Random Insertion of a TetR-Inducing Peptide Tag into *Escherichia coli* Proteins Allows Analysis of Protein Levels by Induction of Reporter Gene Expression

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The insertion element InsTip α was constructed to generate protein expression data. It randomly fuses the TetR-inducing peptide Tip to the affected reading frame. Fusion protein expression is quantified by Tet-regulated reporter gene expression. The expression patterns of tagged *Escherichia coli* genes fully agree with published data from transcriptional fusions or microarrays, validating the Tip tag approach.

Proteome expression profiles provide important information but are cumbersome to obtain as the data are usually collected by two-dimensional electrophoresis (9, 25). When genome sequences are available, proteome-wide sequence-directed tagging approaches are an alternative (7, 13, 33). If directed tagging is not possible, random transposon-mediated tagging approaches are employed to fuse large reporter genes like *lacZ* to reading frames. The expression of these large reporter fusions is indicative of the amount of originally encoded protein expressed under the particular experimental conditions (21, 28). However, large fusion proteins may exhibit different properties than the untagged proteins, e.g., by affecting their solubility (19) or localization through steric hindrance (13). We describe here an approach to obtain a genetic readout of proteomic information by creating fusions with a short hexadecapeptide, called Tip (transcription-inducing peptide), that induces Tet repressor (16). For that purpose we constructed an insertion element, called InsTip α , to generate transposome-mediated random (12) in-frame translational fusions with Tip in *Escherichia coli*. We demonstrate here that this element can be used to determine the expression characteristics of proteins in five such fusion mutants.

Construction and function of InsTip α in *E. coli*. We assembled the 1,816-bp-long insertion element InsTip α (Fig. 1A) from previously published vectors and oligonucleotides, amplified it by PCR, and cloned it into the PvuII-digested vector pWH1866 (17) (see Table 1 for a list of all bacterial strains, plasmids, and phages) to obtain pWH1912. (Construction details and sequences of all plasmids generated in this study are available upon request.) InsTip α is flanked by 19-bp-long mosaic elements (ME) (26), the binding sites of the hyperactive transposase Tn5 (8). The leftward end of InsTip α (Fig. 1A) contains a sequence designed to fuse a tag, termed InsTip1, to any protein encoded at the insertion site. An open reading frame spans the ME encoding 7 amino acids, followed by a sequence encoding a 7-amino-acid linker and the 16-residue Tip element terminated by an ochre stop codon. The insertion element also contains *aphAIII*, which confers kanamycin resistance in gram-

negative and -positive bacteria (10), flanked by two Flp recombinase target sites to allow excision of the antibiotic resistance gene (4), thereby minimizing genomic interference.

To determine if InsTip α insertion into an open reading frame can generate a protein fusion that induces TetR, InsTip α was fused to TrxA of *E. coli*, which had previously been used to express and characterize Tip (16). The constructed plasmid, pWH1909, expressing TrxA-InsTip1 under *P_{lac}* control, and the plasmid pWH1911, expressing TetR constitutively at a low level, were cotransformed into *E. coli* WH207(λ tet50) (35). The addition of IPTG (isopropyl- β -D-thiogalactopyranoside) to the resulting strain leads to expression of the InsTip1 fusion protein, and subsequent induction of TetR should relieve repression of *lacZ* (Fig. 1C). We titrated the strain with IPTG concentrations of 15 to 500 μ M to express increasing amounts of TrxA-InsTip1 (Fig. 1D). Plasmid pWH2101 expressing TrxA-Tip was cotransformed instead of pWH1909 to provide a positive control for induction (16). A plateau of β -galactosidase (β -Gal) activity was obtained for concentrations above 60 μ M of IPTG with both constructs (Fig. 1D). Western blot analyses carried out simultaneously demonstrated nearly identical expression levels of the fusion proteins TrxA-Tip and TrxA-InsTip1 (Fig. 1E). Thus, neither the length nor the primary structure of the peptide segment encoded by the ME and linker affects the TetR-inducing properties of Tip.

