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Review

Tracking bacterial pathogens with genetically-encoded reporters

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ABSTRACT

During the infectious process, bacterial pathogens are subject to changes in environmental conditions such as nutrient availability, immune response challenges, bacterial density and physical contacts with targeted host cells. These conditions occur in the colonized organs, in diverse regions within infected tissues or even at the subcellular level for intracellular pathogens. Integration of environmental cues leads to measurable biological responses in the bacterium required for adaptation. Recent progress in technology enabled the study of bacterial adaptation in situ using genetically encoded reporters that allow single cell analysis or whole body imaging based on fluorescent proteins, alternative fluorescent assays or luciferases. This review presents a historical perspective and technical details on the methods used to develop transcriptional reporters, protein–protein interaction assays and secretion detection assays to study pathogenic bacteria adaptation in situ. Finally, studies published in the last 5 years on gram positive and gram negative bacterial adaptation to the host during infection are discussed. However, the methods described here could easily be extended to study complex microbial communities within host tissue and in the environment.

1. Introduction

In their host, microbes are exposed to varying growth conditions that are chiefly influenced by intrinsic bacterial factors such as the density and aggregation state, the subcellular distribution within host cells, the localization within a given tissue and the organ(s) colonized (Fig. 1). Throughout evolution, bacteria have acquired the capacity to sense their environmental input in order to generate the appropriate biological output for adaptation. This review will focus on genetically encoded reporters used for studying adaptation of bacterial pathogens to their environment within host cells tissues. Examples of the most recent studies on the use of such reporters in gram negative and gram positive pathogens will be presented after a short historical and technical perspective on the design of the various types of reporters that have been developed.

1.1. Transcriptional reporters

The quest for assays to monitor the adaptation of microbes to their environment is an endeavor that originated from the very cradle of molecular biology, as illustrated by the studies on *Escherichia coli* adaptation to growth on galactose that led to the description of the operon model by Jacob and Monod [1]. The potential of β-galactosidase, encoded by the lacZ gene, for monitoring the activity of ectopic promoters was rapidly understood (reviewed in [2]). However, β-galactosidase needs to first be recovered by lysis of the bacterium before it can be assayed, thus β-galactosidase reporters do not preserve contextual information and allow only for interrogating bacterial adaptation or interactions with their host indirectly. With the discovery of Green Fluorescent Protein (GFP), and particularly its optimization for expression and maturation in bacteria [3,4], studies of host pathogen interaction in situ using bacterial transcriptional reporters became a reality and led, for example, to the identification of genes expressed by *Salmonella typhimurium* and *Mycobacterium tuberculosis* specifically inside macrophages [5,6]. In addition, due to its intrinsic fluorescence and therefore the simplicity to measure promoter activity, GFP-based assays are amenable to exhaustive studies of promoters activity within a genetically tractable bacterial model [7].

Currently GFP-based assays are preferable to structurally homologous Red Fluorescent Protein (RFP) from cnidarians because of the slower maturation of the latter [8,9]. Further discovery and optimization of far-red fluorescent proteins (FP) with fast

