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**Title:** *Gaussia princeps* luciferase as reporter for transcriptional activity, protein secretion and protein-protein interactions in *Salmonella enterica* serovar Typhimurium

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**Abstract**

*Gaussia princeps* luciferase (Gluc) is widely used as a reporter in eukaryotes, but data about its applicability in bacteria is very limited. Here we show that a codon-optimized Gluc gene can be efficiently expressed in *Salmonella enterica* sv. Typhimurium (*S*. Typhimurium). To test different Gluc variants as transcriptional reporters, we used the *siiA* promoter of *Salmonella* pathogenicity island 4 (SPI-4) driving expression of either an episomal or a chromosomally integrated Gluc gene. Most reliable results were obtained from lysates of single-copy Gluc reporter strains. Given the small size, high activity and co-factor independence of Gluc, it might be especially suited to monitor secretion of bacterial proteins. We demonstrate its usefulness by luminescence detection of fusion proteins of Gluc and C-terminal portions of the SPI-4-encoded, type I-secreted adhesin SiiE in supernatants. The SiiE C-terminal moiety including immunoglobulin (Ig) domain 53 is essential and sufficient for mediating type I-dependent secretion of Gluc. In eukaryotes, protein-protein interaction studies based on split Gluc protein complementation assays (PCA) could be established. We adapted these methods for use in *Salmonella*, demonstrating the interaction between the SPI-1-encoded effector SipA and its cognate secretion chaperone InvB. In conclusion, the versatile Gluc can be used to address a variety of biological questions, thus representing a valuable addition to the toolbox of modern molecular biology and microbiology.

## Introduction

The luciferase from the marine copepod *Gaussia princeps* (Gluc) is the smallest (~19.9 kDa) coelenterazine (CTZ)-utilizing luciferase known to date. A humanized variant of Gluc (hGluc), which was codon-optimized for expression in cultured mammalian cells, has been widely used as a reporter gene (26). *Gaussia* luciferase exhibits an activity up to 1,000-fold higher than to *Renilla reniformis* luciferase (Rluc), firefly luciferase (Fluc) (26) or bacterial luciferases (LuxAB) (30). The outstanding sensitivity of Gluc-based assays was previously demonstrated detecting as low as $10^{-18}$ mol purified Gluc (27) or one eukaryotic cell transiently expressing Gluc (25). Gluc shares with Rluc its independence of any host-derived cofactors. Additionally, Gluc shows a good robustness regarding changes in pH (12, 30), heat shock and hydrogen peroxide (30). A split form of Gluc was applied as a reporter in protein complementation assays (PCA) to assess protein-protein interactions (15, 23).

The suitability of Gluc as a reporter gene was also shown in certain non-mammalian model organisms. *Gluc* genes codon-optimized for expression in the respective hosts were used in reporter assays with the fungus pathogen *Candida albicans* (5) and the bacteria *Mycobacterium smegmatis* (1, 30) and *M. tuberculosis* (1) as well as the algae *Chlamydomonas reinhardtii* (24). Recently, heterologous expression of highly active and soluble Gluc in *Escherichia coli* was reported, suggesting its usefulness as a reporter gene in *Enterobacteriaceae* (22).

Besides *E. coli*, one of the best characterized member of the *Enterobacteriaceae* is *Salmonella enterica*. *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) is a model pathogen able to cause gastroenteritis in humans and a typhoid fever-like disease in mice which is used to study many aspects of *Salmonella* pathogenicity. Most of its virulence functions are encoded within horizontally acquired regions on the genome called *Salmonella* pathogenicity islands (SPI) (8). SPI-1 and SPI-2 encode type III secretion systems (T3SS) enabling the pathogen to invade non-phagocytic cells and to survive within host cells, respectively. We

recently were able to show that another pathogenicity island, SPI-4, functionally cooperates with SPI-1 to facilitate the invasion of polarized epithelial cells (6). SPI-4 encodes for a type I secretion system (T1SS) consisting of three subunits: the inner membrane ABC (ATP binding cassette) protein SiiF energizing the transport, the periplasmic adaptor protein (PAP) SiiD and an outer membrane pore-forming protein (OMP) SiiC (11). The T1SS substrate SiiE, a giant non-fimbrial adhesion, is secreted via a C-terminal signal sequence and subsequently mediates intimate contact to the apical host cell membrane allowing for SPI-1-dependent effector injection and bacterial invasion (6). SiiE is of repetitive structure comprising 53 domains of the immunoglobulin (Ig) fold as well as beta-sheets and coiled-coils within its N-terminus (28).

With this study, we set out to establish Gluc as a reporter protein for (i) transcriptional activity, (ii) protein secretion and (iii) protein-protein interactions in *S.* Typhimurium. Gluc uniquely combines small size with very high activity, which has great potential for future applications.

**Materials and Methods**

*Cloning*: A description of the cloning procedures can be found in the supplemental material. All primers used in this study can be found in Table S1 in the supplemental material. An overview about the plasmids used is given in Supplementary Table S2. All constructs were initially screened by colony PCR using suitable check primers and finally verified by restriction analysis and sequencing.

*Generation of chromosomal fusions*: Paired primers SiiA-Gluc-for and SiiA-RedRep-rev were used to amplify Gluc or GlucM43I together with a kanamycin resistance cassette from pWRG168 or pWRG215. Insertion of Gluc in the chromosome by using Red recombinase was carried out as described previously (9). Successful integration was screened with primers SiiA-Check-for and Gluc-Check-rev and verified by sequencing. All fusions were transferred in fresh wild-type (WT) background or, in the case of WRG64 and WRG84, in a Δ*sirA* background (MvP896) by means of P22 HT105/1 *int-201* transduction. All strains used are listed in Table 1.

*Preparation of bacterial lysates*: The optical density at 600 nm ($OD_{600}$) of bacterial cultures was determined, and an equivalent of 0.5 $OD_{600}$ was removed. The supernatant was removed by centrifugation (8,000 x g, 5 min, 4 °C) and bacteria were resuspended in 40 µl of assay buffer: 10 mM Tris-HCl pH 7.8, 0.6 M NaCl, 1 mM EDTA (buffer A) (27). An equal amount of 0.1 mm glass beads (BioSpec Products, Bartlesville, OK, USA) was added to the bacterial suspensions on ice and cells were lysed using a bead beater device (TurboMix, Scientific Industries, Bohemia, NY, USA) for 5 min at an 80 % power setting. Lysates were cleared by centrifugation (14,000 x g, 10 min, 4 °C) and could be stored at -20 °C for several days without significant loss of Gluc activity (data not shown).

*Gluc overexpression in* Salmonella: Overnight cultures of strains harboring pBAD24-derived Gluc expression vectors were grown to mid-log phase ($OD_{600}$ = 0.5 to 0.8) and L-(+) arabinose (Sigma-Aldrich, Schnelldorf, Germany) was added to a final concentration of 10 mM

to induce Gluc expression. After 1 h of induction, samples were prepared for luminescence assays.

*Sample preparation to detect secretion of Gluc-SiiE fusions*: Overnight cultures were reinoculated at 1:100 in 3 ml of fresh LB and grown under aeration for 6 hrs. $OD_{600}$ was determined and supernatants and pellets from 1 ml of culture were collected by centrifugation (8,000 x g, 10 min, 4 °C). Supernatants were sterile filtered using 0.2 µm syringe filters (Acrodisc HT Tuffryn, Pall Life Sciences, Dreieich, Germany). Pellets were resuspended in 800 µl of assay buffer and subjected to lysis using a bead beater as described above.

*Sample preparation for split Gluc approach*: Overnight cultures were reinoculated at 1:31 in 3 ml of fresh LB and grown under aeration to mid-log phase ($OD_{600}$ 0.5 to 0.8) phase. After the addition of 50 ng/ml anhydrotetracycline (AHT, Sigma-Aldrich, Schnelldorf, Germany) to induce expression from $P_{tetA}$, cultures were allowed to grow until 4 hrs after reinoculation. $OD_{600}$ was determined and bacterial pellets from 1 ml and 0.5 ml of culture were collected by centrifugation (8,000 x g, 10 min, 4 °C). The pellet from 1 ml of culture was resuspended in 160 µl of assay buffer and subsequently used for luminescence detection in whole cells. The pellet from 0.5 ml of culture was resuspended in 400 µl assay buffer and subjected to lysis using a bead beater as described above.

*Luciferase assays*: To measure Gluc activity of transcriptional fusions and of Gluc expressed from pBAD24-derived vectors, 40 µl of assay buffer containing 12.5 µM native CTZ (Biotium, Hayward, CA, USA or Synchem, Felsberg, Germany) was added to a 10-µl aliquot of the bacterial suspensions or dilutions of bacterial lysates as indicated. To detect luciferase activity of Gluc-SiiE fusion proteins or split Gluc constructs, 10 µl of assay buffer containing 50 µM native CTZ (Synchem) was added to 40 µl undiluted samples. Native CTZ and different CTZ variants (sampler kit, Biotium) stock solutions of 10 mM in methanol were stored at -70 °C. Dilutions of CTZ stocks in assay buffer were stable for several days at -20 °C (data not shown). Luminescence measurements were carried out in 96-well white flat bottom plates

(Thermo Nunc, Langenselbold, Germany) by using an Infinite M1000 plate reader equipped with a photon counter detector (Tecan, Grödig, Austria). The reaction was started by well-wise addition of the substrate either manually or by injection. Signals were integrated over 1 s and those exceeding the detection limit of $10^7$ light counts per second (LCPS) were weakened by an OD1 filter.

*Bacterial two hybrid (BacTH) assay*: *E. coli* reporter strain BTH101 (14) was freshly transformed with combinations of two plasmids each encoding for fusion proteins with the T18 or T25 fragments derived from CyaA under the control of an IPTG-inducible promoter. After transformation, three µl of bacterial suspensions were spotted onto LB plates containing 25 µg/ml kanamycin (Carl Roth, Mannheim, Germany), 50 µg/ml carbenicillin (Carl Roth) and 100 µM isopropyl-β-D-thiogalactopyranoside (IPTG, Fermentas, St. Leon-Rot, Germany) as well as 40 µg/ml X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, Fermentas) as indicator for LacZ activity. Plates were incubated at 30 °C for 42 hrs and colonies were documented using a digital camera.