Purified hyperactive transposase and PvuII-restricted, gel-purified InsTip α DNA were incubated to form transposomes (2) and electroporated (8) into *E. coli* WH207(λ tet50)/pWH1911, resulting in 6,400 kanamycin-resistant candidates on MacConkey agar plates. After 36 h of incubation at 37°C, 5 colonies displayed a yellow color indicating induction of TetR. Chromosomal DNA from the five strains was prepared and sequenced to determine their InsTip α integration sites (2). The diverse sequences obtained at the InsTip α integration loci agree in indicating its random insertion (12). Table 2 lists the strains obtained and the genes with their respective InsTip α insertion sites (see also Fig. 2), together with information about the function and subcellular localization of the affected proteins.

Determining fusion protein expression by InsTip1-induced

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FIG. 1. *InsTipα* architecture, in vivo screen, and in vivo activity. (A) Architecture of the insertion element *InsTipα*. Triangles represent the ME for transposase binding, the white box indicates the linker element, and the gray box encodes Tip. The ochre stop codon is symbolized by "S." The white and black arrows represent the kanamycin resistance gene (*aphAIII*) and its promoter, and a lollipop represents the bidirectional transcriptional terminator from *Tn10* (30). The resistance cassette is flanked by two Flp recombinase target sites (FRT) drawn as black arrows. The amino acid sequence encoded by the mosaic element at the leftward 5' end, the linker, and *tip* is shown in single-letter abbreviations above the nucleotide sequence that is displayed in white type on a black background. The stop codon is marked by an asterisk. (B and C) In vivo screening system for TetR-inducing *InsTip1* fusions. (B) Screening was carried out with *E. coli* WH207(λ *tet50*) containing a chromosomally encoded *tetA-lacZ* transcriptional fusion. TetR (gray ovals) binds *tetO* and represses *lacZ*. (C) Integration of the insertion element *InsTipα* into an expressed open reading frame (*geneX*) results in a *GeneX-InsTip1* fusion protein (light gray oval with X). Induction of TetR by this fusion protein is indicated by β -Gal activity. (D) In vivo activity of the *TrxA-InsTip1* fusion. The leftmost pair of bars shows the repressed (white) and tetracycline-induced (gray) β -Gal activities. The next seven bars show the activities of the *TrxA-Tip* fusion control without (white) and with IPTG at the indicated concentrations (black; 15, 30, 60, 125, 250, and 500 μ M). The right group of bars shows the β -Gal activities obtained with the *TrxA-InsTip1* fusion under the same conditions. The y axis displays the β -Gal activities in Miller units (MU) (23). (E) Western blot of 10 μ g of crude cell lysates separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and analyzed with an anti-thioredoxin A monoclonal antibody (Invitrogen).

β -Gal expression. We first examined the growth curves of the five candidates and their parent strain WH207(λ *tet50*)/pWH1911 at 37°C in MacConkey medium lacking the indicator bromocresol purple (Fig. 2A to E). The growth rates of all

strains were identical (Fig. 2B to E), except that for WH851, which was clearly growth defective (Fig. 2A). We assumed that the *InsTipα* insertion in *atpD* inactivates ATP synthase (29). We therefore analyzed the growth of WH851 and the *atpD*⁺

TABLE 1. Bacterial strains, plasmids, and phage used in this study

Strain, plasmid, or phage	Relevant characteristics	Reference or source
Strains		
RB791	IN[<i>rrnD-rrnE</i>]1 <i>lacI</i> ^q P _{lac} L8; cloning and propagation of pWH1909 and pWH2101	3
WH207	<i>galK rpsL thi ΔlacX74 recA13</i>	35
WH851	WH207(<i>Δtet50</i>)/pWH1911 <i>atpD</i> ₁₁₄₇ ::InsTipα; Km ^r	This study
WH852	WH207(<i>Δtet50</i>)/pWH1911 <i>ppiD</i> ₃₂₈ ::InsTipα; Km ^r	This study
WH853	WH207(<i>Δtet50</i>)/pWH1911 <i>yraI</i> ₆₀₇ ::InsTipα; Km ^r	This study
WH854	WH207(<i>Δtet50</i>)/pWH1911 <i>yicO</i> ₈₅₀ ::InsTipα; Km ^r	This study
WH855	WH207(<i>Δtet50</i>)/pWH1911 <i>yfdX</i> ₄₇₅ ::InsTipα; Km ^r	This study
Plasmids and phage		
pWH1866	Ap ^r Km ^r ; InsTet ^{G-1} ; pUC18 derivative	17
pWH1909	Ap ^r Km ^r P _{lac} TrxA-InsTip1; pBR322 derivative	This study
pWH1911	Cm ^r <i>lacI</i> ^q <i>tetR</i> ; pACYC177 derivative	This study
pWH1912	Ap ^r Km ^r ; InsTipα; pUC18 derivative	This study
pWH2101	Ap ^r P _{lac} <i>trxA-tip</i> ; pBR322 derivative	16
<i>Δtet50</i>	Tn10 <i>tetA-lacZ</i> transcriptional fusion	32

