10 μg/ml of gentamicin. The macrophage cells were washed three times with 1× PBS, and the J774-A.1 cells were lysed with 1 ml of sterile distilled water. The lysate was spread onto LB plates containing kanamycin and incubated overnight at 37°C, and the *Salmonella* serovar Typhimurium mutants were tested for intracellular growth deficiencies by employing the same procedure described above for intracellular selection. The chromosomal locations of the InsTet^{G-}1 insertions in the surviving mutants were determined as described above.

RESULTS AND DISCUSSION

Construction and features of the insertion element InsTet^{G-}1. The features of the insertion element designed and constructed in this work are depicted in Fig. 1. It consists of 1,376 bp flanked by PvuII sites. The element is bordered by two 19-bp transposase recognition sequences (ME) necessary to form the transposase complex. The *tetA* promoter (P_A) containing two *tetO* sequences is located next to ME on the right side of the figure and transcribes out of the insertion element. It was mutated at one position to eliminate P_R promoter activity and prevent TetR-dependent transcription into the element (15). The middle portion of the insertion element consists of the kanamycin resistance cassette, which is needed for selection, flanked by NcoI sites. The left side of Fig. 1 shows the stop codons in all three reading frames and a transcriptional terminator to protect the element from incoming translation or transcription. InsTet^{G-}1 was constructed by hybridizing eight oligonucleotides containing both MEs, the *tetA* promoter, and the translational and transcriptional stop sequences. Cloning of this DNA was followed by insertion of a kanamycin resistance cassette into the NcoI site, as detailed in Materials and Methods.

Testing of the regulatory properties of InsTet^{G-}1 in *E. coli* and *Salmonella* serovar Typhimurium. Plasmid pWH1867 contains the insertion element located upstream of *lacZ*, placing it under the control of the P_A promoter. Two different variants of TetR were used for regulation. pWH1411BD carries wt TetR, which allows transcription only in the presence of atc, while pWH1411^{r2} encodes revTetR^{r2} (3) and shows expression of *lacZ* in the absence and repression in the presence of 0.4 μM atc, an amount that ensures full induction without growth inhibition, when cotransformed with pWH1867 in *E. coli* DH5α and *Salmonella* serovar Typhimurium 12023. Western blot analysis confirmed that the two TetR variants are expressed at the same levels in both organisms (data not shown). The results of β-gal expression for each strain are shown in Fig. 2 and reveal efficient regulation in all four strains. The induction factors are 2,080-fold for TetR and 60-fold for revTetR^{r2} in *E. coli* and 50-fold for TetR and 30-fold for revTetR^{r2} in *Salmonella* serovar Typhimurium. Regulation by TetR is not as tight in *Salmonella* serovar Typhimurium as it is in *E. coli* with this combination of plasmids, but revTetR^{r2} regulation is al-

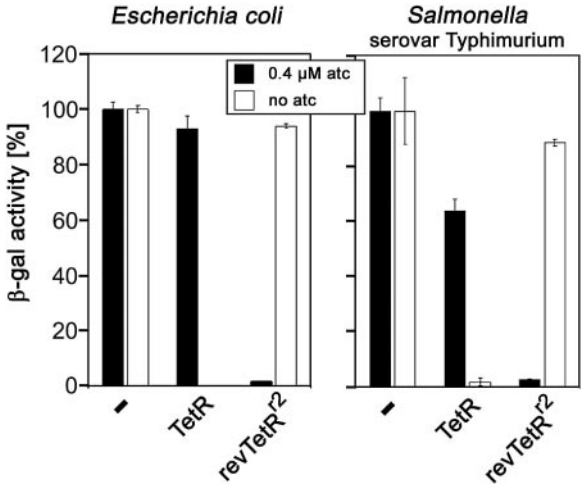

FIG. 2. Test of the regulatory function of the insertion element InsTet^{G-}1 cloned in front of a promoterless *lacZ* gene and cotransformed with TetR-expressing plasmids. The large regulatory window of InsTet^{G-}1 was determined by measurements of β-gal activity. The percent β-gal activity can be seen on the *y* axis. The left panel shows the results for *E. coli* and the right panel shows results for *Salmonella* serovar Typhimurium with atc (black columns) and without atc (white columns). Measurements without a regulator (−) represent the 100% values, with 1,120 Miller units (MU) (with atc) and 1,200 MU (without atc) for *E. coli* and 2,350 MU (with atc) and 1,720 MU (without atc) for *Salmonella* serovar Typhimurium. TetR and revTetR^{r2} represent the two TetR variants expressed from pWH1411.

most the same. These results establish the large regulation window of the atc-inducible promoter. Wild-type TetR efficiently shuts down P_A in the absence of an inducer, as does revTetR^{r2} in the presence of atc, while with the latter regulator, a slightly increased basal expression level is observed. The P_A promoter may be too strong in front of some genes; however, this can be circumvented by the use of lower atc concentrations or by less-efficient inducers (19, 23). We assume that the available options for the adjustment of induction levels will meet the needs of most genes. In an attempt to make use of these properties, a Tn*10*-based system was developed previously (28); however, this transposon has the disadvantage of nonrandom insertion, leading to mutational hotspots (1). Furthermore, a Tn*10* variant in which the tetracycline resistance gene *tetA* was present has been employed (20–22). As a result, tetracycline-induced transcription must proceed from P_A through the resistance gene before reaching the target gene downstream of the insertion site. Since *tetA* overexpression decreases bacterial fitness (9), this may influence the observed phenotype.

