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Integrative elements for *Bacillus subtilis* yielding tetracycline-dependent growth phenotypes

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ABSTRACT

We describe the construction and application of elements for random insertion of promoter containing DNA into the genome of *Bacillus subtilis*. The outward-facing promoter of these integrative elements termed InsTet^{G+} is inducible by tetracycline so that conditional mutants are generated. We constructed three InsTet^{G+} variants using different regulatory windows. In the first, the regulator gene *tetR* is located within the element, allowing one-step mutagenesis. The second contains *tetR* in the chromosome and yields the best regulation efficiency. The third exploits xylose-dependent *tetR* expression from a plasmid, enabling induction of TetR synthesis so that distinct expression levels of an affected gene can be adjusted. We have obtained mutant strains with all three variants. For some of them, growth can be modulated by the presence of effectors. Most growth defects occur in the presence of inducers, presumably due to regulated expression of antisense RNA.

INTRODUCTION

The continuously rising number of sequenced prokaryotic genomes yields many genes with unknown function. Despite the availability of extensive databases for gene annotation by homology to known genes, this is the case for ~30–40% of postulated genes in each genome. Delineation of their function is often achieved by generating targeted disruptions followed by the phenotypic analysis. To circumvent time-consuming gene-by-gene deletion approaches (1), transposons can be used to generate a saturating pool of random insertion mutants. Genes critical for growth, however, require adjacent integration of elements with regulatable outward-facing promoters to obtain conditional knockouts. Transposon-derived elements containing arabinose-, IPTG- or tetracycline-sensitive promoters have been described for Gram negative bacteria [reviewed in (2)]. To avoid the inherent mobility of transposons, the transposase encoding genes have been eliminated after insertion has taken place. For example, a Tn*10* transposase encoding gene together with an integrative element has been placed on a thermosensitively replicating plasmid in *Bacillus subtilis*, which can be removed by growth at the non-permissive temperature (3). Extracellular integration of transposon-like elements, derived from Tn*5*, Tn*7*, *Mu*, *Himar1* or Ty*1*, into DNA has been accomplished using suitable transposase proteins [reviewed in (4)]. The modified DNA must then be introduced into the host.

The most elegant technique involves electroporation of the so-called transposome complexes into cells (5). These consist of two molecules of mutant Tn*5*-type transposase bound to two mosaic elements flanking a DNA sequence of choice. These transposomes can be introduced into a number of unicellular organisms, where they are activated by cytoplasmic Mg^{2+} ions to be inserted into the genome. Since no transposase-encoding DNA enters the cell, the obtained insertion mutants are genetically stable. This approach has meanwhile been described for different bacteria and for single cell eukaryotes [http://www.epicentre.com/transcite.asp; (6,7)].

We describe the construction of integrative elements with tc-sensitive outward promoters and their use for mutagenesis of *B.subtilis*. The efficiency of tc-regulation has been demonstrated in various Gram positive bacteria, such as *Staphylococcus*, *Streptococcus*, *Mycobacterium*, *Streptomyces* and *Bacillus* (8–14). We have obtained a number of conditional growth deficient or auxotrophic *B.subtilis* strains using these constructs.

MATERIALS AND METHODS

Anhydrotetracycline (atc) was purchased from Acros (Geel, Belgium). All other chemicals were from Merck (Darmstadt, Germany), Roth (Karlsruhe, Germany) and Sigma (Munich, Germany) at the highest purity available. Enzymes for DNA restriction and modification were obtained from New England

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Table 1. Bacterial strains used in this study

Strains or plasmids	Relevant characteristics	Reference or source
Escherichia coli DH5α	*recA*1 *endA*1 *gyrA*96 *thi relA*1*hsd*R17 (r_K^-, m_K^+) *supE*44 Φ80d*lacZ*Δ*m15* Δ*lac*U169	(46)
WH601Δ	DH5α carrying InsTet^{G+}1, *aphAIII* cassette excised by Cre recombinase (Km^S)	This study
Bacillus subtilis 168	*trpC2*	Laboratory stock
WH502	*trpC2 amyE*::>InsTet^{G+}1>-*lacZ*	This study
WH502Δ	*trpC2 amyE*::>InsTet^{G+}1Δ*tetR*>-*lacZ*	This study
WH503	*trpC2 amyE*::>InsTet^{G+}1a>-*lacZ*	This study
WH510	*trpC2 yhaO*::>InsTet^{G+}1>	This study
WH511	*trpC2 yaaQ*::<InsTet^{G+}1<	This study
WH512	*trpC2* >InsTet^{G+}1>-*kinA*	This study
WH525	*trpC2 spsB*::>InsTet^{G+}2>	This study
WH526	*trpC2* pWH119 *bla*::<InsTet^{G+}2<	This study
WH527	*trpC2 cysE*::>InsTet^{G+}2>	This study
WH528	*trpC2* >InsTet^{G+}2>-*tagD*	This study
WH529	*trpC2 yufL*::>InsTet^{G+}2>	This study
WH530	*trpC2 yaaO*::>InsTet^{G+}2>	This study
WH531	*trpC2 hisH*::>InsTet^{G+}2>	This study
WH532	*trpC2 lacA*::*Pt17-tetR-aphAIII yvaO*::<InsTet^{G+}2^{Cm}<	This study
WH533	*trpC2 lacA*::*Pt17-tetR-aphAIII yydB*::<InsTet^{G+}2^{Cm}<	This study
WH534	*trpC2 lacA*::*Pt17-tetR-aphAIII yydB*::<InsTet^{G+}2^{Cm}<	This study
WH535	*trpC2 lacA*::*Pt17-tetR-aphAIII proB*::<InsTet^{G+}2^{Cm}<	This study
WH536	*trpC2 lacA*::*Pt17-tetR-aphAIII pbpB*::<InsTet^{G+}2^{Cm}<	This study
WH555	*trpC2 lacA*::*P*-tetR-aphAIII*	This study
WH556	*trpC2 lacA*::*P*-tetR-aphAIII amyE*::>InsTet^{G+}2^{Cm}>-*lacZ*	This study
WH557	*trpC2 lacA*::*Pt17-tetR-aphAIII*	This study
WH558	*trpC2 lacA*::*Pt17-tetR-aphAIII amyE*::>InsTet^{G+}2^{Cm}>-*lacZ*	This study
WH560	*trpC2 amyE*::>InsTet^{G+}2^{Cm}>-*lacZ*	This study
WH570	*trpC2 amyE*::>InsTet^{G+}2>-*lacZ*	This study
Plasmids		
pAC6	Integrative plasmid for *B.subtilis amyE*	(20)
pHT304	Ap^r, Er^r, pUC19 polylinker	(27)
pUC19	Ap^R, *lacZα*, pMB1 ori	(47)
pWH119	pHT304 with *xylA* promoter from *B.subtilis* 168 upstream of *tetR*	(13)
pWH125	pHT304 with synthetic promoter upstream of *revtetR r2*	(13)
pWH1941	pAC6 with InsTet^{G+}0 upstream of *lacZ*	This study
pWH1942	Integrative plasmid for *B.subtilis lacA*	This study
pWH353	Km^r, Ap^r, improved Pr* promoter upstream of *tetR(B)*	(14)
pWH354	Km^r, Ap^r, improved Pr* promoter with second *tetO* upstream of *tetR(B)*	(14)

Square parentheses denote promoter direction in InsTet^{G+}.

Biolabs (Frankfurt/Main, Germany), Fermentas (St Leon-Rot, Germany), Roche (Mannheim, Germany), Stratagene (Heidelberg, Germany) and PeqLab (Erlangen, Germany), and were used according to the manufacturer's recommendations. Isolation and manipulation of DNA was performed using standard techniques. Oligonucleotides were purchased from MWG-Biotech (Ebersberg, Germany) and TIB-Molbiol (Berlin, Germany). Sequencing was carried out according to the protocol provided by the manufacturer for cycle sequencing and analyzed using an ABI PRISM^{TM} 310 Genetic Analyzer (Applied Biosystems, Weiterstadt, Germany).

