Physiological stress of intracellular *Shigella flexneri* visualized with a metabolic sensor fused to a surface-reporter system

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### 1. Introduction

Although the molecular interplay between intracellular bacterial pathogens and their hosts includes sophisticated virulence determinants, the basic scenario for infection is determined by the metabolic and physiological fitness of the invading microorganisms [1]. Limitation of some key nutrients (typically, iron) is one of the first barriers of defense against potential bacterial assailants. In return, nutrient starvation has been evolutionarily recruited as one of the major host signals that cause expression of virulence factors. That many Gram-negative pathogens lacking the major C-starvation sigma factor *rpoS* stop being virulent [2,3] exposes how much the triggering of an infectious program relies not only on responding to specific host signals, but also on the sensing of a generic metabolic standing. In this context, recognizing the overall physiological status of bacteria during invasion of host cells is critical for identifying the limiting steps of the process. Several attempts have been made in the recent years for visualizing (in most cases with the help of transcriptional green fluorescent protein (GFP) fusions) the expression of specific genes during the entire cycle of infection of target cells by *Salmonella*, *Bordetella*, *Listeria* or *Mycobacterium* [4]. Yet, the metabolic status is the result of combining multiple nutritional inputs with responses to the distinct physico-chemical stresses that define specific niches (pH, osmolarity, redox state, temperature, etc.). But no promoter is known to naturally describe by itself the outcome of such many environmental conditions.

During the course of the work with genes dependent on the alternative sigma factor σ54 in Gram-negative bacteria, we and others have documented that the transcriptional outcome of some promoters of this kind is as dependent on the specific signal to which promoters respond as on the physiological status of cells [5–12]. Among other cases, this is the case in the subclass of σ54-promoters that drive expression on operons for consumption of unusual C-sources (*m*-xylene, phenol). For instance, the *m*-xylene transcriptional regulator XylR is able to activate its σ54-promoter *Pu* only when cells are under C-starvation and a low metabolic charge. This is because the productive binding of all regulatory proteins to that promoter depends on a multitude of physiological conditions, including the growth-phase dependent levels of integration host factor (IHF), ppGpp, heat shock and sigma factor competition [6–11]. Although the XylR/*Pu* pair comes originally from the soil bacterium *Pseudomonas putida*, its transcriptional regulation can be grossly reproduced in *Escherichia coli* [9,10]. Deletion of the *m*-xylene binding domain of XylR leaves a truncated transcriptional factor that is receptive to such physiological conditions and thus becomes an operative sensor of the metabolic state [13]. We have exploited this feature to visualize the physiological situation of *Shigella flexneri* during infection of Henle 407 cells in culture. To this end, we have combined the xylRAA-*Pu* sensor system with a novel surface reporter [14] that avoids some of the problems associated to the use of GFP and provides, instead, several advantages. Our results suggest that intracellular *Shigella* cells undergo a considerable metabolic stress.
2. Materials and methods

2.1. Bacterial strains, plasmids and general techniques

The bacterial strains and plasmids used in this study are listed in Table 1. Strains were grown in LB medium [15]. Clones expressing the β-galactosidase (lacZ) gene were selected on MacConkey or IPTG/X-gal agar medium [15]. Where required, media were supplemented with Cm (30 μg/ml), IPTG (1 mM) and X-gal (40 μg/ml). DNA preparation and genetic manipulations were performed according to standard protocols [15]. Plasmid DNA was isolated with the QiAprep Spin Miniprep Kit (Qiagen, USA) according to the manufacturer's instructions. Transformation of bacterial cells with plasmid DNA was performed by electroporation [16]. Restriction and modification enzymes were purchased from New England Biolabs (Schwalbach, Germany). The trp-lacZ gene was excised from plasmid pU8 [17] as a 3.1 kb BamHI/HindIII fragment, blunted with T4 DNA polymerase and inserted into HindIII digested/blunted pLB9-A6 [14].