*Electrophoresis and Western blotting*: Secreted protein fraction was obtained by trichloroacetic acid (TCA) (Carl Roth) precipitation of 10 ml culture supernatants as described before (11). Polyacrylamide gel electrophoresis (PAGE) was carried out according to standard protocols using a Mini-Protean Tetra cell system (Bio-Rad Laboratories, Munich, Germany) or NuPAGE 4 to 12 % Bis-Tris gradient gels (Invitrogen, Karlsruhe, Germany). Semi-dry Western blotting was done in a Trans-Blot SD cell (Bio-Rad) at 1 mA/cm$^2$ for 1 hr. Antibodies against Gluc (New England BioLabs, Frankfurt am Main, Germany), the *Salmonella* flagellar antigen (FliC) (clone 4H2, Biotrend, Cologne, Germany) or DnaK (clone 8E2/2, Enzo Life Sciences, Lörrach, Germany) were applied over night after blotting. Bound antibodies were subsequently detected with anti-mouse and anti-rabbit horseradish peroxidase (HRP)-coupled secondary antibodies (Jackson Immuno Research/Dianova, Hamburg, Germany) using a Chemi-Smart 3000 chemiluminescence system (Vilber Lourmat, Eberhardzell, Germany). Blot

images were processed (marker overlay, tonal range, 16 to 8 bit conversion) using Photoshop CS5 (Adobe Systems, Munich, Germany).

## Results

*Gluc expression and substrate specificity in* S. *Typhimurium*

For establishing *Gaussia* luciferase as a reporter in *S*. Typhimurium, we used a *Gluc* gene variant codon-optimized for expression in *Salmonella enterica* (Geneart, Regensburg, Germany). Expression of full length Gluc including the coding sequence for the eukaryotic secretion signal comprising amino acids (aa) 1 to 17 was previously found to be problematic in bacteria (12, 19). Therefore, we omitted codons 2 to 17 of wild-type Gluc, generating $Gluc_{M18-185}$, which was used as "Gluc" in all subsequent experiments. Inouye and Sahara (12) showed that purified Gluc has a narrow substrate spectrum and that only native CTZ is a useful substrate for Gluc. We tested different commercially available CTZ variants as substrates for Gluc in lysates and whole bacterial cells. *S*. Typhimurium harboring pBAD24-Gluc (GenBank HM241886) was used for the expression of Gluc by the addition of 10 mM arabinose. With Gluc-containing *Salmonella* lysates, native CTZ showed the highest initial light counts, 1 to 2 orders of magnitude higher than those of *f*-CTZ, *h*-CTZ or *i*-CTZ (see Fig. S1A in the supplemental material). With *hcp*-CTZ and *fcp*-CTZ as substrates, which should give highest light emission with RLuc (manufacturer's information), Gluc had the lowest activity, starting with $10^4$ LCPS and decreasing to less than 400 LCPS in about 4 min (see Fig. S1A). Gluc light emission with whole cells and native CTZ was almost 3 orders of magnitude lower than that of lysates (see Fig. S1B). Whereas light emission kinetics with lysates followed the rapid decay typical for flash-type luciferases, a slight increase in LCPS reaching a plateau after 3 min was observed with whole cells (see Fig. S1B).

The amount of light emitted by Gluc depends on the final substrate concentration. With Gluc-expressing *M. smegmatis*, there is a linear relationship between luminescence and substrate concentration between 0.1 μM and 10 μM CTZ (30). Using whole cells or a 1:100 dilution of *Salmonella* lysates, we tested native CTZ in concentrations between 0.01 μM and 100 μM (Fig. 1A). Robust detection of light emission was possible starting from 0.05 μM CTZ.

There was a linear relationship between light counts and substrate concentration in the range between 0.75 µM and 10 µM CTZ ($R^2 > 0.99$) using cell lysates (Fig. 1B). Whole cells exhibited a linear trend between 5 µM and 75 µM ($R^2 > 0.97$) (Fig. 1C). Saturation of light emission could be observed starting from 25 µM or 100 µM CTZ with lysates and whole cells, respectively (Fig. 1A). In PCA, an earlier saturation of light emission, starting already at 10 µM CTZ was observed (23). With increasing concentrations, the background arising from CTZ auto-oxidation might become a problem (27). As a compromise, we therefore used a final concentration of 10 µM CTZ in all further assays.

*Gluc as plasmid-based transcriptional reporter*

To demonstrate the usability of Gluc as a transcriptional reporter, we chose the SPI-4 virulence locus as an example. It was previously shown that the expression of SPI-4 is coregulated with SPI-1-encoded invasion genes and depends on transcriptional regulators SirA and HilA (10, 20). *In vitro*, SPI-1 and SPI-4 expression is induced during late exponential growth in rich medium (10). The six genes of SPI-4, *siiA* to *F*, seem to form one operon whose expression is driven from a promoter upstream of *siiA* (10). To generate the Gluc reporter plasmid pWRG169, we modified the mid-copy vector pGEN-luxCDABE (16) exchanging the *lux* operon with the Gluc gene combined with an optimized ribosome binding site (RBS) (4). Subsequently, a ~1 kb fragment 5' of *siiA* containing the putative *siiA* promoter was cloned in pWRG169, yielding pWRG170. pWRG170 was transferred in *S*. Typhimurium wild-type (WT) and the isogenic *sirA*-deficient strain MvP896 (32). Luminescence activities of lysates (Fig. 2A) and whole cells (see Fig. S2A in the supplemental material) were determined in a kinetics assay over 6 hrs post inoculation. All strains tested showed comparable growth kinetics by means of $OD_{600}$ measurements (data not shown). Using the *siiA* promoter to express Gluc, we observed the characteristic induction peak during late logarithmic growth in WT [$P_{siiA}$::*Gluc*] (Fig. 2A). The *sirA*-deficient strain showed reduced luminescence activity at the level of the WT harboring the promoterless vector. The Gluc reporter plasmid without promoter exhibited

significant background activity (Fig. 2A). Around 4 hrs after reinoculation, maximum activity of SPI-4 expression was observed using both, whole cells and lysates. With whole cells (see Fig. S2A), the maximum luminescence was about 10-fold lower than that of the 1:10 dilution of lysates (Fig.2A). In contrast to previous data obtained with Fluc reporters (10), the differences in activity between the WT and the *sirA* strain were much lower, reaching only about 10-fold (whole cells) and 5-fold (lysates) at 4 h post inoculation. Furthermore, plasmidbased Gluc assays showed significant variability between individual experiments, as indicated by high standard deviations (Fig. 2A, also see Fig. S2A).

*Gluc as chromosomally encoded transcriptional reporter*

To address the problem of high background activity and variability, single-copy Gluc reporter strains were generated. We made use of our previously introduced technique and integrated Gluc within the *Salmonella* genome with the help of λ Red recombinase (9). To test the activity of chromosomally integrated Gluc, we replaced the *siiA* open reading frame (ORF) by the Gluc gene by use targeting construct generated by PCR with template vector pWRG168. The resulting strain, WRG34, harbors a start codon fusion of the Gluc gene to *siiA*, placing the Gluc gene in the same genomic context under the same regulatory elements as *siiA*. As a control, the Gluc reporter construct was transferred in the isogenic *sirA*-deficient strain MvP896 by means of P22 phage transduction yielding strain WRG84. Bacterial lysates (Fig. 2B) and whole cells (see Fig. S2B in the supplemental material) were collected as described above. The maximum activity of SPI-4 expression occurred at 3.5 h and 4 h after reinoculation detecting Gluc in lysates and whole cells, respectively. In contrast to our previous results with the plasmid-based reporter (Fig. 2A), strain WRG84 lacking the global regulator SirA showed nearly no background activity. During late logarithmic growth, lysates of WRG34 (*siiA*::Gluc) show luminescence activity up to 200-fold higher than those seen for WRG84 (*sirA siiA*::Gluc) (Fig. 2B). If whole cells were compared, a 16-fold difference was still observed at peak activity between the two strains (see Fig. S2B).

*Gluc mutant with higher luminescence activity*

Mutational analysis of the Gluc protein was conducted recently in two independent studies to screen for Gluc variants with more favorable light emission kinetics and higher activity (19, 29). Both groups identified methionine residues to be of critical importance. Exchange of methionine to isoleucine (M43I) and leucine (M43L, M110L) both led to an increased half-life of light emission. We modified Gluc with the M43I mutation suggested by Maguire *et al*. (19) which should offer both stabilized and high light emission compared to the WT. Lysates were obtained from *Salmonella* expressing GlucM43I from pBAD-GlucM43I. Comparing the light emission kinetics of lysates containing GlucM43I or WT Gluc over 15 min revealed higher and prolonged activity of GlucM43I (see Fig. S3 in the supplemental material). The M43I mutation led to an initial 2.6-fold increase in luminescence activity compared to the WT. Whereas WT Gluc light emission decreased within 90 s to ~4.1 %, more than one-fourth of GlucM43I activity was still present after the same time (see Fig. S3).

We tested the performance of GlucM43I as a transcriptional reporter by chromosomal exchange of *siiA* ORF in *Salmonella* WT and MvP896 *sirA* backgrounds, resulting in strains WRG42 and WRG64, respectively. When luminescence activity was measured in a 6-h time course, GlucM43I exhibited ~20 % higher peak activity during late logarithmic growth (Fig. 2A).

*Gluc is not naturally secreted in* S. *Typhimurium*

The high sensitivity of Gluc-based reporter systems enables direct extracellular luminescence detection, as demonstrated recently using mouse urine and blood samples (31). These studies showed clearly Gluc being superior, with respect to stability, sensitivity, and detection range, to the established secretion reporter SAP (secretory *a*lkaline *p*hosphatase). Two prerequisites should be fulfilled to apply Gluc as a secretion reporter in bacteria. First, there should be a difference in activity and/or substrate accessibility of intra- and extracellularly localized Gluc. We found in *Salmonella* an ~2,000-fold difference in

luminescence activity comparing whole cells and lysates, demonstrating that the Gram-negative cell envelope represents a diffusion barrier for CTZ, limiting substrate availability for intracellular Gluc (see Fig. S1). Second, Gluc and Gluc fusion proteins must not be secreted naturally in significant amounts. It was shown recently that *M. smegmatis* releases high amounts of Gluc in culture supernatants regardless of the presence or absence of its natural secretion signal (1). We tested the supernatants of *Salmonella* cultures for luminescence activity where Gluc or GlucM43I expression was induced from pBAD24-based vectors. Figure 3A shows luminescence activity of cell pellets, cell lysates and supernatants. We found the vast majority of Gluc activity associated with cells, resulting in a several-thousand-fold difference in luminescence compared to supernatants (Fig. 3A). Although luminescence can be readily detected in supernatants, most of the protein is retained within the cytosol even under the unfavorable condition of very high Gluc expression levels.

