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Design of biological sensors based on the OpvAB phase variation system of *Salmonella enterica*

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Tesis doctoral

Design of biological sensors based on the OpvAB phase variation system of *Salmonella enterica*

Memoria elaborada por David Rodríguez Olivenza para optar al título de Doctor por la Universidad de Sevilla

Tesis realizada bajo la dirección del Dr. Josep Casadesús Pursals y la Dr. Mireille Ansaldi en el Departamento de Genética de la Facultad de Biología de la Universidad de Sevilla

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Sevilla 2019

A mis padres y a mi hermano Antonio

A mi familia

“Messieurs, ce sont les microbes qui auront le dernier mot!”

LOUIS PASTEUR

RESUMEN

Este trabajo se ha basado en investigaciones previas de nuestro grupo sobre el operón *opvAB* de *Salmonella enterica* y ha utilizado dichos conocimientos para diseñar y desarrollar tres aplicaciones biotecnológicas: (i) la formación de subpoblaciones bacterianas que difieran en un fenotipo específico elegido a voluntad, (ii) el desarrollo de un biosensor capaz de detectar bacteriófagos, y (iii) la detección de inhibidores de la metilación del DNA.

(i) Estudios previos habían establecido que la transcripción del operón *opvAB* está sujeta a biestabilidad reversible (cambio de fase). Este trabajo describe que la clonación de un fragmento de 689 pares de bases que contiene el promotor y la región reguladora (UAS) de *opvAB* es capaz de conferir biestabilidad reversible a genes heterólogos (ej., el operón *lac* y diversos genes de resistencia a antibióticos). El sistema de cambio de fase es exportable a *Escherichia coli*. La expresión de genes de resistencia a antibióticos bajo el control de *opvAB* genera subpoblaciones resistentes y sensibles al antibiótico en cuestión, y puede ser útil para el estudio de la heteroresistencia a antibióticos. La heteroresistencia es un fenómeno de gran relevancia clínica ya que la aparición de subpoblaciones resistentes es difícil de detectar y con frecuencia causa un fallo en el tratamiento de las infecciones.

(ii) Los productos del operón *opvAB* son proteínas que acortan el antígeno O del lipopolisacárido, generando resistencia a bacteriófagos que usan el antígeno O como receptor. Esta propiedad ha permitido desarrollar biosensores para la detección de bacteriófagos. Dichos sensores se basan en la detección de fluorescencia emitida por la proteína verde fluorescente, GFP, y han sido generados por manipulación genética de la estirpe silvestre. Además de ser una alternativa al uso de los antibióticos, los bacteriófagos tienen importancia en ecología ya que participan activamente en el control de las comunidades bacterianas y en la transferencia de información genética entre bacterias.

(iii) La transcripción del operón *opvAB* está regulada por metilación Dam, y la ausencia de metilación Dam origina expresión constitutiva. En una estirpe

que produce proteína verde fluorescente (GFP) bajo el control de *opvAB*, la inhibición de la metilación Dam se puede detectar como un aumento de fluorescencia. La funcionalidad de este sistema de detección se ha demostrado en presencia concentraciones muy bajas de sinefungina, un inhibidor comercial de la metilación del DNA. Esta prueba de concepto sugiere que el sensor puede ser útil en la búsqueda de nuevos inhibidores de la metilasa Dam, potencialmente útiles como fármacos antibacterianos.

INTRODUCTION

The genus *Salmonella*

The genus *Salmonella* belongs to the family Enterobacteriaceae, which is classified in the γ-proteobacteria subdivision. *Salmonella* is phylogenetically close to the genera *Escherichia*, *Shigella*, and *Citrobacter*. The genus *Salmonella* includes facultative anaerobic, rod-shaped Gram-negative bacteria. Most Salmonellae are motile and are able to infect a wide variety of animal hosts including amphibians, reptiles, mammals, and birds. Current taxonomy divides the genus *Salmonella* into two species, *Salmonella enterica* and *Salmonella bongori* [1]. In turn, *Salmonella enterica* includes six subspecies [2]: *enterica* (I), *salamae* (II), *arizonae* (IIIa), *diarizonae* (IIIb), *houtenae* (IV) and *indica* (VI).

Salmonella isolates from the same subspecies are further classified into serovars based on the White-Kauffman classification scheme [3], which relies on specific patterns of agglutination reactions based on antisera against two highly variable surface antigens, O (lipopolysaccharide O-antigen) and H (flagellar proteins) [2], [4]. There are more than 2,500 *Salmonella* serovars, most of which belong to subsp. *enterica* [3]. Only serovars of this subspecies regularly colonize warm-blooded vertebrates [5], and so they account for 99% of human infections by *Salmonella*, while serovars of *Salmonella bongori* and other *Salmonella enterica* subspecies are usually associated to cold-blooded vertebrates or found in the environment [6].

Serovars belonging to subsp. *enterica* differ in their host specificity and in the types of diseases they promote. Some serovars are host-restricted, while others can infect a wide variety of animal hosts [7, p. 2]. The diseases caused by subsp. *enterica* serovars vary from self-limiting gastroenteritis to life-threatening systemic infection, and the outcome of the disease depends on the specific serovar-host combination. An example of specialist is *S.* Typhi, the causing agent of typhoid fever. In contrast, *S.* Typhimurium produces mild gastroenteritis in humans but causes a systemic infection similar to human typhoid fever when infecting immunodeficient mice [8].
S. Typhi causes more than 27 millions of cases of typhoid fever worldwide, with 217,000 deaths approximately [9], whereas *S.* Typhimurium and *S.* Enteritidis cause >90 million infections worldwide with more than 150,000 deaths per year [10]. In sub-

Saharian Africa, *S.* Typhimurium and *S.* Enteriditis cause high rates of bacteremia in immunocompromised patients and in children [11], [12].

The interaction between serovar Typhimurium and mice has been extensively used as a model for typhoid fever in humans [13]. A widely used mouse-virulent strain is ATCC 14028 [14].

Evolution of *Salmonella*

Salmonella and *Escherichia* are close relatives, and diverged 120-160 million years ago [15]. Almost 25% of the *Salmonella* genome consists of genetic material that is absent in *Escherichia coli* [5], [16]. The evolution of *Salmonella* pathogenicity (**Figure I.1**) has involved sequential acquisition of genetic elements, each contributing to different aspects of its lifestyle [17], [18]. Amongst those elements are the *Salmonella* pathogenicity islands (SPIs), which are clusters of virulence genes located in the chromosome. More than 10 SPIs have been described [19], including some which are serotype-specific. These regions are absent in the chromosome of other Enterobacteriaceae, and usually have different G+C content than the average of the *Salmonella* chromosome, suggesting that they have been acquired by horizontal gene transfer [16], [18].