Optimizing the insertion reaction in vitro. To determine the conditions that yield the maximal number of insertions with the transposase–InsTet^{G-}1 complex, we performed an in vitro insertion experiment using pUC19 DNA. One hundred nanograms of purified insertion element InsTet^{G-}1 was mixed with an equimolar amount or up to a 20-fold excess of purified transposase. The insertion reaction was done as detailed in Materials and Methods. The products were transformed into *E. coli* DH5α, and the transformants were selected for kanamycin resistance. A fivefold molar excess of the monomeric transposase over insertion element yielded the largest number of kanamycin-resistant candidates (data not shown) and was used for further experiments. Thirty of the candidates were randomly picked, their plasmids were purified, and the InsTet^{G-}1 insertion sites were determined by sequencing with an outward primer. Twenty-four of these candidates exhibited different insertion loci (data not shown). Most of the sites were found within *bla*, *lacZ*, or the polylinker region, while no insertions occurred in *ori*, thus indicating random target site selection.

Creating pools of *E. coli* mutants with InsTet^{G-}1. To explore the insertion properties of InsTet^{G-}1 in *E. coli*, pools of insertion mutants were created and subsequently screened for auxotrophy. For that purpose, 1 μl of the freshly prepared InsTet^{G-}1–transposase complex (for details, see Material and Methods) was electroporated into freshly prepared electrocompetent cells, which were then spread onto kanamycin/chloramphenicol plates (60 mg/liter Km and 25 mg/liter Cm) and incubated overnight at 37°C. All colonies were washed from the agar plates using LB medium, the suspension was centrifuged and resuspended in 1 ml of LB medium containing 10% (vol/vol) dimethyl sulfoxide, and 100-μl aliquots were stored at −70°C as stocks. Following this procedure, one mutant pool with about 25,000 candidates was created from *E. coli* MG1655/pWH1411BD encoding wt TetR, and another mutant pool containing about 105,000 candidates was created from *E. coli* MG1655/pWH1411^{r2} encoding the reverse TetR^{r2}.

Screening for auxotrophic *E. coli* mutants. We developed a screening protocol by employing 96-well plates so that the growth of the candidates could be monitored by reading the OD_{595}. Aliquots of the mutant pools were streaked onto LB plates containing kanamycin and chloramphenicol so that individual colonies could be picked from the plates. Ninety-six-well plates were supplemented with 200 μl of M9 minimal medium containing 0.2% glucose and kanamycin and chloramphenicol for selection, except for the well containing the wild-type strain, for which kanamycin was omitted. Each well was inoculated from one colony, the resulting suspension was mixed, and 100 μl was transferred into a well of a second plate containing 25 μl of the same medium supplemented with 2 μM atc. Subsequently, 25 μl of minimal medium was added to the wells in the first plate so that all wells contained the same volume of 125 μl of broth. The plates were then incubated at 37°C for 24 h on a stroking shaker in the dark to account for the light sensitivity of atc, and their OD_{595} was determined in a well plate reader. Using this procedure, we obtained four different phenotypes. Most of the strains were prototrophic, showing the same OD_{595} in both wells. The second-largest population displayed an unregulated auxotrophic phenotype (a mutant was scored auxotrophic when the OD_{595} was below 0.1) where mutants could not grow in either minimal medium. The third-largest number of mutants displayed a regulatable auxotrophic phenotype only growing in the absence (*E. coli* strain with TetR^{r2}) or presence (*E. coli* strain with TetR) of atc. The fewest candidates had a reverse regulatable phenotype and were able to grow only in the absence of atc. All these candidates originated from the *E. coli* strain with TetR. All mutants with an auxotrophic phenotype were streaked onto LB plates and restreaked onto minimal medium plates with and without atc to verify their phenotypes. After 36 h of incubation in the dark, the mutants with a confirmed phenotype were grown in