*Gluc fusion proteins can monitor T1S*

To test, whether Gluc is suitable monitoring type I secretion (T1S), we set out to characterize the C-terminal signal sequence of the T1SS substrate SiiE. Previously, mutational analyses in conjunction with Western blots and a customized SiiE-specific enzyme-linked immunosorbent assay (ELISA) led to the identification of a C-terminal portion including Ig domain 53 to be essential for mediating SiiE transport via the *siiCDF*-encoded T1SS (11, 28). In a complementary setup, we constructed fusion proteins consisting of an N-terminal GlucM43I and different C-terminal moieties of SiiE to define the minimal secretion signal. A detailed overview of all constructs can be found in Fig. 3B: a fusion protein consisting of Gluc and the last four Ig domains of SiiE (Ig50 to 53 [Ig50-53]) is efficiently secreted as culture supernatants showing high luminescence activity after 6 h of growth (Fig. 3C). If the construct was transferred in a strain lacking the ABC protein SiiF or a stop codon was introduced between Gluc and SiiE Ig50-53, almost no luciferase activity could be detected in the supernatants (Fig. 3C). When only Ig domain 53 of SiiE was fused to the C terminus of Gluc,

luminescence activity could be detected in supernatants, but reaching less than 5 % of the level seen for Ig50-53 fusion protein (Fig. 3C). Deletion of the first 15 N-terminal amino acids of Ig53 abolished the ability to mediate T1S of Gluc, which is consistent with previous results from SiiE mutational analysis (28). As a control, we did Western blots to detect the secreted fusion proteins in precipitated supernatants. In all the supernatants with significant luciferase activity, a signal was detectable with an anti-Gluc antibody (see Fig. S4 top panel, in the supplemental material). The theoretical molecular masses were calculated as 69.7 kDa and 34.8 kDa for Gluc::SiiE-Ig50-53 and Gluc::SiiE-Ig53, respectively. The signal intensity of the smaller fusion protein was very low compared to that of Gluc::SiiE-Ig50-53 (see Fig. S4). As a loading control, the flagellar antigen FliC was detected in the supernatant samples (see Fig. S4, middle panel). This result is in good correlation with the decreased luminescence activity of this construct detected in supernatants (Fig. 3C). To check for significant bacterial lysis, which might lead to unspecific Gluc release, samples from supernatants were probed for the cytosolic marker protein DnaK. DnaK was not detected except in a bacterial pellet sample included as a positive control (see Fig. S4, bottom panel).

Given the big difference in luminescence activity comparing intra- and extracellular Gluc (Fig. 3A), we speculated that this effect based on substrate availability enables detection of secreted Gluc in whole cultures. Indeed, we measured very similar Gluc activities regardless of using whole cultures or sterile-filtered culture supernatants (Fig. 3C). Comparing individual fusion proteins in quantitative assays might be difficult due to differences in the expression levels and/or protein stabilities of the respective fusions. Measuring the Gluc activity in cell lysates is a convenient way to detect such differences and enables easy normalization. We detected comparable luciferase activties in bacterial lysates expressing different Gluc fusion proteins, which indicates similar protein stabilities and expression levels (Fig. 3C).

*Split-Gluc approach to detect protein-protein interactions in* Salmonella

The high sensitivity, good signal-to-noise ratio and simplicity of assays demonstrated in our previous results prompted us to evaluate the usability of the split-Gluc PCA to investigate protein-protein interactions in *Salmonella*. In PCA, a reporter protein is split into two halves and these parts are fused to putative interaction partners. If interaction via the fusion proteins occurs, the protein is reconstituted from its parts, thereby restoring reporter activity (2). Two reports suggested two different split sites for optimal complementation results with Gluc: Remy and Michnick (23) split Gluc between G109 and E110 whereas Kim *et al*. (15) divided the enzyme between Q105 and G106 (Fig. 4B). We compared both variants side by side employing a known *Salmonella* protein-protein interaction as an example. The interaction of T3SS effectors with their cognate type III secretion chaperones is absolutely essential for the subsequent translocation through the T3SS needle complex (3). The SPI-1-encoded secretion chaperone InvB was shown to bind the effectors SipA, SopA, SopE and SopE2 (3, 17). The chaperone binding domain (CBD) of SipA, which is recognized by InvB, could be localized to the first 47 N-terminal amino acids and truncated SipA variants lacking this part were unable to form stable complexes with InvB *in vitro* (18).

To test this interaction in our split-Gluc approach, we fused full-length SipA or an N-terminally truncated $SipA_{48-685}$ with either $Gluc_{106-185}$ or $Gluc_{110-185}$. Correspondingly, $Gluc_{18-105}$ or $Gluc_{18-109}$ was fused to the C-terminus of full-length InvB lacking its stop codon. An overview about the constructs is given in Fig. 4C. The corresponding Gluc fusion proteins were cloned together in a low-copy-number plasmid as an operon structure under the control of a tetracycline-inducible promoter (see supplemental material). After induction with 50 ng/ml AHT, the luminescence activity of bacterial lysates (Fig. 4D, top, gray bars) and whole cells (Fig. 4D, bottom, black bars) was measured. In lysates from bacteria expressing full-length SipA together with InvB (pWRG299 and pWRG300), we measured high luminescence activity indicating split-Gluc complementation through chaperone-effector interaction (Fig. 4D). As expected, deletion of the SipA CBD in $SipA_{48-685}$ abrogated InvB binding which resulted in

significantly reduced Gluc activity (pWRG374 and pWRG375) (Fig. 4D). By use of bacterial lysates, a 10-fold (Q105-G106) or 13-fold (G109-E110) decrease in signal could be measured with SipA lacking its CBD compared to full-length SipA (Fig. 4D, top). Even with whole cells, a 2.3-fold (G109-E110) or 4.8-fold (Q105-G106) difference was observed (Fig. 4D, bottom). To rule out that the differences measured are the result of lower expression levels of the $SipA_{48-685}$ fusion proteins, we used a polyclonal anti-Gluc antibody to detect the split-Gluc fusions in bacterial lysates by Western blotting. All SipA fusion proteins showed comparable expression levels (see Fig. S5A in the supplemental material).

To compare our approach to an established technique, we used a bacterial two-hybrid (BacTH) assay to show the interaction between InvB and SipA. BacTH is based on a PCA of fragments T18 and T25 derived from *Bordetella pertussis* CyaA (13). Coexpression of the T25-InvB fusion protein together with $SipA_{1-685}$-T18 in *E. coli* BTH101 resulted in dark blue colonies (see Fig. S5B in the supplemental material). This indicated efficient CyaA fragment complementation through $SipA_{1-685}$-InvB interaction. If the $SipA_{48-685}$-T18 fusion protein was used instead, CyaA PCA was much less efficient, as indicated by virtual absence of LacZ activity (see Fig. S5B). This essentially confirmed our results obtained with split Gluc.

**Discussion**

We conducted a comprehensive study to evaluate the usefulness of *Gaussia princeps* luciferase as a reporter in *Salmonella enterica*. Comparing the Gluc luminescence activity obtained from whole cells and cell lysates indicates that the Gram-negative envelope represents a diffusion barrier for coelenterazines. If Gluc was highly expressed in *Salmonella*, cell wall permeability of CTZ seems to be the limiting factor because luciferase activity reaches a constant plateau after 3 min (see Fig. S1B in the supplemental material). Substrate accessibility of intracellular luciferases seems to be an issue in different organisms. When whole cells of the fungus *C. albicans* were compared to cell lysates in Rluc activity, signals were enhanced about 60-fold when lysates were used (5). The extremely high enzymatic activity of Gluc compared to that of other luciferases enables the detection of genes with very low expression at their endogenous levels. When we used Gluc to report transcriptional activity of the *siiA* promoter, the lowest background activity was observed with Gluc integrated in a single copy within the *Salmonella* genome (Fig. 2B). Care should be taken if Gluc is used in multi-copy reporter plasmids, since significant background activity occurred (Fig. 2A). As an alternative to established transcriptional reporters, Gluc might be used as highly sensitive, fast option with comparably low costs.

Recently, new Gluc variants were developed promising higher activity in combination with increased signal stability (19, 29). The GlucM43I mutant showed a higher and more glow-type light emission kinetics in *Salmonella* lysates (see Fig. S3 in the supplemental material). We used this improved Gluc variant in fusion proteins together with domains of the SPI-4-encoded adhesin SiiE to quantify T1SS-dependent secretion. Previous results from the analysis of different SiiE deletion mutants with ELISA underscored the importance of the C-terminal part including the complete Ig53 domain for secretion of the adhesin (28). We addressed this question in a different way, defining the SiiE C-terminal region including Ig53 to be essential and sufficient for mediating T1S of a heterologous N-terminal domain (Gluc). No T1SS-

dependent secretion of Gluc was detected if 15 N-terminal amino acids from SiiE Ig53 were deleted (Fig. 4C). Interestingly, we observed an approximately 25-fold reduced secretion when Gluc was fused to Ig53 alone compared to Ig50-53 (Fig. 4C). Recent results from studies with the type I secreted hemophore HasA of *Serratia marcescens* indicate that there is a role of linear sequence stretches within HasA in increasing secretion efficiency. The C-terminal secretion signal might play an important role inducing ATP hydrolysis by the ABC protein which subsequently leads to disassembly of the T1SS and release of the substrate (21). Our results indicate an influence of domains located N-terminally of Ig53 on the secretion efficiency of SiiE. The characterization of these signals has to be addressed in future experiments. Gluc-based secretion reporters might open a venue for the identification and characterization of secreted proteins without stable periplasmic intermediates, which is a great advantage over alkaline phosphatase (PhoA)-based secretion assays. Besides type I also type III, type IV, and type VI secretion systems feature structures bridging the periplasm (7). Whether Gluc can be secreted via these systems has to be elucidated.

To demonstrate that the split-Gluc method can be used in *Salmonella* to detect protein-protein interactions, we have chosen the binding of the T3SS effector SipA to its cognate secretion chaperone InvB as an example (18). With both published splitting sites (Fig. 4B), we got a highly significant, up-to-13-fold increase of luminescence activity with full-length SipA and InvB (Fig. 4D). The two alternative split variants had both advantages and disadvantages. In our assay, the G109-E110 split variant (23) produced about 50 % higher signal with $SipA_{1-685}$ and InvB in lysates (Fig. 4D, top), but led to a 3.8-fold increased background for $SipA_{48-685}$ which lacks the CBD, in whole cells (Fig. 4D, bottom). Although InvB is a typical class I chaperone forming homodimers, the complex binds only one SipA effector molecule. Within this chaperone-effector complex, the CBD of SipA is bound by only one InvB protein (18). With our approach, we cannot determine whether there is an alternation between the two InvB monomers donating the Gluc N terminus or whether one InvB protein provides the Gluc part

exclusively (Fig. 4A). A unique feature of split Gluc is its reversible complementation, enabling investigation of dynamic and transient protein-protein interactions *in vivo* (15, 23). We could not demonstrate if Gluc reconstitution is reversible in *Salmonella*, because the interaction between SipA and InvB is thought to be stable upon binding of the chaperone-effector complex to the T3SS apparatus (18). Further experiments should prove if dynamic interactions can be reproduced by split-Gluc complementation in *Salmonella*.