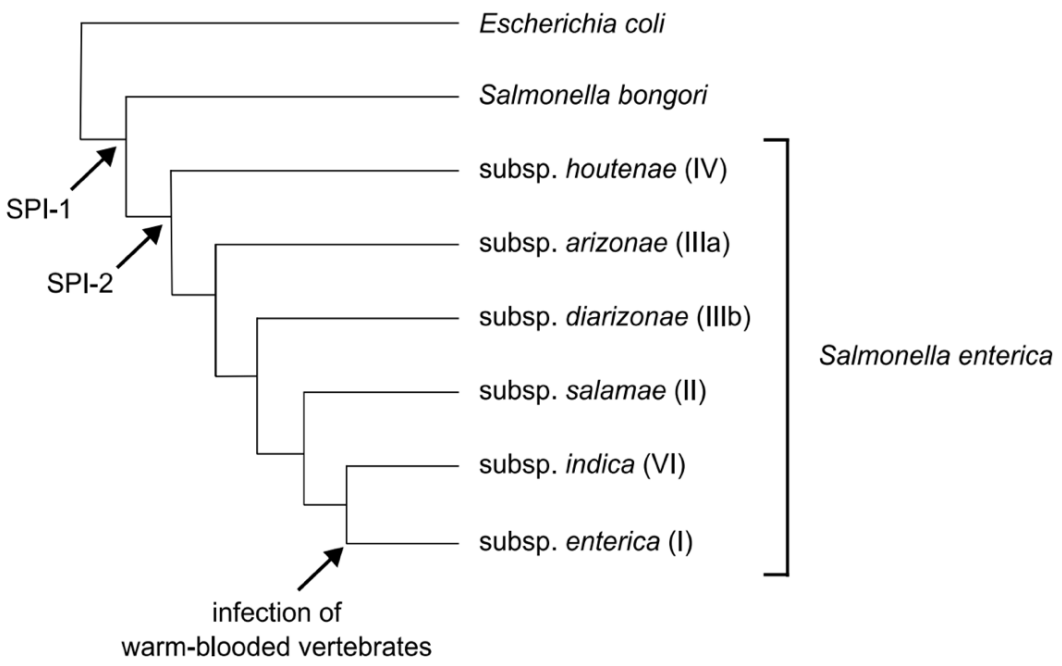


Figure I.1. **Phylogeny of the genus *Salmonella*.** Modified from [20].

The best characterized SPIs are *Salmonella* pathogenicity islands 1 (SPI-1) and 2 (SPI-2). SPI-1 was acquired first by the common ancestor of the two *Salmonella* species, and is involved in the invasion of intestinal epithelial cells in the animal host [21]. SPI-1 acquisition likely allowed *Salmonella* to become an intracellular pathogen associated with cold-blooded vertebrates [20].

SPI-2 allows *Salmonella* to survive in macrophages and colonize deep tissues [22], and its acquisition marked the split of the two *Salmonella* species [20]. Hence, only members of *Salmonella enterica* have the ability to reach deep tissues and organs to produce systemic infections. The ancestor of subsp. *enterica* acquired the capacity to infect warm-blooded vertebrates, and different lineages subsequently evolved to colonize a variety of hosts. Even though the mechanisms of host specificity are not fully understood, the presence of a virulence plasmid in some serovars of subsp. *enterica* has suggested the potential involvement of plasmid functions [6]. Another factor that may be involved in host specificity is the presence of different sets of fimbrial operons in different serovars [6], [23].

Salmonella infection

Infection with *Salmonella enterica* is usually caused by ingestion of contaminated food or water. Along the digestive track, *Salmonella* must endure adverse conditions that serve as protective mechanisms against bacterial infections. In the stomach, acid pH destroys the majority of microorganisms [24]. Activation of the acid tolerance response enables *Salmonella* to endure periods of severe acid stress [25], [26]. In the small intestine, *Salmonella* finds high concentrations of bile, secreted in the duodenum during digestion. Bile has two main antibacterial activities: as a detergent that disrupts the cell envelope [27] and as a DNA damaging agent [28]. However, *Salmonella* and other enterobacteria are intrinsically resistant to high concentrations of bile [27]. Bile resistance is a complex phenomenon and involves multiple mechanisms including formation of physical barriers such as the lipopolysaccharide [29] and the enterobacterial common antigen [30], active efflux [31], [32], activation of the general stress response [33] and modification of the peptidoglycan structure [34].

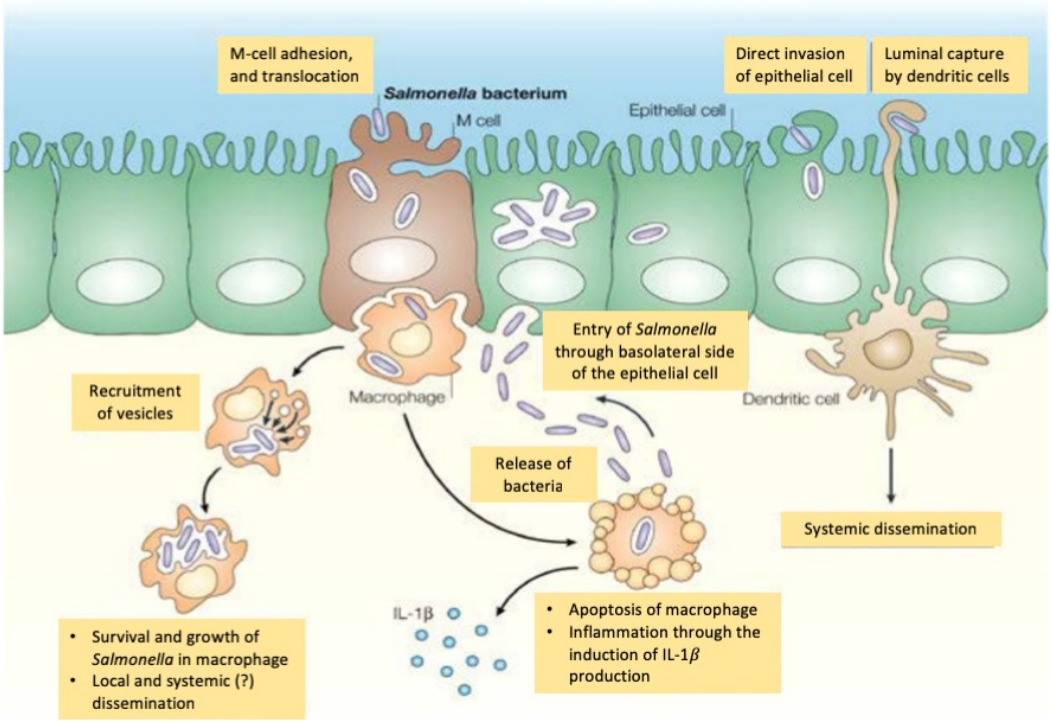


Figure I2. **Diagram of *Salmonella* infection.** The three main routes of *Salmonella* invasion of the intestinal epithelium are represented: adhesion and translocation through M cells, direct invasion of intestinal epithelial cells, and capture by dendritic cells. Reproduced from [35].

