FEBS 29219 FEBS Letters 579 (2005) 813–818

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

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Received 17 September 2004; revised 29 November 2004; accepted 23 December 2004

Available online 11 January 2005

Edited by Pascale Cossart

Abstract When deleted of its N-terminal signal-reception domain, the broad host range σ^{54}-dependent transcriptional regulator XylR, along with its cognate promoter *Pu*, becomes a sensor of the metabolic stress of the carrier bacteria. We have employed a surface reporter system to visualize the physiological status of intracellular *Shigella flexneri* during infection of Henle 407 cells in culture. To this end, the *xylRΔA* gene has been engineered adjacent to a bicistronic transcriptional fusion of *Pu* to a *lamB* variant tagged with a short viral sequence (*cor*) and β-galactosidase (*lacZ*). The accessibility of the *cor* epitope to the external-most medium and the expression of *Pu* in the bacterial population was confirmed, respectively, with immunomagnetic beads and the sorting of *Escherichia coli* cells treated with a fluorescent antibody. Intracellular *Shigella* cells expressed the *Pu–lamB|cor–lacZ* reporter at high levels, suggesting that infectious cells endure a considerable metabolic constraint during the invasion process.

Keywords: σ^{54}-Promoter; Surface reporter; XylR; *cor* epitope; LamB; *Shigella*

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 σ^{54} 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 σ^{54}-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 σ^{54}-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 *xylRΔA-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.

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Abbreviations: IHF, integration host factor; LacZ, β-galactosidase; BSA, bovine seroalbumin; PBS, phosphate-saline buffer; IMBs, immunomagnetic beads; Mab, monoclonal antibodies; GFP, green fluorescent protein; PCR, polymerase chain reaction; FACS, fluorescence-activated cell sorting

0014-5793/$30.00
doi:10.1016/j.febslet.2004.12.062

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 pUJ8 [17] as a 3.1 kb *Bam*HI/*Hin*dIII fragment, blunted with T4 DNA polymerase and inserted into *Hin*dIII digested/blunted pLBB9-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-A6* [14] and *trp-lacZ*. To facilitate the cloning of fragments in front of the reporter genes, a double strand oligonucleotide (5′-GAATTCTGCAGGATCCAAGCTTAATTAATTAAC-3′) encompassing the sequences recognized by the restriction endonucleases *Eco*RI, *Pst*I, *Bam*HI, *Hin*dIII and translational terminators in all three potential open reading frames was inserted into the pre-blunted *Eco*RI restriction site of pA6LZ1, thereby generating pA6LZ3. To prevent read-through transcription into the reporter genes, the ribosomal RNA *rrnB* T1 transcriptional terminator was amplified by polymerase chain reaction (PCR) from pKK232-8 [18] using forward (5′-GCAAATTTCCAGGCATCAAATAA-3′) and reverse (5′-GGGAATTCCCTGGCAGTTTATGG-3′) primers, digested with *Apo*I (underlined) and ligated to *Eco* RI-digested pA6LZ3, thereby eliminating one *Eco*RI 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 *Not*I 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 *Bam*HI fragment of plasmid pRS/MAD2 [19] encoding a constitutive mutated form of the prokaryotic enhancer-binding 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 (bacteria:cell). 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 LamB-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 (Ingenasa 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 LamB-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 $\approx 10^7$–10^8 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 seroalbumin (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 dilutions were plated 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 10^7–10^8 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 FACScan device (Benton Dickinson, Erembodegen-Aalst, Belgium).