Although C-terminal tagging of SipA and InvB was functional, other interaction partners might require N-terminal fusion of split Gluc portions for functionality. A future task is to develop a modular expression system allowing convenient cloning and simultaneous expression of two proteins fused to the N- and C-terminal parts of Gluc, respectively. In summary, with the split-Gluc approach we could obtain results comparable to those obtained by a bacterial two-hybrid assay based on *Bordetella pertussis* CyaA fragment complementation (13) (see Fig. S5B in the supplemental material). Apart from this, split-Gluc PCA has several advantages: (i) It does not require special reporter strains for analysis. (ii) There is no change of endogenous cyclic AMP (cAMP) levels throughout the experiment. (iii) Gluc complementation is very fast and can easily be quantified. (iv) The split Gluc fragments are smaller and hence less likely to interfere with functions of the fusion partner. Therefore, this approach might be of particular interest for investigating protein-protein interactions *in vivo* in the natural host with small impact on general cellular functions.

**Acknowledgements:**

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**Figure Legends**

Figure 1. Influence of substrate concentration on luminescence activity.

(A) Gluc was expressed from pBAD24-Gluc in *S*. Typhimurium WT and lysates were prepared as described in Materials and Methods. Lysates were finally diluted 1:100 in assay buffer and whole cells were collected in parallel. Different concentrations of native CTZ were added and luminescence activity was determined. (B) Linear regression of data from lysates for the concentration range indicated. (C) Linear regression of data from intact cells for the concentration range indicated. In (B) and (C) a subset of the data from (A) is depicted.

Figure 2. Expression of Gluc reporter fusions to the SPI-4 promoter quantified from bacterial lysates.

(A) Overnight cultures of the strains indicated were reinoculated 1:31 in fresh LB and grown under aeration for 6 hrs. Samples were collected every 30 min. A plasmid harboring a transcriptional fusion of Gluc to a fragment 5' of *siiA* containing the putative SPI-4 promoter (pWRG170) as well as a promoterless control (pWRG169) were transferred in *S*. Typhimurium WT and the *sirA*-deficient strain MvP896. Lysates were prepared as described in Materials and Methods, diluted 1:10 and normalized to $OD_{600}$. (B) The *siiA* ORF was exchanged by *Gluc* within the chromosome in WT (WRG34) and MvP896 (*sirA*, WRG84). *Gluc*M43I was used in a similar exchange experiment to generate WRG42 (WT) and WRG64 (*sirA*). Luminescence activities in culture lysates were determined as described for panel A. The data represent mean and standard deviation of three independent experiments done in triplicates.

Figure 3. Gluc as marker to monitor type I secretion in *S*. Typhimurium.

(A) Gluc and GlucM43I were expressed from pBAD24-derived plasmids and luminescence activities of whole cells (black bars), supernatants (open bars) and 1:10 diluted lysates (gray bars) were determined. (B) Various translational fusions of Gluc to the C-terminal

moiety of SiiE were generated as indicated. The corresponding plasmid names are given in brackets. For construction details refer to the Supplementary Information. (C) The reporter gene fusions were expressed under control of the *siiA* promoter in *S*. Typhimurium WT or the *siiF* strain deficient in the SPI-4 T1SS. After 6 hrs of growth in LB, samples were collected and Gluc activity was quantified for whole cells, sterile-filtered supernatants and bacterial lysates. The data represent mean and standard deviation of three independent experiments done in triplicates.

Figure 4. A split Gluc protein complementation assay (PCA) for detection of chaperone-effector interaction

(A) Schematic representation of the principle. The N-terminus of GlucM43I was fused to the C-terminus of the secretion chaperone InvB. The corresponding C-terminal portion of GlucM43I was fused to the cognate T3SS substrate SipA. Upon interaction via the N-terminal SipA chaperone binding site (CBD), both Gluc parts reconstitute the functional enzyme and light is emitted with addition of coelenterazine (CTZ). Two split Gluc variants as depicted were generated and tested (B). InvB was fused to two N-terminal Gluc split variants while SipA and a SipA variant lacking its CBD ($SipA_{48-685}$) were both fused to the two variants of the C-terminal split Gluc portions (C). (D) All fusion constructs and the split Gluc variants without fusion partner were transferred into *S*. Typhimurium and expression was induced with 50 ng/ml AHT. Gluc activity was quantified from bacterial lysates (upper panels, gray bars) and intact cells (lower panels, black bars). The gray line in the lower graphs represents the background luminescence measured with an empty vector control (pWSK29). Mean and standard deviation of one representative out of three independent experiments done in triplicates is shown. Statistical analysis by Student's *t*-test was done by comparing individual strains as depicted: *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$.

Figure S1. Substrate specificity of Gluc and membrane permeability of different substrates.

(A) Gluc was overexpressed from pBAD24-Gluc in *S*. Typhimurium WT and lysates were prepared as described in Materials and Methods. After 1:10 dilution of lysates, different coelenterazines (CTZs) were added to a final concentration of 10 μM. Light emission after substrate addition was recorded every 30 seconds over 15 min using one second integration time. (B) Gluc was expressed from pBAD24-Gluc in *S*. Typhimurium WT and whole cells were collected as described in materials and methods. Gluc quantification with different CTZs was carried out as described in (A). The data represent mean and standard deviation of three independent experiments done in triplicates.

Figure S2. Expression of Gluc reporter fusions to the SPI-4 promoter quantified from intact bacteria

The strains used are described in Fig. 1. The experiments were essentially carried out as described for Fig. 1 except whole cells were used as described in Materials and Methods. The data represent mean and standard deviation of three independent experiments done in triplicates.

Figure S3. Light emission kinetics of Gluc WT and GlucM43I.

Gluc was expressed from pBAD24-Gluc in *S*. Typhimurium WT and lysates were prepared as described in Materials and Methods. Lysates were finally diluted 1:1,000 in assay buffer and light emission was recorded after substrate addition every 30 seconds over 15 min using one second integration time. The data represent mean and standard deviation of three independent experiments done in triplicates.

Figure S4 Western blot detecting Gluc fusion proteins in supernatants.

Samples for Western blot were obtained from precipitated supernatants as described in Material and Methods. Gluc-specific bands were detected with constructs Gluc::SiiE-Ig50-53 (*) and Gluc::SiiE-Ig53 (#) using a polyclonal anti-Gluc antibody (upper panel). As controls, FliC (middle panel) and DnaK (lower panel) were detected with monoclonal antibodies.

Figure S5 Western blot detecting Gluc fusion proteins in lysates and bacterial two hybrid.

(A) Samples shown in Fig. 4D upper panels were subjected to protein electrophoresis using Bis-Tris gradient gels. In subsequent Western blot, a polyclonal anti-Gluc antibody was used to detect the different SipA fusion proteins: $SipA_{1-685}$::C-$Gluc_{106}$ (82.66 kDa), $SipA_{48-685}$::C-$Gluc_{106}$ (77.86 kDa), $SipA_{1-685}$::C-$Gluc_{110}$ (82.37 kDa) and $SipA_{48-685}$::C-$Gluc_{110}$ (77.58 kDa). InvB::N-$Gluc_{105/109}$ fusion proteins could not be detected by this antibody. (B) The CyaA-T18 and -T25 fragments were expressed in *E. coli* BTH101 either alone ("without") or fused to proteins as indicated. Cells were spread on plates containing IPTG and X-Gal. Blue colonies indicated CyaA fragment complementation. A strain co-expressing T18 and T25 fused to GCN4-ZIP (13) was included as positive control.

