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 Tn5, Tn7, Mu, Himar1 or Ty1, 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 Tn5-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...
Table 1. Bacterial strains used in this study

<table>
<thead>
<tr>
<th>Strains or plasmids</th>
<th>Relevant characteristics</th>
<th>Reference or source</th>
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<tr>
<td><em>Escherichia coli</em> DH5α</td>
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<td>WH601A</td>
<td>DH5α carrying InsTetGα, aphIII cassette excised by Cre recombinase (Km²)</td>
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**Plasmids**

- pAC6: Integrative plasmid for *B.subtilis* amyE
- pH304: Ap², Blt, pUC19 polylinker
- pUC19: Ap², lacZα, pMB1 ori
- pWH119: pH304 with yvaA promoter from *B.subtilis* 168 upstream of tetR
- pWH125: pH304 with synthetic promoter upstream of revsR12
- pWH1941: pAC6 with InsTetG10 upstream of lacZ
- pWH1942: Integrative plasmid for *B.subtilis* lacA
- pWH353: Km², Ap², improved Pr* promoter upstream of tetR(B)
- pWH354: Km², Ap², improved Pr* promoter with second tetO upstream of tetR(B)

Square parentheses denote promoter direction in InsTetGα.

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™ 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: InsTetGα (see below) was brought into *E.coli* DH5α via transposone 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 InsTetGα1 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 InsTetGα for insertable tetracycline responsive promoter elements for Gram positive bacteria. Figure 1 schematically illustrates the key steps in construction. The backbone of InsTetGα 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. Ligations of all four double-stranded fragments was performed with T4-DNA ligase at 15°C overnight. The gel-purified fragment
was cloned into pUC19, cut with NdeI and HindIII, giving rise to pWH1935. The contained element carries mosaic elements (MEs) for Tn5 transposase binding. Three successive stop codons are symbolized by capital ‘S’ and are given below. The Tn50 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 Pxy/tytet (as regulatable outward-facing promoter). Boxes marked as ‘O’ represent tet-operators.

Figure 1. Key steps of InsTetG construction. Closed bars symbolize single-stranded oligonucleotides for hybridization. Closed triangles represent the MEs for Tn5 transposase binding. Three successive stop codons are symbolized by capital ‘S’ and are given below. The Tn50 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 Pxy/tytet (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 Tn5 transposase binding at both ends, stop codons in all three forward reading frames, a Tn50 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 digested with NdeI and cloned into the likewise digested pWH1935. This plasmid is a derivative of 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 pWH1935(BD), bearing chimeric tetR(BD) and the divergent promoters P* and Pxy/tytet (14), were subsequently restricted with KpnI and Stul. Then thereby excised P*/Pxy/tytet 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 Hpal and cloned into the plasmid described above and the resulting vector was termed pWH1935-1 and contained InsTetG-1 (Figures 1, lower 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/Stul 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 InsTetG-1a, containing two tet-operators outside the outward promoter region (Figure 2A, lower part). pWH1935-2Cm (with InsTetG-2Cm, 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 InsTetG-2 (Figure 5A), was obtained by the deletion of P*/Pxy/tytet region of InsTetG-1a via XhoI and NcoI, creating blunt ends with the Klenow fragment, and religation. Cloning of both InsTetG-2 elements was performed in E.coli WH601Δ. The nucleotide sequences of InsTetG-1, InsTetG-1a, InsTetG-2 and InsTetG-2Cm 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, InsTetG-0 was cloned into pAC6 via Stul and SmaI. One candidate with aphAIII divergent to lacZ was termed pWH1941. The relevant new portions of the other InsTetG 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 InsTetG-1 was cloned via KpnI and Sacl 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 GAATTCCGGGAAATAAAAAACTA-GTTTGCCAAATAACTCCACCAATGATAATGTGCACAAAGGAGGTATTAATGATGTCTAGA, which was termed Pt17.

Transposome formation
Transposase was cloned from IS50R 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 InsTetG+ 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 InsTetG+ 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 OD600 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 overnight on ice and resuspended in 20 µl TE. The chromosomal DNA was fragmented by digestion with EcoRI, which cuts once only in most versions of InsTetG+, at the very 5′ end distal to the outward promoter, except InsTetG+2Cm, for which StuI was used. Efficiency of restriction was assayed by gel-electrophoresis of an aliquot. After restriction, the outward promoter of InsTetG+ and the sequence downstream of it were obtained as one fragment, which was sequenced using Tnp_out2, binding immediately downstream of P_syl/ser. 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
InsTetG+ elements harboring tetR and tc-sensitive outward promoters
The basic InsTetG+ variant contains MEs as binding sites for mutant Tn5 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_syl/ser 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 InsTetG+1 (one tetO) and InsTetG+1a (two tetO), as shown in Figure 2A. InsTetG+1 and InsTetG+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 InsTetG+1 variant with a non-functional tetR variant (lacking 16 bp in the middle of the orf). The generated strains were termed WH502 (InsTetG+1-lacZ), WH502A (InsTetG+1 ΔtetR-lacZ) and WH503 (InsTetG+1a-lacZ) (Table 1). Their β-gal activities are shown in Figure 2B. InsTetG+1 yields complete atc-dependent induction, but only a moderate 5-fold repression. InsTetG+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
InsTetG\(^{+}\) insertion disrupts yaaQ in strain the WH511, with the promoter oriented towards the 3’ end of the essential thymidyylate 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.

InsTetG\(^{+}\) in WH512 is located immediately upstream of kiaA (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\(^{+}\).