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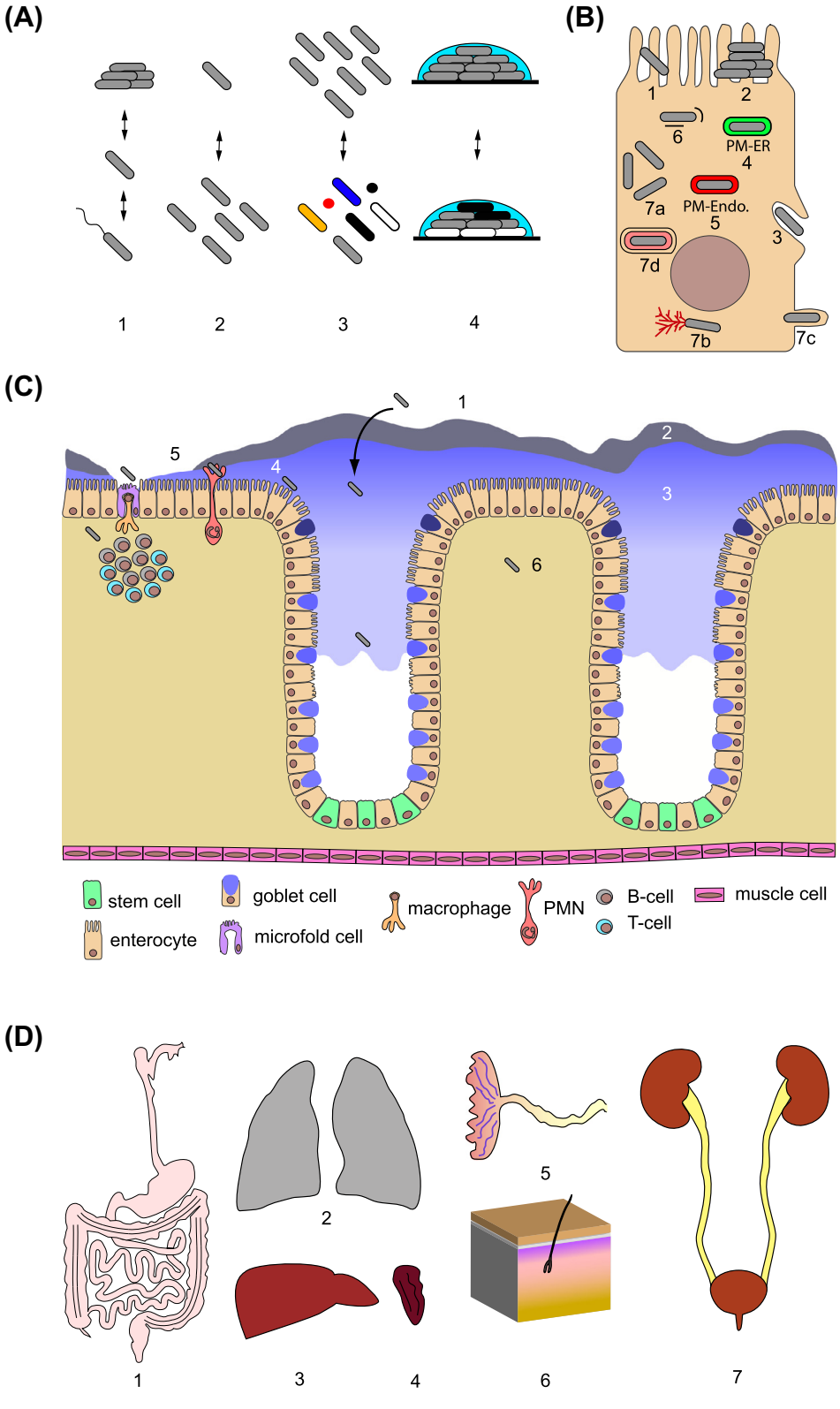
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maturation rate (half-time for maturation < 10 min) would expand the palette of experiments, including intravital observations that would be achievable with FP-based transcriptional reporters [10–13]. On the other hand, bioluminescence imaging (BLI) relying on the expression of luciferases has some advantages over GFP fluorescence for intravital whole-body imaging of bacterial infections due mainly to its greater sensitivity, as it is an enzymatic process, and better preservation of the living tissue due to the absence of excitation laser beams [14–16]. The lux operon from *Photorhabdus luminescens* in particular have been used in several bacteria because it produces its own substrate and hence, it does not require co-injection of substrates such as is the case with other luciferases [17,18]. In addition, many efforts have been made to improve the signal intensity and to red-shift the emission from the lux operon to minimize interference from the blue–green light absorbing components of the tissue [19,20]. Specific virulence promoters are starting to be used to determine the key features of the infection process such as the anatomical sites that are linked to the expression of critical virulence traits [21,22]. Indeed, transcriptional reporters are relatively easy to design and to implement in vivo, and since bacteria adapt to their environment mostly through transcriptional regulation, reporters monitoring virtually any type of environmental changes can be imagined with minimal a priori information about the signaling pathway of interest. Limiting factors to consider in the application of transcription-based assays are the time necessary to accumulate detectable levels of reporting molecules (for GFP only fast maturing and the brightest variants should be used [8]) and the stability of the reporter signal upon reduction of transcription activity. In this respect, ssrA peptide carboxy-terminal fusions that are recognized by the ClpX protease (ClpXP) are useful to reduce the half-life of reporter proteins (from 8 h down to approximately 40 min in *E. coli*) in both gram-negative and gram-positive bacteria [23–26]. In addition, the reporter half-life depends on the rate of division of the bacterial species of interest in a given context [27] (Fig. 2; Tables 1 and 2). It is important to also consider that both luciferase, GFP and RFP are inactive in the total absence of oxygen (<0.02% O_2 for GFP; [28,29]); this is important as pathogens particularly in the gastro-intestinal tract are submitted to varying levels of oxygen, as illustrated by the key oxygen-sensing role of FNR that regulates the T3SA assembly and efficacy in *Shigella flexneri* in vivo [29]. However, fluorescent reporters for performing experiments in anoxia relying on Flavin Binding Proteins (FbP) and SNAP-f have also been recently reported [30–34], but further applications will be required to judge upon their relative merits particularly for in vivo studies. To obtain more sensitive transcriptional reporters, specific care should also be given to optimizing expression by ensuring that the translation initiation site and the codon usage of the reporter gene (e.g. GFP variants for mammalian expression often have a high G-C content) are adequate for the bacteria of interests [35]. This remark is also true for other approaches described below.