Bacterial strains and plasmids

The bacterial strains and plasmids used and generated in this study are listed in Table 1. All *B.subtilis* strains are based upon the wild-type strain 168. Chromosomal integration into the *amyE* locus was verified on starch containing media plates by the loss of amylase activity, whereas integration into the *lacA* locus was checked by the PCR analysis. Cloning was performed in *Escherichia coli* DH5α unless stated otherwise. For manipulation of InsTet elements without encoded *tetR*, we used *E.coli* WH601Δ, which was obtained as follows: InsTet^{G+}1 (see below) was brought into *E.coli* DH5α via transposome mutagenesis (5). One mutant strain was subsequently transformed using the thermosensitive Cre recombinase expressing plasmid p2266 (W. Hammerschmidt, unpublished data). By adjusting appropriate temperatures, the kanamycin resistance marker of InsTet^{G+}1 was excised by Cre recombinase, since it was flanked by asymmetric *loxP* sites (15). The resulting strain WH601Δ expresses *tetR* and temporarily represses *tetO* vested promoters.

Construction of the integrative elements

The integrative elements were designated InsTet^{G+} for insertable tetracycline responsive promoter elements for Gram positive bacteria. Figure 1 schematically illustrates the key steps in construction. The backbone of InsTet^{G+} was assembled using oligonucleotides for hybridization with an overlap of 12 bp. Sequences of oligonucleotides are given in Supplementary Table 1. Single-stranded oligonucleotides were phosphorylated by T4 polynucleotide kinase before hybridization, which was performed by heating 32.5 pmol of each of the two complementary oligonucleotides to 95°C and cooling them down to room temperature within 2 h. Ligation of all four double-stranded fragments was performed with T4-DNA ligase at 15°C overnight. The gel-purified fragment

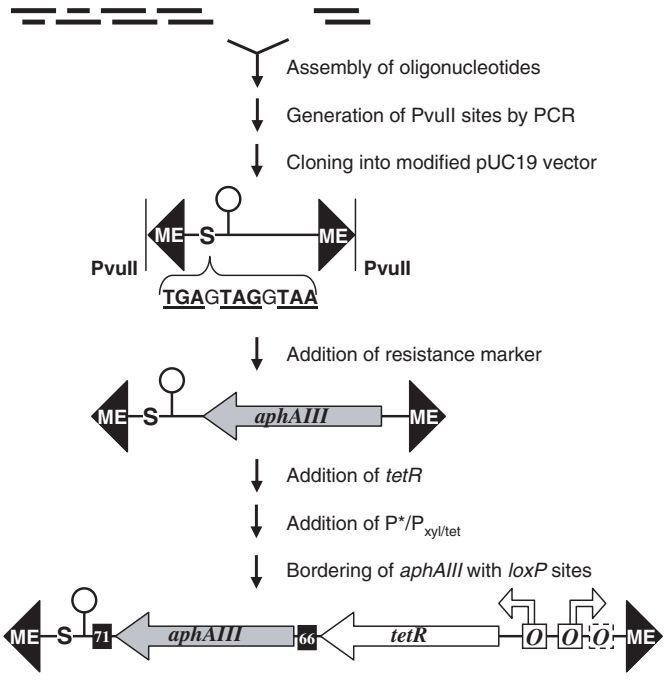

Figure 1. Key steps of InsTet^{G+} construction. Closed bars symbolize single-stranded oligonucleotides for hybridization. Closed triangles represent the MEs for Tn*5* transposase binding. Three successive stop codons are symbolized by capital 'S' and are given below. The Tn*10* derived transcriptional terminator is drawn as a hairpin. The numbers 71 and 66 represent the *loxP* sites *lox71* and *lox66*, flanking the kanamycin resistance cassette (*aphAIII*). Open arrows denote P* (for *tetR* expression) and $P_{xyl/tet}$ (as regulatable outward-facing promoter). Boxes marked as '*O*' represent *tet*-operators.

was cloned into pUC19, cut with NdeI and HindIII, giving rise to pWH1934. The contained element carries mosaic elements (MEs) for Tn*5* transposase binding at both ends, stop codons in all three forward reading frames, a Tn*10* originated transcriptional terminator (16) and several restriction sites for cloning. After that, the phosphorylated and hybridized oligonucleotides RBTns_fw_2 and RBTns_rev_2b were integrated into the NcoI site. One candidate was chosen that contained the newly inserted PacI site positioned adjacent to the transcriptional terminator. In order to flank the element with PvuII restriction sites, the sequence was amplified by PCR using oligonucleotides RBTns_Pvu_fw and RBTns_Pvu_rev. To reduce restriction sites in the vector backbone, pUC19 was modified such that the singular NdeI site became an additional PvuII site. This was achieved by site-directed mutagenesis, using oligonucleotides Nde_Pvu_fw and Nde_Pvu_rev, and a method similar to that described as QuikChange-Mutagenesis (Stratagene, La Jolla, CA); however, by using Pwo Polymerase and adding a ligation step before to the transformation of *E.coli*. The PvuII cut plasmid and the likewise digested PCR product of the InsTet element were ligated and one candidate with the KpnI site of InsTet close to the 5′ end of the *bla* gene was termed pWH1935. As a marker for kanamycin selection, an *aphAIII* gene, originally cloned from plasmid pDG792 (17), was cut out of pWH1866 (M. Köstner, unpublished data) using NcoI and cloned into the likewise digested pWH1935. This gave rise to pWH1935-0, which contains InsTet^{G+}0, with the *aphAIII* cassette in the opposite direction, as *bla* in the carrier vector (Figure 1, middle part). *TetR* was PCR-amplified from pWH1925 (18) and modified for cloning by PCR using oligonucleotides DP6mut and qac_bw. The product was restricted with XbaI and BstEII and cloned into pWH1935-0. The resulting plasmid and pWH353(BD), bearing chimeric *tetR(BD)* and the divergent promoters P* and $P_{xyl/tet}$ (14), were subsequently restricted with KpnI and StuI. Then thereby excised P*/$P_{xyl/tet}$ region was cloned into the pWH1935 derivative. An *aphAIII* cassette was amplified by PCR from pDG792 with oligonucleotides loxP71_KmR and loxP66_KmR containing asymmetric *loxP* sites to enable Cre recombinase mediated excision of *aphAIII* (19). The product was restricted with PacI and HpaI and cloned into the plasmid described above and the resulting vector was termed pWH1935-1 and contained InsTet^{G+}1 [Figures 1 (lower part) and 2A (upper part)]. In order to measure the maximal expression exerted by the outward promoter located on the element, we substituted *tetR* in pWH1935-1 for a non-functional *tetR* sequence lacking 16 bp within the gene. This was achieved by cloning via ApaI/StuI and was carried out using *E.coli* WH601Δ as a host.

pWH1935-1a was obtained by the restriction of pWH1935-1 with XhoI and KpnI and ligation of a 129 bp fragment released from pWH354, upon restriction with the same enzymes. As a result, pWH1935-1a harbours InsTet^{G+}1a, containing two *tet*-operators within the outward promoter region (Figure 2A, lower part). pWH1935-2^{Cm} (with InsTet^{G+}2^{Cm}, see Figure 4A) was constructed as follows: a *cat* cassette was amplified from the plasmid pWH105 (13) using oligonucleotides CmR_fw1 and CmR_rev1 thereby inserting restriction sites for XhoI and PacI. The resistance marker was cloned into the likewise restricted pWH1935-1a. pWH1935-2, carrying InsTet^{G+}2 (Figure 5A), was obtained by the deletion of P* and the *tetR* gene of pWH1935-1a via XhoI and NcoI, creating blunt ends with the Klenow fragment, and religation. Cloning of both InsTet^{G+}2 elements was performed in *E.coli* WH601Δ. The nucleotide sequences of InsTet^{G+}1, InsTet^{G+}1a, InsTet^{G+}2 and InsTet^{G+}2^{Cm} are available upon request.

Construction of strains for β-galactosidase measurements

For *in vivo* quantification of the regulatory capacities of the elements, they were cloned into a plasmid pAC6 background, which carries a promoterless *lacZ* gene flanked by *amyE* sequences for integration into *B.subtilis* (20). Since this plasmid has an origin of replication for *E.coli* only, it needs to be integrated by homologous recombination in order to confer kanamycin resistance to *Bacillus*. Initially, InsTet^{G+}0 was cloned into pAC6 via StuI and SmaI. One candidate with *aphAIII* divergent to *lacZ* was termed pWH1941. The relevant new portions of the other InsTet^{G+} variants were cloned into pWH1941 via PacI and KpnI. Owing to detrimental effects of the strong promoters encountered during cloning in *E.coli*, ligation products were directly used for the transformation of *B.subtilis* 168, as described previously (21). β-galactosidase measurements of mid-log cultures were carried out as described previously (13).