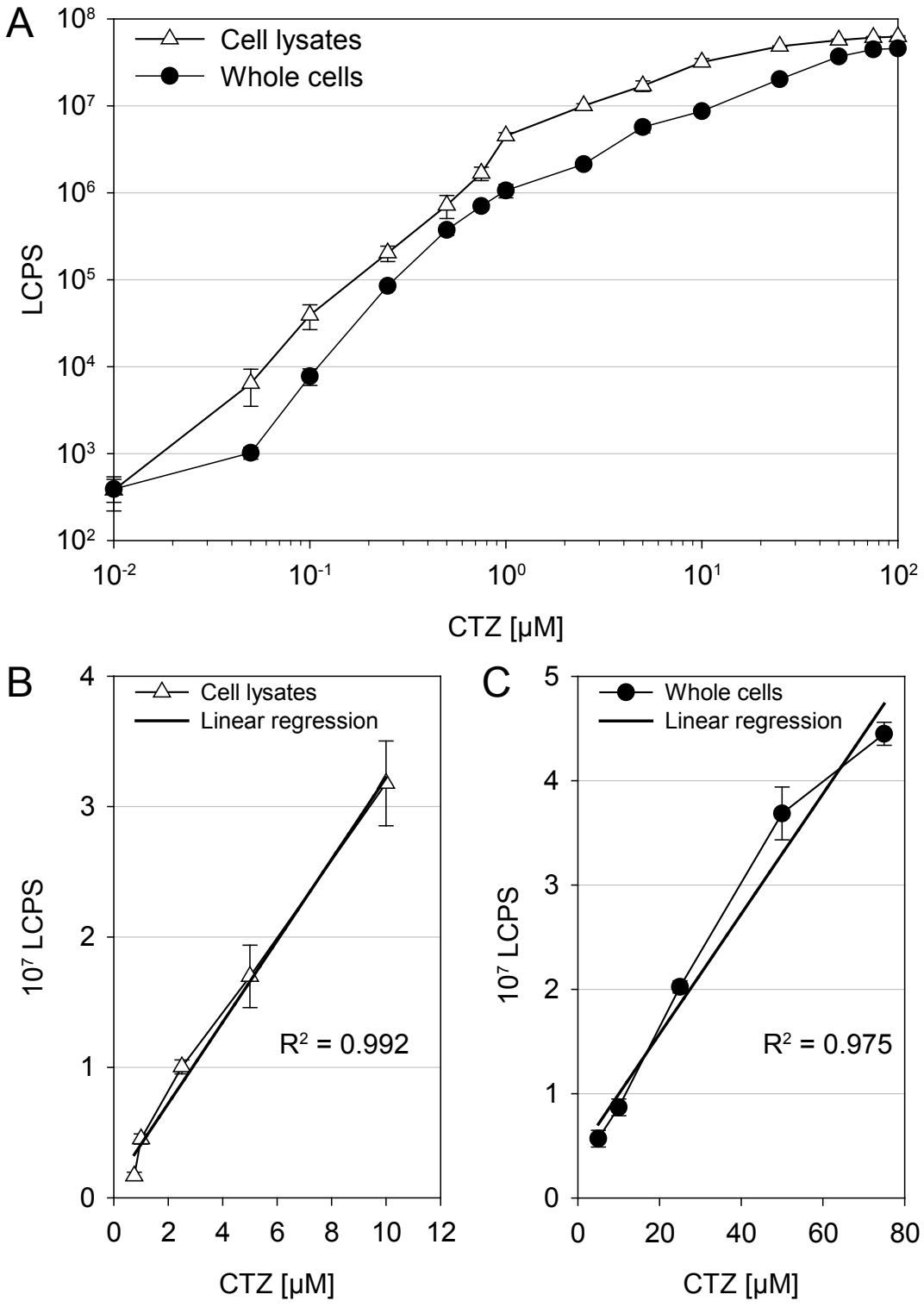
**Tables**

Table 1. Bacterial strains used in this study

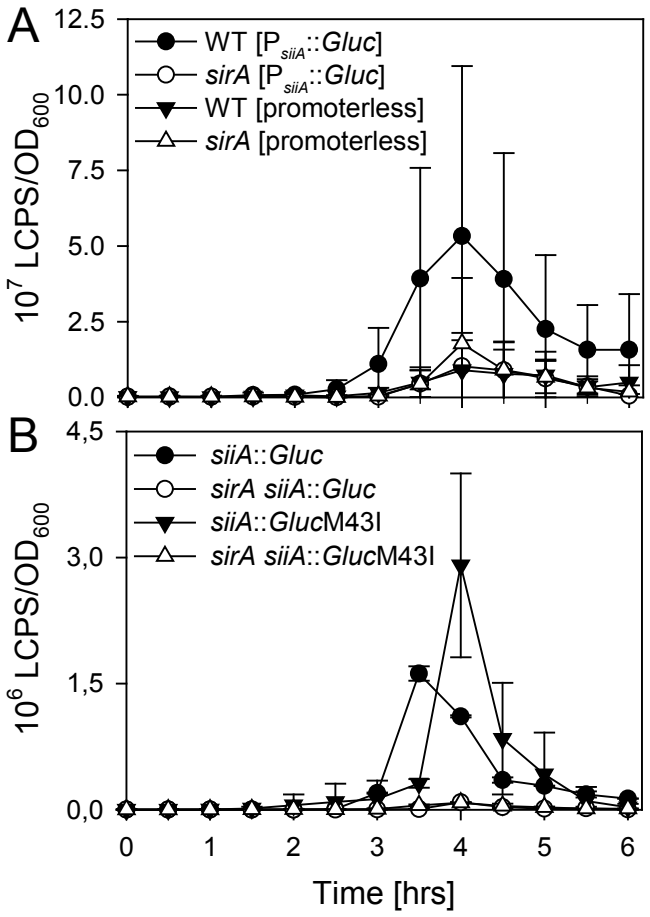
| *S. enterica* serovar Typhimurium strains | Relevant characteristic(s) | Source or Reference |
|---|---|---|
| NCTC 12023 | Wild-type, Nal$^s$, isogenic to ATCC 14028 | NCTC, Colindale, UK |
| MvP812 | *siiF* FRT | 11 |
| MvP818 | *invC* FRT | 6 |
| MvP896 | *sirA* FRT | 32 |
| WRG34 | *siiA*::*Gluc aph*, Kan$^r$ | This study |
| WRG42 | *siiA*::*Gluc*M43I *aph*, Kan$^r$ | This study |
| WRG64 | *sirA* FRT *siiA*::*Gluc*M43I *aph*, Kan$^r$ | This study |
| WRG84 | *sirA* FRT *siiA*::*Gluc aph*, Kan$^r$ | This study |
| *E. coli* strains | | |
| BTH101 | F-, *cya-99*, *araD139, galE15, galK16, rpsL1* (Str$^r$), *hsdR2, mcrA1, mcrB1* | 14 |

[a] FRT, FLP recombination target.

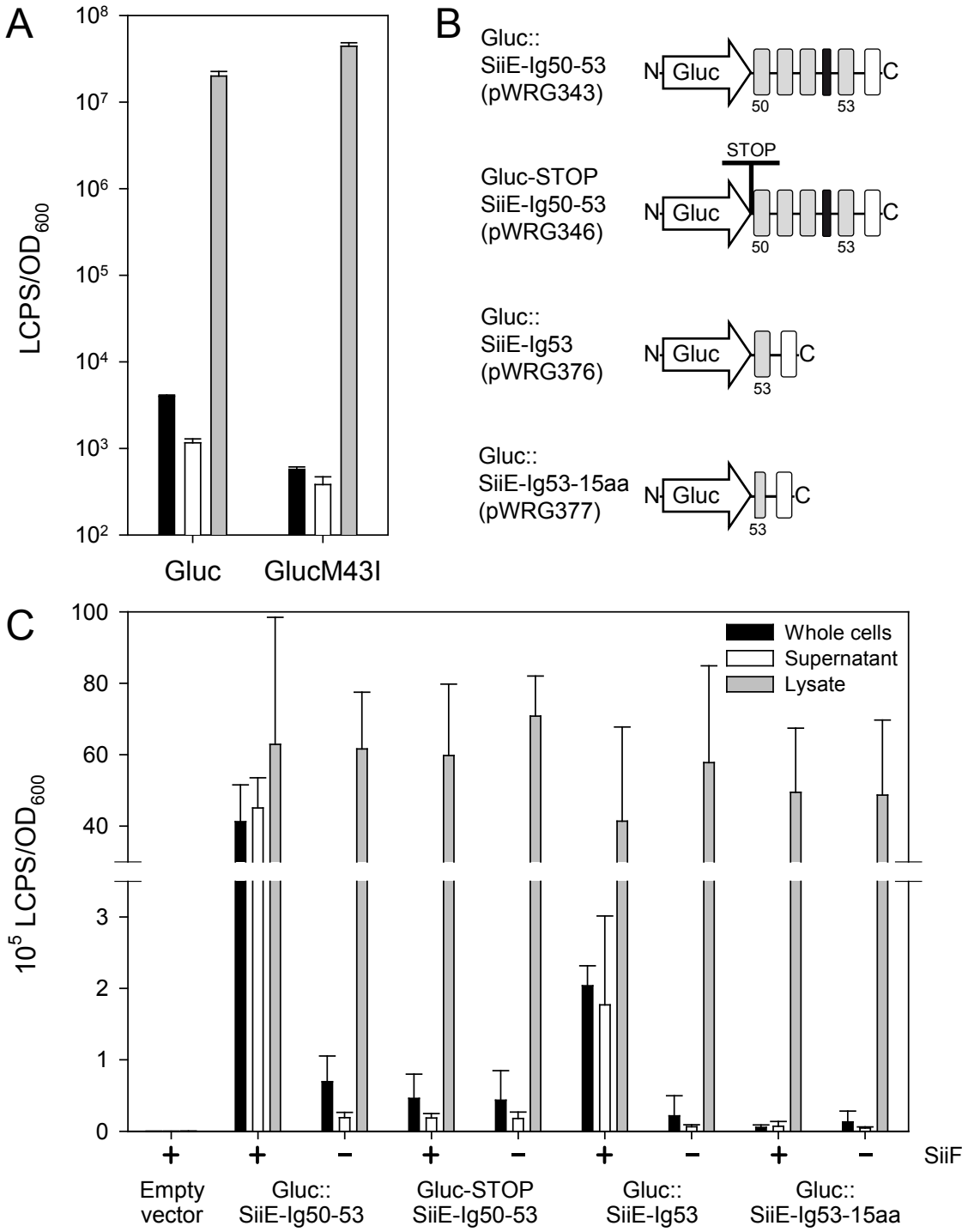
Wille *et al.* Fig. 1



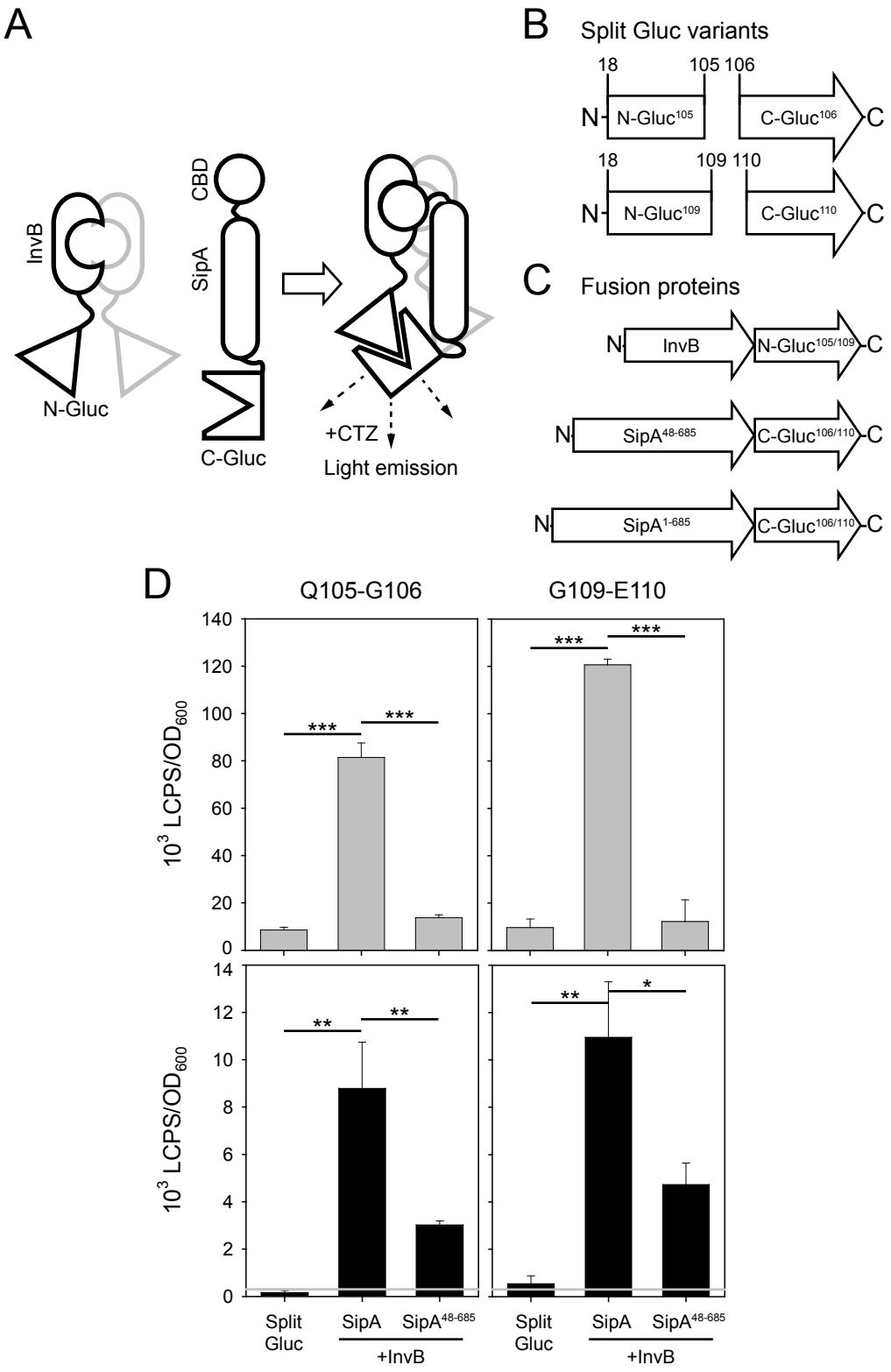
Wille *et al.* Fig. 2



Wille *et al.* Fig. 3



Wille *et al.* Fig. 4



# Supplementary Information

## *Gaussia princeps* luciferase as reporter for transcriptional activity, protein secretion and protein-protein interactions in *Salmonella enterica* serovar Typhimurium