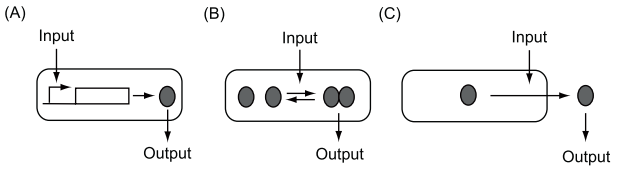

Fig. 2. The main types of genetically encoded reporters used for studying adaptation of bacteria to their environment. (A) Transcription-based assay; the box region represent reporter gene under the control of the promoter responding to the input; grey circle is the reporter protein; angled arrow indicate transcription initiation; straight arrows indicate stimulation of reaction or the direction of reactions. (B) Protein–protein/RNA/DNA interaction assays. (C) Secretion-based assays. Input represents the environmental change detectable by the bacteria. Output is the measurable signal (e.g. luminescence or fluorescence) produced by the reporter that allows for measuring the adaptation of the bacteria to its changing environment.

Table 1
Relative properties of the three different classes of genetically-encoded bacterial reporters.

	Transcriptional	PPI	Secretion
Ease of design	+++	+	++
Versatility in applications	+++	+	++
Dynamic response	+	+++	++
In vivo use	+++	+	+
Intravital imaging	+++	–	–
Examples (Refs.)	[27,35,67,70,91,92]	[37,47,53]	[56,57,60,61,74,78]

1.2. Protein–protein interaction reporters

The activation of different signaling pathways induced by environmental changes encountered by pathogens inside their host leads to direct or RNA-/DNA-mediated protein–protein dimerization events that are necessary to trigger the appropriate biological response. Critical protein–protein interactions (PPI) within a signaling pathway of interest can be monitored by very sensitive assays. For example, fluorescence resonance energy transfer (FRET) assays relying on expression of dimerizing partners fused to cyan and yellow fluorescent proteins (CFP and YFP, respectively), were used to study the chemotaxis pathway in *E. coli* with exquisite space and time resolution [36,37]. New pairs of fluorescent protein (FP) optimized for FRET have been introduced recently; in the future, they should both simplify the design and improve the sensitivity of bacterial FRET reporters [38–40]. Bioluminescence Resonance Energy Transfer (BRET) PPI assays are based on the expression of dimerizing partners fused to luciferase and YFP. In principle, BRET could be used in place of FRET assays, but BRET is more difficult to image and thus, entails some loss of contextual information. Protein-fragments complementation assays (PCA) were described in bacteria based on the murine dihydrofolate