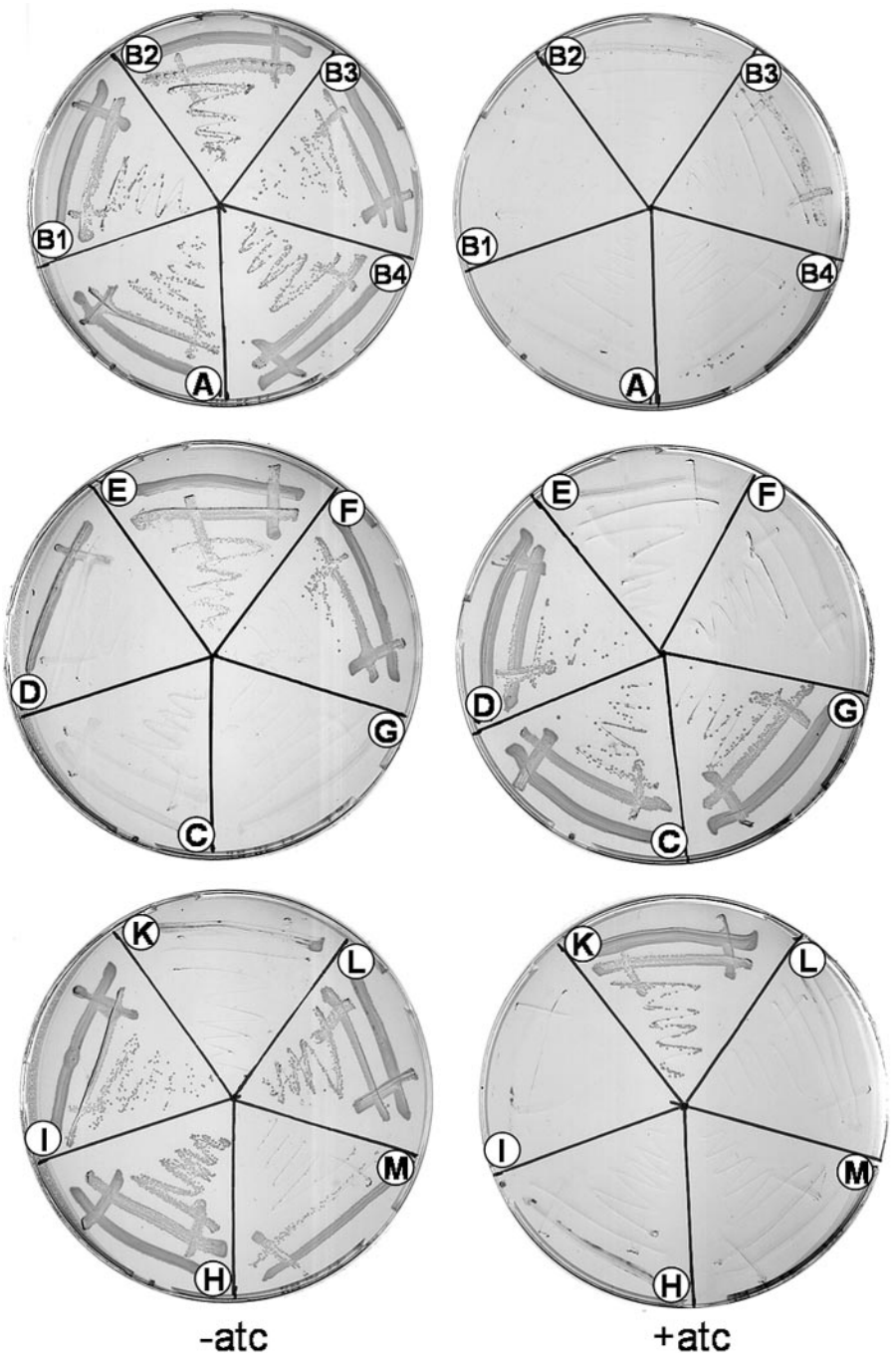

FIG. 3. M9 minimal medium agar plates containing 2% glucose as a carbon source with and without 0.4 μM atc (+atc and −atc, respectively) showing the growth phenotypes of the regulatable *E. coli* candidates. Letters A to M refer to the candidates described in Table 3.

liquid LB medium to obtain stock cultures. The screening procedure described above was carried out twice for each of the mutant pools so that a total of 9,920 candidates were examined, leading to 396 candidates with one of the three auxotrophic phenotypes. However, only 106 of the candidates were confirmed on plates. This is a somewhat disappointing result, which may be related to the different growth conditions in the well plates compared to agar plates. Ninety-one of these mutants had a nonregulatable auxotrophic phenotype, and 15 mutants displayed atc-regulatable auxotrophy, as shown in Fig. 3.

Locations of the InsTet^{G-}1 insertions in *E. coli*. From the 106 mutants with a confirmed phenotype, chromosomal DNA was prepared and restricted with EcoRI, and the insertion sites were determined by sequencing with an InsTet^{G-}1-specific primer. The resulting sequences were analyzed using the BLAST server at the EcoCyc database (http://ecocyc.org). The affected genes are shown in Table 2, where they are grouped

TABLE 2. Insertion mutants of *E. coli* and functions of the affected genes[a]

