Epitope-Tagged Autotransporters as Single-Cell Reporters for Gene Expression by a *Salmonella Typhimurium* _wbaP_ Mutant

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Abstract

Phenotypic diversity is an important trait of bacterial populations and can enhance fitness of the existing genotype in a given environment. To characterize different subpopulations, several studies have analyzed differential gene expression using fluorescent reporters. These studies visualized either single or multiple genes within single cells using different fluorophores. However, variable maturation and folding kinetics of different fluorophores complicate the study of dynamics of gene expression. Here, we present a proof-of-principle study for an alternative gene expression system in a _wbaP_ mutant of *Salmonella Typhimurium* (S. Tm) lacking the O-sidechain of the lipopolysaccharide. We employed the hemagglutinin (HA)-tagged inverse autotransporter invasin (_invA_ _HA_) as a transcriptional reporter for the expression of the type three secretion system 1 (T1) in S. Tm. Using a two-reporter approach with GFP and the InvAHA in single cells, we verify that this reporter system can be used for T1 gene expression analysis, at least in strains lacking the O-antigen (_wbaP_), which are permissive for detection of the surface-exposed HA-epitope. When we placed the two reporters _gfp_ and _invA_ _HA_ under the control of either one or two different promoters of the T1 regulon, we were able to show correlative expression of both reporters. We conclude that the _invA_ _HA_ reporter system is a suitable tool to analyze T1 gene expression in S. Tm and propose its applicability as molecular tool for gene expression studies within single cells.

Introduction

Most bacteria in the environment live in communities (e.g. biofilms). A characteristic of these communities is the presence of microorganisms with different phenotypes that can take over complementary roles [1]. However, different phenotypes have increasingly been recognized within bacterial cultures that harbor the same genotype and share the same microenvironment.
This phenotypic diversity (heterogeneity) within isogenic bacteria has been recognized as common trait among bacterial species and has recently developed into an important research field [2–5]. The functional importance of such phenotypic heterogeneity has remained poorly understood. Phenotypic diversity within isogenic bacterial populations is caused by stochastic (random) events during gene expression, molecular segregation during cell division and/or metabolic activity. In a given system (e.g. a virulence regulatory system), this stochastic behavior, combined with a threshold requirement and a subsequent positive feedback reinforcement that results in a non-linear response can lead to the formation of two (or more) stable phenotypes (= bistable or multistable states (for detailed information on the causes of phenotypic diversity see [6–8]). Phenotypic diversity is thought to have important implications, i.e. in situations of bet hedging and division of labor [9, 10].

In traditional microbial research, bacterial cultures were assumed to be homogenous as they were derived from genetically identical cells. Therefore, outcomes from experiments were usually averaged and researchers completely neglected the influence of different subpopulations in the obtained results. Since the awareness about phenotypic diversity within isogenic bacterial cultures increased, microscopy and flow cytometric analysis in combination with fluorescently labeled molecules of interest, has improved the knowledge of single cell behavior [3, 11–14].

For Salmonella enterica subspecies enterica serovar Typhimurium (S. Tm), both bet hedging and division of labor have been discussed in the context of the expression of the type three secretion system 1 (T1) virulence factor [15, 16]. S. Tm is an enteropathogenic, Gram-negative bacterium that causes self-limiting gastroenteritis [17]. For successful infection, S. Tm requires the T1 apparatus, which is encoded on the Salmonella pathogenicity island 1 (SPI-1) at centisome 63 of the chromosome [18]. T1 is a key virulence factor for invasion and is expressed in a bistable fashion. Thus, even under inducing conditions in vitro and in the host’s intestine only a minority of cells are expressing T1 (T1+ cells) [3, 16, 19]. The regulation of T1 has been the subject of numerous studies and the key regulatory factors controlling its expression are well established (for detailed information see [20, 21]). However, the detailed mechanism that leads to bistable expression is still not completely understood. To gain mechanistic insights into the T1 regulation, single cell studies analyzing multiple genes simultaneously would be of interest. Previous studies have used fluorescent protein reporters for analyzing gene expression of one or two genes of interest [2, 4, 22]. Mechanistic analysis would benefit from expanding the number of genes measured in the individual cell. Also, the different maturation and folding properties of different fluorophores have complicated the study of dynamics of gene expression. Thus, new reporter systems with uniform maturation kinetics and the potential to analyze more than two genes per cell would be of significant interest.

Autotransporters are classified as a family of virulence factors that employ a type V secretion system and are capable of transporting their own extracellular passenger domain through the outer membrane. The type V secretion system is comprised of five classes, type Va through to Ve. The Type Ve autotransporter subfamily is characterized by an inverted topology and was only recently described as own class [23–25]. Oberhettinger and colleagues demonstrated the inverted topology of this family using epitope-tagged intimin and invasin [25]. The adhesins intimin and invasin, found in enteropathogenic E. coli and Yersinia species (spp.), respectively, represent two members of the type Ve autotransporter subfamily [26, 27].

Here, we performed a proof-of-concept study to assess if autotransporters may be used as reporters for gene expression in Gram-negative bacteria. In particular, we tested the hemagglutinin (HA)-tagged autotransporter invasin to assess gene expression by S. Tm. To establish this system, we chose a two-reporter approach using the fluorophore GFP and the epitope-tagged invasin. Both reporters were placed under the control of promoters present on SPI-1 and thus under the control of the T1 regulon. We demonstrate here that both reporters can be
successfully used in one single cell. We show correlative expression of both reporters when driven under the control of one or two different promoters of the T1 regulon. This expands the molecular toolbox for future studies of gene expression within a single bacterial cell.