When *Salmonella* reaches the distal small intestine, adhesins and fimbriae allow the pathogen to adhere to the intestinal epithelium [36]. *Salmonella* is able to invade the intestinal epithelium through three different routes (**Figure I2**): (i) by inducing a phagocytosis-like process in non-phagocytic enterocytes, (ii) through specialized epithelial M cells, and (iii) through dendritic cells that intercalate epithelial cells by extending protrusions into the gut lumen [37], [38]. The two first routes are mediated by the virulence-associated type 3 secretion system encoded on *Salmonella* pathogenicity island 1 (SPI-1) [39], and invasion of M cells is the predominant route of intestinal traversal [40]. After invasion, and depending on the host-serovar combination, *Salmonella* can cause two main kinds of infection: gastroenteritis and systemic infection. In gastroenteritis, the infection is localized in the intestine, and induces an inflammatory reaction in the intestinal mucosa. Accumulation of liquid in the intestinal lumen leads to diarrhea [41], [42]. The inflammatory response creates a novel luminal niche, which favors growth of *Salmonella* over the resident microbiota of the intestine. Remarkably, the cascade of events that takes place as consequence of inflammation produces the accumulation of tetrathionate ($S_4O_6^{2-}$) in the intestinal lumen [43]. *Salmonella* can use tetrathionate as electron acceptor for respiration to obtain energy for growth in the inflamed gut lumen, taking advantage over the resident microbiota, which must rely on less efficient fermentation processes. In systemic infection, *Salmonella* crosses the epithelial barrier and can survive inside phagocytes due to the possession of a second type 3 secretion system encoded on *Salmonella* pathogenicity island 2 (SPI-2). Dissemination through the lymphatic system permits colonization of multiple target organs, particularly the spleen, the liver, the gall bladder and the bone marrow [44]. A fraction of individuals recovering from systemic infection become asymptomatic, life-long carriers of *Salmonella*, acting as reservoirs for future infections. In humans, serovar Typhi can establish chronic carriage in the gall bladder [44].

Formation of bacterial lineages

The study of differentiation in bacterial species that undergo developmental programs has played a historic role in biology [45]–[47]. In addition, phenotypic differences between colonies [48] and within colonies [49] were described many years ago in bacterial species that do not undergo development. Despite their technical limitations, these early studies contributed to bring about the idea that phenotypic heterogeneity might be a common phenomenon in the bacterial world [50]. This view has been confirmed by single cell analysis technologies [51]–[55]. Furthermore, theoretical analysis has provided evidence that phenotypic heterogeneity can have adaptive value, especially in hostile or changing environments [56]–[58]. In certain cases, the adaptive value of subpopulation formation is illustrated by experimental evidence [33], [59], [60].

In general, the evolutionary significance of the formation of bacterial subpopulations can be interpreted as the result of two different strategies: division of labor and bet-hedging [53], [61], [62]. Division of labor, also known as cooperation, implies that there is an interaction between different phenotypes, and in a given environment both subpopulations together are fitter than any of them separately. In turn, bet-hedging occurs when each subpopulation is fitter than the other in a particular environment, so that the population as a whole is fitter in a variety of conditions and prepared to adapt to an environmental change.

Formation of bacterial lineages is governed by diverse mechanisms, including programmed genetic rearrangement [63] and contraction or expansion of DNA repeats at genome regions known as contingency loci [64], [65]. In other cases, however, lineage formation is controlled by epigenetic mechanisms [54], [66]. Although the known examples of non-genetic heterogeneity show disparate levels of complexity, epigenetic formation of bacterial subpopulations typically fits in the following, simplified model: certain cell-to-cell differences can serve as physiological signals, and signal propagation by a feedback loop generates a heritable phenotype [54], [67]. Cell-to-cell differences can be a consequence of environmental inputs or result from the noise intrinsical to many cellular processes [52], [54], [58]. An important factor that contributes to gene expression noise is the finite number effect: noise is more prevalent

if the number of molecules involved in a process is limited [68]–[70]. This is relevant in gene expression since transcription and translation events are relatively infrequent events, and also because transcription factors are often present in small numbers [71]. In turn, the feedback loops that propagate the initial state can be relatively simple (e. g., the perpetuation of autogenous control beyond cell division) or involve complex mechanisms like the formation of inheritable DNA adenine methylation patterns in the genome [54], [66], [72]. Some feedback loops are stable enough to cause bistability, the bifurcation of a bacterial population into two distinct phenotypic states [67]. If a feedback loop is metastable, reversion of the epigenetic state will occur after a certain number of cell divisions. Reversible bistability is usually known as phase variation, and typically involves reversible switching of gene expression from OFF to ON or from low to high expression [73]–[75]. Examples of phase variation have been described mostly in bacterial pathogens, and subpopulation formation is frequently viewed as a strategy that may facilitate evasion of the immune system during infection of animals [73]–[75]. This view is supported by the observation that phase-variable loci often encode envelope components or proteins involved in modification of the bacterial envelope [73], [75].

Dam methylation

Base methylation is a DNA modification present in all kingdoms of life. C5-methylcytosine and N6-methyl-adenine are found in bacterial, archaeal and eukaryotic genomes, whereas N4-methyl-cytosine is found only in bacteria [76], [77]. The methyl group of modified bases protrudes from the major groove of the double DNA helix, which is a typical place for recognition of DNA motifs by DNA-binding proteins [78]. Consequently, the methylation state of critical adenosine or cytosine moieties can regulate the interaction between DNA-binding proteins and their cognate DNA sequences [78], [79]. Formation of N6 -methyl-adenine (an probably of the other methylated bases) lowers the thermodynamic stability of DNA [80] and alters DNA curvature [81], which could further influence DNA-protein interactions.