Classification	Gene(s)	Pathway
Biosynthetic pathways	*argC*, *D*, *E*, *G*	Arginine biosynthesis
	aroB	Chorismate biosynthesis
	bioA, *B*	Biotin biosynthesis
	carB	Pyrimidine and arginine biosynthesis
	cvpA	Colicin V biosynthesis
	cysD, *G*, *J*, ***Q***	Sulfur metabolism
	entB, *F*	Enterobactin biosynthesis
	hflD	Membrane protein in operon with *purB*
	hisB	Histidine biosynthesis
	ilvA, *C*, *D*	Valine, isoleucine biosynthesis
	leuA, *B*, *C*	Leucine biosynthesis
	menD	Menaquinone biosynthesis
	metA, *B*, *C*, *E*	Methionine biosynthesis
	panB, *C*	Pantothenate biosynthesis
	pdxB	Pyridoxal 5′-phosphate biosynthesis
	proA	Proline biosynthesis
	purC, *H*	Purine nucleotide biosynthesis
	pyrD, ***F***	Pyrimidine ribonucleotide biosynthesis
	rfaG	Lipopolysaccharide core biosynthesis
	serA, *C*	Serine biosynthesis
	thiC	Thiamine biosynthesis
	thrA	Homoserine biosynthesis
	trpA, *B*, *C*, ***E***	Tryptophan biosynthesis
	tyrA	Phenylalanine/tyrosine biosynthesis
Catabolism	*fruK*	1-Phosphofructokinase
	gltA	Citrate synthase
	mtlA	Subunit of EIImtlA
	pgi	Phosphoglucose isomerase
	ppc	Phosphoenolpyruvate carboxylase
Regulation	*argP*	Transcriptional regulator
	cspA	Transcriptional activator
	cysB	Transcriptional regulator
	ilvY	Transcriptional regulator
Transporters	***nirC***	Nitrite transporter
	cysP	Thiosulfate ABC transporter
	glcA	Glycolate transporter
	xylE	Xylose major facilitator superfamily transporter
Essential genes	*lhr*	Member of ATP-dependent helicase superfamily II
	mraZ	Member of *dcw* cluster (cell division)
	prmA	Methylation of 50S ribosomal subunit protein L11
	rplM	L13 protein of 50S ribosomal subunit
	sfmH	Involved in fimbrial assembly
	truA	Pseudouridylate synthase I
Unknown function	*b3557*	IS*5*-related protein
	ydeO	Putative AraC-type regulatory protein
	yfgL	Putative dehydrogenase
	yhdP	Putative transport protein
	yidL	Putative AraC-type regulatory protein
	yjhF	Member of Gnt transporter family
	ykgC	Putative oxidoreductase
	yrbK	Conserved hypothetical protein

[a] Genes are classified depending on their function; regulatable candidates are shown in boldface type.

according to their functions. The regulatable mutants are depicted in boldface type. Seventy-four different genes were mutated, with 32 of them showing more than one insertion site. Two to three identical mutants were found only in the genes *ilvC*, *pgi*, and *b3557*. The insertion sites of all mutants are randomly distributed in the *E. coli* chromosome (data not shown). The 9 bp of the short direct repeat flanking the insertion sites and the 1 bp immediately adjacent to it (sequences not shown) agree with the previously published consensus sequence (11), in which only the first of the 9 bp shows a clear preference for one nucleotide. For all other positions, no single nucleotide is present in more than 40% of all isolates. This result clearly demonstrates the random distribution of the insertions and the low redundancy obtained with this mutagenesis protocol.

TABLE 3. All regulatable auxotrophic *E. coli* mutants[a]

Designation	Gene	Function	Phenotype	Regulator
A	*aroB*	3-Dehydroquinate synthase	regaux	revTetR^{r2}
B1	*b3557*	IS*5* protein	revreg	TetR
B2	*b3557*	IS*5* protein	revreg	TetR
B3	*b3557*	IS*5* protein	revreg	TetR
B4	*cspA*	Intergenic region downstream of *cspA ccw*	revreg	TetR
C	*cysP*	Thiosulfate ABC transporter	regaux	TetR
D	*cysQ*	Involved in sulfite synthesis	regaux	TetR
E	*ilvY*	Transcriptional dual regulator	regaux	revTetR^{r2}
F	*mraZ*	Involved in cell wall synthesis and cell division	regaux	revTetR^{r2}
G	*nirC*	Nitrite FNT transporter	regaux	TetR
H	*pyrF*	Subunit of orotidine-5′-phosphate-decarboxylase	regaux	revTetR^{r2}
I	*rplM*	Upstream of *rplM* (product, 50S ribosomal subunit protein L13)	regaux	revTetR^{r2}
K	*thiC*	Thiamine biosynthesis	regaux	TetR
L	*trpE/trpL*	Subunit of anthranilate synthase	regaux	revTetR^{r2}
M	*yrbK*	Conserved hypothetical protein	regaux	revTetR^{r2}

[a] The involved genes are depicted with their functions. "regaux" indicates the regulatable auxotrophic phenotype, whereas "revreg" denotes the reverse regulatable phenotype. "revTetR^{r2}" denotes the reverse TetR^{r2} variant present in the respective strains.

Regulation of expression of the *E. coli* InsTet^{G-}1 insertion mutants. The 15 atc-regulatable mutants are summarized in Table 3 in alphabetical order of their affected genes. The two different phenotypes (regulated auxotrophy [regaux] and reverse regulated auxotrophy [revreg]) and the regulator type, TetR or revTetR^{r2}, are depicted as well. They clearly demonstrate the advantages resulting from the variation of regulation for obtaining different types of mutants. Most notably, regulation by revTetR^{r2} opens the window for the direct detection of essential genes that reveal their auxotrophy only in the presence of atc. Until now, this was only possible by negative selection (18). The genes surrounding the insertion sites are depicted in Fig. 4.