Materials and Methods

Plasmid constructions

A list with all plasmids is provided in Table 1. The suicide plasmid pZ503 harboring invAHA204 (encoding the invasin gene from Yersinia enterocolitica O:8 [25]) downstream of a truncated sipA (*sipA; nucleotide 1156–2058 of the open reading frame (orf)) was constructed as follows. The *sipA fragment and invAHA204 fragments were amplified by PCR from pM1300 and pInvA, respectively, using the primer combinations #52/#53 and #54/#55, respectively. Both fragments were fused by a subsequent amplification step using primers with overlapping restriction sites for BamHI (primer #52) and NotI (primer #55) and digestion by the restriction endonucleases BamHI and NotI. The *sipA-invAHA204 carrying fragment was ligated into pSB377, which was previously cleaved by the same restriction enzymes, resulting in pZ503.

The suicide plasmid pZ526, carrying the sipA-invAHA204-gfp insert, was constructed by cleaving the gfp fragment from pM972 using NotI and SacII and its ligation into the previously NotI- and SacII- digested plasmid pZ503. The suicide plasmid pZ522, harboring the sipA-gfp-invAHA204 insert, was constructed in three steps, following a modified protocol from An and colleagues [28]. First, the three fragments *sipA, gfp and invAHA204 were amplified using primers with specific overhanging restriction sites to allow directed ligation of all fragments. The amplification of *sipA was done using the primers #124/#125 (overlapping restriction sites SpeI and AflII, respectively). The gfp fragment was created by amplification with the primers #126/#127 (overlapping restriction sites AflI and SalI, respectively). Finally, invAHA204 was produced using the primers #128/#129 (overlapping restriction sites SalI and SacII, respectively). The *sipA and gfp fragments were each transferred into the pGEM-T easy cloning vector, resulting in pZ523 and pZ524, respectively. In the second step, the *sipA and gfp fragments were combined. The *sipA fragment was cut out from pZ523 using the restriction enzymes SpeI and SalI. The gfp fragment was cut out from pZ524 using AflI and SalI. Both fragments were combined by ligation and subsequent PCR amplification using the primers #124/#127 (SpeI and SalI overhangs). The resulting *sipA-gfp containing PCR fragment was introduced into the pGEM-T easy vector (Promega), creating pZ527. In the third step, the *sipA-invAHA204 fragment was retrieved from pZ527 by SpeI and SalI and ligated with the previously amplified and SalI- and SacII- digested invAHA204 fragment. After the following PCR amplification step using the primers #124/#129 (harporing SpeI and SacII overhangs, respectively), the *sipA-gfp-invAHA204 fragment was digested with SpeI and SacI and ligated into the pSB377 vector, cut with the same restriction endonucleases. The final ligation step resulted in pZ522. All plasmids were verified by sequencing.

Bacterial strains

All strains used in this study were derived from Salmonella Typhimurium SL1344 [33]. The Tables 2 and 3 list all bacterial strains and primer sequences.

The suicide plasmid pZ503 was introduced into M3142 and the unflagellated non-motile strain χ8602 [34] by conjugation, thereby creating Z531 and Z532, respectively. In the strains Z531, Z532 and M3142, the wbaP gene was deleted as previously described [35]. In short, the primers #62 and #63, harboring the λ recombinase recognition sites and sequences adjacent to the wbaP gene, were used to amplify the kanamycin resistance cassette from pKD4. The PCR product was electroporated into Z531, Z532 and M3142, each harboring pKD46 to create an
in-frame deletion of \( wbaP \) directly in the desired strains. The pKD46 plasmid was directly transformed into the strains of interest to avoid the need for an additional P22 phage transduction step (note: P22 phage binds to the O-antigen, which is deleted in these strains). Thereby, the final strains Z536, Z537 and Z555, respectively, were constructed.

### Table 1. Plasmids used in this study.

| Plasmid | Characteristics | Resistance | Reference |
|---------|----------------|------------|-----------|
| pM1300  | sipA; pSB377 derivative | tet [9] | |
| pM972   | sica-gfpmut2; pBR322 derivative with gfpmut2 [29] expression under the promoter control of sica | amp [3] | |
| pM965   | rpsM-gfpmut2; pBR322 derivative with constitutive gf expression under the promoter control of rpsM | amp [30] | |
| pTet-gfp| pGM-Tet-GFP; AHTC-inducible expression of gf; high copy | amp [31] | |
| pInvAHA | pASK-IBA2-invAHA204; HA-tagged invA in pASK-IBA2 vector; AHTC-inducible promoter | amp [25] | |
| pZ503   | *sipA-invAHA204; pSB377 derivative with InvAHA under the control of the sica promoter which controls the sicAsipBCDA operon | tet This study | |
| pZ522   | *sipA-gfpmut2-invAHA204; pSB377 derivative with GFPmut2 and InvAHA under the control of the sica promoter which controls the sicAsipBCDA operon | tet This study | |
| pZ523   | *sipA; sipA in pGEM-T easy vector (Promega) | amp This study | |
| pZ524   | gfpmut2; gfpmut2 in pGEM-T easy vector (Promega) | amp This study | |
| pZ526   | sipA-invAHA204-gfpmut2; pZ503 (pSB377) derivative with InvAHA and GFPmut2 under the control of the sica promoter which controls the sicAsipBCDA operon | tet This study | |
| pZ527   | *sipA-gfpmut2; sipA-gfpmut2 in pGEM-T easy vector (Promega) | amp This study | |
| pZ543   | pASK-IBA2 expression vector; AHTC-inducible promoter | amp This study | |
| pASK-IBA2 | Expression vector; AHTC-inducible promoter | amp [32] | |
| Int wt  | eaeA gene in pASK-IBA2 vector | amp [32] | |
| Int wt-Strep | Int wt with C-terminal Streptag in pASK-IBA2 vector | amp [32] | |