Base modification in bacterial genomes is performed by two kinds of DNA methyltransferases: (i) associated with restriction-modification systems that protect the

cell from foreign unmethylated DNA [82], [83]; (ii) solitary methyltransferases that do not have a restriction enzyme partner [79]. A paradigm of solitary methyltransferase is the Dam methylase of Gamma-proteobacteria. Dam is only found in a particular clade of bacteria consisting of the orders Enterobacteriales, Vibrionales, Aeromonadales, Pasteurellales, and Alteromonadales [84]. It is essential for viability in *Vibrio cholerae* and certain strains of *Yersinia* [85]. In *Escherichia coli* and *Salmonella enterica*, a *dam* mutation causes pleiotropic defects but is not lethal [86], [87]. Dam shares significant sequence identity with DNA methyltransferases such as MboI and DpnII, both of which have a restriction enzyme counterpart [84], [88]. This relatedness suggests that Dam has evolved from an ancestral restriction-modification system. A crucial difference, however, is that the Dam methylase is highly processive, able to perform multiple methylation reactions before dissociating from the DNA molecule, whereas restriction-modification DNA methylases are distributive [89]. The Dam methylase transfers a methyl group from S-adenosyl-methionine to the N6 amino group of the adenosine moiety of 5'GATC3' sites [86]. Methylation occurs shortly after DNA replication, which means that hemimethylated GATC sites are the natural substrate of the Dam methylase. However, Dam methylates hemimethylated and unmethylated GATC sites with similar efficiency [86]. DNA binding and/or methyl transfer efficiency are influenced by the flanking sequences of the GATC sites [90].

N6 -methyl-adenine can be used as a signal for genome defense, DNA replication and repair, nucleoid segregation, regulation of gene expression, control of transposition, and host-pathogen interactions [84], [88], [91] [86] (**Figure I3**). Dam plays a crucial role in the correction of replication errors by providing a way to identify the template DNA strand [92]. The formation of a mismatched base pair results in recognition by the MutS protein and the recruitment and assembly of the MutHLS complex. Methyl-directed mismatch repair is initiated by MutH, which nicks the nonmethylated (newly replicated) DNA strand at the nearest hemimethylated GATC site, ensuring that the parental template strand is not altered [93]. Degradation and resynthesis of the mismatched daughter strand eventually result in correction of the mismatched sequence.

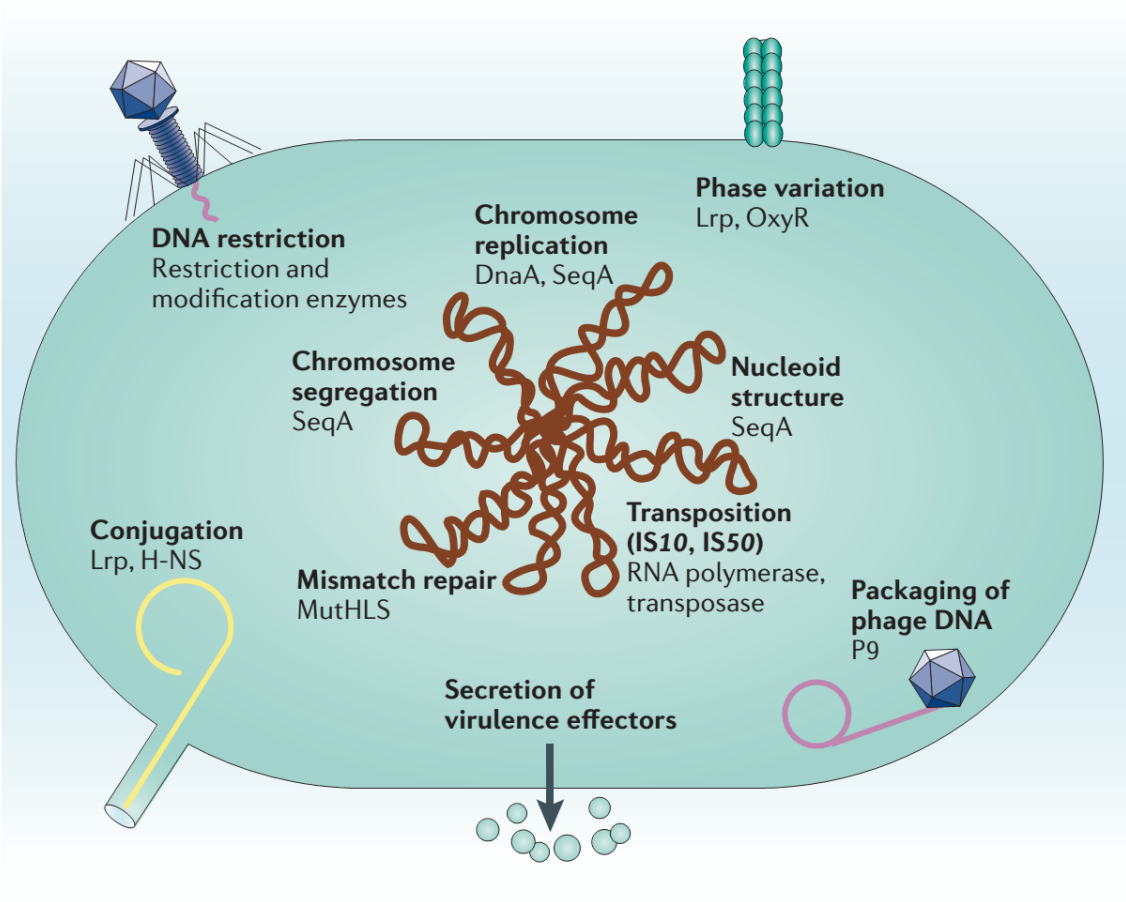


Figure I3. Examples of roles of N6-methyl-adenine in enteric bacteria. When known, the methylation-sensitive DNA-binding proteins involved in each process are indicated. Reproduced from [79].

Initiation of chromosome replication in *E. coli* requires binding of an ATP-bound form of the initiator protein DnaA to the replication origin (*oriC*), followed by separation of the two strands of the double helix and loading of DNA helicase. However, binding of DnaA at the *oriC* region is only possible if the GATCs located in the region are methylated; a hemimethylated origin is inactive [94]. Interestingly, the density of GATC sites in the *oriC* region is roughly tenfold higher than the average in the *E. coli* chromosome (11 GATC sites within 245 base pairs) [95]. Dam-dependent timing of DNA replication and nucleoid organization are controlled by a protein known as SeqA, which binds hemimethylated GATC sites in the *oriC* region, inhibiting further rounds of replication [96]. SeqA also binds to newly generated hemimethylated GATC sites along the chromosome, organizing the daughter chromosomes into nucleoid domains [97], [98].

The involvement of Dam methylation in bacterial virulence was first described in *Salmonella*: the 50% lethal dose (LD50) of a *dam* mutant is 10,000 fold higher than that of the wild type upon oral inoculation [99], [100]. Pleiotropic effects caused by absence of Dam methylation might explain this extreme phenotype: *dam* mutants display lower invasion capacity, reduced motility, envelope instability, ectopic expression of fimbriae, sensitivity to bile salts, lower expression of virulence genes, and altered LPS O-antigen chain length [88], [101], [102].