Fig. 1. Bacterial and host factors directly impacting on the environmental conditions to which bacterial pathogens are exposed. (A) Intrinsic intra- and inter-bacterial factors: (1) bacteria can grow individually, express a planktonic phenotype or aggregate; (2) bacterial density; (3) bacterial species diversity; (4) biofilm formation (mono or plurispecies). Double arrows emphasize the equilibrium between the prototypical conditions depicted in each category. (B) Subcellular distribution (intracellular pathogens): (1) adhesion to the PM; (2) adhesion to the PM and formation of microcolonies; (3) adhesion and invagination of the PM to penetrate inside the cell; (4) association with vacuole of PM and endoplasmic reticulum origin, or of (5) PM and endosome/lysozome origin; (6) partially lysed vacuole; (7) cytoplasmic bacteria (a), which can moved in the cytoplasm using actin comets (b), form PM protrusions (c), or get trapped in autophagosomes (d). In addition, the environmental conditions sensed by the bacteria can also be affected by the specific cell types infected. (C) Tissue distribution, here demonstrated with a scheme of the colon showing regions of interest: (1) devoid of mucus or mucus surface (dense commensal bacteria population); (2) highly cross-linked mucus; (3) poorly cross-linked mucus; (4) adjacent to the epithelial cells; (5) adjacent or within lymphoid follicules (interactions with immune cells); (6) in the lamina propria. (D) Whole body distribution; which organs are colonized and what are their properties: (1) gastro-intestinal tract (great change in environmental conditions from mouth to stomach, small intestine and colon; high variation in nutrients, pH, density in bacteria of the resident microbiota etc.); (2) lungs; (3) liver; (4) spleen; (5) placenta; (6) skin; (7) urinary tract. The 4 different factors presented in A–D will affect the environmental inputs (e.g. pH, nutrients, immune response challenges, virulence trait expression) to which the bacteria respond by the appropriate signaling pathway to trigger the epigenetic and genetic output necessary for adaptation.

Table 2
Relative properties of some key fluorescent or luminescent proteins for the development of bacterial reporters.

	GFP[a]	RFP[b]	FbP[c]	SNAP–f	Bact. Luc (lux operon)	Firefly luciferas e[d]	Renilla/Gaussia luciferases
Folding/maturation time (<10 min)	Yes	No	Yes	ND	ND	Yes	ND
In vivo (fix. sample)	+++	+++	ND	ND	–	–	–
Single-cell	+++	+++	+++	+++	–	–	–
Intravital whole body imaging	+	++	ND	ND	+++	++1/2	++1/2
Microscopy	+++	+++	+++	+++	+	+	+
Anoxia	–	–	+++	+++	–	–	–
Reversible PCA	–	–	ND	ND	ND	+++	+++

[a] Half-time for maturation of GFPmut2 and GFPmut3, the faster maturing variant of GFP express as soluble protein in bacteria is approximately 5 min.
[b] Half-time for maturation mKate2, the faster maturing variant of RFP is approximately 20 min.
[c] Half-time for maturation of iLov is approximately 3 min.
[d] Half-time for maturation is approximately 1 min.

reductase (DHFR) [41–43], where DHFR fragments are brought together and folded by the assistance of dimerizing partners tethered to each moiety. A fluorescent assay for the DHFR PCA based on fluorescent methotrexate has been described in mammalian cells [44], but has not been adapted to bacteria so far. Conversely, the bimolecular fluorescence complementation (BiFC) of GFP was originally described in bacteria [45,46], but it has not been used much to detect activation of specific bacterial signaling pathways [47]. In addition, there is strong evidence that the complementation of GFP from the BiFc fragments is irreversible [45,46,48], hence precluding monitoring of highly dynamic PPI events in real-time. Results obtained by Waldo and coll. suggest that it might be possible to refine fragments design to obtain a reversible BiFC GFP [49], but this remains to be shown. In contrast, the IFP1.4, Renilla and Gaussia luciferases PCA are reversible, but have not yet been exploited much in bacteria [16,50–52]. In the case of the assembly and activation of large macromolecular structure, such as the Type 6 secretion apparatus (T6SA), straightforward FP-fusions to monitor localization changes and assembly of substructures composed of repeated monomers, yielded great functional insights [53], similarly to the "direct" PPI assays described above. Overall, PPI assays could yield highly dynamic information and contextual information about the adaptation of bacterial pathogens to environmental changes, yet, their applications have been rather limited and appear particularly challenging for in vivo studies (Fig. 2; Tables 1 and 2).