*truncated sipA carrying only nucleotides 1156–2058 of the orf; sm = streptomycin, cm = chloramphenicol, tet = tetracycline, kan = kanamycin, amp = ampicillin

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### Table 2. Strains used in this study.

| Strain | Relevant genotype | Derivative of | Resistance | Reference |
|--------|------------------|---------------|------------|-----------|
| SB300  | wt S. Tm | SL1344 | sm | [33] |
| SKI-12 | ΔwbaP | SL1344 | sm | [36] |
| M3142  | prgH-gfp+ | JH3010, SL1344 [37] | cm | Diard et al., unpublished; gfp+: [38] |
| Z531* | prgH-gfp+, sipA-invAHA204 | M3142 | cm, tet | This study |
| Z536* | prgH-gfp+, sipA-invAHA204 wbaP::aphT | Z531 | cm, tet, kan | This study |
| Z532* | sipA-invAHA204 | χ8602, SL1344 [34] | sm, tet | This study |
| Z537* | sipA-invAHA204 wbaP::aphT | Z532 | sm, tet, kan | This study |
| Z555* | prgH-gfp+ wbaP::aphT | M3142 | sm, kan, cm | This study |
| Z562  | sipA-invAHA204-gfpmut2 | SB300 | sm, tet | This study; gfpmut2: [29] |
| Z565* | sipA-invAHA204-gfpmut2 | X8602 | sm, tet | This study |
| Z567* | sipA-invAHA204-gfpmut2 wbaP::cat | Z565 | sm, tet, cm | This study |
| Z569  | sipA-gfpmut2-invAHA204 | SB300 | sm, tet | This study |
| Z572* | sipA-gfpmut2-invAHA204 | X8602 | sm, tet | This study |
| Z574* | sipA-gfpmut2-invAHA204 wbaP::cat | Z572 | sm, tet, cm | This study |

* Unflagellated non-motile \( \chi8602 \) background (\( \Delta flIC, \Delta flJB \); wt = wild type

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The suicide plasmids pZ526 and pZ522, carrying the sipA-invA-HA204-gfp and sipA-gfp-invA-HA204 inserts, respectively, were each individually integrated into the chromosome of SB300. This created the strains Z562 and Z569, respectively. Subsequently, P22 phage transduction was performed to introduce the inserts of pZ526 and pZ522 individually into the χ8602 background, resulting in Z565 and Z572, respectively. To obtain Z567 and Z574, the wbaP gene was replaced by a chloramphenicol resistance gene cassette by amplification of the resistance cassette from pKD3, as described before. The integration of the two inserts was verified by PCR using the primers #68/#70. The primer combinations #64/#65 and #64/#66, respectively, were used to confirm the wbaP deletion.

The suicide plasmids pZ526 and pZ522, carrying the sipA-invA-HA204-gfp and sipA-gfp-invA-HA204 inserts, respectively, were each individually integrated into the chromosome of SB300. This created the strains Z562 and Z569, respectively. Subsequently, P22 phage transduction was performed to introduce the inserts of pZ526 and pZ522 individually into the χ8602 background, resulting in Z565 and Z572, respectively. To obtain Z567 and Z574, the wbaP gene was replaced by a chloramphenicol resistance gene cassette by amplification of the resistance cassette from pKD3, as described before. The integration of the two inserts was verified by PCR using the primers #68/#70. For confirming the wbaP exchange by the chloramphenicol cassette, the primer combination #64 and #65 was used.

Table 3. Primer sequences used in this study.

| # | Primer | Primer sequence 5`-3` | Purpose |
|---|--------|---------------------|---------|
| 52 | SipA-BamHI-fw | GATGACGATCCATGATGCGTACATCCGAACGCGG | 1) strain/plasmid construction, |
| 53 | SipA-InvA-rv | CACAGTTGGTGTTCTATGATGCGTACATCCGAACGCGG | 1) PCR verification, |
| 54 | InvA-fw | ATGTTATTGATGCGTACATCCGAACGCGG | 1) sequencing |
| 55 | InvA-Notl-rv | GACGATGCGTACATCCGAACGCGG | 1) |
| 60 | SipA-seq | TATCGGTCAGGATCCAAGAATTCAGG | 2) |
| 61 | SipA-seq2 | GCCGTTGAGGATCCAAGAATTCAGG | 2) |
| 62 | SipA-ko-ctrl-fw | ATGTTATTGATGCGTACATCCGAACGCGG | 1) |
| 63 | SipA-ko-ctrl-rv | CCAACTGTTATCATCCAAGAATTCAGG | 2) |
| 64 | wbA-pkd-fw | CTATATATGCTTTATTGATGCGTACATCCGAACGCGG | 1) |
| 65 | wbA-pkd-rv | GACGATGCGTACATCCGAACGCGG | 2) |
| 66 | aphT-ctrl-rv | CCGTCAGAAGAGGAGAATTCAGG | 2) |
| 67 | aphT-ctrl-fw | CCGTCAGAAGAGGAGAATTCAGG | 2) |
| 124 | _sipA-SpeI-fw | GATGACGCTAGGATTTGATGCGTACATCCGAACGCGG | 1) |
| 125 | _sipA-AflII-rv | GACGATCTTAAGCTACGCGTACATCCGAACGCGG | 1) |
| 126 | GFP-AflII-fw | GATGACGCTAGGATTTGATGCGTACATCCGAACGCGG | 1) |
| 127 | GFP-Sall-rv | GACGATGCGTACATCCGAACGCGG | 2) |
| 128 | InvA-Sall-fw | GATGACGCTAGGATTTGATGCGTACATCCGAACGCGG | 1) |
| 129 | InvA-SacII-rv | GACGATGCGTACATCCGAACGCGG | 2) |
| 144 | sipA-seq-rv | CGTGCAATGCGAAGGCAATCCGAACGCGG | 3) |
| 145 | InvA-seq-rv | ACGGTCAGCACTGAGGCAATCCGAACGCGG | 3) |