Eight mutants regulated by TetR were found. Four of them showed a normal phenotype, and four showed a reverse regulated phenotype in which the mutants cannot grow in the presence of atc in minimal medium. The reverse regulated mutants, designated B1 to B4, show insertion sites in the same orientation behind *hokA* (Fig. 4). This gene encodes a small toxic peptide and originates from plasmid R1, where the *hok/sok* locus mediates plasmid stabilization by the killing of plasmid-free cells. The *hokA* gene on the *E. coli* chromosome is not active due to an insertion of an IS*150* element just upstream of the reading frame (27). We therefore assume that P_A drives transcription of the toxin gene, causing cell death in the presence of atc. The observation that these mutants also die on rich medium with atc (data not shown) corroborates this interpretation. The other four TetR-regulated mutants, designated C, D, G, and

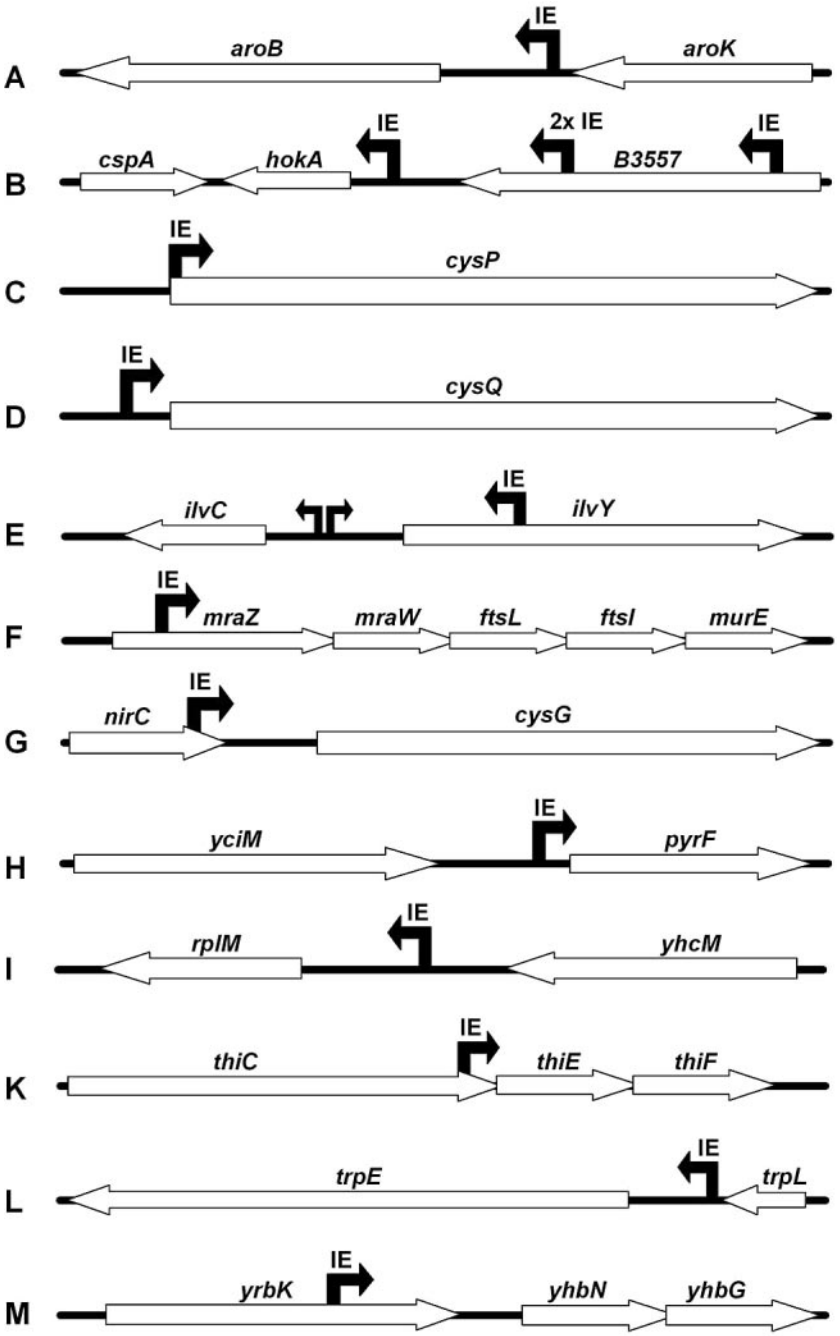

FIG. 4. Schematic overview of all insertion sites of the regulatable *E. coli* candidates found in the screens. "IE" depicts the insertion site of the element. The black arrow represents the direction of the *tetA* promoter.

K in Fig. 4 and Table 3, show a normal auxotrophic behavior due to mutations in biosynthetic pathways.

Seven revTetR^{r2}-regulated mutants were found. Four of them, designated A, E, H, and L in Fig. 4 and in Table 2, contain insertions in genes encoding biosynthetic pathways. The E mutant shows an interesting genotype, with the promoter of the insertion element oriented towards *ilvC* (36). Thus, InsTet^{G-}1 located in *ilvY* has replaced the regulator of *ilvC*, obviously making its expression dependent on the presence of atc. The other three mutants, designated F, I, and M, contain insertions in front of essential genes, as verified by their lack of growth on LB plates with atc. Mutant F contains InsTet^{G-}1 in *mraZ*, the first gene in the *dcw* cluster. This operon is essential for cell wall synthesis and cell division (35). The I mutant contains the 50S ribosomal subunit protein L13 under atc control, and the M mutant contains InsTet^{G-}1 at the 3′ end of *yrbK*, with P_A directed towards *yhbG* and *yhbN*, encoding a putative ABC transporter for which an arabinose-regulated mutant with the same phenotype has been described previously (32). The *yrbK* knockout does not have a detectable

phenotype on glucose minimal medium. Thus, InsTet^{G-}1 seems to block transcription from an upstream promoter, while P_A drives the transcription of the two ABC transporter-encoding genes *yhbNG*. This is an example of identifying unknown genes and enabling their direct analysis by regulated expression using InsTet^{G-}1.