Construction of strains with chromosomally located *tetR*

The P*-*tetR-aphAIII* region of InsTet^{G+}1 was cloned via KpnI and SacII into pWH1942. This plasmid is a derivative of

pBluescript and as such cannot replicate in *Bacillus*. It contains the abovementioned restriction sites in a region between the 5′ and the 3′ regions (500 bp each) of the *B.subtilis lacA* gene. Hence, pWH1942-P*-*tetR-aphAIII* could be integrated into *B.subtilis lacA* via homologous recombination. Thereby, *B.subtilis* WH555 was obtained. The resulting plasmid was further modified through cloning of a pool of synthetic promoter fragments (13) via EcoRI and XbaI and inserted into *lacA*. Thereby, one obtained and analyzed strain carried the promoter sequence GAATTCCCGGGAAATAAAAAACTAGTTTGCCAAATAACTCCACCAATGATATAATGTCAACAAAAAGGAGGTATTAATGATGTCTAGA, which was termed Pt17.

Transposome formation

Transposase was cloned from IS*50*R and mutated at three positions to obtain a hyper- and transactive protein encoding gene (22). Overexpression of transposase was achieved through IPTG induction and purification was performed using the IMPACT-CN system of New England Biolabs (Frankfurt/Main, Germany).

The InsTet^{G+} elements were obtained upon PvuII restriction of the respective carrier plasmids, all of which are derivatives of pWH1935. Fragments were gel-purified, reconstituted using GFX (Pharmacia, Freiburg, Germany) and DNA concentrations were determined by UV-spectroscopy. Formation of transposomes was conducted by mixing appropriate amounts of InsTet^{G+} DNA (see Results) with a 5-fold molar excess of monomeric transposase in a reaction tube, after adjustment to a final concentration of 5% glycerol in deionized water. Transposomes were allowed to assemble during the 30–60 min incubation period at ambient temperature or at 37°C. The reaction setups were subsequently dialyzed against demineralized water by incubation on floating nitrocellulose filter plates (25 nm; Millipore, Billerica, MA) on Petri dishes for 30 min at room temperature.

Preparation and electroporation of bacterial cells

The procedure for electroporation of transposome complexes into *E.coli* has been described previously (5). *B.subtilis* cells were treated as follows. An aliquot of 1 ml of an overnight *B.subtilis* Luria–Bertani (LB) culture (supplemented with antibiotics when appropriate) was inoculated into 200 ml of fresh LB. The culture was shaken at 37°C until an OD_{600} of 1.5–2 was reached and harvested at 4°C by centrifugation for 10 min at 6000 ***g***. Cells were washed three times with decreasing amounts of ice-cold demineralized water and then resuspended in 2 ml of 30% PEG6000 at 4°C. An aliquot of 200 µl of this suspension and an appropriate amount of transposomes (up to 30 µl) were transferred into 2 mm gapped disposable electroporation cuvettes (PeqLab, Erlangen, Germany) and mixed. Electroporation was performed using a Bio-Rad GenePulser at 25 µF, 200Ω and 2.5 kV, and 0.2 ml of prewarmed SOC medium was added immediately afterwards, and the cells were allowed to grow for 90 min in an orbital shaker at 37°C. Subsequently, cells were spread on LB plates containing appropriate antibiotics for selection.

Preparation and sequencing of chromosomal DNA and determination of InsTet insertion loci

The presence of InsTet^{G+} elements in transformed *Bacilli* was confirmed by PCR using single-stranded ME as a sole primer. For locating the insertion sites of the respective element in these strains, we prepared chromosomal DNA of saturated overnight 20 ml LB cultures grown at 37°C using the Qiagen DNA-mini-kit (Qiagen, Hilden, Germany). Spin-column eluates were concentrated by precipitation with 10% (v/v) PEG6000 and 500 mM NaCl overnight on ice and resuspended in 20 µl TE. The chromosomal DNA was fragmented by digestion with EcoRI, which cuts only once in most versions of InsTet^{G+}, at the very 5′ end distal to the outward promoter, except InsTet$^{G+}2^{Cm}$, for which StuI was used. Efficiency of restriction was assayed by gel-electrophoresis of an aliquot. After restriction, the outward promoter of InsTet^{G+} and the sequence downstream of it were obtained as one fragment, which was sequenced using Tnp_out2, binding immediately downstream of $P_{xyl/tet}$. The promoter region of WH557 was sequenced with DP3. Sequencing was performed according to the supplier's recommendations (Applied Biosystems, Darmstadt, Germany).

Western blot experiments

Western blotting was performed as described previously (13), using the commercially available ECL+ kit (Amersham Biosciences, Freiburg, Germany). Polyclonal antibodies raised against TetR were diluted 1:20 000.

RESULTS

InsTet^{G+} elements harboring *tetR* and tc-sensitive outward promoters

The basic InsTet^{G+} variant contains MEs as binding sites for mutant Tn*5* transposase, stop codons in all three reading frames, a transcriptional terminator and an *aphAIII* cassette for kanamycin selection (see Figure 1, middle part). The *tet*-regulation cassettes contain *tetR* fused to promoter P* and a divergently oriented $P_{xyl/tet}$ hybrid promoter, harboring one (source pWH353) or two *tet*-operators (source pWH354) (14). They were inserted together with an *aphAIII* cassette flanked with asymmetric *loxP* sites (15), to yield InsTet^{G+}1 (one *tetO*) and InsTet^{G+}1a (two *tetO*), as shown in Figure 2A. InsTet^{G+}1 and InsTet^{G+}1a were fused to a promoterless *lacZ* and integrated into *amyE* of *B.subtilis* 168 to determine their efficiency of regulation. We also integrated an InsTet^{G+}1 variant with a non-functional *tetR* variant (lacking 16 bp in the middle of the *orf*). The generated strains were termed WH502 (InsTet^{G+}1-*lacZ*), WH502Δ (InsTet^{G+}1Δ*tetR-lacZ*) and WH503 (InsTet^{G+}1a-*lacZ*) (Table 1). Their β-gal activities are shown in Figure 2B. InsTet^{G+}1 yields complete atc-dependent induction, but only a moderate 5-fold repression. InsTet^{G+}1a leads to incomplete atc induction combined with tighter repression, resulting in a regulation factor of ∼28. Steady-state levels of TetR in mid-log cultures were determined in the absence or presence of atc. Owing to the presence of *tetO* within P*, autoregulation of TetR expression occurs in the strains WH502 and WH503, as shown by the immunoblot analysis (Figure 2B, lower panel). According to their regulatory

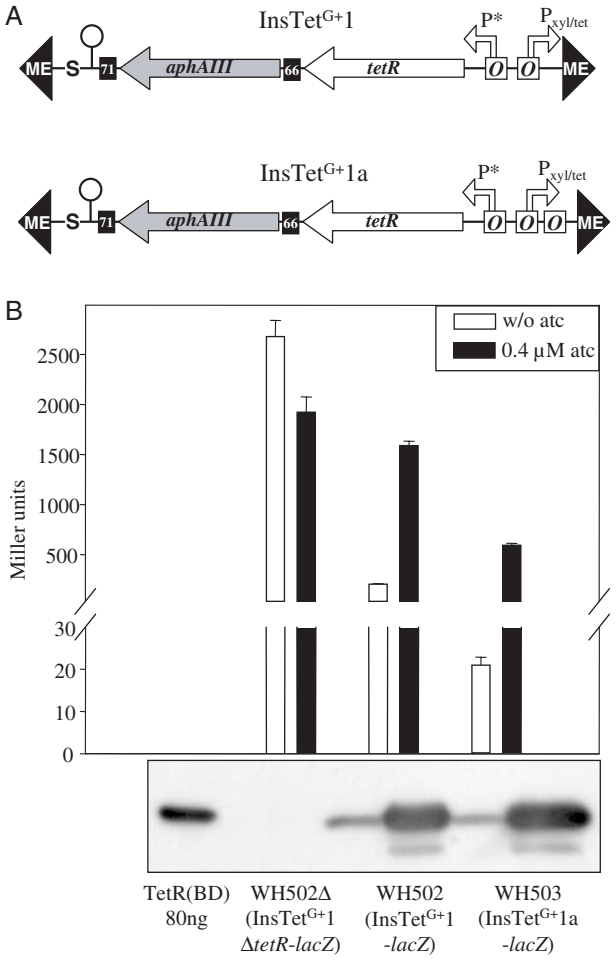

Figure 2. (**A**) Depiction of the elements InsTet^{G+}1 and InsTet^{G+}1a. The relevant promoters for the expression of *tetR* and outward regions are indicated. (**B**) Regulatory capacities of elements InsTet^{G+}1 and InsTet^{G+}1a as transcriptional *lacZ* fusions within the *amyE* locus. Values obtained without atc are given as open bars; closed bars display values with atc. WH502Δ (carrying InsTet^{G+}1 without functional *tetR*) was used as a 100% control and yielded ~2700 (without atc) and ~1900 (with 0.4 µM atc) Miller units. Lower panel shows the corresponding TetR-directed western blots obtained with the respective soluble proteins. Purified TetR is shown as a control on the left.

capacities, both elements seemed suitable for generating mutant *Bacillus* strains, albeit with small regulatory windows.