parent strain on minimal medium with a nonfermentable carbon source. In contrast to the *atpD*⁺ strain, WH851 displayed no growth on agar plates containing minimal medium with succinate as the sole carbon source (data not shown). Since growth on this medium depends on a functional oxidative phosphorylation system, this phenotype is consistent with a nonfunctional ATP synthase (14, 18). This assumption was further supported by the growth phenotype in liquid minimal medium supplemented with limiting amounts of glucose as the sole carbon source (18, 34); in this medium, WH851 reached a maximal optical density of 0.3, in contrast to an optical density of 1.0 for the *atpD*⁺ reference strain (data not shown), again indicating the absence of oxidative phosphorylation.

At regular intervals during the growth periods, cells were collected from all candidate strains and the parent strain to determine the β-Gal activities during exponential and stationary growth (Fig. 2A to E). The parent strain showed no change

in β-Gal activity throughout the entire experiment, although the β-Gal activity could be induced 40-fold by the addition of Tc (Fig. 1D) (35). Only background levels of β-Gal activity were detected for the candidate strains during exponential growth (Fig. 2A to E). However, for PpiD-InsTip1, YraI-InsTip1, YicO-InsTip1, and YfdX-InsTip1, a rapid increase in β-Gal activity was observed at the onset of the stationary growth phase, reaching a plateau by the 20-h time point of the experiment (Fig. 2B to E). With a value of approximately 1,100 units (23), WH852 has demonstrated the highest level of β-Gal activity. In contrast, we detected an increase in β-Gal activity within the stationary growth phase for WH851, but it was delayed and not as pronounced as the increases for the other strains. In addition, it did not reach a plateau during the 40-h time frame of the experiment (Fig. 2A).

We calculated an approximately sixfold increase in β-Gal activity of WH853 with the YraI-InsTip1 fusion for the transition from mid-exponential growth (4 h) to full stationary phase (20 to 40 h) (Fig. 2C). This observation is in excellent agreement with published data from transcriptome profiling in which a 4.1-fold increase in the mRNA level was observed due to the growth phase shift (11).

In WH854, we established expression of the YicO-InsTip1 fusion in stationary phase. *yicO* promoter activity was previously reported to be induced in the early stationary phase by overexpression of the two-component regulatory system BaeSR using a transcriptional reporter gene fusion (1). In contrast, a whole-genome microarray analysis of BaeSR-dependent gene expression from mid-exponential-phase cultures showed no increase of *yicO* expression (24). Our results for YicO-InsTip1 expression are in full agreement with the results of both of these earlier reports and explain their apparently diverse results.

In WH855, InsTipα had integrated into the *yfdX* gene. Since *yfdX* is an essential gene (6), both the insertion and the identical growth curves of WH855 and the *yfdX*⁺ reference strain at 37°C indicate that residues in a C-terminal position from Gly158 are not needed for growth under these conditions (Fig. 2E). A transcriptome comparison of exponentially grown cells with stationary-phase cells of *E. coli* showed that *yfdX* is induced significantly in the stationary phase (see supplemental data from reference 31 as cited in reference 22). Our data from the YfdX-InsTip1 fusion corroborate this result (Fig. 2E).

TABLE 2. Annotation of InsTipα insertion strains

Strain	Gene name ^a	Gene essentiality ^b	Protein function ^c	Subcellular localization ^c	Insertion site ^d	Fusion protein (aa) ^e
WH851	<i>atpD</i> (1,383)	Nonessential	Membrane-bound ATP synthase; F ₁ sector; β-subunit	Cytoplasmic	1147	412
WH852	<i>ppiD</i> (1,872)	Nonessential	Peptidylprolyl- <i>cis-trans</i> -isomerase D	Membrane anchored; periplasmic	328	138
WH853	<i>yraI</i> (696)	Nonessential	Unknown; belongs to the periplasmic pilus chaperone family	Periplasmic	607	232
WH854	<i>yicO</i> (1,335)	Nonessential	Unknown; predicted xanthine/uracil permease	Integral, inner membrane protein	850	313
WH855	<i>yfdX</i> (636)	Essential	Unknown	Periplasmic	475	188

^a Values in parentheses are lengths in bp.