Purpose:

1) strain/plasmid construction,  
2) PCR verification,  
3) sequencing

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Culturing of bacterial strains

Overnight cultures were diluted 1:20, except when stated otherwise, in fresh LB broth (0.1 M NaCl) and grown for 4 h at 37°C, before aliquots for Western blot analysis were taken and subsequent staining was performed. For the growth-curve experiments, overnight cultures were diluted 1:100 and the optical density (OD$_{600}$) was measured at the indicated time points. In all induction experiments, we either used anhydrotetracycline (AHTC, Chemie Brunschwig AG) or L-arabinose at the stated concentrations. Except otherwise stated, the induction was done immediately after dilution of the overnight culture.

Immunofluorescence measurements

Subcultures were grown for 4 h as described before. 10 $\mu$l of the subculture containing approximately 1x10$^7$ cells, were transferred onto V-shaped 96-well plates (Nunc 96-well polypropylene MicroWell Plates, Thermo Scientific). Before staining, cells were washed 2x with 200 $\mu$l washing buffer (PBS, 4% sucrose, 0.02% sodium azide) on the plate by centrifuging at 4°C at 4000 rpm for 10 min. Blocking was done with 200 $\mu$l blocking buffer (washing buffer containing 3% bovine serum albumin (BSA)) for 1 h at 4°C, before cells were stained in 25 $\mu$l blocking buffer containing first the monoclonal mouse anti-HA (1:1000, clone HA-7, Sigma) and then the secondary goat anti-mouse-Cy5 antibody (1:200, Jackson ImmunoResearch) for 1 h, 4°C. Washing between and after the staining steps was done with PBS as described above. Finally, the cells were resuspended in 200 $\mu$l PBS and surface staining of the HA tag was analyzed by flow cytometry and fluorescence microscopy.

Flow cytometric analysis was done using an LSR II analyzer (BD Biosciences) by measuring forward and sideward scatter of bacterial cells in addition to GFP, representing the respective transcriptional fusions, and the surface-stained HA tag (GFP and Alexa Fluor 647 laser, respectively). The data was analyzed with FlowJo software (version 10.0.8).

For microscopy, 150 $\mu$l of the previously stained cells were transferred onto gelatin (0.2%) coated cover slips. Cover slips were prepared as previously described [39]. The cover slips harboring stained bacterial cells were mounted onto glass slides using Mowiol and kept in the dark at RT overnight before placing them at 4°C to store for further analysis. The fixed slides were analyzed the next day using an Axiovert 200m microscope with a spinning disc confocal laser unit (Visitron) and a solid state laser unit (Toptica). The samples were analyzed using the lasers for either GFP (488 nm) or Cy5 (647 nm) excitation. Data analysis was done using Velocity 6.3 and GraphPad Prism (version 6.07). The correlation analysis was performed using the Spearman correlation analysis in GraphPad Prism. Spearman rank correlation is a non-parametric test to measure the degree of association between two variables. In contrast to linear regression analysis, this test does not make assumptions about the distribution of the samples or the dependency of the analyzed parameters and is used for ordinal or nominal data.

For Fig 1B, a quick staining protocol was applied. In short, bacteria from 1.5 ml of the 4 h subculture were pelleted and washed in blocking buffer (PBS containing 5% BSA). Blocking was done in 1 ml volumes for 1 h at 4°C, before the mouse anti-HA (1:5000, clone 12CA5, Roche) and goat anti-mouse-Cy5 (1:200, Jackson ImmunoResearch) antibodies were added. Antibody incubation was done at 4°C for 1 h. Cells were then transferred onto agarose pads (1% agarose in PBS) and analyzed using the Zeiss Axioplan 2 microscope (Zeiss) using the same software as described above.

Western blot analysis

Whole cell lysates of bacteria were prepared by resuspending bacterial pellets in Laemmli Sample Buffer to obtain 1x10$^9$ bacteria/ml, before incubation at 95°C for 5 min. The proteins were
Fig 1. Scheme of HA epitope topology in invasin and characterization of plasmid-based InvaHA expression. A) Schematic drawing of the HA epitope tag position within the β-barrel of invasin. Six extracellular loops extrude to the extracellular space, with loop 4 displaying the HA epitope tag. The C-terminal part of the passenger domain reaches out into extracellular space. Figure adapted from [25]. B) Microscopic analysis of HA-specific staining in SB300 and SKI-12 (ΔwbaP; O-antigen-deficient strain, [36]). The indicated strains harboring the plnvAHA plasmid were stained using the quick staining protocol described in the material and methods section. The scale bar represents 10 μm. C) Western blot analysis.
resolved by SDS-PAGE and subsequently transferred onto nitrocellulose membranes. Subsequently, the membranes were Coomassie-stained to obtain protein loading controls. In short, membranes were washed 3x for 2 min in ddH2O, before they were stained with the filter stain (Coomassie brilliant blue R 0.05% final concentration). Membranes were de-stained for 5 min with the de-stain solution to allow protein visualization and dried at RT. For the antibody staining, the membranes were blocked overnight at 4°C or 1 h at room temperature (RT) in PBS/T (PBS, 0.1% Tween20) containing 5% milk powder. The blots were stained with monoclonal mouse anti-HA (1:1000, clone HA-7, Sigma), monoclonal mouse anti-GFP (1:2000, clone 7.1 and 13.1, Roche) and secondary peroxidase-conjugated goat anti-mouse antibody (1:4000, Sigma). The Page Ruler Plus Prestained Protein Ladder (10–250 kDa, Thermo Scientific) was used as molecular size marker.