1.3. Protein secretion assays

Several assays have been specifically developed to follow the secretion of proteins by bacteria, a phenomenon of vital importance for the virulence of several bacterial pathogens. The original reporter used for such assays was based on alkaline phosphatase [54]. Later Cornelis and coll. used fusions of the calmodulin-dependent adenylate cyclase CyaA to bacterial effectors of interest to identify the minimal domain of T3SS-effectors required for their translocation into infected cells [55]. More recently a fluorescent assay based on the hydrolysis of the CCF2/CCF4 by the β-lactamase TEM-1 was introduced to follow the activity of the T3SS of pathogenic *E. coli* upon host cell attachment in non-disruptive conditions [56]. Enzyme-based assays such as those are extremely sensitive, but often at the cost of loosing contextual information. For example, it is possible using enzymatic assays to determine the kinetics of secretion inside a given infected cell, but it is often impossible to determine which given bacterium is secreting let alone to locate secreted proteins inside the host cell. Another approach based on the strong affinity of chaperones for their cognate effectors and the expression in host cells of the former fused to FP has been used to detect secretion of a *S. typhimurium* effector [57]. Interestingly, this strategy could be easily combined with enzyme-based secretion assay to provide the missing contextual information discussed above. Assays based on the detection of the secretion of tetracystein-tagged effectors or on the self-assisted complementation of a large and a small GFP fragments (full-length GFP fully or partially clog the T3SA [58,59]), respectively expressed in the host cell and tagged to the secreted bacterial effectors, were designed to locate secreting bacteria and secreted effectors [49,60,61]. Generally, the secretion assays described so far are not easily implementable in situ (Fig. 2; Tables 1 and 2).

2. Recent progress in the tracking of the virulence process of gram-negative bacterial pathogens in situ

2.1. BLI for studying bacterial pathogenesis

Progress in the detection of bioluminescence has revealed the promise of BLI to follow in real-time the progression of infection. For example, a basic strain that expresses constitutively the lux operon was developed to follow the progression of infection by *Yersinia pestis* in a mouse model of infection [62]. The luciferase signal not only approximated the CFU counts obtained by classical methods but also allowed following in real-time the infection in anesthesized animals and to observe the relapse of infection following antibiotic treatment. Putting the lux operon under the control of specific promoters involved in the regulation of expression of selected virulence genes has also been done [22,63]. For example, colonization of the upper urinary tract was shown to be coincident with the expression of the lux operon placed under the control of the promoter of fliC, a flagellar component [22]. Similarly, the lux operon was put under the control of the invasion promoters of various serotypes of *Yersinia enterocolitica* to show for the first time expression of the invasins in various tissues [63]. Further developments of BLI in the context of bacterial infection include the use of multiple luciferases with compatible green and red signal to perform competition experiments [64]. Recently, the concomitant injection in mice of near-infra red fluorescent particles with non-pathogenic *E. coli* expressing the lux operon was shown to yield a red shifted signal, in a physical process dubbed fluorescence by unbound excitation from luminescence (FUEL) [20]. Furthermore, protein fusions between either of the luciferase subunits of the lux operon and YFP or RFP to permit intramolecular BRET are promising [65]. The development of alternative approaches using the far-red fluorescent bacteriophytochrome IFP1.4 and enabling single-bacteria analyses from tissue samples in combination with whole body imaging is also an exciting prospect [12].

2.2. Bacterial transcriptional reporter for single-cell analyses

A few years ago, Helaine et al. reported a simple transcriptional reporter based on the dilution of the GFP signal inside infected cells after interruption of the synthetic transcription-inducing signal

(e.g. IPTG/lacI and/or arabinose/araC) [27]; this system was used as a simple integrating reporter of the bacterial cell metabolism since dividing bacteria quickly diluted their reservoir of GFP molecules. Recently, this reporter system was used to identify a sub population of not replicative, metabolically active, hence viable *S. typhimurium* found within resident macrophages of the secondary infected organs such as the Peyer's Patch, mesenteric lymph nodes, spleen or liver [66]. These non-replicative bacteria, called persisters, were resistant to several antibiotics and quickly resumed growth in LB medium and to some extent in secondary infected macrophages, indicating their potential role in relapsing infection. The harsh conditions, such as low pH and nutrient scarcity, encountered by the bacteria within the host vacuoles were shown to lead to the rapid appearance of the persisters sub-population in macrophages. The elegant simplicity of the reporter plasmids used should be amenable for addressing similar questions in several other bacterial pathogens.