B.subtilis mutants generated with InsTet^{G+}1

Electroporation of InsTet^{G+}1 transposomes into *B.subtilis* 168 using 2 µg of DNA yielded 387 kanamycin-resistant candidates, and three of them displayed atc-dependent growth phenotypes (Figure 3A). Their insertion loci are shown in Figure 3B.

WH510 has InsTet^{G+}1 inserted into *yhaO*, with the promoter facing downstream towards *yhaN* and *yhaM*. None of these three genes forming a putative operon has been categorized as essential (http://bacillus.genome.jp). YhaN displays moderate similarity to an ATPase involved in DNA repair, and YhaM is a 3′–5′ exoribonuclease. Deletion of *yhaM* had not yielded any phenotype (23). The results described here suggest that overexpression effects in the presence of atc may cause the growth defect observed on CSK minimal-media plates (24).

InsTet^{G+}1 insertion disrupts *yaaQ* in strain the WH511, with the promoter oriented towards the 3′ end of the essential thymidylate kinase encoding *tmk* (1). We assume that atc-induced antisense RNA reduces *tmk* expression, resulting in an atc-dependent lack of growth in rich and minimal media.

InsTet^{G+}1 in WH512 is located immediately upstream of *kinA* (inserted between the putative −10 and Shine Dalgarno sequences), encoding a two-component sensor histidine kinase involved in the initiation of sporulation (25). However, the affected gene could also be *patA*, encoding a putative aspartate amino transferase, necessary for amino acid biosynthesis (26), which could be downregulated by atc-induced antisense RNA, thus explaining the growth defect on CSK^{atc}.

Taken together, WH510 and WH512 display regulated auxotrophy, whereas WH511 is a conditional growth-defective strain.

InsTet^{G+} elements with improved regulation

We constructed new InsTet^{G+} elements to broaden the regulatory window. *TetR* was removed from InsTet^{G+}1a and integrated into *lacA* on the *B.subtilis* chromosome (see Materials and Methods). To increase expression, the P* promoter was replaced in one construct by the promoter Pt17, obtained from a synthetic promoter pool (13). Furthermore, we exchanged the resistance cassette to *cat*, which yielded InsTet^{G+}2^{Cm}. Figure 4A shows this element, together with the two *tetR* expression constructs integrated in *lacA*. The *B.subtilis* strains carrying *tetR* in the chromosome are referred to as WH555 (P*-*tetR*) and WH557 (Pt17-*tetR*). Strains additionally vested with InsTet^{G+}2^{Cm}-*lacZ* transcriptional fusions integrated into *amyE* were designated WH556 (based upon WH555) and WH558 (based upon WH557).

We quantified β-galactosidase (β-gal) activities in the presence or absence of atc in both strains. The results are shown in Figure 4B. Apparently, the autogeneous control of TetR expression from P* in WH556 does not yield enough protein for tight repression. The synthetic Pt17 promoter is not subject to autoregulation and mediates much better repression. Western blots confirmed that this is due to increased amounts of TetR in the absence of atc (Figure 4B, lower panel). Thus, *lacZ* regulation in WH558 resulted in an induction/repression ratio of ~314.

We also constructed strains in which transcriptional control of the outward promoter is accomplished by plasmid encoded TetR. The strains WH560 (InsTet^{G+}2^{Cm}-*lacZ*) and WH570 (InsTet^{G+}2-*lacZ*) do not contain *tetR* in their chromosomes and differ only by their resistance markers (see Figures 4A and 5A). They were transformed with the *tetR* expression plasmid pWH119, with *tetR* under xylose-inducible P_{xylA} control, or with pWH125 bearing *revtetRr2*, expressed from a synthetic promoter termed Pt16 (Figure 5A). Both plasmids had yielded a broad regulatory range in a different genetic context (13). pHT304 served as the control plasmid without *tetR* (27). The β-gal activities for WH570 are given in Figure 5B. The regulatory windows of these strains are also larger compared with those with *tetR* located in the insertion element, with a regulation factor of ~140 for pWH119 and of ~80 for pWH125. It should be noted that the β-gal activity in the strain carrying pWH119 is tightly repressed only in the presence of xylose. This is due to increased intracellular

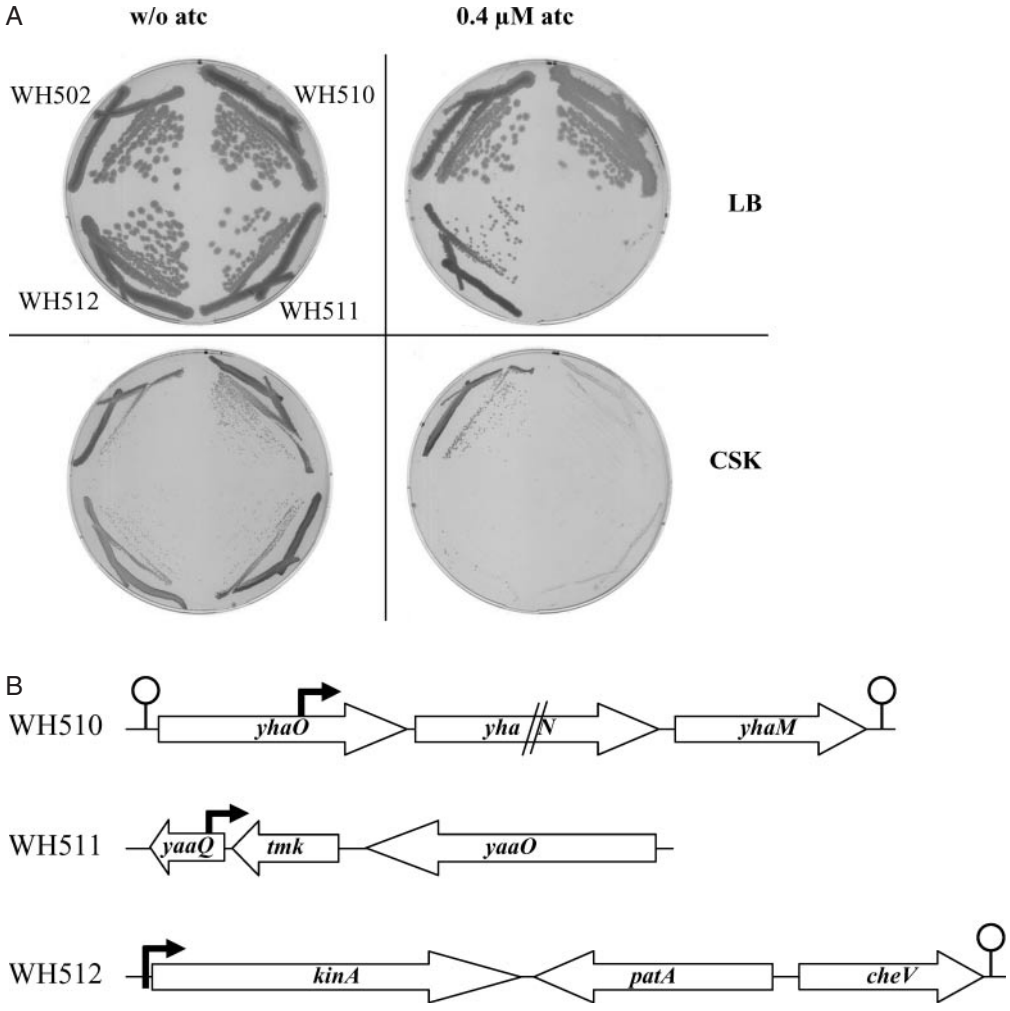

Figure 3. (**A**) Atc-dependent growth phenotypes of InsTet^{G+}1 integration mutants. The upper row shows strains WH502, WH510, WH511 and WH512 on LB plates without and with 0.4 µM atc, as indicated. The lower row shows the same strains on CSK minimal medium plates. Strain WH502 represents a positive control. (**B**) Schematic depiction of InsTet^{G+}1 insertion loci in strains WH510–WH512. The closed arrow symbolizes $P_{xyl/tet}$.

amounts of TetR obtained through induction of the *tetR* driving P_{xylA} promoter, which was confirmed by the western blot analysis (Figure 5B, lower panel).