Dam methylation-dependent transcriptional control of gene expression can be classified into two main types [103]:

(I) Clock-like controls that use the methylation state of the DNA as a signal to couple gene expression to a particular stage of the cell cycle. Examples of transcriptional activation by GATC hemimethylation include the conjugal transfer gene *traJ* and the IS10 transposase gene [104], [105]. An example of repression by hemimethylation is provided by the gene *dnaA,* which encodes an initiator of chromosome replication [106].

(II) Switch-like controls that turn on and off gene expression upon formation of differential methylation patterns at specific GATC sites. Such sites are typically found forming clusters at or near the promoters of phase variation loci [73].

Because active demethylation is not known to occur in bacteria, competition between DNA-binding proteins and Dam methylase is the only mechanism known to generate nonmethylation [66]. Successful exclusion of the Dam methylase from GATC sites requires a decrease in processivity, which occurs at GATC sites flanked by specific, AT-rich DNA sequences [90].

Phase variation systems regulated by Dam methylation

We will briefly discuss three phase variation systems under Dam methylation control: *pap* and *agn43* from *Escherichia coli* and *opvAB* from *Salmonella enterica*.

pap

The *pap* operon encodes pyelonephritis-associated pili that mediate adhesion of uropathogenic *E. coli* to the urinary mucosa [107]. Synthesis of Pap pili is subjected to phase variation, and Dam methylation controls switching between ON and OFF states [72]. The regulatory region of the *pap* operon contains six binding sites for the leucine-responsive regulatory protein, Lrp. Sites 5 and 2 contain GATC motifs known as $GATC^{dist}$ and $GATC^{prox}$, respectively. In the OFF state, Lrp binds cooperatively and with high affinity to sites 1-3, and prevents RNA polymerase binding [66], [72], [107]. Lrp binding at sites 1-3 reduces the affinity of Lrp for sites 4-6, and preserves the nonmethylated state of $GATC^{prox}$ while $GATC^{dist}$ remains methylated. The high affinity of Lrp for nonmethylated $GATC^{prox}$ and its incapability to bind a methylated $GATC^{dist}$ generates a feedback loop that propagates the OFF state [72], [107]. Switching to the ON state needs translocation of Lrp to sites 4-6. Translocation involves the auxiliary protein PapI. The PapI/Lrp complex has higher affinity for GATC sites 4-6 than for 1-3 GATCs [72]. Binding of Lrp to sites 4-6 hinders methylation of $GATC^{dist}$ and permits methylation of $GATC^{prox}$, favoring the propagation of the ON state [107].

agn43

The *agn43* gene of *E. coli* encodes an autotransporter protein whose expression is subjected to phase variation under control of Dam methylation and the transcription factor OxyR [108]. Three GATC sites present within the binding site of OxyR at the *agn43* regulatory region. OxyR binding to nonmethylated *agn43* GATCs represses transcription and prevents GATC methylation. In turn, methylation of GATC sites avoids OxyR binding [109]–[111]. *agn43* expression thus depends on the competition between OxyR binding and Dam methylation, and switching may occur upon DNA replication [111], [112].

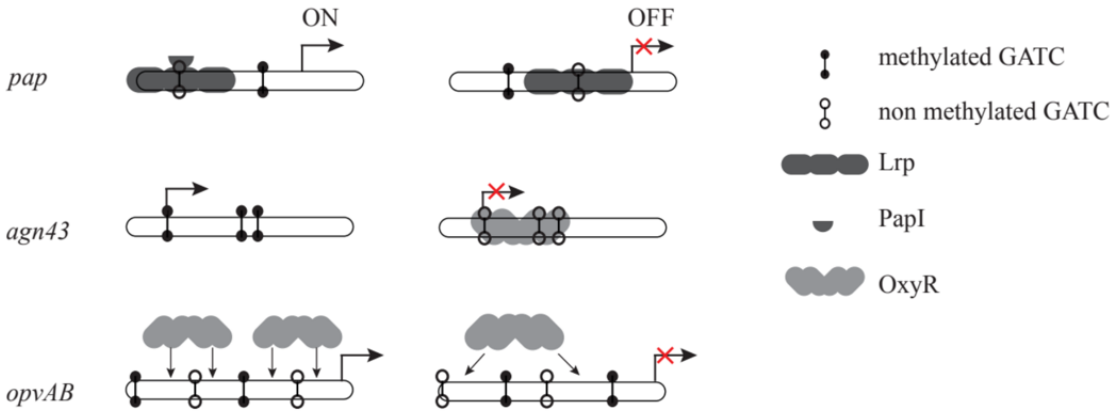


Figure I4. Diagrams for Dam methylation-dependent regulation of *pap*, *agn43* and *opvAB* phase variation. For simplicity, binding sites, Dam methylase and RNA polymerase are not represented. Adapted from [113], [114].

opvAB

The *Salmonella enterica opvAB* operon shows phase variation, which produces bacterial lineages with standard (OpvAB^{OFF}) and shorter (OpvAB^{ON}) O-antigen chains in the lipopolysaccharide. Transcription of *opvAB* is controlled by the LysR-type factor OxyR and by Dam methylation. The *opvAB* regulatory region contains four sites for OxyR binding (OBS^{A-D}), and four GATC motifs (GATC$^{1-4}$) which are susceptible to be methylated by the Dam methylase. Regulation by OxyR is oxygen-independent [115]. OpvAB^{OFF} and OpvAB^{ON} cell lineages present opposite DNA methylation patterns in the *opvAB* regulatory region: (i) in the OpvAB^{OFF} state, GATC^{1} and GATC^{3} are non-methylated, whereas GATC^{2} and GATC^{4} are methylated; (ii) in the OpvAB^{ON} state, GATC^{2} and GATC^{4} are non-methylated, whereas GATC^{1} and GATC^{3} are methylated [114].

The OBS^{A} and OBS^{C} sites of *opvAB* are identical to the consensus sequence for OxyR binding while OBS^{B} and OBS^{D} are not. This difference may explain the higher stability of the OpvAB^{OFF} lineage, resulting in a ~600-fold difference between ON→OFF and OFF→ON transition rates. The predominant OFF state involves binding of OxyR to the OBS^{A} and OBS^{C} sites, which protects GATC^{1} and GATC^{3} from methylation. In this configuration, GATC^{2} and GATC^{4} are unprotected and therefore are methylated by

Dam. In the ON state, OxyR binds to the OBS^B and OBS^D sites. As a consequence, $GATC^2$ and $GATC^4$ are protected from methylation and remain non-methylated, while $GATC^1$ and $GATC^3$ are methylated. In this configuration, RNA polymerase is recruited to the *opvAB* promoter and transcription of *opvAB* takes place. Like other LysR-like transcription factors, OxyR may contact RNA polymerase within the DNA region occupied by the regulator [116]. Additional factors involved in the formation of OpvAB cell lineages are the GATC-binding protein SeqA which contributes to the stability of the $OpvAB^{OFF}$ lineage, and the nucleoid protein HU which stabilizes the $OpvAB^{ON}$ lineage [114].