The resulting plasmid (pA6LZ1) carries the trp-lacZ gene oriented in the sense of transcription of the lac promoter. Plasmid pA6LZ1 (Fig. 1) carried two different reporter genes positioned in tandem, namely lamB-α6 [14] and trp-lacZ. To facilitate the cloning of fragments in front of the reporter gene, a double strand oligonucleotide (5'-GAATTCTCGAGATCCAGGCTTAAATTTAC-3') encompassing the sequences recognized by the restriction endonucleases EcoRI, PstI, BamHI, HindIII and translational terminators in all three potential open reading frames was inserted into the pre-blunted EcoRI restriction site of pA6LZ1, thereby generating pA6LZ3. To prevent read-through transcription into the reporter gene, the ribosomal RNA rrnB T1 transcriptional terminator was amplified by polymerase chain reaction (PCR) from pKK232-8 [18] using forward (5'-GCAATTTTCGACCGTAGATTATTAC-3') and reverse (5'-GGGAATTCGGTGCAGTTTATGG-3') primers, digested with Apol (underlined) and ligated to EcoRI-digested pA6LZ3, thereby eliminating one EcoRI restriction site and leaving intact the other restriction sites present in the polylinker. The resulting plasmid was named pA6LZ4 (Fig. 1). An equivalent plasmid (pA6LZ4R) bears the same 5.2 kb NotI fragment containing the reporter genes and the polylinker in the opposite orientation with respect to the lac promoter (Fig. 1). Finally, the plasmid pA6LZ4-6 was constructed by fusing the 1.35 kb BamHI fragment of plasmid pRS/MAD2 [19] encoding a constitutive mutated form of the prokaryotic enhancer-enhancer protein XylR (XylRΔA2) and the Pu promoter into pA6LZ4.

2.2. Tissue culture methods, infection and immunofluorescence studies

The human cell line Henle 407 (ATCC CCL-6) was maintained in DMEM supplemented with 10% FCS and 2 mM glutamine (GIBCO Laboratories, Germany). For immunofluorescence studies, cells were seeded onto 12 mm-diameter glass coverslips in 24-well tissue culture plates (Inter Med Nunc) and infected during 3 h with overnight grown cells of S. flexneri aroD strain SFL124-27 at a multiplicity of infection of 100:1 (bacteriaceal). This aroD strain was preferred in the invasion experiments because of the ease of handling with a relatively lower level of containment. Infected cells were fixed with 3.7% paraformaldehyde in phosphate-saline buffer (PBS) and permeabilized with 0.2% Triton X-100 in the same PBS. Bacteria and the Lamb6-A6 protein were stained using a rabbit polyclonal antiserum against S. flexneri Y (Behringwerke, Marburg, Germany) and the monoclonal antibodies (Mab) 6A.A6 that binds to the coronavirus A6 antigen (Engenasa SA, Madrid) as first antibodies and fluorescein-labeled goat-anti-rabbit immunoglobulin G (IgG) (Jackson ImmunoResearch Laboratories) and TRITC-conjugated goat-anti-mouse (Dianova, Germany) as secondary antibodies. Coverslips were washed, mounted, and cells were examined by epifluorescence with a Zeiss axiophot microscope (Carl Zeiss, Germany). For immunoseparation experiments, cells were grown on 100 mm Petri dishes (Inter Med Nunc) and infected with bacteria as described above.

2.3. Immunoseparation and flow cytometry analysis

Bacterial subpopulations expressing the Lamb6-A6 protein were recovered by using the Mab 6A.A6 and sheep anti-mouse IgG IMBs (Dynabeads M280, Dynal, Germany). The method of separation was either direct, when the immunomagnetic beads (IMBs) were first incubated with the Mab 6A.A6 and subsequently with bacteria, or indirect, when the Mab 6A.A6 was first incubated with bacteria and then with IMB. In brief, bacteria were grown until they reached the exponential growth phase, mixed in determined proportions and 0.05–10 μg of the Mab 6A.A6 was added for 1 ml of bacterial suspension containing ~109 bacteria. The mixture was then incubated for 10–30 min at 4°C. Bacteria were collected and the antibody excess was washed with 0.1% bovine serum albumin (BSA) in PBS. IMBs prepared according to the manufacturer's instructions were added and the mixture was incubated for additional 30 min at 4°C. The IMBs were then separated with a Dynal magnetic particle concentrator, washed with 0.1% BSA in PBS and diluted on MacConkey or IPTG/X-gal agar.

For recovering bacteria from S. flexneri-infected Henle 407 cells, the invasion test was made as above but, after 1.5 h of incubation at 37°C, gentamicin (30 μg/ml) was added to kill extracellular bacteria and monolayers were further incubated for 4–6 h. Cells were lysed with distilled water and intracellular bacteria were recovered from supernatant fluids by centrifugation, resuspended in 0.1% BSA in PBS and processed for immunoseparation as before. For detection of different subpopulations of bacteria by flow cytometry, the samples with the cell suspensions were incubated with the Mab 6A.A6 as the primary antibody (0.05–10 μg/ml for 10−106 bacteria) for 30 min at 4°C. Then, bacteria were collected, washed with 0.1% BSA in PBS and resuspended in the same solution. Anti-mouse IgG-FITC conjugated secondary antibody was added to the bacterial suspension and cells were further incubated for 30 min at 4°C. After washing, bacteria were analyzed on a FACSscan device (Benton Dickinson, Erembodegen-Aalst, Belgium).