Mutagenesis of *B.subtilis* WH557 with InsTet^{G+}2^{Cm}

We have used InsTet^{G+}2^{Cm} for transposome mutagenesis of *B.subtilis* WH557, using 1000 ng of InsTet^{G+}2^{Cm} DNA, which yielded 486 chloramphenicol-resistant candidates. Screening for regulated growth impairments on LB or CSK plates with or without atc (data not shown) yielded five strains, designated as WH532–WH536.

The element disrupted *yvaO* in WH532, with $P_{xyl/tet}$ directed towards *yvaN*. This strain shows atc-induced growth defects on LB and CSK plates, which may be due to the overexpression of *yvaJ* (*rnr*), encoding a 3′–5′ exoribonuclease (28).

Two insertions, 102 bp apart, occurred in *yydB* in WH533 and WH534. The regulated promoter is collinear with *yydB* in both the cases. Therefore it can be assumed that the regulated gene, yielding the atc-dependent growth defect on LB, but not on CSK, is the same in both strains. This could be *fbp*, encoding fructose-1,6-bisphosphatase, involved in gluconeogenesis (29), but the observed growth defect only on LB but not on CSK is difficult to be rationalized with this assumption.

The *proB* gene is disrupted in WH535, which is unable to form colonies on CSK without the inducer. *ProB* forms a putative operon with *proA*, encoding glutamyl-γ-semialdehyde dehydrogenase and is involved in proline biosynthesis (30). *B.subtilis* contains a *proB* paralogue, called *proJ*, which probably compensates for the disrupted *proB* (31). Thus, atc-dependent *proA* expression would explain the regulated auxotrophy of this strain.

Integration of the element into *pbpB* occurred in WH536. This strain is able to grow, although *pbpB*, encoding the penicillin-binding protein PBP 2B, has been described as essential, as this protein catalyzes the final stages of peptidoglycan synthesis (32,33). Growth of WH536 ceases on plates with atc. It is possible that InsTet^{G+}2^{Cm} insertion does not affect a relevant region of the PBP 2B. The gene *ftsL* downstream from *pbpB* is essential (34), which is in agreement with our findings: *FtsL* should be repressed in the absence of the inducer, resulting in growth deficiency in both media. This effect can be abolished by adding atc.

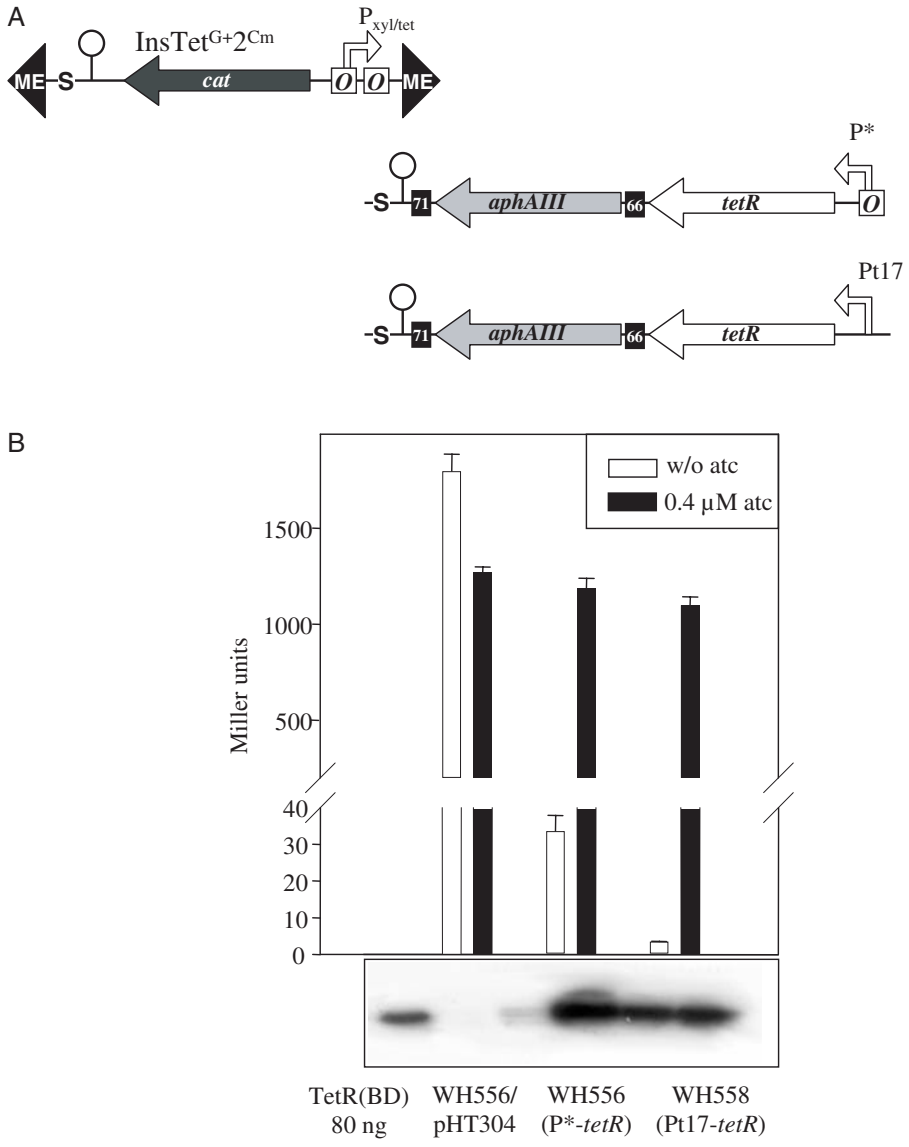

Figure 4. (**A**) Depiction of the element InsTet$^{G+}2^{Cm}$ and the InsTet$^{G+}1$ derived regions for *tetR* expression integrated into *lacA*. The promoter P* for *tetR* expression (in WH555) is derived from pWH353, the Pt17 designated promoter (in WH557) is a synthetic construct. (**B**) Regulatory capacities of InsTet$^{G+}2^{Cm}$ as transcriptional *lacZ* fusion within the *amyE* loci of WH555 or WH557, yielding WH556 or WH558, respectively. β-Gal measurements are depicted under different growth conditions: open bars indicate no atc, whereas closed bars represent 0.4 µM atc. Values obtained with WH556/pHT304 represent the maximal *lacZ* expression exerted by InsTet$^{G+}2^{Cm}$, resulting in Miller units of ∼1200 to ∼1700. Lower panel shows corresponding TetR-directed western blots obtained with the respective soluble proteins. Purified TetR is shown as a control on the left.

Thus, two strains display regulated growth deficiency in both media, two strains show diminished growth only on LB in the presence of atc, and one strain exhibits a conditional auxotrophy, since it is incapable of forming colonies in the minimal medium without atc.

Mutagenesis of *B.subtilis* 168/pWH119 with InsTet$^{G+}2$

Since pWH119 contains *tetR* under xylose control, we created a third mutant pool with InsTet$^{G+}2$ in *B.subtilis* 168 bearing this plasmid. About 1200 kanamycin-resistant candidates were obtained using 850 ng of DNA bound to transposase. Of these 432 were analyzed for growth on LB and CSK plates containing no effector, 0.2% xylose or 0.4 µM atc. Addition of xylose would lead to enhanced repression of a *tet*-regulated gene (see Figure 5B), allowing to analyze the effects of three different expression levels of the gene. We have obtained seven strains with conditional growth phenotypes (Figure 6A). The insertion loci are schematically given in Figure 6B.

WH525 contains InsTet$^{G+}2$ inserted in *spsB*, with the promoter facing towards the *spsCDEFGIJKL* operon involved in spore coat polysaccharide synthesis. This strain is unable to grow on LB and CSK plates with atc, however, it is presently not evident why the induction of these genes leads to growth defects.

WH526 carries InsTet$^{G+}2$ integrated into the plasmid pWH119 so that $P_{xyl/tet}$ transcribes antisense RNA of the erythromycin resistance gene. This strain is incapable of growing in erythromycin containing media when induced by atc.