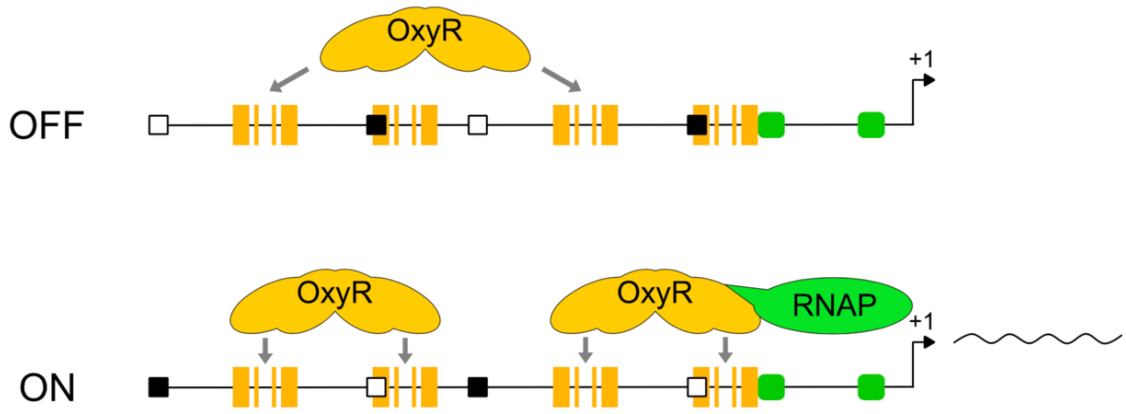


Figure I5. Model of *opvAB* phase variation. The diagram shows the Dam methylation states found in $OpvAB^{OFF}$ and $OpvAB^{ON}$ cell lineages and the hypothetical patterns of OxyR binding to cognate sites. Black and white squares represent methylated and nonmethylated GATC sites, respectively. Adapted from [114].

The *opvA* and *opvB* genes encode inner membrane proteins that modulate the O-antigen chain length. The dramatic change in LPS structure caused by *opvAB* expression (see a diagram in **Figure I6**), renders *S. enterica* resistant to bacteriophages 9NA, Det7, and P22 due to the fact that the O-antigen is the bacterial surface receptor used by these bacteriophages [117], [118], [119, p. 22], [120]

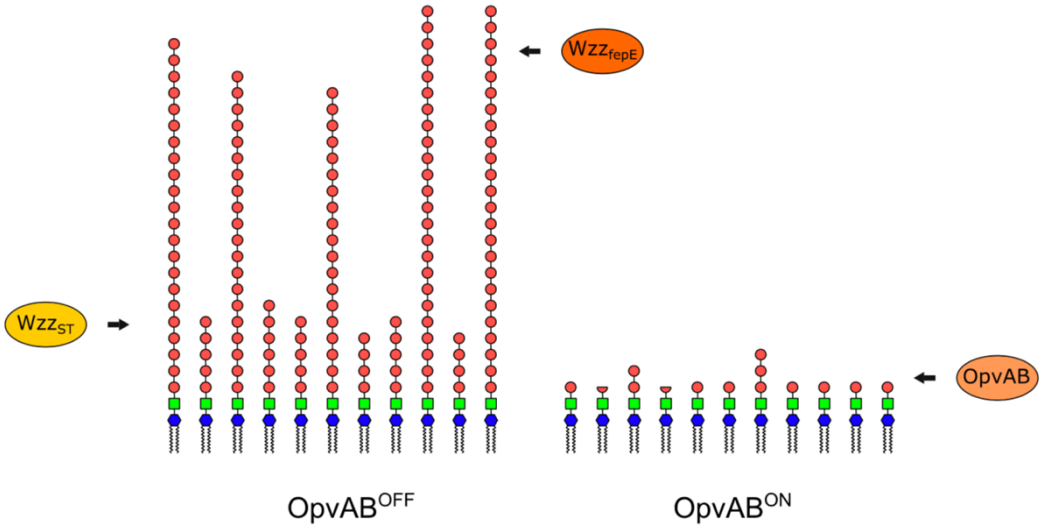


Figure I6. Diagrams of lipopolysaccharide structure in the OpvA^{OFF} and OpvAB^{ON} subpopulations. Lipid A is represented in blue and core oligosaccharide in green. Every red circle represents five O-antigen repeat units. Modal lengths conferred by Wzz$_{ST}$, Wzz$_{fepE}$ and OpvAB are indicated. Adapted from [114].

Because *opvAB* phase variation is skewed towards the OFF state, *S. enterica* populations contain a major subpopulation of OpvAB^{OFF} (phage-sensitive) cells and a minor subpopulation of OpvAB^{ON} (phage-resistant) cells. In the presence of a bacteriophage, the OpvAB^{OFF} subpopulation is killed and the OpvAB^{ON} subpopulation is selected (**Figure I7**). Hence, the existence of a small subpopulation of phage-resistant cells preadapts *S. enterica* to survive phage challenge. In OpvAB^{-} *S. enterica*, acquisition of phage resistance is mutational only, and a frequent mechanism is alteration of LPS structure. Because the LPS plays major roles in bacterial physiology including resistance to environmental injuries and host-pathogen interaction [121], *opvAB* phase variation may have selective value by providing *S. enterica* with a non-mutational, reversible mechanism of phage resistance. This mechanism offers the additional advantage of protecting *Salmonella* from multiple phages, perhaps from all phages that bind the O-antigen.

Acquisition of phage resistance in OpvAB^{ON} cells requires a payoff: reduced virulence in the mouse model. In a phage-free environment, this payoff may be irrelevant because the avirulent subpopulation is minor as a consequence of skewed switching of *opvAB* expression toward the OFF state: only 1/1,000 *S. enterica* cells can be expected to be avirulent in a phage-free environment. The virulence payoff is therefore enforced in the

presence of phage only, and its adaptive value may be obvious as it permits survival. On the other hand, the fitness cost of OpvAB-mediated phage resistance can be expected to be temporary only because phase variation permits resuscitation of the virulent OpvAB^{OFF} subpopulation as soon as phage challenge ceases. Resuscitation may actually be rapid as a consequence of skewed switching towards the OpvAB^{OFF} state.