Application of InsTet^{G-}1 to *Salmonella* serovar Typhimurium. In order to analyze genes in *Salmonella* serovar Typhimurium, a well-understood pathogenic relative of *E. coli*, it was transformed with plasmid pWH1411BD or pWH1411^{r2}. Mutant pools of *Salmonella* serovar Typhimurium were created with InsTet^{G-}1 according the *E. coli* protocol described above, except that a fivefold-higher amount of InsTet^{G-}1–transposase complexes had to be used for electroporation to obtain large pools of insertion mutants. One pool of about 156,000 candidates contained TetR-regulated mutants. The screening of 1,880 randomly chosen candidates on microtiter plates yielded 33 auxotrophic strains. Twenty-eight of those strains were confirmed on solid medium. Of these, 25 mutants exhibited constitutive auxotrophy, while 1 mutant displayed auxotrophy in the absence of atc and 2 mutants showed auxotrophy in the presence of atc. The screening of 1,600 randomly chosen candidates from another pool containing about 10,200 candidates yielded 15 auxotrophic strains. This rather low yield of auxotrophic mutants prompted the application of a more efficient screen. After an ampicillin enrichment of the pool containing 10,200 candidates, 700 candidates were screened. One hundred ninety-four of these were auxotrophs, and four showed auxotrophy in the absence of atc. Thirty-seven of these 198 mutants were sequenced, with the results displayed in Table 4.

Ampicillin enrichment of intracellular growth-deficient *Salmonella* serovar Typhimurium mutants. We next attempted to enrich growth-deficient mutants of *Salmonella* serovar Typhimurium with ampicillin in the murine macrophage-like cell line J774-A.1 using an approach similar to the ones previously described for *Listeria monocytogenes* (6) and *Salmonella* serovar Typhimurium (24). J774-A.1 cells were infected with the insertion mutant library (for details, see Materials and Methods). We found two different *Salmonella* serovar Typhimurium mutants that survived the ampicillin treatment in J774-A.1 cells. Mutant A has an InsTet^{G-}1 insertion in *pyrE*, encoding orotate phosphoribosyltransferase, and mutant B has the insertion in *argA*, encoding *N*-acetylglutamate synthase. These two genes have not been specifically recognized as essential for intracellular growth previously, although pyrimidine auxotrophy leads to an intracellular growth deficiency (24).

Conclusion. The constructed insertion element InsTet^{G-}1 generates conditional knockout mutants in *E. coli* and *Salmonella* serovar Typhimurium that respond to the presence of atc. The use of revTetR^{r2} or TetR leads to the generation of mutants that can grow only in the presence or in the absence of atc. In principle, induction of transcription can lead to the induction of expression of a gene fused downstream of the P_A promoter. However, it may also lead to repression of expression when the affected gene is silenced by antisense RNA. The yield of regulated mutants is expected to be much lower than that of obtaining unregulated auxotrophs, but the successful enrichment by counterselection, even in intracellularly growing *Salmonella*, should greatly facilitate the isolation of a sufficiently high number of regulated auxotrophs. Since the inducer is able to enter most bacterial cells without requiring an uptake protein, this insertion mutagenesis should be widely applicable, as has recently been demonstrated for *Bacillus subtilis* (2). Moreover, since atc and its analogs are not metabolized by higher organisms or bacteria, the regulation should also work fine in in vivo models of pathogenicity, like infected mice, which can be provided with atc in their drinking water or by infusion. Thus, this approach should be a useful tool for the functional analysis of known genes under various conditions or for elucidating the function of unknown genes.

TABLE 4. Insertion mutants found in *Salmonella serovar* Typhimurium[a]

Gene	Function	Phenotype
argC	*N*-Acetyl-γ-glutamylphosphate reductase	aux
argE	Acetylornithine deacetylase	aux
argG	Argininosuccinate synthetase	aux
argI	Ornithine carbamoyltransferase 1	aux
aroB	3-Dehydroquinate synthase	aux
carB	Carbamoyl-phosphate synthase large subunit	aux
cysC	Adenylylsulfate kinase	aux
cysH	Phosphoadenosine phosphosulfate reductase	aux
cysI	Sulfite reductase α subunit	aux
cysJ	Sulfite reductase β subunit	aux
cysP	Thiosulfate transport protein	aux
cysW	Thiosulfate permease W protein	aux
glnA	Glutamine synthetase	aux
hisC	Histidinol-phosphate aminotransferase	aux
hisD	Histidinol dehydrogenase	aux
ilvA	Threonine dehydratase	aux
ilvC	Ketol acid reductoisomerase	aux
ilvD	Dihydroxy acid dehydratase	aux
ilvE	Branched-chain amino acid aminotransferase	aux
ilvY	Regulatory protein	aux
leuA	2-Isopropylmalate synthase	aux
leuC	Isopropylmalate isomerase large subunit	aux
metA	Homoserine *O*-succinyltransferase	aux
metR	***metE*/*metH* regulator**	**regaux**
nadB	L-Aspartate oxidase	aux
panC	Pantoate-β-alanine ligase	aux
ppc	Phosphoenolpyruvate carboxylase	aux
purC	Phosphoribosylaminoimidazole-succinocarboxamide synthase	aux
purD	Phosphoribosylglycinamide synthetase	aux
purF	Amidophosphoribosyltransferase	aux
purH	Bifunctional phosphoribosylamino-imidazolecarboxamide formyltransferase/IMP cyclohydrolase	aux
pyrD	Dihydroorotate dehydrogenase	aux
pyrF	Orotidine 5′-phosphate decarboxylase	aux
recE	**Exodeoxyribonuclease VIII**	**revreg**
rfbI	CDP-6-deoxy-Δ-3,4-glucoseen reductase	aux
STM 4450	**Putative inner membrane protein**	**revreg**
serA	D-3-Phosphoglycerate dehydrogenase	aux
serB	3-Phosphoserine phosphatase	aux
serC	**Phosphoserine aminotransferase**	**regaux**
thrC	Threonine synthase	aux