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

**InsTetG\(^{+}\) elements with improved regulation**

We constructed new InsTetG\(^{+}\) elements to broaden the regulatory window. TetR was removed from InsTetG\(^{+}\)1a and integrated into lacA on the *B. subtilis* chromosome (see Materials and Methods). To increase expression, the P\(^{\star}\) 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 InsTetG\(^{+}\)2C\(_{m}\). 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\(^{\star}\)-tetR) and WH557 (Pt17-tetR). Strains additionally vested with InsTetG\(^{+}\)2C\(_{m}\)-lacZ transcriptional fusions integrated into amyE were designated WH556 (based upon WH555) and WH558 (based upon WH557).

We quantified \(\beta\)-galactosidase (\(\beta\)-gal) activities in the presence or absence of atc in both strains. The results are shown in Figure 4B. Apparently, the autogenous control of TetR expression from P\(^{\star}\) 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 (InsTetG\(^{+}\)2C\(_{m}\),lacZ) and WH570 (InsTetG\(^{+}\)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\(_{\text{xyh}}\) control, or with pWH125 bearing revet/\(\text{tet}R\)2, 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 \(\beta\)-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 \(\beta\)-gal activity in the strain carrying pWH119 is tightly repressed only in the presence of xylose. This is due to increased intracellular capacities, both elements seemed suitable for generating mutant *Bacillus* strains, albeit with small regulatory windows.

**B. subtilis mutants generated with InsTetG\(^{+}\)**

Electroporation of InsTetG\(^{+}\) transposomes into *B. subtilis* 168 using 2 \(\mu\)g 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 InsTetG\(^{+}\) inserted into yhaQ, 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).

![Figure 2](image_url)
amounts of TetR obtained through induction of the tetR driving P\textsubscript{xylA} promoter, which was confirmed by the western blot analysis (Figure 5B, lower panel).

Mutagenesis of \textit{B. subtilis} WH557 with InsTet\textsuperscript{G+2\textsubscript{Cm}}

We have used InsTet\textsuperscript{G+2\textsubscript{Cm}} for transposome mutagenesis of \textit{B. subtilis} WH557, using 1000 ng of InsTet\textsuperscript{G+2\textsubscript{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 \textit{yvaO} in WH532, with Pxyl/tet directed towards yvaN. This strain shows atc-induced growth defects on LB or CSK plates, which may be due to the overexpression of \textit{yvaJ} (\textit{rnr}), encoding a 3'-5' exoribonuclease (28).

Two insertions, 102 bp apart, occurred in \textit{yydB} in WH533 and WH534. The regulated promoter is collinear with \textit{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 \textit{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 \textit{proB} gene is disrupted in WH535, which is unable to form colonies on CSK without the inducer. \textit{ProB} forms a putative operon with \textit{proA}, encoding glutamyl-g-semialdehyde dehydrogenase and is involved in proline biosynthesis (30). \textit{B. subtilis} contains a \textit{proB} paralogue, called \textit{proI}, which probably compensates for the disrupted \textit{proB} (31). Thus, atc-dependent \textit{proA} expression would explain the regulated auxotrophy of this strain.

Integration of the element into \textit{pbpB} occurred in WH536. This strain is able to grow, although \textit{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\textsuperscript{G+2\textsubscript{Cm}} insertion does not affect a relevant region of the PBP 2B.

The gene \textit{ftsL} downstream from \textit{phpB} is essential (34), which is in agreement with our findings: \textit{FisL} should be repressed in the absence of the inducer, resulting in growth deficiency in both media. This effect can be abolished by adding atc.
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\textsuperscript{G+2}**

Since pWH119 contains tetR under xylose control, we created a third mutant pool with InsTet\textsuperscript{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\textsuperscript{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\textsuperscript{G+2} integrated into the plasmid pWH119 so that P\textsubscript{xyltet} transcribes antisense RNA of the erythromycin resistance gene. This strain is incapable of growing in erythromycin containing media when induced by atc.
The insertion locus in strain WH527 is the serine-acetyltransferase, encoding \textit{cysE} (35). It shows constitutive auxotrophy on CSK plates. Interestingly, this strain also displays diminished growth on LB with xylose or with atc. As \textit{cysE} is the second gene in a tricistronic operon, regulated expression of \textit{cysS}, encoding a cysteinyl-tRNA synthetase downstream of \textit{cysE}, is likely to cause the observed effects. Our results suggest that repression and overexpression of \textit{cysS} leads to growth defects, maybe by an imbalanced amount of cysteinyl-tRNA.

We assume that \textit{P_xyltet} leads to conditional \textit{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 \textit{tagD} for this phenotype.

\textit{YufL} is the insertion locus in WH529. This gene, also referred to as \textit{malK}, encodes a malate kinase sensor, which, together with the downstream encoded \textit{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 \textit{YufL} so that the truncated protein might still exert phosphorylation of \textit{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 \textit{tmk}.
WH530 does not grow on LB *xy* but spreads well on CSK *xy*. 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 *xy*. The reason for this effect not being observed on CSK supplemented with xylose is unclear.

<table>
<thead>
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<th>w/o atc/xy</th>
<th>0.2% xy</th>
<th>0.4 µM atc</th>
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<tr>
<td>WH570</td>
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<td>WH528</td>
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**Figure 6.** (A) Apc or xylose-dependent growth phenotypes of *InsTetG*+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 *InsTetG*+2 insertion loci in strains WH525–WH531. The closed arrow symbolizes *P* *xyl*.
WH531 carries an InsTetG<sup>Gr</sup> 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 InsTetG<sup>Gr</sup>-1 and InsTetG<sup>Gr</sup>-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 element 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 InsTetG<sup>Gr</sup>-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 InsTetG<sup>Gr</sup>-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**