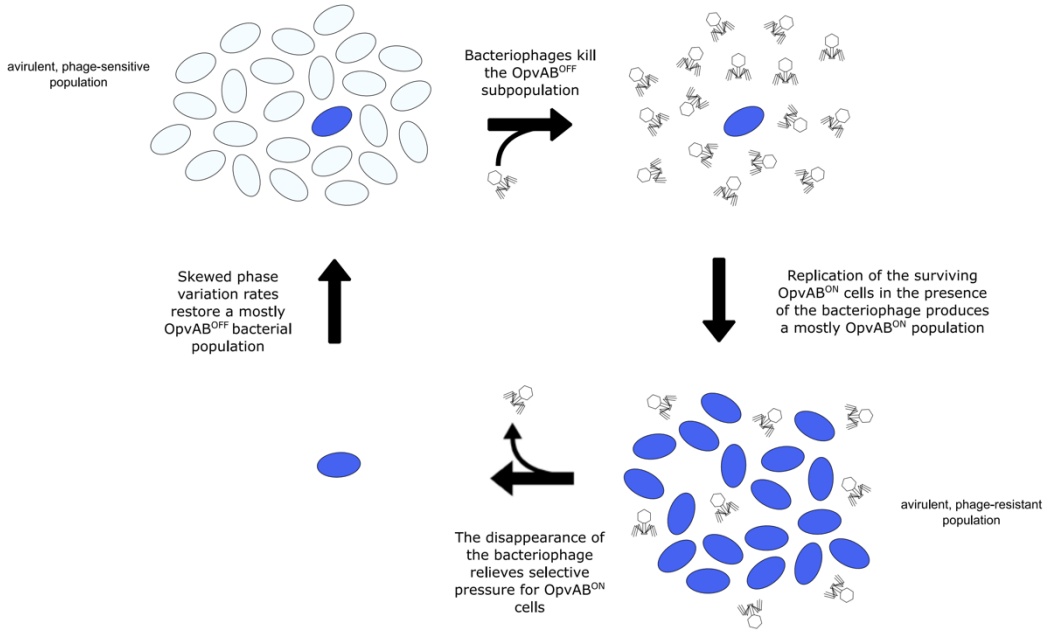


Figure I7. Phase variation of *opvAB* as a reversible bacteriophage resistance mechanism. Diagram for the selection of the OpvAB^{ON} subpopulation in the presence of a bacteriophage that uses the O-antigen as receptor. OpvAB^{OFF} cells are represented in white, OpvAB^{ON} cells in blue. Adapted from [122]

Lack of Dam methylation locks *opvAB* expression in the ON state [114]. Introduction of point mutations in the GATCs sites alters the regulation of the operon and produces different sizes of OFF and ON subpopulations [123].

Other phase-variable systems controlled by DNA adenine methylation play roles in bacteriophage resistance. In *Haemophilus influenzae*, DNA adenine methylation controls phase-variable resistance to bacteriophage HP1c1 but the underlying mechanism remains hypothetical [124]. Phase variation can also contribute to phage resistance without alteration of the bacterial surface. For instance, certain genes

encoding restriction-modification systems show phase variation [125], [126]. In other cases, Dam-dependent gene regulation controls the synthesis or modification of envelope structures such as fimbriae or the LPS O-antigen [127].

Biosensors

Biosensors are devices that combine biological materials and transducers for the detection of specific signals (e. g., chemical components, heavy metals, metabolites with economic interest, contaminants, microorganisms, control parameters, etc.). This occurs through the conversion of biochemical signals into quantifiable physicochemical signals that are proportional to the initial amount of sample. The first biosensor, developed in 1962, consisted of a biological device able to detect the presence of glucose [128]. Since then, biosensors of different types have been developed and marketed for various applications. Biosensors often provide a more specific, sensitive, rapid, real and reproducible results compared to chemical sensors. Indeed, conventional chromatographic methodologies such as gas chromatography (GC) or high-performance liquid chromatography (HPLC) are tedious and not portable while biosensors can offer rapid and on-site/point-of-care monitoring of even trace levels of targets [129]–[131]. Additional advantages are high specificity and tight interaction between biological molecules and target compounds. Several studies have focused on developing biosensors with high accuracy and sensitivity using enzymes or receptor proteins as the recognition elements, RNA, DNA, antibodies or whole cells [129], [132]–[134]. Although biosensors that use enzymes are widely used, the cost of production and purification is high, and cofactors are typically required to generate a measurable signal for certain targets [131].

The use of whole cells as sensing devices presents numerous advantages over enzymatic and protein systems due to the low cost and improved stability. Cofactors are present inside the cells, and production is reduced to one step, where the cell grows. Moreover microbes present high stability under harsh environments [131], [135]. Whole-cell biosensors usually measure changes in cellular metabolism such as pH or gene expression [136], [137].

Microorganisms have been used with a variety of sensing techniques including electrochemical and optical detection. The first monitors the electroactive species consumed or produced by these microorganisms by conductimetric, amperometric, impedimetric or potentiomentric methods. In contrast, optical techniques are based on the quantification of fluorescence, luminescence, color, etc. [131]. Microbial auxotrophy was used by Pfleger et al. in order to monitor growth-limiting small molecules such as mevalonate [136].

Reporter gene expression under the control of a specific regulatory network is another powerful readout with high sensitivity. Commonly used reporter genes are enzymes with activities detected through colorimetric, fluorescent, or luminescent readout methods [138]–[140]. A relatively large number of biosensors use either β-galactosidase (β-gal) or luciferase as reporter [138], [141]–[143].

Fluorescent proteins are also widely utilized in microbial sensors because they do not need to be supplied with any substrate due to their autofluorescence. For example, the green fluorescent protein (GFP) has been widely used to measure gene expression and to study cell-trafficking mechanisms [144]. GFP has been used, for instance, in a number of whole-cell biosensors in order to detect environmental contaminants. However, a limitation is that the original GFP takes time to mature and is inactive under anaerobic conditions. Moreover, the goal of multiplexing different signal detection at the same time led to the engineering of fluorescent protein versions with different colors and enhanced stability and folding properties [145].

The design of sensors for the detection of pathogenic bacteria is a rapidly developing field, due to the importance of a specific and accurate diagnosis to prevent infectious diseases. Some biosensors are based on nucleic acids and have the advantage of being able to amplify the signal using PCR, which considerably increases their sensitivity. A limitation, however, is that PCR-based methods are not able to discriminate live and dead bacteria. Antibodies are also used in this kind of biosensors, and are useful to detect spores and toxins [146].

Development of biosensors to monitor bacterial levels in food products and water samples is a promising research topic. Phage-based biosensors have been successfully

used for detection of bacteria directly in fresh products such as milk [139], [140], broth [147], fresh tomato [148], and water [149].

Most phages recognize their host very specifically to the bacterial strain level, with exceptions like *Listeria* phage A511 that identifies, binds and kills within an entire genus [150]. The inherent ability of the phages to bind to their target pathogen has been exploited to design biosensor surfaces using physical and chemical functionalization [151].