Table 1

| Strain or plasmid | Relevant genotype and characteristics | Reference or origin |
|-------------------|--------------------------------------|---------------------|
| **Bacterial strains** |                                       |                     |
| CC118             | Δ(ara-leu) araD lac lacZ4 galE galK phoA thi-l rpsE rpoB argE (Am) recA1 | [17]                |
| CC118 E* SURE     | CC118 F*::Tc; lacZ4::TcflacZ4-153    |                     |
| SFL124-27         | TeC, S. flexneri aroD serotype Y     | [27]                |
| **Plasmids**      |                                       |                     |
| pRS/MAD2          | Kmr, Ap, promoter probe plasmid carrying the xylRA42 constitutive form of the transcriptional activator xylR and the Pu promoter |                     |
| pU8J              | Ap, promoter probe plasmid for transcriptional fusions |                     |
| pKK232-8          | Ap, Cm, pBR322 derivative containing tandem rrnB terminators |                     |
| pLLB9             | Cm, plasmid carrying the promoterless lamB-153 sequence |                     |
| pLLB9-A6          | Cm, pLLB9-A6 epitope inserted in its own BamHI site |                     |
| pA6LZ1            | Cm, pLLB9-A6 derivative with the trp-lacZ gene inserted in the HindIII restriction site | This work |
| pA6LZ3            | Cm, pA6LZ1 derivative with a multiple cloning site inserted into the EcoRI restriction site | This work |
| pA6LZ4            | Cm, pA6LZ1 derivative with the rrnB T1 transcriptional terminator cloned upstream of the multiple cloning site |                     |
| pA6LZ4-R          | Cm, pA6LZ4-R derivative cloned in the opposite orientation |                     |
| pA6LZ4-6          | Cm, pA6LZ4 derivative carrying the BamHI 1.35 kb fragment from pRS/MAD2 | This work |
Fig. 1. Schematic representation of the main plasmids used in this study. All DNA segments shown were assembled on the single-copy vector pVL8T, a derivative of pSC101 plasmid. Abbreviations: B, BamHI; E, EcoRI; H, HindIII; N, NotI; P, PstI; Plac, lac promoter; lamB-153, promoterless structural gene encoding the LamB protein; lamB-A6, promoterless structural gene encoding a LamB derivative carrying the antigenic site C of glycoprotein S of transmissible gastroenteritis coronavirus (A6 antigen); trp-lacZ, promoterless hybrid LacZ gene; rrnB T1, transcriptional terminator of the ribosomal RNA operon. Pr, promoter of the xylR gene; Pu, promoter of the upper pathway of the xyl operon. Sizes are symbolic.

3. Results and discussion

3.1. Rationale of the metabolic sensing system

The visualization of the physiological state of intracellular Shigella is based on two molecular tools, namely, a sensor regulator/promoter pair and a dedicated reporter system. For the first, we exploited the known ability of multiple metabolic and environmental signals to downregulate the σ^54-dependent promoter during its activation by XylR and m-xylene. In its natural context, non-limited growth at fast rate inhibits Pu, while slow growth, C-limitation and general environmental stress activate the promoter at very high level [5–11,13]. On this basis, we have put together a gross metabolic sensor cassette in which the gene for the XylR protein, deleted of its m-xylene binding domain, has been assembled adjacent to Pu in one-copy number plasmid pA6LZ4-6 (Fig. 2). While this promoter does not respond any more to aromatic inducers, it does maintain its sensitivity to metabolic stress. The other component of the setup is a bicistronic reporter system which includes, as a single transcriptional unit, a promoterless variant of the lamB gene (named lamB-A6) and lacZ (Fig. 2). The LamB protein is the maltose outer membrane receptor in E. coli [20,21] which, in the case of the LamB-A6 variant, bears the antigenic site C of the glycoprotein S of the transmissible gastroenteritis coronavirus (A6 antigen or cor antigen) displayed on its outside-facing surface [14]. Its expression makes bacteria to present the cor antigen in their surface, where it is readily bound by cognate Mab. For our purposes, we argue that such a surface reporter is superior to GFP. This is because of the difficult folding pathway of the protein prior to fluorescence emission [22] and the requirement of oxygen for GFP activation, which may flaw its detection in specific reducing intracellular environments [23–25]. Furthermore, the stability of GFP also renders the timing of gene activation difficult to monitoring [26]. On the contrary, the LamB-A6 protein enjoys a rapid turn-over, efficient expression in a wide range of bacterial hosts and A6-specific antibodies are available. Co-transcription of lamB-A6 with lacZ in our reporter system (Fig. 2) expands the utility of our reporter system to the analysis of promoter strength, if required.