In *Vibrio cholerae*, the tcpA-H and ctxAB operons, which are crucial for virulence, are regulated by the master transcriptional activator ToxT. Using DNA amplicon microarrays, the tcp operon was shown to be up-regulated at the surface of the mucosa in the rabbit ileal loop model [67]. To decipher further the regulation of the tcp operon during in vivo infection, these authors designed a transcriptional reporter inserted in a single copy in the chromosome and composed of the tcpA promoter and of the ClpXP destabilized variant of GFPmut3 (half-life $\approx$ 40 min) to make the transcriptional reporter more responsive upon transcription interruption. Using the intrinsic fluorescence of this reporter, Nielsen et al. were able to show that the tcpA promoter was more active within 5 μm of the mucosa than further away in the mucus layer. Furthermore, bacteria adopted a bimodal expression (low or high level) of the tcpA reporter at late time points during in vivo infection and in the stationary phase when cultured in broth in the presence of bicarbonate. This bifurcation phenomenon was fully reversible, persisted during subculturing experiments and depended on the relative concentration of ToxT and the activation of the CRP/cAMP, in a manner consistent with a bistable switch; notably, this phenotype could have important consequence for inter-individual transmission of the infection [67].

2.3. Tracking the activity of secretion systems

Secretion systems, such as the Type III, IV and VI are essential elements of the virulence strategy of numerous gram-negative bacteria. The structure and activity of the Type Three Secretion Apparatus (T3SA) has been one of the most studied (reviewed in [59,68,69]). Recently, transcriptional reporters using a fast maturing variant of GFP to determine the in vivo context of *Salmonella enterica* secreting through SPI-I or SPI-II T3SA were reported [70]. In this study, transcriptional reporters based on the expression of GFP (ClpXP destabilized; half-life $\approx$ 40 min) were placed under the control of the invF and ssaG promoters, which are upregulated by SPI-I and SPI-II secretion systems, respectively. The reporter strains were used in a bovine ligated ileal loop model of infection to demonstrate that bacteria found in the enterocytes at 2 h post-infection (PI) are SPI-I and flagella-expressing, while at 8 h PI, bacteria found inside the lamina propria are SPI-2 expressing. However, GFP expression was detected using a polyclonal antibody due to low intrinsic fluorescence.

We used a similar approach in *S. flexneri* to monitor the activity of the T3SA within infected cells and to determine the cellular context in which the T3SA was active [35]. A transcription based secretion activity reporter (TSAR) was developed by introducing into GFPsf the chromophore sequence of GFPmut2, in order to obtain the fast folding and maturing variant GFPsfm2 (ClipX destabilized; half-life $\approx$ 40 min). This reporter was found to produce reliably detectable level of intrinsic fluorescence enabling live-imaging. Using bacterial genetics and fluorescence recovery after photobleaching (FRAP), we were able to circumvent the intrinsic limitation of transcriptional reporters (e.g. the lag in detecting the signal after actual activation of the transcription) to determine that the T3SA of *S. flexneri* is activated upon entry and during cell-to-cell spread in membrane compartments derived from the plasma membrane. In between these discrete steps, the T3SA is inactivated, which permits the replenishment of bacterial stores of translocators/effectors necessary for lysis of the secondary vacuole during cell-to-cell spread. Finally, proof of principle experiment demonstrated the capacity of the TSAR to be used for in vivo studies on fixed colonic sample recovered from infected Guinea pig and may well be suited to track interactions of *S. flexneri* with immune cells using two-photon microscopy [71,72].

Rosenshine and coll. have exploited further the β-lactamase secretion assay that they previously reported [73], to measure precisely the dynamics of secretion of 20 effectors from their genomic locus in enteropathogenic *E. coli* (EPEC), using single and whole population analyses [74]. Bacteria found within microcolonies, in contrast with planktonic bacteria, were shown to be the secreting subpopulation. The Tir protein responsible with Intimin for the intimate attachment of EPEC to cells is the first to be secreted around 20 min post-adhesion, while the secretion of other effectors was detectable around 60 min post-adhesion. The application of the β-lactamase secretion assay during in vivo infection was also validated in two studies. Following in vivo infection, cells of interest were recovered from infected animal and loaded with the fluorescent β-lactamase substrate CCF2-AM. This method demonstrated that *Y. pestis* infected immune cells, mostly phagocytes, but also lymphocytes at a low frequency [75], while *S. typhimurium* infected lymphocytes, dendritic cells, monocytes and particularly neutrophiles, but not mature macrophages [76]. Finally, near-infrared fluorescent substrates for the β-lactamases have been described for performing intravital imaging experiments [77] (see Section 3.3 for detailed description).