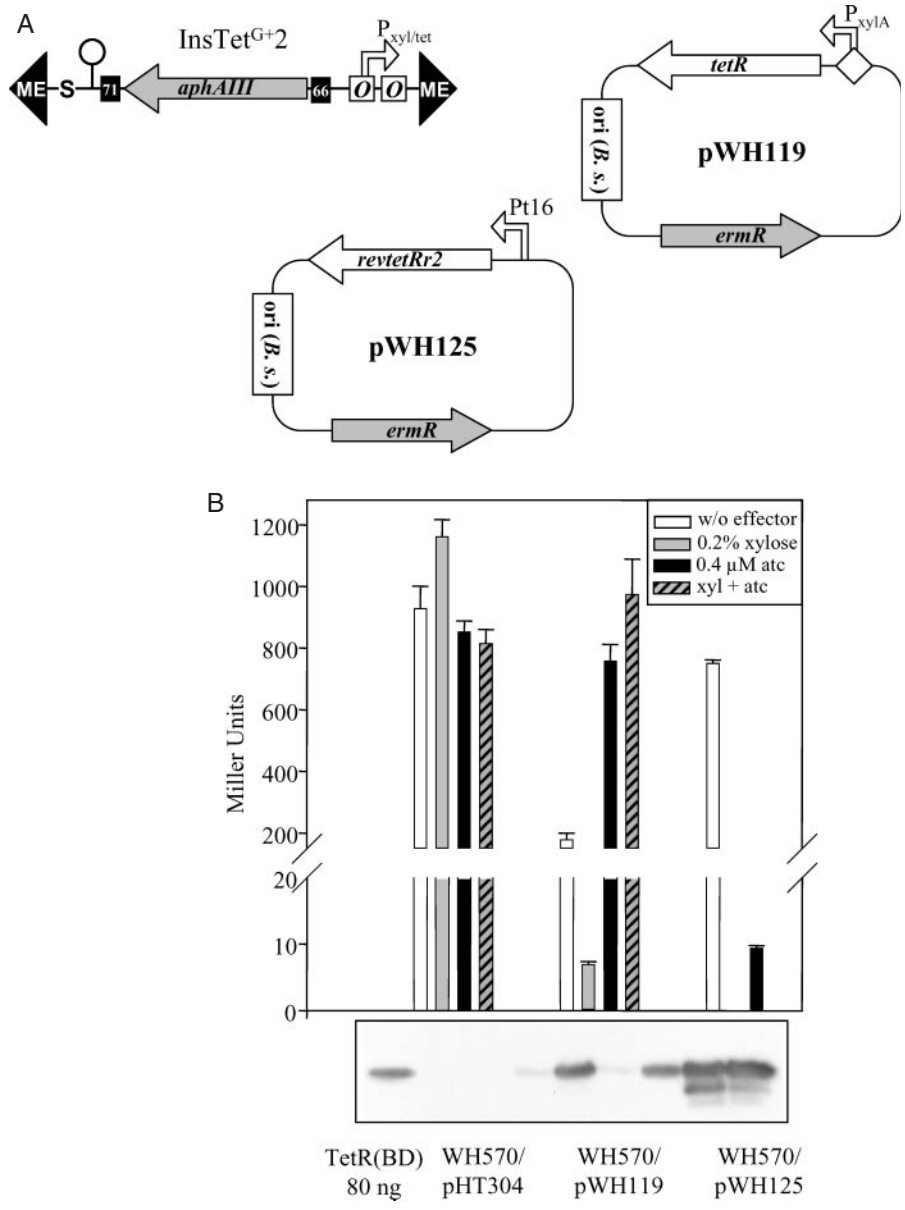

Figure 5. (**A**) Depiction of the element InsTet^{G+}2 and the plasmids pWH119 and pWH125 for the expression of *tetR* or *revtetRr2*, respectively. The promoter for *tetR* expression in pWH119 contains a *xyl*-operator represented by a rhomb. Transcription of *revtetRr2* is exerted by the constitutive synthetic promoter pt16 (13). (**B**) Regulatory capacities of InsTet^{G+}2 as transcriptional *lacZ* fusion within the *amyE* locus, yielding WH570. Measurements with the auxiliary plasmids pWH119 and pWH125 are depicted under different growth conditions, open bars, no atc or xylose; gray bars, 0.2% xylose; closed bars, 0.4 µM atc; hatched black/gray, 0.2% xylose and 0.4 µM atc. Values obtained with WH570/pHT304 represent the maximal *lacZ* expression of ~800 to ~1200 Miller units obtained with InsTet^{G+}2. Lower panel shows corresponding TetR-directed western blots with the strains' soluble proteins. Purified TetR is shown as a control on the left.

The insertion locus in strain WH527 is the serine-acetyltransferase, encoding *cysE* (35). It shows constitutive auxotrophy on CSK plates. Interestingly, this strain also displays diminished growth on LB with xylose or with atc. As *cysE* is the second gene in a tricistronic operon, regulated expression of *cysS*, encoding a cysteinyl-tRNA synthetase downstream of *cysE*, is likely to cause the observed effects. Our results suggest that repression and overexpression of *cysS* leads to growth defects, maybe by an imbalanced amount of cysteinyl-tRNA.

We assume that $P_{xyl/tet}$ leads to conditional *tagD* expression in WH528. This gene encodes a glycerol-3-phosphate cytidyltransferase and is indispensable for growth (36). Interestingly, the growth defect of this strain is brought about by xylose and relieved by atc, indicating that high levels of TetR are required to sufficiently repress *tagD* for this phenotype.

YufL is the insertion locus in WH529. This gene, also referred to as *malK*, encodes a malate kinase sensor, which, together with the downstream encoded YufM (MalR), constitutes a two-component system for the regulation of malate utilization (37,38). The insertion is located downstream of the kinase domain of YufL so that the truncated protein might still exert phosphorylation of YufM. At present, ceasing of growth in the presence of atc on both media remains obscure for this strain.

The gene affected in WH530 is presumably the same as in WH511, namely the thymidylate kinase encoding *tmk*.