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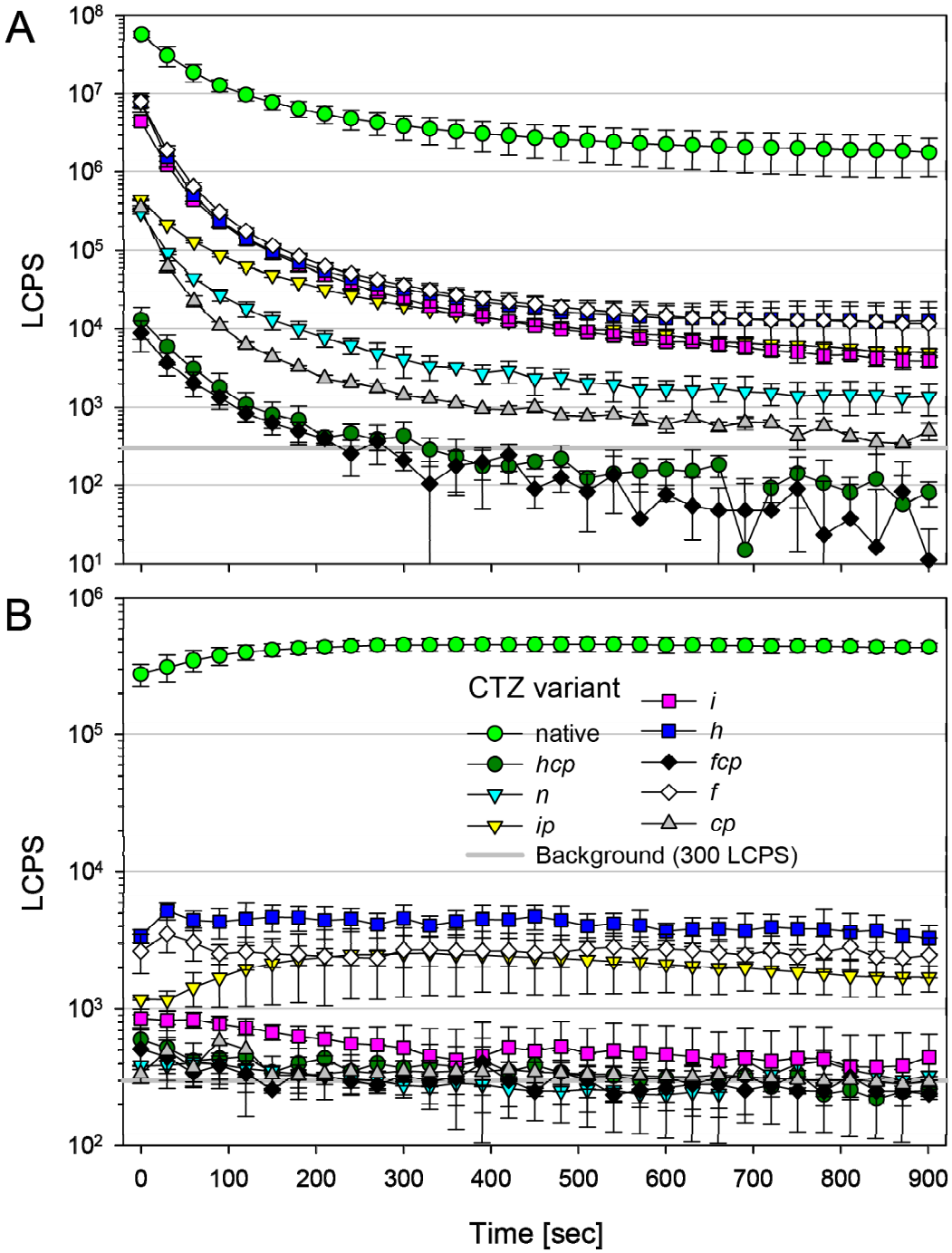
**Supplementary Figures:**

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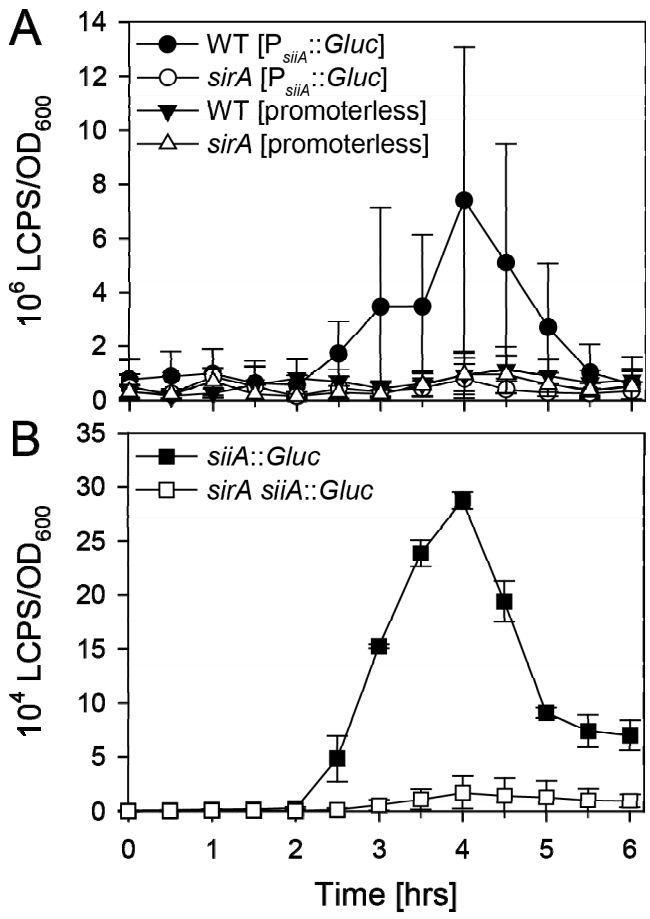


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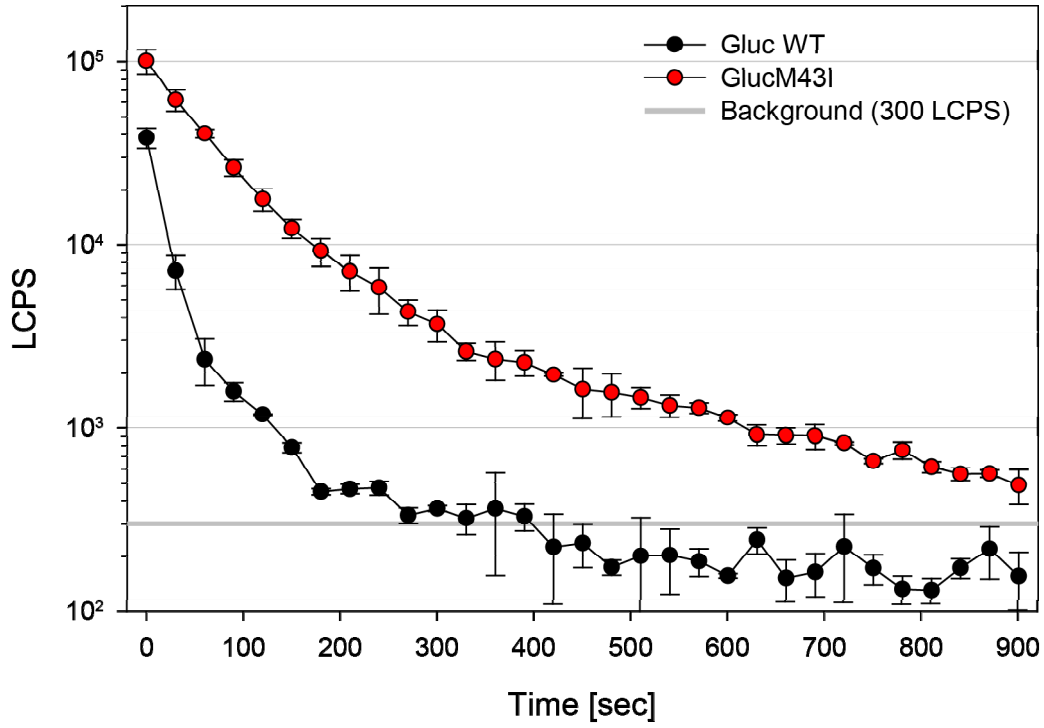
The strains used are described in Fig. 1. The experiments were essentially carried out as described for Fig. 1 except whole cells were used as described in Materials and Methods. The data represent mean and standard deviation of three independent experiments done in triplicates.

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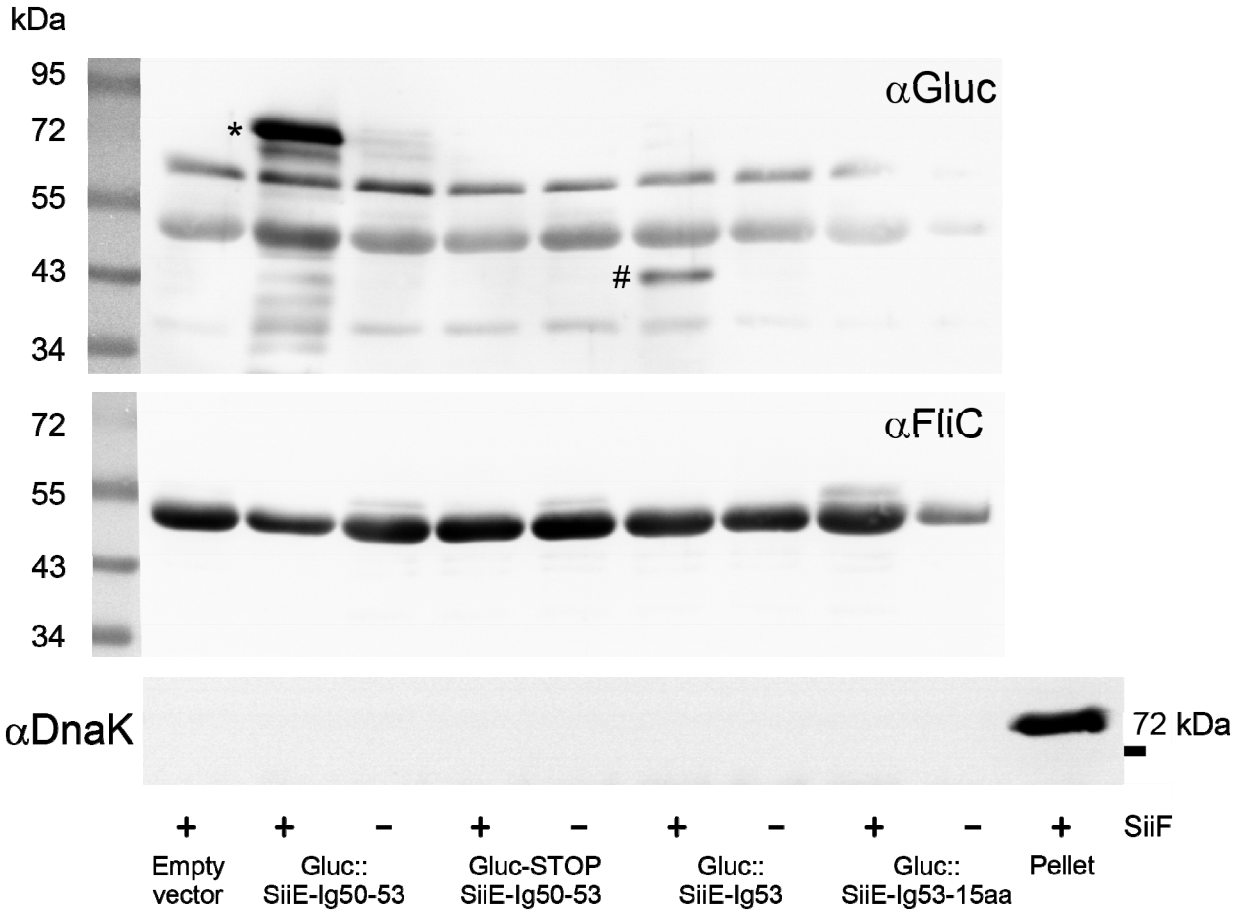