Table 1
Bacterial strains and plasmids

Strain or plasmid	Relevant genotype and characteristics	Reference or origin
Bacterial strains		
CC118	Δ(*ara-leu*) *araD* Δ*lacX*74 *galE galK phoA thi-1 rpsE rpoB argE* (Am) *recA1*	[17]
CC118 F′ SURE	CC118 F′::[Tc^r, *lacI*^{q}ZΔM15]	[17]
SFL124-27	Tc^r, *S. flexneri aroD* serotype Y	[27]
Plasmids		
pRS/MAD2	Kmr, Ap^r, promoter probe plasmid carrying the *xylR*Δ*A*2 constitutive form of the transcriptional activator *xylR* and the *Pu* promoter	[28]
pUJ8	Ap^r, promoter probe plasmid for transcriptional fusions	[17]
pKK232-8	Ap^r, Cm^r, pBR322 derivative containing tandem *rrnB* terminators	[18]
pLBB9	Cm^r, plasmid carrying the promoterless *lamB-153* sequence	[14]
pLBB9-A6	Cm^r, pLBB9 A6 epitope inserted in its single *Bam*HI site	[14]
pA6LZ1	Cm^r, pLBB9-A6 derivative with the *trp-lacZ* gene inserted in the *Hin*dIII restriction site	This work
pA6LZ3	Cm^r, pA6LZ1 derivative with a multiple cloning site inserted into the *Eco*RI restriction site	This work
pA6LZ4	Cm^r, pA6LZ3 derivative with the *rrnB* T1 transcriptional terminator cloned upstream of the multiple cloning site	This work
pA6LZ4-R	Cm^r, as pA6LZ4 but with the 5.2 kb *Not*I fragment cloned in the opposite orientation	This work
pA6LZ4-6	Cm^r, pA6LZ4 derivative carrying the *Bam*HI 1.35 kb fragment from pRS/MAD2	This work

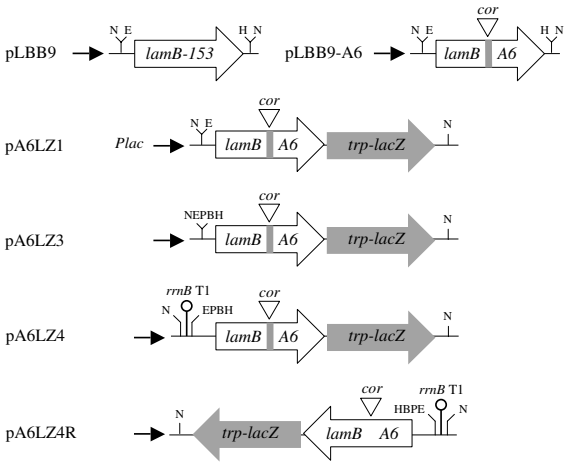

Fig. 1. Schematic representation of the main plasmids used in this study. All DNA segments shown were assembled on the single-copy vector pVLT8, a derivative of pSC101 plasmid. Abbreviations: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; N, *Not*I; P, *Pst*I; *Plac*, *lac* promoter; *lamB-153*, promoterless 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.

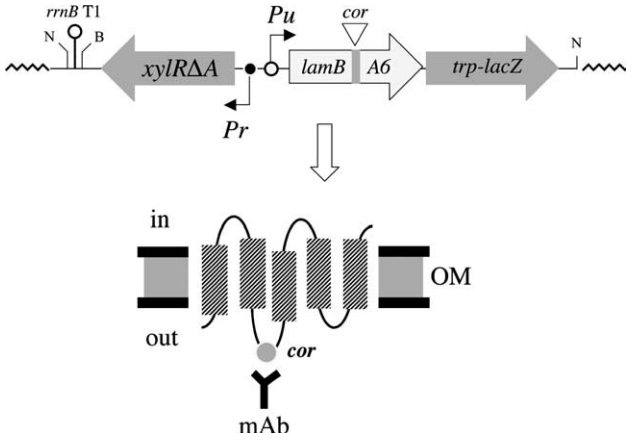

Fig. 2. Organization and functioning of the metabolic sensor. The insert of plasmid pA6LZ4-6 is sketched. The segment contains *xylRΔA2*, a deletion derivative of the *xylR* gene encoding a truncated form of the XylR regulator expressed through its own promoter *Pr* and located adjacent to the *Pu* promoter. This in turn drives expression of the surface reporter LamB protein, which has been genetically endowed with a viral *cor* antigen at one permissive site facing the exterior and thus amenable to recognition by an anti-*cor* Mab. A *lacZ* gene is placed next to the lamB-A6 gene for further analysis of promoter strength. Expression of the *Pu* promoter brought about by metabolic stress ends in the presentation of the cor antigen on the bacterial cell surface, which can then me submitted to immunomagnetic separation, FACS or immunofluorescence analysis.