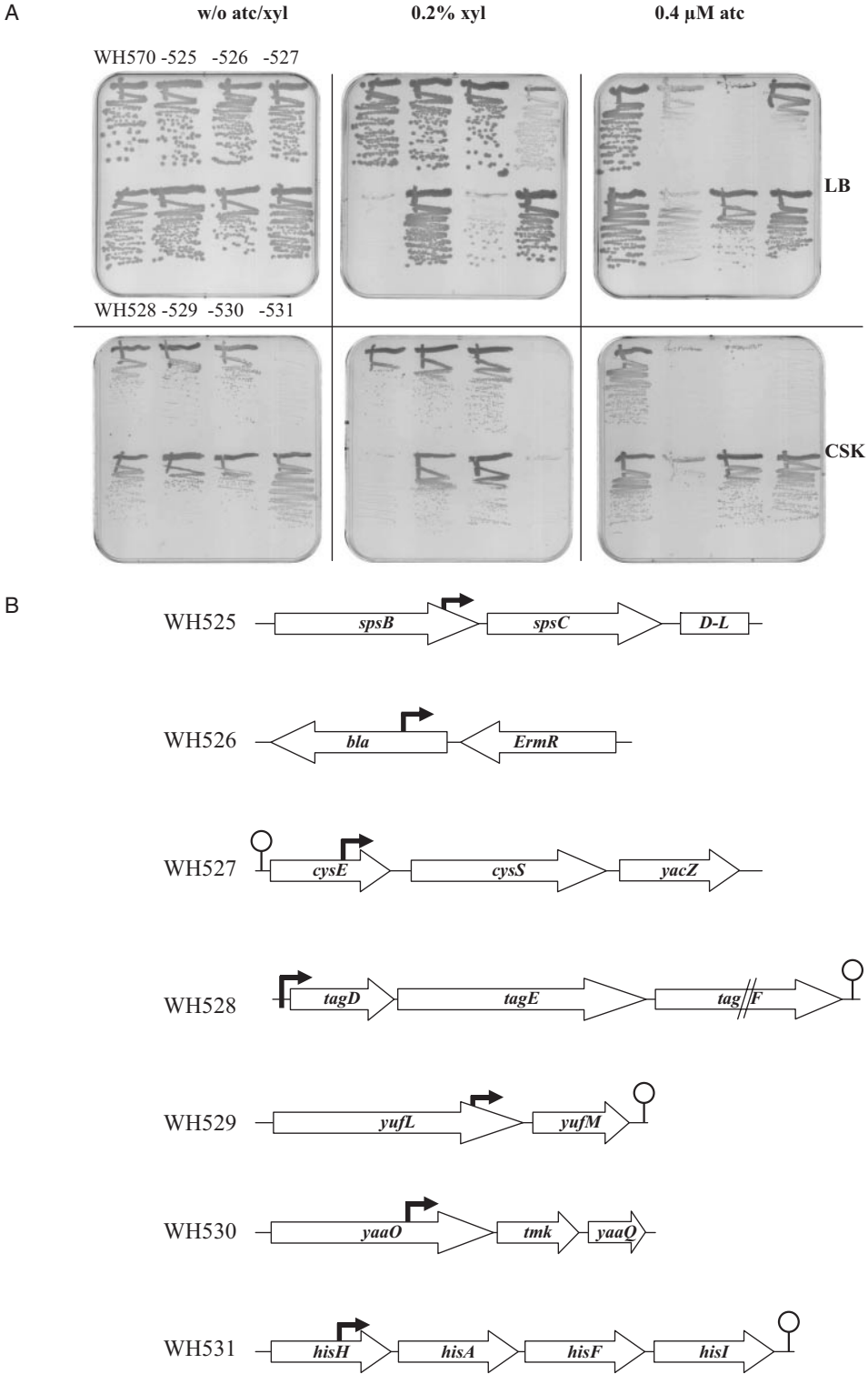

Figure 6. (**A**) Atc or xylose-dependent growth phenotypes of InsTet^{G+}2 derived *B.subtilis* mutants. The upper row shows strains WH570 and WH525–WH531 (each harboring pWH119) on LB plates without atc and xylose, with 0.2% xylose or with 0.4 µM atc, as indicated. The lower row demonstrates growth on CSK minimal medium with the same strains. WH570/pHT304 represents a positive control. (**B**) Schematic depiction of InsTet^{G+}2 insertion loci in strains WH525–WH531. The closed arrow symbolizes $P_{xyl/tet}$.

WH530 does not grow on LB^{xyl} but spreads well on CSK^{xyl}. The insertion is located in *yaaO* and $P_{xyl/tet}$ fires toward *tmk* in the presence of atc. This is in contrast to WH511, where the promoter orientation suggests conditional antisense regulation of the gene. In the presence of xylose, *tmk* should be tightly repressed in WH530, leading to diminished growth on LB^{xyl}. The reason for this effect not being observed on CSK supplemented with xylose is unclear.

WH531 carries an InsTet^{G+}2 insertion in *hisH* involved in histidine biosynthesis. This has no consequences for growth on LB plates, but no colonies appeared on CSK plates with xylose. Apparently, tight repression of the downstream genes *hisAFI* is responsible for this phenotype.

Taken together, six strains of this pool show conditional growth defects, while WH531 displays a conditional auxotrophy.

DISCUSSION

Transposomes have previously been used for mutagenesis of some Gram positive bacteria from the genera *Rhodococcus*, *Mycobacterium* and *Corynebacterium*, using a Tn5-derived system (39–43), and from *Staphylococcus* and *Streptococcus*, using Mu transposase (7). We improve this technology by incorporating regulated outward promoters, thereby for the first time generating conditional mutant strains by transposome mutagenesis. The InsTet^{G+}1 and InsTet^{G+}1a constructs (Figure 2A) enable one-step mutagenesis, since they deliver all components necessary for regulation. However, they exhibit only a small range of regulated expression (Figure 2B). An expanded regulatory window with tighter repression and higher induction was obtained by moving *tetR* from the insertion elements into the chromosome or on a plasmid having the regulator expressed from stronger promoters. Mutants obtained with WH557, containing Pt17-*tetR* in the *lacA* locus, demonstrate that a single copy of *tetR* can mediate tight repression and complete induction by atc.

The use of pWH119 carrying *tetR* under xylose control enables an additional regulatory feature: the amounts of TetR needed to tightly repress target gene expression are only provided when xylose is applied. This yields also mutants, where InsTet^{G+}2, one of the elements without *tetR*, controls essential genes without the need to add atc. The isolation of mutants with xylose-dependent auxotrophy or growth defects underlines the usefulness of this approach. Since the revTetR variant also works fine in concert with the InsTet^{G+}2 elements, conditional null-mutant strains may be obtained in which growth is ceased upon administration of atc. With TetR, we also mostly obtained mutants demonstrating impaired growth with atc. This may be caused by the overexpression of downstream gene(s) or by the induction of antisense transcription. The notion that antisense RNA induction may be the prevalent regulatory mechanism is not surprising, because the promoter-out element needs to integrate into a small region around the natural promoter of a gene for it to exert forward (sense) control. In contrast, insertion into a larger region downstream and opposite to the affected gene would lead to antisense regulation (2). Hence, antisense control may be rather the rule than the exception for the mutants found with this approach. In fact, regulated expression of antisense RNA has been used to identify essential *Staphylococcus aureus* genes (44).

Although many of the integrative promoter-out elements described to date rely on regulation using IPTG or arabinose as effectors, we here exploit tc-based regulation. It has recently been shown that this confers tight repression and sensitive induction in *B.subtilis* (13). Many tc-analogues freely permeate biological membranes without the need for uptake systems and induce TetR far below antibiotically active concentrations (45). In addition, tc and derivatives thereof are not metabolized and can be used in animal models. The versatility of *tet*-regulation in combination with insertion elements described here should be very useful for studying gene function in pathogenic bacteria.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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REFERENCES

1. Kobayashi,K., Ehrlich,S.D., Albertini,A., Amati,G., Andersen,K.K., Arnaud,M., Asai,K., Ashikaga,S., Aymerich,S., Bessieres,P. *et al*. (2003) Essential *Bacillus subtilis* genes. *Proc. Natl Acad. Sci. USA*, **100**, 4678–4683.
2. Judson,N. and Mekalanos,J.J. (2000) Transposon-based approaches to identify essential bacterial genes. *Trends Microbiol*., **8**, 521–526.
3. Steinmetz,M. and Richter,R. (1994) Easy cloning of mini-Tn*10* insertions from the *Bacillus subtilis* chromosome. *J. Bacteriol*., **176**, 1761–1763.
4. Hayes,F. (2003) Transposon-based strategies for microbial functional genomics and proteomics. *Annu. Rev. Genet*., **37**, 3–29.
5. Goryshin,I.Y., Jendrisak,J., Hoffman,L.M., Meis,R. and Reznikoff,W.S. (2000) Insertional transposon mutagenesis by electroporation of released Tn*5* transposition complexes. *Nat. Biotechnol*., **18**, 97–100.
6. Lamberg,A., Nieminen,S., Qiao,M. and Savilahti,H. (2002) Efficient insertion mutagenesis strategy for bacterial genomes involving electroporation of *in vitro*-assembled DNA transposition complexes of bacteriophage mu. *Appl. Environ. Microbiol*., **68**, 705–712.
7. Pajunen,M.I., Pulliainen,A.T., Finne,J. and Savilahti,H. (2005) Generation of transposon insertion mutant libraries for Gram-positive bacteria by electroporation of phage Mu DNA transposition complexes. *Microbiology*, **151**, 1209–1218.
8. Ji,Y., Marra,A., Rosenberg,M. and Woodnutt,G. (1999) Regulated antisense RNA eliminates alpha-toxin virulence in *Staphylococcus aureus* infection. *J. Bacteriol*., **181**, 6585–6590.
9. Stieger,M., Wohlgensinger,B., Kamber,M., Lutz,R. and Keck,W. (1999) Integrational plasmids for the tetracycline-regulated expression of genes in *Streptococcus pneumoniae*. *Gene*, **226**, 243–251.
10. Ehrt,S., Guo,X.V., Hickey,C.M., Ryou,M., Monteleone,M., Riley,L.W. and Schnappinger,D. (2005) Controlling gene expression in mycobacteria with anhydrotetracycline and Tet repressor. *Nucleic Acids Res*., **33**, e21.
11. Blokpoel,M.C., Murphy,H.N., O'Toole,R., Wiles,S., Runn,E.S., Stewart,G.R., Young,D.B. and Robertson,B.D. (2005) Tetracycline-inducible gene regulation in mycobacteria. *Nucleic Acids Res*., **33**, e22.
12. Rodriguez-Garcia,A., Combes,P., Perez-Redondo,R. and Smith,M.C. (2005) Natural and synthetic tetracycline-inducible promoters for use in the antibiotic-producing bacteria *Streptomyces*. *Nucleic Acids Res*., **33**, e87.
13. Kamionka,A., Bertram,R. and Hillen,W. (2005) Tetracycline-dependent conditional gene knockout in *Bacillus subtilis*. *Appl. Environ. Microbiol*., **71**, 728–733.