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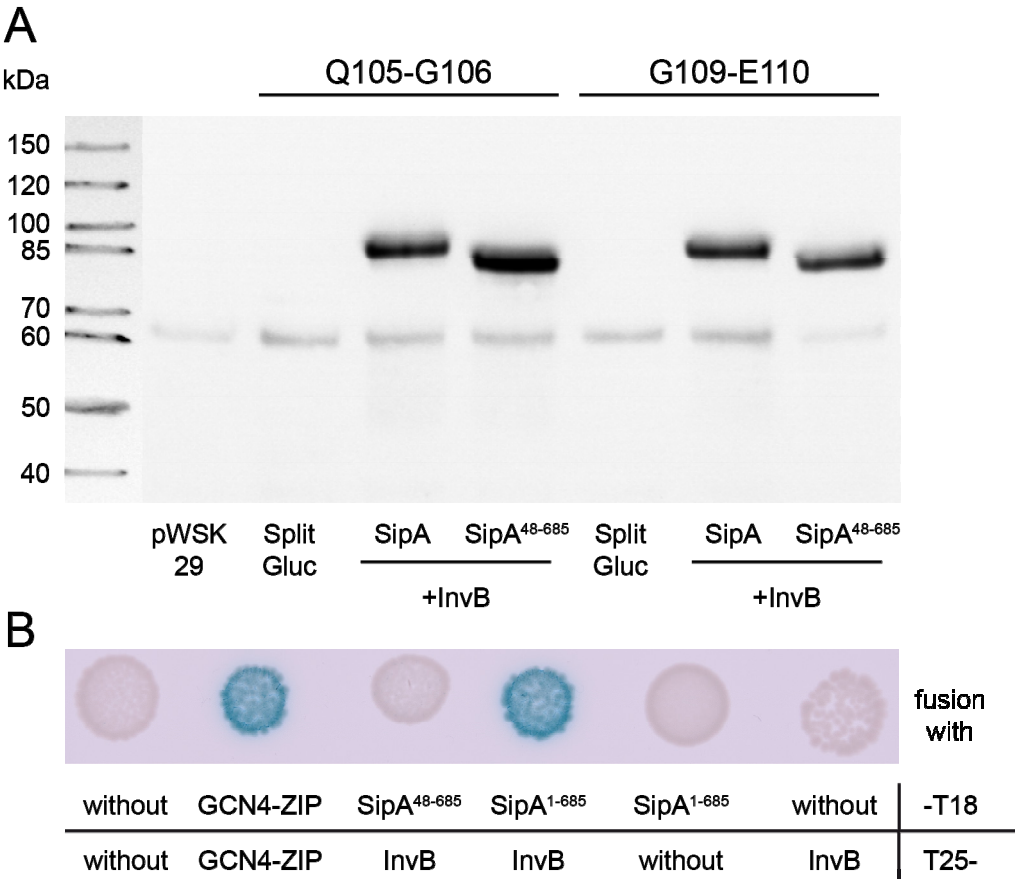


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**Supplementary Materials and Methods:**

*Cloning*: All primers used in this study can be found in Supplementary Table 1. An overview about the plasmids used is given in Supplementary Table 2. The *Gluc* gene and its variants *Gluc*-LVA and *Gluc*M43I-LVA were synthesized without the eukaryotic secretion signal codon-optimized for *Salmonella enterica* (Geneart, Regensburg, Germany). The *Gluc* ORFs were cloned into pBAD24 via *Eco*RI/*Hin*dIII yielding pBAD24-Gluc (Genbank HM241886) and pBAD24-GlucM43I.
Primers NotI-Gluc-for and XhoI-Gluc-rev were used to amplify Gluc from pBAD24 derivatives and the resulting fragments were cloned into p2795 (4) leading to pWRG168 and pWRG215, respectively. pGEN-luxCDABE (7) was digested *Eco*RI/*Sna*BI to remove the EM7 promoter. Complementary oligos MCS-RBS-for and MCS-RBS-rev were annealed and the resulting dsDNA fragment was digested *Eco*RI/*Eco*RV and ligated into digested pGEN-luxCDABE generating pWRG15. The *luxCDABE* operon of pWRG15 was replaced by a *Gluc* PCR fragment generated from pBAD24-Gluc as template with primers SmaI-RBS-Gluc-for and Gluc-NcoI-rev. In the resulting pWRG169 reporter plasmid, the *siiA* promoter ($P_{siiA}$) was cloned via *Eco*RI/*Sma*I after amplification from *Salmonella* genomic DNA with primers EcoRI-Pro-SPI4-for and Pro-SPI4-SmaI-rev, yielding pWRG170.
For generation of Gluc-SiiE fusion proteins, $P_{siiA}$ was amplified from chromosomal DNA with primers XhoI-SmaI-ProSPI4-for and ProSPI4-XbaI-HindIII-rev and cloned into pWSK29 (9) using *Xho*I/*Hind*III. With primers XbaI-Gluc-for and Gluc-HindIII-rev (with stop codon) or Gluc-HindIII-rev2 (without stop codon) *Gluc*M43I was amplified from pBAD24-GlucM43I and cloned via *Xba*I and *Hind*III behind $P_{siiA}$. Different parts of the SiiE C-terminus were amplified from chromosomal DNA using the common reverse primer SiiE-C-NotI-rev in combination with HindIII-siiE-C-for (Ig50-53), HindIII-siiE-Ig53-for or HindIII-siiE-Ig53-aa15-for. Final transfer of the *siiE* fragments via *Hind*III and *Not*I behind *Gluc*M43I resulted in plasmids pWRG343 (Ig50-53), pWRG376 (Ig53) and pWRG377 (Ig53-15aa). Cloning of the *siiE* fragment corresponding to Ig50-53 behind *Gluc*M43I-Stop yielded pWRG346.
The operons for the split Gluc approach were constructed as follows: The tetracycline-inducible promoter $P_{tetA}$ was amplified from pST98-AS (8) with primers Acc65I-SmiI-Pro-tet-for and Pro-tet-EcoRV-XhoI-rev. The promoter was cloned in pWSK29 via *Kpn*I/*Xho*I. *sipA* and $sipA_{48\text{-}685}$ were amplified from *Salmonella* genomic DNA using primer SipA-HindIII-XhoI-rev in combination with primer EcoRV-RBS-sipA-for or EcoRV-RBS-sipA48-for, respectively. The *sipA* fragments were cloned behind $P_{tetA}$ using *EcoR*V/*Xho*I. The two C-terminal Gluc portions C-$Gluc_{106}$ and C-$Gluc_{110}$ were amplified from pBAD-GlucM43I with primer Gluc-XhoI-rev in combination with primer HindIII-C-Gluc106-for or HindIII-C-Gluc110-for, respectively. After digestion with *Hind*III/*Xho*I, C-*Gluc* fragments were cloned behind *sipA* or $sipA_{48\text{-}685}$.
A fusion of *invB* with N-$Gluc_{105}$ or N-$Gluc_{109}$ was generated in pBluescript II SK (+) (pSK, Fermentas): N-$Gluc_{105}$ and N-$Gluc_{110}$ were amplified from pBAD24-GlucM43I using primer XhoI-BglII-Gluc-for in combination with primer N-Gluc105-NotI-rev or N-Gluc109-NotI-rev, respectively. After that, the fragments were cloned in pSK via *Xho*I/*Not*I. The *invB* gene was amplified from *Salmonella* genomic DNA with primers XhoI-RBS-invB-for and InvB-BglII-rev. The *invB* gene was fused to the two N-*Gluc* fragments after cloning with *Xho*I/*Bgl*II. The *invB*::N-$Gluc_{105}$ and *invB*::N-$Gluc_{109}$ fragments were released from pSK after digestion with *Xho*I/*Not*I and subsequently cloned in the previously generated pWSK-based vectors harboring the *tetA* promoter and combinations of *sipA*/C-$Gluc_{106}$, $sipA_{48\text{-}685}$/C-$Gluc_{106}$, *sipA*/C-$Gluc_{110}$ and $sipA_{48\text{-}685}$/C-$Gluc_{110}$, yielding vectors pWRG300, pWRG374, pWRG299 and pWRG375, respectively.
As a control, the unfused split Gluc parts were cloned in pWSK29 $P_{tetA}$. C-terminal Gluc portions were amplified from pBAD24-GlucM43I with primer Gluc-XhoI-rev in combination

with EcoRV-RBS-C-Gluc106-for or EcoRV-RBS-C-Gluc110-for. Fragments were cloned behind $P_{tetA}$ using EcoRV/XhoI. The N-terminal parts were amplified from the same template using primer XhoI-RBS-Gluc-for in combination with N-Gluc105-NotI-rev or N-Gluc109-NotI-rev. An operon structure was generated with transfer of the N-terminal fragments into vectors harbouring the respective C-terminal fragments under control of $P_{tetA}$ using *Xho*I/*Not*I, yielding vectors pWRG354 (Q105-G106) and pWRG355 (G109-E110).
To generate reporter plasmids for bacterial two hybrid, full length *sipA* was amplified from chromosomal DNA with primers HindIII-sipA-for and SipA-BamHI-rev. $sipA_{48\text{-}685}$ was amplified using primer XbaI-sipA48-for in combination with SipA-BamHI-rev. Both fragments were cloned in pUT18 (6) resulting in plasmids pWRG426 and pWRG428. The *invB* gene was amplified by PCR from *Salmonella* chromosomal DNA using primers XbaI-invB-for and InvB-KpnI-rev. The fragment was cloned into p25-N (1) via *Xba*I/*Kpn*I leading to pWRG430.
All constructs were initially screened by colony PCR using suitable check primers and finally verified by restriction analysis and sequencing.