^b As defined by Gerdes et al. (6).

^c As annotated by Riley et al. (27).

^d After the indicated nucleotide position.

^e Deduced length in amino acids (aa).

FIG. 2. InsTip α insertion sites and expression of the resulting InsTip1 fusion proteins. (A to E) Results for the five strains with InsTip α insertions in (A) *atpD*, (B) *ppiD*, (C) *yraI*, (D) *yicO*, and (E) *yfdX*. Top panels, schematic depiction of the insertion site and orientation of InsTip α into each target gene. The respective gene is represented by a black arrow and its designation, and its total length is indicated in bp. InsTip α is drawn schematically above the open reading frame, and its insertion site is indicated by a dotted line with the exact position of the nucleotide preceding the inserted sequence. Bottom panels, growth curves (filled squares) and β -Gal activities (filled bars) of WH207(λ tet50)/pWH1911 and the respective candidate strains (open squares and open bars) grown at 37°C in MacConkey medium without indicator. Growth is expressed by the optical density at 600 nm (OD₆₀₀) on the left y axis. The right y axis displays the β -Gal activities in Miller units (MU) (23). Each value was determined in triplicate, and the standard deviations are shown (error bars).

FIG. 3. Carbon source-dependent differential expression of PpiD-InsTip1. β -Gal activities of WH207($\Delta tet50$)/pWH1911 (closed bars) and WH852 (open bars) grown at 37°C to an optical density at 600 nm of 0.2 in M9 minimal medium supplemented with 50 mM glycerol or pyruvate. The y axis displays the β -Gal activities in Miller units (MU) (23). Each value was determined in triplicate and is shown with standard deviations (error bars). The dotted lines indicate the background β -Gal activities determined for the untagged parent strain, which were subtracted from the β -Gal activities of the tagged strain to determine the induction factor.

Besides being able to detect the expression of InsTip1 fusion proteins on MacConkey and minimal media, we can also use InsTip1 to determine quantitative differences in protein expression. A threefold-higher expression level of a *ppiD-lacZ* transcriptional fusion has been reported for exponentially growing *E. coli* in minimal medium with pyruvate as the sole carbon source than with glycerol as the sole carbon source (5). Therefore, we analyzed β -Gal activity of WH852 cells under these conditions and found a twofold-higher β -Gal activity for growth with pyruvate than with glycerol within the exponential growth phase. Thus, the result obtained with this translational fusion is in excellent agreement with the data from a transcriptional fusion (Fig. 3).

Conclusions. We have successfully used the peptide Tip (16) as a protein expression tag. We constructed an in-frame fusion which indicates the expression of the tagged target gene by readout of a separated *lacZ* gene. In contrast to direct *lacZ* fusions, this introduces only a 30-amino-acid-long extension after the insertion site, turning the protein fusion into an inducer for TetR. The induction of a separated reporter gene should lead to amplification of the signal. We constructed an InsTip α insertion element, formed the complex with hyperactive transposase (8), randomly mutated (12) the model organism *E. coli*, and obtained five candidates out of 6,400 insertion mutants. The tagged proteins were detected during the stationary growth phase, extending and confirming published expression data from transcriptional fusions or microarrays for *atpD*, *ppiD*, *yraI*, *yicO*, and *yfdX*, thereby validating the Tip tag approach. We could also measure expression of *ppiD* during the exponential growth phase. Together with the ability to detect proteins of essential and nonessential genes, as well as proteins localized in the cytoplasm, the inner membrane, or the periplasm with the tag, our results clearly demonstrate the applicability of InsTip α as a global tag. These results encourage us to further improve the insertion efficiency and to expand the detection of active InsTip1 fusion proteins by avoiding polar effects on downstream genes through inclusion of an outward promoter in the element (15). We also aim to improve

detection of protein expression within the exponential phase via enhanced screening, for example, by reduction of the relatively high basal level of β -Gal activity under repressed conditions to enlarge the detection window (20) or by the use of selection systems.

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