Results
Analysis of a plasmid-based invA<sub>HA</sub> expression construct

An HA-tagged invasin (invA<sub>HA</sub>) reporter plasmid had previously been designed for topological studies of the type Ve autotransporters in E. coli [25]. This previous work had verified that the invasin gene from Yersinia enterocolitica O:8 can efficiently be displayed on the surface of other bacterial species. The invA<sub>HA</sub> fusion gene harbored a double HA tag linked by the amino acid triplet GSG (GSG-HA-GSG-HA-GSG) within loop 4 of the invasin’s β-barrel at the position A204. The double HA tag is referred to as HA tag throughout the paper and its location is illustrated in Fig 1A. The fusion gene was located on a pASK-IBA2 plasmid backbone and its expression was controlled by a tetracycline-regulated promoter. This promoter can be induced using anhydrotetracycline (AHTC), a tetracycline derivative, which lacks antibiotic activity. To verify the expression of the HA epitope tag in Salmonella spp., we transformed the invA<sub>HA</sub>-harboring plasmid into the SL1344 S. Tm derivative SB300 [33]. However, while Western blots of bacterial lysates verified the expression of InvA<sub>HA</sub> in SB300, the staining of the intact bacteria with anti-HA antibodies did not yield any HA-specific signal (Fig 1B and 1C). As the HA tag was located at the extracellular loop of the invasin β-barrel, we reasoned that the absence of anti-HA antibody staining might be attributable to shielding by the lipopolysaccharide (LPS) layer that prevented antibody access to the HA epitope.

We therefore decided to test for HA staining using S. Tm SKI-12, an isogenic mutant that was lacking the O-antigen polysaccharide moiety of LPS. The expression of the O-antigen was ablated due to the deletion of wbaP, which encodes a phosphogalactosyltransferase, essential for the initiation of the O-antigen biosynthesis [36]. The earlier work had demonstrated that this mutation can be complemented. As expected, SKI-12 pInvA<sub>HA</sub> expressed the InvA<sub>HA</sub> protein upon induction with AHTC, as shown by surface staining of induced bacterial cells and Western blot (Fig 1B and 1C). In conclusion, these data suggested that the O-antigen layer of the SB300 strain prevented antibody staining of the β-barrel-positioned HA epitope of invasin. Therefore, we have chosen SKI-12 as the background strain for the following experiments.

The expression of some proteins, including the expression of the T1 apparatus or even GFP can impose a significant burden upon the bacterial cell [39, 40]. This can affect bacterial physiology as indicated by reduced growth rates or reduced colonization. To investigate whether the expression of the invA<sub>HA</sub> reporter had an influence on the growth of the bacterial cells, we
performed a growth experiment and measured the growth of the cells upon induction of InvAHA expression. As shown in Fig 1D, the induction of InvAHA in the SKI-12 background did not significantly affect growth. SKI-12 harboring an empty control vector served as an additional control. In contrast, a tetracycline-inducible gfp expressing plasmid, which was known to inflict a burden to S. Tm, showed a much lower growth rate. In addition, we tested the effect of gradually increasing the expression of the invAHA reporter. In line with the data shown above, gradual induction increased the reporter surface staining on SKI-12 (S1 Fig). This suggested that the invAHA reporter can be used to monitor gene expression in SKI-12 and that reporter expression can be monitored over a wide range of induction levels.

Deletion of wbaP has a slight effect on T1 expression

In order to probe the suitability of invAHA as a transcriptional reporter, we wanted to compare it to the well-established gfp reporter. In particular, we wanted to assess SPI-1 expression. In the past, the expression of the T1 was mostly analyzed in the S. Tm background with O-antigen-proficient LPS (smooth LPS, SB300) [3, 19]. In order to use the T1-gfp reporter in an O-antigen-deficient wbaP background, we first had to establish SPI-1 expression and monitor T1-gfp in this strain. To this end, we used the plasmid-based T1 reporter, expressing gfp under the promoter control of the SPI-1-encoded chaperone SicA [3]. The sicA promoter controls the expression of the operon, encoding sicAsipBCDA [20]. Flow cytometric analysis revealed that T1 expression was reduced (from ~ 55% to ~ 45%) in the absence of the O-antigen polysaccharide (Fig 2). Nevertheless, T1 is expressed at appreciable levels. Therefore, the ΔwbaP strain could be used for our proof-of-concept work to test if autotransporters can be used in principle as transcriptional gene expression reporters. The use of the ΔwbaP strain of InvAHA was of advantage, as the HA epitope could be stained in the O-antigen-deficient strain, in contrast to the SB300 strain harboring a smooth LPS layer (Fig 1B).

Chromosomal invAHA reporters for the expression of the SPI-1 promoters PprgH and PsicA

To assess the suitability of the autotransporter approach, we generated strains carrying the invAHA reporter in the chromosome. For this purpose, we focused on T1. First, we intended to verify that the T1 expression displayed by the invAHA reporter is comparable to the gfp-based T1 reporter system used in previous studies [3, 15, 19, 37]. We therefore used the previously published prgH promoter-driven gfp (PprgH-gfp) reporter strain JH3010 used by the Hinton laboratory to visualize T1 expression [37]. PrgH is a structural protein of the T1 needle and is encoded on one of the three large operons of SPI-1. We constructed all strains in a non-motile phenotype [34] to allow future analysis of these strains in single cell setups, such as the microfluidic setup [41]. By P22 transduction we introduced the PprgH-gfp allele of the JH3010 strain into the flagella-mutated background strain (χ8602), before we introduced the invAHA reporter downstream of the SPI-1 encoded effector sipA [42]. SipA is the last gene of the sicAsipBCDA operon and expressed under the control of the sicA promoter. Hence, both reporters, PprgH-driven gfp and PsicA-driven invAHA, were under the control of the T1 regulon and should be expressed by the same cells. Finally, to allow HA-specific staining, we introduced wbaP deletions into all strains used for the following experiments (see Table 1).