**Supplementary Tables:**

Supplementary Table 1. Oligonucleotides used in this study

| Oligonucleotide | Sequence (5'→3'), restriction sites underlined |
|---|---|
| Cloning primers | |
| Acc65I-SmiI-Pro-tet-for | GGTACCATTTAAATTCGATGGGTGGTTAACTC |
| EcoRI-Pro-SPI4-for | GCCGAATTCAAAGCGTTATTTGCATTTTCG |
| EcoRV-RBS-C-Gluc106-for | CGCGATATCAGGAGGACTACAATGGGTGGCATTGGCGAAGCGATTG |
| EcoRV-RBS-C-Gluc110-for | CGCGATATCAGGAGGACTACAATGGAAGCGATTGTGGATATTCC |
| EcoRV-RBS-sipA48-for | CGCGATATCAGGAGGACTACAATGGGCCCGCAACTGGAAGATTTTC |
| EcoRV-RBS-sipA-for | CGCGATATCAGGAGGACTACAATGGTTACAAGTGTAAGGAC |
| Gluc-HindIII-rev (with Stop codon) | GCCAAGCTTTTATTAATCGCCACCCGCACCTTTAATT |
| Gluc-HindIII-rev2 (without Stop codon) | CCGAAGCTTATCGCCACCCGCACCTTTAATTTTATC |
| Gluc-NcoI-rev | CCGCCATGGTTATTAATCGCCACCCGCAC |
| Gluc-XhoI-rev | ATCCTCGAGTTATTAATCGCCACCCGCAC |
| HindIII-C-Gluc106-for | GATAAGCTTGGTGGCATTGGCGAAGCG |
| HindIII-C-Gluc110-for | GATAAGCTTGAAGCGATTGTGGATATTCC |
| HindIII-siiE-C-for | TCGAAGCTTACGCCGCCAAATGCTCCGGTC |
| HindIII-siiE-Ig53-aa15-for | TCGAAGCTTATAGATCGTTCAATTAGTCAGAC |
| HindIII-siiE-Ig53-for | TCGAAGCTTGTGACAGCCTATAGTATTACATTG |
| HindIII-sipA-for | GACAAGCTTGGTTACAAGTGTAAGGACTCAGC |
| InvB-BglII-rev | CGCAGATCTTCTCATTAGCGACCGACTAAAAAC |
| InvB-KpnI-rev | CTTAGGTACCCTCATTAGCGACCGACTAAAAAC |
| MCS-RBS-for | AATGAATTCCTGCAGACTAGTTCTAGAGCGGCCGCCACCGCGGTGGAGCTCGGATCCCCGGGAAAGAGGAGAGATATCTCC |
| MCS-RBS-rev | GGAGATATCTCTCCTCTTTCCCGGGGATCCGAGCTCCACCGCGGTGGCGGCCGCTCTAGAACTAGTCTGCAGGAATTCATT |
| N-Gluc105-NotI-rev | ATCGCGGCCGCTTATTACTGCGCGCTTTCTTTATCGC |

| | |
|---|---|
| N-Gluc109-NotI-rev | ATCGCGGCCGCTTATTAGCCAATGCCACCCTGCGCGC |
| NotI-Gluc-for | ATAGCGGCCGCATGAAACCGACCGAAAACAAC |
| Pro-SPI4-SmaI-rev | GAGCCCGGGTTGTCTCCTGATATTACATTGTG |
| ProSPI4-XbaI-HindIII-rev | GCGAAGCTTCTAGAGTCTCCTGATATTACATTGTG |
| Pro-tet-EcoRV-XhoI-rev | GCGCTCGAGGATATCTTTCTCTATCACTGATAGGGAG |
| SiiE-C-NotI-rev | GCGGCGGCCGCTTATTATGCGTGTTCTTCTTGATTATC |
| SipA-BamHI-rev | CTTGGATCCCGCTGCATGTGCAAGCCATC |
| SipA-HindIII-XhoI-rev | TCACTCGAGAAGCTTACGCTGCATGTGCAAGCCATC |
| SmaI-RBS-Gluc-for | CGGCCCGGGAAAGAGGAGAAAAGTATGAAACCGACCGAAAACAAC |
| XbaI-Gluc-for | CGCTCTAGAATGAAACCGACCGAAAACAAC |
| XbaI-invB-for | GACTCTAGAGCAACATTTGGATATCGCTG |
| XbaI-sipA48-for | GACTCTAGACCCGCAACTGGAAGATTTTC |
| XhoI-BglII-Gluc-for | CTGCTCGAGAGATCTATGAAACCGACCGAAAACAAC |
| XhoI-Gluc-rev | CGACTCGAGTTATTAATCGCCACCCGCAC |
| XhoI-RBS-Gluc-for | CTGCTCGAGAGGAGGACTACAATGAAACCGACCGAAAACAAC |
| XhoI-RBS-invB-for | CTGCTCGAGAGGAGGACTACAATGCAACATTTGGATATCGC |
| XhoI-SmaI-ProSPI4-for | GATCTCGAGCCCGGGAAAGCGTTATTTGCATTTTCG |
| Primers for Red reporter | Ends homologous to template plasmids pWRG168/215 italic |
| SiiA-Gluc-for | AAAACATTTTATTCACAATGTAATATCAGGAGACAACATG*AAACCGACCGAAAACAACGA* |
| SiiA-RedRep-rev | AACAGACAAACAAATAGCGGTAATGATTTATATATTTCAC*GTGTAGGCTGGAGCTGCTTC* |
| Control primers | |
| Gluc-Check-for | GTCGATCTGTGCGTGGATTG |
| Gluc-Check-rev | ACAATCGCTTCGCCAATGCC |
| LuxC-Check-rev | CTTTGCCCTACCGTATAGAG |
| MCS-RBS-Check-rev | GCTCACGCTCTGTCATTTTCT |
| SiiA-Check-for | CGGGTGGTTTTAATTTGCTG |
| SipA-Check-for | CCCGGCTTACGAGTCATACC |

Supplementary Table 2. Plasmids used in this study

| Plasmid | Relevant characteristic(s) | Source or Reference |
|---|---|---|
| p25-N | Cloning and expression vector; encodes the T25 fragment (aa 1–224 of CyaA); $Km^r$ | (1) |
| p2795 | High-copy-number vector, with kanamycin resistance cassette flanked by FRT sites, $Ap^r$, $Km^r$ | (4) |
| pBAD24 | High-copy expression vector with arabinose-inducible promoter ($P_{BAD}$) and optimized ribosome binding site (RBS), $Ap^r$ | (5) |
| pBAD24-Gluc | pBAD24 vector with *Gaussia princeps* luciferase (*Gluc*); codon-optimized for expression in *Salmonella enterica*, $Ap^r$ | This study<br>Genbank HM241886 |
| pBAD24-GlucM43I | pBAD24 vector with codon-optimized enhanced *Gluc*, $Ap^r$ | This study |
| pGEN-*luxCDABE* | Medium-copy-number vector (ori p15A), *Photorhabdus luminescens* luciferase operon (*luxCDABE*) under control of EM7 synthetic promoter (Invitrogen), $Ap^r$ | (7) |
| pKD46 | λ Red-expression under control of $P_{BAD}$, temperature-sensitive, $Ap^r$ | (2) |
| pKNT25-ZIP | Derivative of pKT25 (6); leucine zipper of GCN4 is fused in frame to the T25 fragment; $Km^r$ | (6) |
| pST98-AS | Low-copy-number vector, I-*Sce*I endonuclease under control of tetracycline-inducible promoter ($P_{tetA}$), $Ap^r$ | (8) |
| pUT18 | Cloning and expression vector; encodes the T18 fragment (aa 225–399 of CyaA); $Ap^r$ | (6) |
| pUT18C-ZIP | Derivative of pUT18C (6); leucine zipper of GCN4 is fused in frame to the T18 fragment; $Ap^r$ | (6) |
| pWRG15 | pGEN-*luxCDABE* with multiple cloning site (MCS) and optimized RBS (3), without promoter, $Ap^r$ | This study |
| pWRG168 | Codon-optimized *Gaussia princeps* luciferase (*Gluc*) in p2795, $Ap^r$, $Km^r$ | This study |
| pWRG169 | *luxCDABE* operon of pWRG15 exchanged by codon-optimized *Gaussia princeps* luciferase (*Gluc*), without promoter, $Ap^r$ | This study |
| pWRG170 | $P_{siiA}$ in pWRG169, $Ap^r$ | This study |
| pWRG215 | *Gluc*M43I in p2795, $Ap^r$, $Km^r$ | This study |
| pWRG299 | $P_{tetA}$ *sipA*::C-*Gluc*$_{109}$ *invB*::N-*Gluc*M43I$_{110}$ in pWSK29, $Ap^r$ | This study |
| pWRG300 | $P_{tetA}$ *sipA*::C-*Gluc*$_{106}$ *invB*::N-*Gluc*M43I$_{105}$ in pWSK29, $Ap^r$ | This study |

| | | |
|---|---|---|
| pWRG343 | Pro-SPI-4 *Gluc*M43I::*siiE*-Ig50-53 in pWSK29, $Ap^r$ | This study |
| pWRG346 | Pro-SPI-4 *Gluc*M43I-Stop *siiE*-Ig50-53 in pWSK29, $Ap^r$ | This study |
| pWRG354 | $P_{tetA}$ C-$Gluc_{106}$ N-*Gluc*$M43I_{105}$ in pWSK29, $Ap^r$ | This study |
| pWRG355 | $P_{tetA}$ C-$Gluc_{110}$ N-*Gluc*$M43I_{109}$ in pWSK29, $Ap^r$ | This study |
| pWRG374 | $P_{tetA}$ $sipA_{48\text{-}685}$::C-$Gluc_{106}$ *invB*::N-*Gluc*$M43I_{105}$ in pWSK29, $Ap^r$ | This study |
| pWRG375 | $P_{tetA}$ $sipA_{48\text{-}685}$::C-$Gluc_{109}$ *invB*::N-*Gluc*$M43I_{110}$ in pWSK29, $Ap^r$ | This study |
| pWRG376 | Pro-SPI-4 *Gluc*M43I::*siiE*-Ig53 in pWSK29, $Ap^r$ | This study |
| pWRG377 | Pro-SPI-4 *Gluc*M43I::*siiE*-Ig53-15aa in pWSK29, $Ap^r$ | This study |
| pWRG426 | $sipA_{1\text{-}685}$:: *T18* in pUT18, $Ap^r$ | This study |
| pWRG428 | $sipA_{48\text{-}685}$::*T18* in pUT18, $Ap^r$ | This study |
| pWRG430 | *T25*::*invB* in p25-N, $Km^r$ | This study |
| pWSK29 | Low copy number vector, $Ap^r$ | (9) |

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