[a] Regulatable candidates are shown in boldface type. The involved genes are depicted with their encoded functions. "aux" indicates a constitutive auxotrophic phenotype, and "regaux" denotes a regulatable auxotrophic phenotype, whereas "revreg" indicates the reverse regulatable phenotype. TetR was used as the regulator in the screen.

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REFERENCES

1. **Bender, J., and N. Kleckner.** 1992. Tn*10* insertion specificity is strongly dependent upon sequences immediately adjacent to the target-site consensus sequence. Proc. Natl. Acad. Sci. USA **89:**7996–8000.
2. **Bertram, R., M. Köstner, J. Müller, J. Vazquez Ramos, and W. Hillen.** 2005. Integrative elements for *Bacillus subtilis* yielding tetracycline-dependent growth phenotypes. Nucleic Acids Res. **33:**e153.
3. **Bertram, R., C. Kraft, S. Wisshak, J. Mueller, O. Scholz, and W. Hillen.** 2004. Phenotypes of combined tet repressor mutants for effector and operator recognition and allostery. J. Mol. Microbiol. Biotechnol. **8:**104–110.
4. **Bethesda Research Laboratories.** 1986. BRL pUC host: *E. coli* DH5α competent cells. Bethesda Res. Lab. Focus **8:**9.
5. **Blattner, F. R., G. Plunkett III, C. A. Bloch, N. T. Perna, V. Burland, M. Riley, J. Collado-Vides, J. D. Glasner, C. K. Rode, G. F. Mayhew, J. Gregor, N. W. Davis, H. A. Kirkpatrick, M. A. Goeden, D. J. Rose, B. Mau, and Y. Shao.** 1997. The complete genome sequence of *Escherichia coli* K-12. Science **277:**1453–1474.
6. **Camilli, A., C. R. Paynton, and D. A. Portnoy.** 1989. Intracellular methicillin selection of *Listeria monocytogenes* mutants unable to replicate in a macrophage cell line. Proc. Natl. Acad. Sci. USA **86:**5522–5526.
7. **Chong, S., F. B. Mersha, D. G. Comb, M. E. Scott, D. Landry, L. M. Vence, F. B. Perler, J. Benner, R. B. Kucera, C. A. Hirvonen, J. J. Pelletier, H. Paulus, and M. Q. Xu.** 1997. Single-column purification of free recombinant proteins using a self-cleavable affinity tag derived from a protein splicing element. Gene **192:**271–281.
8. **Chow, W. Y., and D. E. Berg.** 1988. Tn5tac1, a derivative of transposon Tn5 that generates conditional mutations. Proc. Natl. Acad. Sci. USA **85:**6468–6472.
9. **Eckert, B., and C. F. Beck.** 1989. Overproduction of transposon Tn*10*-encoded tetracycline resistance protein results in cell death and loss of membrane potential. J. Bacteriol. **171:**3557–3559.
10. **Goryshin, I. Y., J. Jendrisak, L. M. Hoffman, R. Meis, and W. S. Reznikoff.** 2000. Insertional transposon mutagenesis by electroporation of released Tn*5* transposition complexes. Nat. Biotechnol. **18:**97–100.
11. **Goryshin, I. Y., J. A. Miller, Y. V. Kil, V. A. Lanzov, and W. S. Reznikoff.** 1998. Tn*5*/IS*50* target recognition. Proc. Natl. Acad. Sci. USA **95:**10716–10721.
12. **Goryshin, I. Y., and W. S. Reznikoff.** 1998. Tn*5* in vitro transposition. J. Biol. Chem. **273:**7367–7374.
13. **Gossen, M., and H. Bujard.** 2002. Studying gene function in eukaryotes by conditional gene inactivation. Annu. Rev. Genet. **36:**153–173.
14. **Guerout-Fleury, A. M., K. Shazand, N. Frandsen, and P. Stragier.** 1995. Antibiotic-resistance cassettes for *Bacillus subtilis*. Gene **167:**335–336.
15. **Gülland, U., and W. Hillen.** 1992. The Tn*10*-encoded *tetR* mRNA has heterogeneous 5′ ends in vivo and in vitro. Gene **114:**97–101.
16. **Hare, R. S., S. S. Walker, T. E. Dorman, J. R. Greene, L. M. Guzman, T. J. Kenney, M. C. Sulavik, K. Baradaran, C. Houseweart, H. Yu, Z. Foldes, A. Motzer, M. Walbridge, G. H. Shimer, Jr., and K. J. Shaw.** 2001. Genetic footprinting in bacteria. J. Bacteriol. **183:**1694–1706.
17. **Hayes, F.** 2003. Transposon-based strategies for microbial functional genomics and proteomics. Annu. Rev. Genet. **37:**3–29.