In this way, we obtained a non-motile strain in an O-antigen-deficient background (wbaP deletion), harboring PprgH-driven gfp and PsicA-driven invAHA expression (Z536, Fig 3A). Furthermore, we constructed two control strains (Z537 and Z555, respectively; Fig 3A), harboring either the PsicA-driven invAHA or the PprgH-driven gfp reporter. These latter strains should allow us to control for potential effects caused by the expression of two reporters within...
the same cell. The cells were grown in LB medium and bacteria were stained for the HA epitope tag. The GFP levels could be measured without any additional staining. Analysis of the stained cells was done by microscopy and by flow cytometry. Single cell expression analysis of Z536 verified that the gfp and invAHA reporters are indeed co-expressed ($r = 0.651$, $p < 0.0001$). As expected, the control strains Z537 and Z555 displayed either the InvAHA or the GFP-specific signal, respectively (Fig 3B). Quantification of the GFP signal revealed that the expression of both reporters within the same cell (i.e. in Z536) was virtually equivalent to that observed in the strains expressing just one reporter at a time (i.e. Z537 and Z555). Only the PprgH-gfp reporter showed a slight reduction in strain Z536 (Fig 3C).

To obtain further evidence, we also performed FACS analysis of the same samples that had been used for microscopy. Again, we observed strong co-expression of PprgH-gfp and of PsicA-invAHA and a slight reduction of PprgH-gfp expression in Z536 (compare to Z555, Fig 3D). This indicated that the invAHA reporter is indeed suitable for monitoring T1 expression.

Next, we analyzed whether the integration of the different reporters resulted in negative effects on the growth of cells at the population level. We did not observe any adverse effects on growth of the new reporter strains (Fig 3E). However, as only a subpopulation of cells expresses T1 [3], the absence of a growth phenotype in this bulk analysis does not reflect the single cell situation of cells harboring the newly introduced reporters. Single cell analysis e.g. by using agar pads [3] or by using microfluidics [15, 41] may help to further back this up.

**Growth phase-dependent T1 expression is detected by the gfp and the invAHA reporter.**

To test the performance of the invAHA reporter, we analyzed the well-established effect of the growth phase on T1 expression at the single cell level [3]. Thus, cultures of the reporter strain were inoculated from overnight cultures (1:400; this avoids spillover T1 expression that can occur in the overnight culture) and cultures were grown for 2 h (non-inducing condition) or for 4 h (T1 induction). Then, the HA epitope was stained and GFP and HA-staining were analyzed by flow cytometry. As expected, the reporter-less control strain SKI-12 did not show any fluorescence signal, while Z555 and Z536 expressed higher GFP levels at 4 h than at 2 h.
Fig 3. Scheme of chromosomal constructs of Z536, Z537 and Z555, expressing the gfp and invAHA reporters under the control of two SPI-1 encoded promoters and analysis of the constructs. A) Schematic drawing of the chromosomal transcriptional fusions in the indicated strains. The reporters were under the control of either the prgH promoter (P\text{prgH}; Z555) or the sicA promoter (P\text{sicA}; Z537) or both (Z536). The cells were stained as described in the material methods section. We used cells from the same preparation for either microscopic (B, C) or flow cytometric (D) analysis. Triplicates were measured.
by flow cytometry (D), whereas these samples were combined for microscopic analysis (B, C). B) Analysis of gfp and invAHA expression of the indicated strains, as measured by microscopy. The invAHA staining was measured by the Cy5 exciting laser for microscopy. The GFP and Cy5 fluorescence values of all individual cells were extracted and the log10 transformed values were plotted against each other. The three colors indicate the different independent experiments. Correlation was analyzed by Spearman correlation analysis (see material and methods for detailed information). Fluorescence of GFP and InvAHA (Cy5) is indicated in arbitrary units (AU). Insert: Microscopy images from the original data set. This illustrates the staining strategy and the nature of the primary data used for the expression analysis. Images represent the phase contrast, GFP (green) and Cy5 (red; HA epitope) channels and the merge (orange; from left top to right bottom). White bar: 5 μm. C) Quantitative analysis of the data set from B. The upper and lower graphs display the percentages of cells displaying GFP or Cy5 (for InvAHA) fluorescence, respectively. The thresholds for GFP and Cy5 (for InvAHA) fluorescence were set by the maximum values in the GFP or Cy5 channel of the wt strain, respectively. Z555 and Z537 are the controls, harboring only the gfp or the invAHA reporter, respectively. Z536 harbors both reporters, gfp and invAHA. D) Flow cytometric analysis of the same cells as in B. The upper and lower graphs display the percentages of T1+ cells, measured by the GFP and Alexa Fluor 647 (for InvAHA) excitations. Statistical significance was assessed using the Mann-Whitney test. E) Growth analysis of the chromosomally tagged strains. Mean and standard deviation of three independent experiments are shown. “wt” = wild type SKI-12. ns = not significant; ** p < 0.0002.

Fig 4. GFP and InvAHA expression during different growth phases. Flow cytometric analysis of gfp (A) and invAHA expression (B) of the indicated strains, measured by GFP and Alexa Fluor 647 (for InvAHA) excitations, respectively. Z555 and Z537 are the controls, harboring only the gfp or the invAHA reporter, respectively. Z536 harbors both reporters, gfp and invAHA. The subcultures were diluted 1:400 prior to incubation for 2 or 4 h, respectively. Statistical significance was tested using the Mann-Whitney test. ** p = 0.0022.