14. Geissendörfer,M. and Hillen,W. (1990) Regulated expression of heterologous genes in *Bacillus subtilis* using the Tn*10* encoded tet regulatory elements. *Appl. Microbiol. Biotechnol.*, **33**, 657–663.
15. Albert,H., Dale,E.C., Lee,E. and Ow,D.W. (1995) Site-specific integration of DNA into wild-type and mutant *lox* sites placed in the plant genome. *Plant J.*, **7**, 649–659.
16. Schollmeier,K., Gärtner,D. and Hillen,W. (1985) A bidirectionally active signal for termination of transcription is located between *tetA* and *orfL* on transposon Tn*10*. *Nucleic Acids Res.*, **13**, 4227–4237.
17. Guérout-Fleury,A.M., Shazand,K., Frandsen,N. and Stragier,P. (1995) Antibiotic-resistance cassettes for *Bacillus subtilis*. *Gene*, **167**, 335–336.
18. Scholz,O., Henssler,E.M., Bail,J., Schubert,P., Bogdanska-Urbaniak,J., Sopp,S., Reich,M., Wisshak,S., Köstner,M., Bertram,R. *et al.* (2004) Activity reversal of Tet repressor caused by single amino acid exchanges. *Mol. Microbiol.*, **53**, 777–789.
19. Abremski,K. and Hoess,R. (1984) Bacteriophage P1 site-specific recombination. Purification and properties of the Cre recombinase protein. *J. Biol. Chem.*, **259**, 1509–1514.
20. Stülke,J., Martin-Verstraete,I., Zagorec,M., Rose,M., Klier,A. and Rapoport,G. (1997) Induction of the *Bacillus subtilis ptsGHI* operon by glucose is controlled by a novel antiterminator, GlcT. *Mol. Microbiol.*, **25**, 65–78.
21. Kraus,A., Hueck,C., Gärtner,D. and Hillen,W. (1994) Catabolite repression of the *Bacillus subtilis xyl* operon involves a cis element functional in the context of an unrelated sequence, and glucose exerts additional *xylR*-dependent repression. *J. Bacteriol.*, **176**, 1738–1745.
22. Goryshin,I.Y. and Reznikoff,W.S. (1998) Tn5 *in vitro* transposition. *J. Biol. Chem.*, **273**, 7367–7374.
23. Oussenko,I.A., Sanchez,R. and Bechhofer,D.H. (2002) *Bacillus subtilis* YhaM, a member of a new family of 3′ to 5′ exonucleases in gram-positive bacteria. *J. Bacteriol.*, **184**, 6250–6259.
24. Martin-Verstraete,I., Stülke,J., Klier,A. and Rapoport,G. (1995) Two different mechanisms mediate catabolite repression of the *Bacillus subtilis* levanase operon. *J. Bacteriol.*, **177**, 6919–6927.
25. Jiang,M., Shao,W., Perego,M. and Hoch,J.A. (2000) Multiple histidine kinases regulate entry into stationary phase and sporulation in *Bacillus subtilis*. *Mol. Microbiol.*, **38**, 535–542.
26. Berger,B.J., English,S., Chan,G. and Knodel,M.H. (2003) Methionine regeneration and aminotransferases in *Bacillus subtilis*, *Bacillus cereus*, and *Bacillus anthracis*. *J. Bacteriol.*, **185**, 2418–2431.
27. Arantes,O. and Lereclus,D. (1991) Construction of cloning vectors for *Bacillus thuringiensis*. *Gene*, **108**, 115–119.
28. Oussenko,I.A. and Bechhofer,D.H. (2000) The *yvaJ* gene of *Bacillus subtilis* encodes a 3′ to 5′ exoribonuclease and is not essential in a strain lacking polynucleotide phosphorylase. *J. Bacteriol.*, **182**, 2639–2642.
29. Fujita,Y., Yoshida,K., Miwa,Y., Yanai,N., Nagakawa,E. and Kasahara,Y. (1998) Identification and expression of the *Bacillus subtilis* fructose-1, 6-bisphosphatase gene (*fbp*). *J. Bacteriol.*, **180**, 4309–4313.
30. Ogura,M., Kawata-Mukai,M., Itaya,M., Takio,K. and Tanaka,T. (1994) Multiple copies of the *proB* gene enhance *degS*-dependent extracellular protease production in *Bacillus subtilis*. *J. Bacteriol.*, **176**, 5673–5680.
31. Belitsky,B.R., Brill,J., Bremer,E. and Sonenshein,A.L. (2001) Multiple genes for the last step of proline biosynthesis in *Bacillus subtilis*. *J. Bacteriol.*, **183**, 4389–4392.
32. Daniel,R.A., Harry,E.J. and Errington,J. (2000) Role of penicillin-binding protein PBP 2B in assembly and functioning of the division machinery of *Bacillus subtilis*. *Mol. Microbiol.*, **35**, 299–311.
33. Yanouri,A., Daniel,R.A., Errington,J. and Buchanan,C.E. (1993) Cloning and sequencing of the cell division gene *pbpB*, which encodes penicillin-binding protein 2B in *Bacillus subtilis*. *J. Bacteriol.*, **175**, 7604–7616.
34. Daniel,R.A. and Errington,J. (2000) Intrinsic instability of the essential cell division protein FtsL of *Bacillus subtilis* and a role for DivIB protein in FtsL turnover. *Mol. Microbiol.*, **36**, 278–289.
35. Pelchat,M. and Lapointe,J. (1999) *In vivo* and *in vitro* processing of the *Bacillus subtilis* transcript coding for glutamyl-tRNA synthetase, serine acetyltransferase, and cysteinyl-tRNA synthetase. *RNA*, **5**, 281–289.
36. Bhavsar,A.P., Zhao,X. and Brown,E.D. (2001) Development and characterization of a xylose-dependent system for expression of cloned genes in *Bacillus subtilis*: conditional complementation of a teichoic acid mutant. *Appl. Environ. Microbiol.*, **67**, 403–410.
37. Tanaka,K., Kobayashi,K. and Ogasawara,N. (2003) The *Bacillus subtilis* YufLM two-component system regulates the expression of the malate transporters MaeN (YufR) and YflS, and is essential for utilization of malate in minimal medium. *Microbiology*, **149**, 2317–2329.
38. Doan,T., Servant,P., Tojo,S., Yamaguchi,H., Lerondel,G., Yoshida,K., Fujita,Y. and Aymerich,S. (2003) The *Bacillus subtilis ywkA* gene encodes a malic enzyme and its transcription is activated by the YufL/YufM two-component system in response to malate. *Microbiology*, **149**, 2331–2343.
39. Fernandes,P.J., Powell,J.A. and Archer,J.A. (2001) Construction of *Rhodococcus* random mutagenesis libraries using Tn*5* transposition complexes. *Microbiology*, **147**, 2529–2536.
40. Mangan,M.W. and Meijer,W.G. (2001) Random insertion mutagenesis of the intracellular pathogen *Rhodococcus equi* using transposomes. *FEMS Microbiol. Lett.*, **205**, 243–246.
41. Philalay,J.S., Palermo,C.O., Hauge,K.A., Rustad,T.R. and Cangelosi,G.A. (2004) Genes required for intrinsic multidrug resistance in *Mycobacterium avium*. *Antimicrob. Agents Chemother.*, **48**, 3412–3418.
42. Laurent,J.P., Hauge,K., Burnside,K. and Cangelosi,G. (2003) Mutational analysis of cell wall biosynthesis in *Mycobacterium avium*. *J. Bacteriol.*, **185**, 5003–5006.
43. Oram,D.M., Avdalovic,A. and Holmes,R.K. (2002) Construction and characterization of transposon insertion mutations in *Corynebacterium diphtheriae* that affect expression of the diphtheria toxin repressor (DtxR). *J. Bacteriol.*, **184**, 5723–5732.
44. Ji,Y., Zhang,B., Van,S.F., Horn,Warren,P., Woodnutt,G., Burnham,M.K. and Rosenberg,M. (2001) Identification of critical staphylococcal genes using conditional phenotypes generated by antisense RNA. *Science*, **293**, 2266–2269.
45. Hillen,W. and Berens,C. (1994) Mechanisms underlying expression of Tn*10* encoded tetracycline resistance. *Annu. Rev. Microbiol.*, **48**, 345–369.
46. Hanahan,D. (1983) Studies on transformation of *Escherichia coli* with plasmids. *J. Mol. Biol.*, **166**, 557–580.
47. Yanisch-Perron,C., Vieira,J. and Messing,J. (1985) Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene*, **33**, 103–119.