Two further advances in detecting effector secretion were recently described. Following the adaptation of tetracystein tags (4xCys) to measure the secretion of Shigella translocators in real time [60], it was shown that the sensitivity of this method could be further optimized by using three tandem repeats of the 4xCys [78,79]. This modification allowed for tracking the secretion of effectors expressed from the native chromosomal locus in *S. enterica* rather than from a high copy plasmid. In addition, the spontaneous complementation of a short alpha-helix of GFP fused to bacterial effectors secreted into the host cell cytosol with a large GFP fragment expressed in infected host cells to form a functional GFP molecule was also recently demonstrated [61]. The GFP complementation enabled Van Engeleburg et al. to measure the secretion and localization of the effectors within the host cells, albeit with significant delays due to the time required for the GFP to fold from the complementing fragments.

The Type IV Secretion Apparatus (T4SA) is capable of injecting DNA in addition to protein effectors into host cells. The transfer of GFP cDNA placed under the control of a mammalian promoter has been used to monitor DNA injection by *Bartonella henselae* into tissue culture cells [80]. While detectable DNA transfer events occurred at a low rate (<0.02%), it was possible to increase this rate by approximately 100-fold by expressing in the bacteria the protein fusion composed of the MOB relaxase, responsible for targeting plasmidic DNA to the T4SA, and the effectors BID. Notably, expression of GFP from DNA injected by *B. henselae*-required host cell division to allow access of the DNA to the nucleus. It will be interesting to see if this type of reporter can be useful to follow the activity of the T4SA in vivo. Developing protein secretion assays such as those described for the T3SA would be highly complementary to this approach.

FP fusions with components of the Type 6 secretion system (T6SS) VipA and ClpV have recently been used to understand the structure and activation of the T6SA of *V. cholerae* and *Pseudomonas aeruginosa* [53,81–83]. Fusion with the VipA proteins, which forms with VipB the tubules of the T6SS, was used to show the contractile nature of the T6SS [53]. FP-fusions to VipA and ClpV were further used to reveal T6SA dueling between bacteria, which consisted in cycles of attack and counterattack by bacteria in close contacts on a solid surface [81,82]. These studies provided a very elegant demonstration of the power of simple FP-fusion system to decipher the response of large macromolecular systems to environmental changes. This approach should be useful to eventually explore the activation of the T6SA within more complex environments such as within multispecies biofilms or within host cells in real-time.

3. Recent progress in the tracking of the virulence process of gram-positive bacterial pathogens in situ

The use of fluorescent and luminescent assays in gram positive bacteria have been hindered in some instance by the poor expression level of the reporter genes due to their G-C content deviating too strongly from the G-C content of the organism of interest. Nonetheless, several studies recently described new plasmids for expressing many FP, codon optimized or not, in *Staphylococcus aureus* [84–87], indicating that experiments previously difficult to realize have become feasible.

3.1. Intravital imaging in gram-positive bacteria

In the case of superficial skin infection, blue–green–yellow fluorescence (400–515 nm) can be used to perform intravital imaging, despite higher interference from tissue components at these low wavelengths as compared to the far red spectrum. Indeed, *S. aureus* skin infection was tracked by GFPmut2 fluorescence using two-photon fluorescence microscopy [3,87]. In addition, the Cerulean and YFP mutations were reintroduced in the GFPmut2 background to ensure good expression in *S. aureus* and other gram-positive organisms. Two types of promoters were used to generate the final reporter plasmids: (1) the SarA-dependent constitutive promoter and (2) the Agr-dependent promoter regulated by the autoinducer AIP, whose expression is strongly upregulated when bacterial density is high during growth in broth. Interestingly, in the skin, FP-expression by Agr is observed in the periphery of the infection foci, suggesting that the growth of bacteria in clusters specifically in those regions and/or host factors might modulate quorum sensing in vivo. The generation of the variously labelled *S. aureus* strains allowed for studying the behaviour and recruitment of polymorphonuclear leukocytes (PMN) labelled with GFP (LysM-EGFP mice) to the infection foci in the skin in real-time [87]. The PMN moves rapidly to the center of the infection foci disregarding or failing to stop when encountering low number of bacteria. Once in the center of the foci, PMN migration slows down and they contributed strongly to controlling the infection as shown by the large bacterial foci formed when their recruitment is inhibited by injection of the bacterial effector Ecb. Since Ecb inhibits complement activation, these results support a role of complement in recruiting PMN to skin infections by *S. aureus* [87]. Far-red fluorescent vancaomycin has also been recently used to perform intravital imaging of myositis in a mouse model of *S. aureus* infection [88].