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(Fig 4A). Equivalent results were obtained for the invAHA reporter expression by Z537 and Z536 (Fig 4B). In conclusion, these experiments showed that invAHA reporters are suitable for monitoring gene expression and suggested that this construct can be combined with established gfp reporters to monitor two different promoters in the same bacterial cell.

Expression of the gfp and invAHA reporters under the control of the same promoter

So far, we have demonstrated that the use of the PsicA-driven invAHA reporter can monitor T1 expression. However, the correlation with the PprgH-gfp reporter was below 100%. It had remained unclear if slight levels of divergence might arise from the different folding/maturation kinetics of the two reporters, the noisiness in promoter expression or to other so far unidentified effects. In a first attempt to address this, we wanted to test whether the combination of both reporters under the control of the same promoter will result in a similar pattern of correlation. For this purpose, we generated a transcriptional fusion of both constructs downstream of sipA, thus placing both constructs under the control of the sicA promoter (Fig 5A).
Fig 5. Expression of the two reporters gfp and invAHA under the control of the same PsicA promoter. 
A) Scheme of the transcriptional fusions of the gfp and invAHA reporters, which were inserted downstream of sipA. B) Microscopic analysis of GFP and InvAHA fluorescence levels (measured by the Cy5 exciting laser for microscopy) of the indicated strains. The three colors indicate the data from different experiments. The correlation was analyzed by Spearman correlation analysis. Fluorescence of GFP and InvAHA (Cy5) is
To control for any artefacts caused by the arrangement of reporters, we generated two strains, one carrying the gfp downstream of the invA HA- and one carrying invA HA downstream of the gfp reporter. This yielded the two strains harboring either sipA-invA HA-gfp (Z567) or sipA-gfp-invA HA (Z574; Fig 5A). Single cell expression analysis revealed that both reporters are expressed and that their expression was correlated (Z567: \( r = 0.630, p < 0.0001 \); Z574 \( r = 0.654, p < 0.0001 \)). This correlation was similar to that observed in Z536 (\( r = 0.518, p < 0.0001 \)) (Fig 5B). The two control strains Z537 and Z555 displayed exclusive invA HA and gfp expression, respectively. However, a few cells displayed false positive signals. This was most likely attributable to noise or to slight cross-contaminations during the handling procedures of the staining protocol.

By and large, all strains showed equivalent levels of T1+ cells, irrespective of whether we used gfp or invA HA as transcriptional reporter. Only the gfp expression under the control of the PprgH promoter showed \( \approx 2 \)-fold differences between the different strains, as the strain Z555 (only one reporter, i.e. PprgH-gfp) expressed a higher fraction of GFP+ cells than the strain Z536 (two reporters, i.e. PprgH-gfp and PsicA-invA HA) (Fig 5C). However, the average GFP fluorescence intensity was quite similar between Z555 and Z536 (Fig 5B). It remains unclear whether the 2-fold difference in the fraction of gfp-expressing cells might be attributable to the presence of two reporters in the same cell. However, this difference was much smaller when focusing on the InvA HA expression (Fig 5C, bottom panel).

In conclusion, we could see similar correlation patterns in the strains harboring the different reporter constructs. Our data confirmed that InvA HA can be used as reporter system to visualize gene expression and that this system can be used in combination with the gfp reporters.

**Discussion**

Here, we established a novel system for the analysis of S. Tm wbaP gene expression at the single cell level. This strategy employs the epitope-tagged autotransporter invasin (InvA HA). Most previous reports studying the expression of individual promoters of interest within single cells have used fluorophores as reporter system [2, 22, 37, 43]. Using strains expressing gfp and invA HA reporters from the same promoter (PsicA, Fig 5) or from two SPI-1 promoters (PprgH and PsicA, Fig 3), which were thought to be co-regulated, we could verify the suitability of this approach.

In theory, autotransporters like InvA may therefore provide distinct advantages when compared to gfp-based reporters. This is particularly relevant for cases when more than one promoter has to be analyzed per cell. First, it might offer a strategy to circumvent problems associated with the different folding/maturation half-lives observed with different fluorescent protein variants. Current approaches using different fluorophores for expression analysis within a single cell [2] impair accuracy due to different folding or maturation times. In future applications, it is conceivable that establishing a set of InvA HA variants with equivalent maturation dynamics may allow such multi-gene expression analysis if one inserts multiple invA
genes, harboring different epitope tags, e.g. \textit{invA}\textsubscript{HA} for \textit{PprgH} and \textit{invA}\textsubscript{Strep} for \textit{PsicA} expression analysis. Thus, it will be an important task for future work to establish the folding/matura-
tion kinetics of the \textit{InvA}\textsubscript{HA} reporter (or similar reporters with longer extracellular extensions; see below) and verify that the kinetics are not affected by exchanging the HA epitope for other
epitope members.

Clearly, there are important limitations that would have to be overcome before one can gen-
erally apply autotransporter reporters. It should be pointed out that the biological activity of wt
autotransporters can have profound effects on pathogen-host interactions \cite{24}. Thus, for
extending the use of autotransporters to infection assays, it will be necessary to disrupt the
host-cell binding. It seems conceivable that this can be achieved by introducing the epitope tag
into the binding domain that is exposed on the bacterial surface. However, this would need to
be addressed in future work.