18. **Hensel, M., J. E. Shea, C. Gleeson, M. D. Jones, E. Dalton, and D. W. Holden.** 1995. Simultaneous identification of bacterial virulence genes by negative selection. Science **269:**400–403.
19. **Henssler, E. M., R. Bertram, S. Wisshak, and W. Hillen.** 2005. Tet repressor mutants with altered effector binding and allostery. FEBS J. **272:**4487–4496.
20. **Hidalgo, A. A., A. N. Trombert, J. C. Castro-Alonso, C. A. Santiviago, B. R. Tesser, P. Youderian, and G. C. Mora.** 2004. Insertions of mini-Tn*10* transposon T-POP in *Salmonella enterica* sv. Typhi. Genetics **167:**1069–1077.
21. **Karlinsey, J. E., S. Tanaka, V. Bettenworth, S. Yamaguchi, W. Boos, S. I. Aizawa, and K. T. Hughes.** 2000. Completion of the hook-basal body complex of the *Salmonella typhimurium* flagellum is coupled to FlgM secretion and *fliC* transcription. Mol. Microbiol. **37:**1220–1231.
22. **Kofoid, E., C. Rappleye, I. Stojiljkovic, and J. Roth.** 1999. The 17-gene ethanolamine (*eut*) operon of *Salmonella typhimurium* encodes five homologues of carboxysome shell proteins. J. Bacteriol. **181:**5317–5329.
23. **Krueger, C., K. Pfleiderer, W. Hillen, and C. Berens.** 2004. Tetracycline derivatives: alternative effectors for Tet transregulators. BioTechniques **37:**546, 548, 550.
24. **Leung, K. Y., and B. B. Finlay.** 1991. Intracellular replication is essential for the virulence of *Salmonella typhimurium*. Proc. Natl. Acad. Sci. USA **88:**11470–11474.
25. **Miller, J. H.** 1992. A short course in bacterial genetics: laboratory manual, p. 72–74. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
26. **Moir, D. T., K. J. Shaw, R. S. Hare, and G. F. Vovis.** 1999. Genomics and antimicrobial drug discovery. Antimicrob. Agents Chemother. **43:**439–446.
27. **Pedersen, K., and K. Gerdes.** 1999. Multiple hok genes on the chromosome of *Escherichia coli*. Mol. Microbiol. **32:**1090–1102.
28. **Rappleye, C. A., and J. R. Roth.** 1997. A Tn*10* derivative (T-POP) for isolation of insertions with conditional (tetracycline-dependent) phenotypes. J. Bacteriol. **179:**5827–5834.
29. **Schneider, K., and C. F. Beck.** 1986. Promoter-probe vectors for the analysis of divergently arranged promoters. Gene **42:**37–48.
30. **Scholz, O., E. M. Henssler, J. Bail, P. Schubert, J. Bogdanska-Urbaniak, S. Sopp, M. Reich, S. Wisshak, M. Köstner, R. Bertram, and W. Hillen.** 2004. Activity reversal of Tet repressor caused by single amino acid exchanges. Mol. Microbiol. **53:**777–789.
31. **Scholz, O., M. Köstner, M. Reich, S. Gastiger, and W. Hillen.** 2003. Teaching TetR to recognize a new inducer. J. Mol. Biol. **329:**217–227.
32. **Serina, S., F. Nozza, G. Nicastro, F. Faggioni, H. Mottl, G. Deho, and A. Polissi.** 2004. Scanning the *Escherichia coli* chromosome by random transposon mutagenesis and multiple phenotypic screening. Res. Microbiol. **155:**692–701.
33. **Sigler, A., P. Schubert, W. Hillen, and M. Niederweis.** 2000. Permeation of tetracyclines through membranes of liposomes and *Escherichia coli*. Eur. J. Biochem. **267:**527–534.
34. **Thanassi, D. G., G. S. Suh, and H. Nikaido.** 1995. Role of outer membrane barrier in efflux-mediated tetracycline resistance of *Escherichia coli*. J. Bacteriol. **177:**998–1007.
35. **Vicente, M., M. J. Gomez, and J. A. Ayala.** 1998. Regulation of transcription of cell division genes in the *Escherichia coli dcw* cluster. Cell. Mol. Life Sci. **54:**317–324.
36. **Wek, R. C., and G. W. Hatfield.** 1988. Transcriptional activation at adjacent operators in the divergent-overlapping *ilvY* and *ilvC* promoters of *Escherichia coli*. J. Mol. Biol. **203:**643–663.
37. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33:**103–119.
38. **Zhu, Z., T. Zheng, C. G. Lee, R. J. Homer, and J. A. Elias.** 2002. Tetracycline-controlled transcriptional regulation systems: advances and application in transgenic animal modeling. Semin. Cell Dev. Biol. **13:**121–128.