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 strain. 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 turnover, 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 *Bam*HI fragment encoding the metabolic sensor system consisting of *xylRΔA*, 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
Immunoseparation of *E. coli* subpopulations expressing the LamB-A6 protein

pA6LZ3:pLBB9 ratios[a]	Viable cell recovery[b]	LacZ$^+$ colonies (%)
1:1	1.1×10^6	100
1:10	1.8×10^5	78
1:100	2.4×10^4	9.5
1:1000	4.2×10^3	5

[a]Approximately 8.0×10^8 bacteria were processed, i.e., treated with IMBs as described in Section 2.
[b]Counted as colonies in selective media. A control 1:1 sample of pA6LZ3:pLBB9 cells yielded 7.5×10^8 bacteria with 46% of LacZ$^+$ colonies. Numbers are the average of two separate experiments.

latter (close to 100%) happened when 0.5 μg of the Mab 6A.A6 antibody was used per ml of bacterial suspension (10^7–10^8 bacteria). These reference conditions were instrumental to set up the best assay for examining intracellular cells as discussed below. A second approach involved the analysis of bacterial populations expressing different levels of the LamB-A6 protein by flow cytometry. To this end, *E. coli* strains carrying either pLBB9-A6 (*lamB-A6* expressed through the *Plac* promoter of the plasmid, Fig. 1) or pA6LZ4-6 (*lamB-A6* expressed from *Pu*, Fig. 2) were mixed in different ratios, labeled with the Mab 6A.A6, and processed by flow cytometry as described in Section 2. As shown in Fig. 3, the analysis by flow cytometry discriminated clearly between wholes of bacterial cells with different expression levels of the surface reporter. The areas corresponding to the specific bacterial populations correlated perfectly with the ratios present in the original bacterial suspensions (Fig. 3). Taken together, these results indicated that the surface reporter system is instrumental to separate gross levels of expression of the cognate promoter by means of the presentation of the *cor* antigen on the cell surface of *E. coli*.

3.3. Activity of the *xylRΔA/Pu/lamB-A6/lacZ* metabolic sensor system in S. flexneri

On the basis of the results above, we transformed *S. flexneri* strain SL124-27 with plasmid pA6LZ4-6 (bearing the *xylRΔA/Pu/lamB-A6/lacZ* metabolic sensor, Fig. 2) as well as the control constructs pA6LZ4 and pA6LZ4R (Fig. 1). Since *E. coli* and *Shigella* share a high proportion of similarity in their respective genomes, we can safely assume that regulation-wise, the sensor and the reporter system of pA6LZ4-6 do describe identical metabolic and environmental conditions. To examine such conditions during the intracellular lifestyle of the bacterium, transformants of *S. flexneri* with each of the plasmids were used to infect Henle cells as explained in Section 2. Following incubation for 3 h, cells were fixed and treated with either a Mab against the O antigen, or the anti-*cor* 6A.A6 Mab. Samples were then treated with a second antibody for visualizing bacterial cells, with the results shown in Fig. 4. The strong fluorescence observed in most intracellular bacteria bearing the *xylRΔA/Pu/lamB-A6/lacZ* plasmid pA6LZ4-6 was indicative of a considerable expression of the metabolic sensor. This result visualized a degree of physiological stress in cells that inhabit the eukaryotic host under the conditions of the assay. A control experiment was run in parallel to ascertain whether the *cor* antigen presented by the *lamB-A6/lacZ* reporter was kept surface-accessible following intracellular infection. To this end, Henle cells co-infected with different proportions of *S. flexneri* (pA6LZ4R) and *S. flexneri* (pA6LZ4-6) were lysed and *cor*-displaying bacteria recovered with IMB as above. The results of Table 3 indicate that cells expressing the LamB-A6 protein were efficiently enriched and thus that presentation of the *cor* antigen was not fouled by its passage through the eukaryotic host.