Our current \textit{InvA}\textsubscript{HA} reporter system is limited to O-antigen-deficient \textit{S}. \textit{Tm} strains, as the
full-length LPS layer prevents antibody binding of the HA epitope (Fig 1B). The same was true
when we used an alternative reporter, i.e. the \textit{E}. \textit{coli} intimin carrying a Strep-tag reporter at its
C-terminus (S2 Fig, \cite{32}). Presumably, this is attributable to shielding by the long \textit{S}. \textit{Tm} O-
antigen that includes up to 100 repeating units and has been estimated to form a 100 nm thick
layer \cite{44}. This may shield not only the HA-epitope of the \textit{InvA}\textsubscript{HA} reporter, but also the C-ter-
minally located Strep-tag of intimin. In fact, changes in O-antigen layer thickness in \textit{Shigella}
spp. were reported to have dramatic effects on host cell invasion by shielding (or exposing) the
tip of the invasion-mediating type three secretion system \cite{45}. Thus, for designing autotran-
sporter reporters, one may have to identify autotransporters that match the LPS O-sidechain
length of the respective bacterium. In our pilot study, we have bypassed this problem by using
an O-antigen deficient \textit{wbaP} strain background. However, for many applications, the O-anti-
gen-deficient background is not optimal, including our studies of T1 expression in wt \textit{S}. \textit{Tm}
(Fig 2).

In previous reports analyzing the role of the O-antigen in \textit{S}. \textit{Tm}, an increased susceptibility
to complement and antimicrobial peptides as well as a reduced colonization of mice was dem-
onstrated in strains lacking the O-antigen \cite{36, 46}. Moreover, these mutants show reduced
swimming motility and an altered invasion efficiency in HeLa tissue culture models \cite{36}. Thus,
the O-antigen-deficient background is not very well suited for \textit{in vivo} studies. In order to cir-
cumvent this problem, one would probably have to employ autotransporters that extend
beyond the O-side chain and add tags to the surface-exposed moiety. This should alleviate the
technical problems arising from the use of the O-antigen-deficient strain.

\textit{S}. \textit{Tm} is exquisitely sensitive to the expression of T1. The reduced growth rate of T1\textsuperscript{+} \textit{S}. \textit{Tm}
cells is attributable (at least in part) to the metabolic costs associated with the expression of the
T1 apparatus and the large pools of the pre-formed effector proteins \cite{3, 16, 19}. It is known
that the co-expression of T1 with a \textit{gfp} reporter further enhances these costs. Thus, monitoring
T1 expression represents a highly sensitive system to probe the "burden" imposed by any
reporter construct. It is interesting to note that the \textit{invA}\textsubscript{HA} and the \textit{gfp} reporters yielded equiva-
 lent results (one possible exception, Fig 5; Z536 vs Z555). This may suggest that the \textit{invA}\textsubscript{HA}
reporter perturbs the system no more (or less) than the \textit{gfp} reporter. As all reporters can poten-
tially affect the system under scrutiny, one should carry out suitable control experiments to
identify the most suitable system. Our \textit{invA}\textsubscript{HA} reporter thus expands the toolbox that one can
choose from. Altogether, our data suggests that \textit{InvA}\textsubscript{HA} reporters should be quite well suited to
replace \textit{gfp} reporters for applications like the parallel analysis of several reporters of bacterial
cells featuring a particular expression pattern, e.g. by MACS sorting.

In \textit{S}. \textit{Tm}, phenotypic diversity in the expression of the T1 virulence factor is well known \cite{3,
16, 19, 22, 37, 43} and the core regulation factors controlling and fine-tuning this system are
established (for reviews see [20, 21]. However, the exact mechanism behind the bistable behavior of the T1 system remains unknown. Analyzing the dynamics of the multiple transcription factors controlling the T1 regulatory cascade (e.g. HilD, HilC, HilA) within single cells would allow a deeper analysis of the fundamental organization of the complex regulatory cascade. An InvA system combining multiple \textit{invA} genes (or similar reporters that allow surface detection even in the presence of wt LPS) with different epitope tags located at the outmost C-terminal region may provide a pioneering system for accurate assessment of T1 regulatory circuits (Fig 6). This would give further insights into the mechanisms behind the phenotypic diversity of the \textit{S}. Tm T1 expression. We propose that the \textit{invA}_{HA} reporter system, or variants thereof, can be used in a similar fashion for multi-dimensional gene expression analysis in Gram-negative bacteria at the single cell level.

**Supporting Information**

\textbf{S1 Fig. Comparison of InvA\textsubscript{HA} induction at the single-cell level by anhydrotetracycline-and arabinose-inducible plasmids.} SKI-12 carrying either a tetracycline-inducible or an arabinose-inducible \textit{invA}_{HA} cassette were cultured for 4 h in LB and exposed to the indicated concentrations of inducer. Living cells were stained using a HA-specific antibody, before they were analyzed by flow cytometry. Induction of InvA\textsubscript{HA} expression was performed either by addition of AHTC or arabinose at the indicated concentrations. DH5\textsubscript{α} pInvA\textsubscript{HA} was used as positive control for InvA\textsubscript{HA} induction. Four to six measurements were performed in two independent
experiments.

(TIF)

S2 Fig. Surface staining of C-terminally Strep-tagged intimin. Flow cytometric analysis of the LPS-proficient SB300 strain, harboring either the wild type (Int wt) or Strep-tagged intimin (Int wt-Strep; [32]). The empty vector control pASK-IBA2 served as negative control for staining. The LPS-deficient strain SKI-12, carrying the Strep-tagged Intimin (SKI-12 Int wt-Strep) served as positive control for Strep-tag staining. Strep-tag expression was induced by addition of 200 ng/ml AHTC.

(TIF)

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Author Contributions
Conceived and designed the experiments: IC WDH. Performed the experiments: IC. Analyzed the data: IC. Contributed reagents/materials/analysis tools: MD PO MS DL MC. Wrote the paper: IC WDH.

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