Listeria monocytogenes invades the small intestine to subsequently disseminate to a variety of secondary organs including the placenta. Using lux operon-expressing *L. monocytogenes* strains, the combined role of the invasins InlA and InlB in targeting the placenta of pregnant gerbils was demonstrated [89]. The infection process was followed intravitally during several days until the animal was sacrificed to measure the bacterial load in the placenta by luminescence. The gerbil and humanized E-CAD mouse model [89] were furthermore exploited, using high quality microscopic imaging, for determining the host cell types targeted by *L. monocytogenes* during invasion of the small intestine epithelium [90]. These developments are paving the way for detailed studies of the adaptation of *L. monocytogenes* to the diverse organs or cell types it may colonize during infection.

3.2. A transcriptional reporter for the streptococcal invasion locus

The streptococcal invasion locus (sil) is regulated by quorum sensing through a two-component system that is activated by the autoinducer SilCR peptide. SilCR is inactive in clinical isolates of pathogenic Group A Streptococcus (GAS), but not in the mostly commensal Group G Streptococcus. Despite inactivation of SilCR, GAS sil still responds to exogenously added SilCR as demonstrated by a transcriptional reporter composed of GFP placed under the control of a SilCR-responding promoter [91]. These observations indicated that sil from GAS could be cross-activated by neighboring GGS when those bacteria colonized the same tissue, although this has not been confirmed in vivo yet. In addition, using a GAS with activated SilCR, Hanski and coll. have shown, using tissue culture cells and a cutaneous mice model of GAS infection, that adhesion and streptolysins-induced endoplasmic reticulum stress are critical factors in the activation of the sil locus in the early stages of infection (e.g. between 6 and 12 h) [92].

3.3. M. tuberculosis imaging with P-lactamase fluorescent assays

M. tuberculosis naturally expresses the β-lactamase BlaC at its surface. This property allows for using validated fluorescent assays to detect β-lactamase activities inside infected cells and tissues without the need for transformation and genetic manipulation. Therefore, it is immediately applicable to most *M. tuberculosis* strains. The β-lactamase CCF4 FRET assay described above has also been used as a vacuole-rupture assay [93]. Recently, it was applied to demonstrate the vacuolar rupture induced by *M. tuberculosis* in human macrophages three to four days post-challenge in an ESX-1 dependent manner [94]. Vacuole lysis by *M. tuberculosis* was shown to lead to macrophage cell death by necrosis while the avirulent BCG strain failed to lyse its vacuole and induce necrosis.

Intravital imaging of *M. tuberculosis* with β-lactamase have recently been rendered possible through the development of novel cephalosporin-based substrates harboring Cy5 and Cy5.5 fluorophores in the vicinity of a quencher that can absorb its radiation by FRET in the intact molecule but not in the hydrolyzed cephalosporin [77]. The most efficient of these substrates, CNIR5, allowed for detection of as low as 10^4 bacteria in the lung and could measure the reduction in bacterial load within 24–48 h after treatment with antibiotics. This study highlights the potential of developing substrates for enzymatic activities unique to the bacterial domain, without the need to genetically manipulate or introduce plasmids into strains of interest, in order to develop highly specific assays to track bacteria in situ.

4. Conclusions

Transcriptional reporters are the most versatile genetically-encoded assays to measure the adaptation of bacteria to their environment. They are generally easy to implement in genetically-tractable bacteria with codon adaptation to the organism of interest and we expect that they will become a cornerstone of host–pathogen, microbiota and environmental bacteria in situ

studies in the future. Protein–protein/RNA/DNA interaction assays have so far been underexploited but their capacity to measure highly dynamic events represents an asset to understand the detailed mechanisms that transform environmental inputs into the specific biological outputs necessary for adaptation. To fulfill their promises, these approaches will require the development of new fluorescent, luminescent and possibly alternative assays that will fill the gaps in the microbiologist toolbox, including far-red fluorescent proteins, oxygen-independent FP, red-shifted lux operon luciferases and specific fluorescent substrates for bacterial enzymes.

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