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 σ^{54}-dependent system has allowed us to visualize the intracellular physiological state of *S. flexneri* during infection of cells in culture. Since the sensor module *xylRΔA/Pu* 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-*

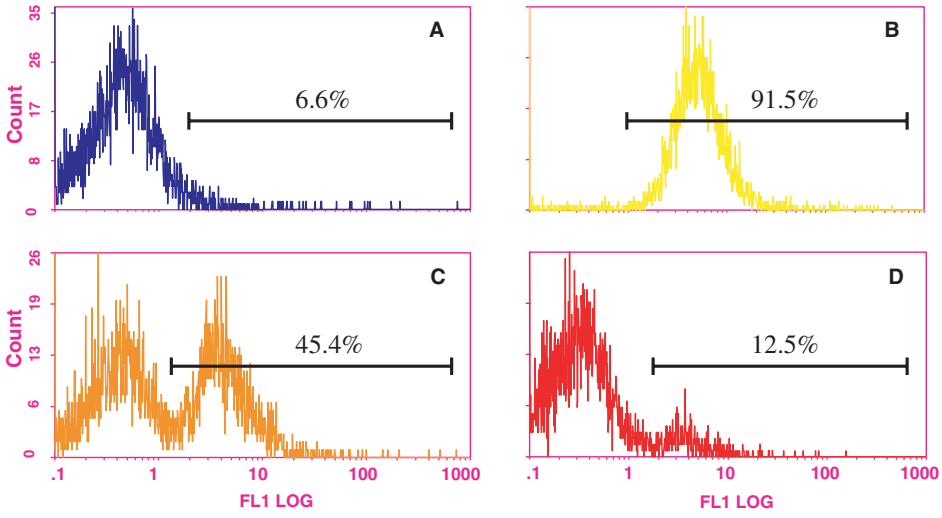

Fig. 3. Separation of bacterial cells expressing LamB-A6 by flow cytometry. Bacteria were grown as explained in the text, mixed in different proportions, treated with the *cor* epitope-specific Mab 6A.A6, labeled with FITC-conjugated rabbit anti-mouse antibodies and analyzed with a cell sorter. Panel 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.

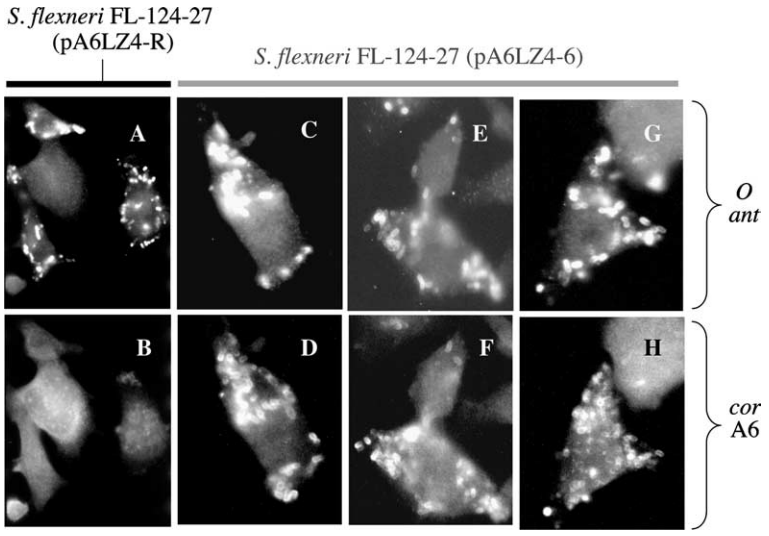

Fig. 4. Intracellular detection of *S. flexneri* cells expressing the *xylRΔA/Pu/lamB-A6/lacZ* metabolic sensor system. Henle 407 cells were infected during 3 h with the *S. flexneri* strain SFL124-27 carrying either pA6LZ4R (panels A and B) or (panels C–F). Then, monolayers were fixed and bacteria were 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.

Table 3
Immunoseparation of *S. flexneri* subpopulations expressing the LamB-A6 protein

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

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.

Acknowledgments: We are grateful to B. Karge for her skillful technical assistance and K.N. Timmis for his support and encouragement during this study. This work was supported in part by a grant from the Lower Saxony-Israel Cooperation Programme founded by the Volkswagen Foundation (21.45-75/2), by EU grants BIOCARTE, LINDANE and ACCESS and by Project BIO2001-2274 of the Spanish CICYT.

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