3.2. Validation of the lamB-A6/lacZ reporter for in situ analysis of promoter output

An essential aspect of the issue at stake in this work is the performance of the surface reporter system and the faithfulness with which it converts promoter activity into a physical property that can be quantified. To this end, we first investigated whether the LamB-A6 protein presents the cor antigen on its surface to the point of allowing recovery of bacterial subpopulations expressing the chimeric protein using either IMBs combined with an A6 antigen-specific Mab or by fluorescence-activated cell sorting (FACS) analysis. To examine this, different proportions of growing E. coli cells (strain CC118 F’SURE, Table 1) harboring either pA6LZ3 or pLBB9 (Fig. 1) were mixed and processed as described in Section 2. In this assay, lamB-A6^− cells are readily distinguishable from cor-less lamB^+ cells, as pA6LZ3 endows its hosts with a blue colony phenotype when plated on IPTG/X-gal medium. Table 2 shows the efficiency of the enrichment, which demonstrated the physical exposure of the cor antigen on the cell surface and the robustness of the interaction with the Mab. In a second series of experiments, the plasmid pA6LZ3 was added to a transcriptional terminator upstream of the reporter cassette and inserted with a 1.35 kb BamHI fragment encoding the metabolic sensor system consisting of xylRAA, and its target promoter Pu facing the bicistronic reporter lamB-A6/lacZ (pA6LZ4-6, see above and Fig. 2). The expression levels of LamB-A6 in stationary cells of E. coli were ~20-fold higher than the counterparts during exponential growth, and the best enrichment factor for the
Table 2

| pA6LZ3:pLBB9 ratios | Viable cell recovery | LacZ⁺ colonies (%) |
|---------------------|----------------------|--------------------|
| 1:1                 | 1.1 × 10⁶            | 100                |
| 1:10                | 1.8 × 10⁵            | 78                 |
| 1:100               | 2.4 × 10⁴            | 9.5                |
| 1:1000              | 4.2 × 10³            | 5                  |

*Approximately 8.0 × 10⁶ bacteria were processed, i.e., treated with IMBs as described in Section 2.

3.4. Conclusion

The combination of a reporter system detectable by physical means (rather than enzymatically or optically) with a metabolic stress sensor derived from a σ⁵₂-dependent system has allowed us to visualize the intracellular physiological state of *S. flexneri* during infection of cells in culture. Since the sensor module *xyIAA* has been shown in various hosts to be a descriptor of multiple environmental conditions and suboptimal metabolic charge, our results picture the metabolic pressure that *Shi-

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Fig. 3. Separation of bacterial cells expressing LamB-A6 by flow cytometry. Bacteria were grown as explained in the text, mixed in different proportions, treated with the cor epitope-specific Mab 6A.A6, labeled with FITC-conjugated rabbit anti-mouse antibodies and analyzed with a cell sorter. Panel A: *E. coli* (pLBB9-A6), lamB-A6 expressed through the Plac promoter of the vector. Panel B: *E. coli* (pA6LZ4-6), lamB-A6 expressed through the Pu promoter. Panels C and D: mixtures of *E. coli* (pLBB9-A6) and *E. coli* (pA6LZ4-6) at 1:1 and 10:1 ratios, respectively. Horizontal bars specify the percentage of the whole bacterial population included in the range of fluorescence levels indicated.
gella cells endure during their intracellular existence. On the other hand, the ease of recovery of specific sub-populations of cells expressing the lamB-A6/lacZ cassette makes this bicistronic reporter an appealing tool for deconstructing the molecular host-pathogen interplay. In particular, the possibility to randomly clone promoters in vector pA6LZ4 (Fig. 2) and separate in vivo the population of those which are expressed in a given site or infection stage opens interesting possibilities for studying new features of cell-bacteria interactions. In any case, our observations imply that any dealings between pathogens and their hosts are framed by gross metabolic boundaries that can in some cases determine the outcome of the entire process.

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Table 3

| Ratio pA6LZ4-6/pA6LZ4R Shigella transformants | IMBs added | LacZ+ colonies (%) |
|---------------------------------------------|------------|-------------------|
| 1:1                                         | –          | 42.3              |
| 1:1                                         | +          | 94.5              |
| 1:4                                         | –          | 35                |
| 1:4                                         | +          | 92.3              |
| 1:40                                        | –          | 2.9               |
| 1:40                                        | +          | 46.2              |

Fig. 4. Intracellular detection of S. flexneri cells expressing the xylRAA/PullamB-A6lacZ metabolic sensor system. Henle 407 cells were infected during 3 h with the S. flexneri strain SFL124-27 carrying either pA6LZ4R (panels A and B) or (panels C–F). Then, monolayers were fixed and bacteria were labeled with polyclonal antibodies against S. flexneri O antigen (panels A, C and E) and the Mab 6A.A6 (panels B, D and F). Coverslips were examined by immunofluorescence microscopy. Note the strong fluorescence of cells bearing the reporter plasmid pA6LZ4-6 when treated with the anti-cor antibody.