Due to the importance of detecting pathogenic strains in a more effective way, new biosensors are being developed that share many components with those developed years ago. Ansaldi et al., 2015, developed a bacteriophage-based biosensor that use a fluorescent reporter protein that will be synthesized, and thus detected, only once the specific recognition step between a genetically modified temperate bacteriophage and its bacterial host has occurred [152].

Bacteriophages

Bacteriophages are classified into eight different major phyla, with different molecular lifestyles and distant relatedness, if any [153]. Tailed bacteriophages include 95% of all the phages reported so far in the scientific literature, and probably make up the majority of phages in nature [154]. It is estimated that there are at least 10^{31} tailed virions in the biosphere, and that phages are 10 to 25-fold more abundant than their bacterial hosts [155]–[157], which makes phage the predominant biological entity on our planet [155], [158]. Their abundance and ability to kill their bacterial hosts means that they play a critical role in virtually all natural processes. Phages are known to alter competition between bacterial strains and species [159], [160], to maintain bacterial diversity [161], [162], and to mediate horizontal gene transfer between bacteria [163], [164]. Phages and bacteria are in a constant arms race that results in continuous cycles of mutually influenced co-evolution [165], [166]. As an example, the adsorption machinery is the most rapidly evolving part of the tailed phage genome [167]. In turn, bacterial defense systems also show high variability (including phase variation) [127], rapid evolution,

and frequent horizontal gene transfer [168]. The specificity in the interaction between a bacteriophage and a bacterial cell is mostly determined by the specificity of adsorption, which is in turn dependent on the nature and structural peculiarities of the receptors that are recognized on the surface of the bacterial envelope [169]. Known receptors of *Salmonella* phages are the LPS O-antigen [170], [171, p. 7], [172], [173], flagellar proteins [174], [175], [176, p. 5], the outer membrane protein for vitamin B12 uptake BtuB [175], [177], the outer membrane protein OmpC [178], [179], the outer membrane protein for drug efflux pump TolC [180], the outer membrane transport protein FhuA [181], and the Vi capsular antigen [182].

A common feature of bacteriophages using the O-antigen as receptor is that their tail spike proteins not only recognize but also hydrolyze the O-antigen, allowing the phage to penetrate through the O-antigen layer during infection [183]. Tailed phages have been traditionally assigned to the order Caudovirales and share a common structure consisting of a polyhedral, often icosahedral, head that contains a double-stranded DNA genome and is attached to a flexible tail. They also share a similar DNA packaging system relying on an ATP cleavage-powered DNA translocase [184]. Tailed phages can undergo a lytic or lysogenic life cycle. Lytic infection results in production of phage progeny and destruction of the host. During lysogenic infection, formation of a prophage may involve integration of the phage genome into the host chromosome or persistence as an extrachromosomal element. Genes that are expressed from the prophage can alter certain traits of the bacterial host. Under stressful conditions (e. g., DNA damage), prophages can be induced and resume a lytic cycle [185]. The bacteriophages used in this work belong the traditional three families in the order Caudovirales: P22 is a Podoviridae (which includes 14% of tailed phages), 9NA, a Siphoviridae (62%), and Det7, a Myoviridae (24%) [153]. These families are defined by their morphology: the Podoviridae have short non-contractile tails, the Siphoviridae have long non-contractile tails, and the Myoviridae have long contractile tails (**Figure I8**).

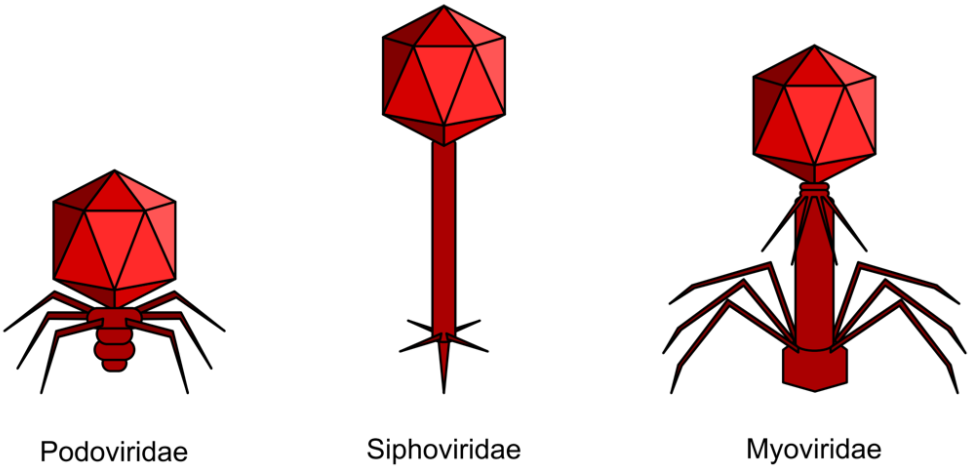


Figure I8. **Idealized structures of bacteriophages belonging to the three families in the order Caudovirales.** Podoviridae have short non contractile tails. Siphoviridae have long non-contractile tails. Myoviridae have long contractile tails. Modified from [WIKIPEDIA].

A recent classification based on sequence similarity of 337 fully sequenced tailed phages infecting 18 bacterial genera belonging to the Enterobacteriaceae family [186] identified 56 different clusters and confirmed that, although tail morphology has been shown not to be the best indicator of relatedness [187], [188], P22, 9NA and Det7 belong to different groups indeed. P22 belongs to the lambda supercluster of temperate phages. P22_H5 is a virulent version of P22 called carrying a mutation in the c2 gene that prevents lysogeny [189]. 9NA constitutes the prototype phage of an isolated cluster which no close relatives. It forms clear plaques with no indication of a lysogenic lifestyle [118]. Det7 is also lytic [190, p. 7] and belongs to the Vi01-like cluster, which is distantly related to T4 [186], [191]. Clustering of the bacteriophages also showed a correlation with genome size and lifestyle. Again, the three phages were placed in three different groups: small temperate phages (P22, 44 Kb genome), small lytic phages (9NA, 53 Kb) and large phages (Det7, 158 Kb). Resistance to bacteriophages can be attained by a variety of mechanisms [192], including inhibition of DNA penetration, production of restriction endonucleases and blockage of the receptor by increased production of the extracellular matrix. However, the most frequent cause of phage resistance is a mutation that affects phage receptors [120], [192], [193]. Resistance to

phages comes at a fitness cost due to deleterious effects of resistance mutations [194], decreased ability to metabolize carbon [195], reduced competitive ability [196], increased susceptibility to other phages [197] and reduction in virulence [198], [199]. This may explain the adaptive value of phase variation in bacterial defense mechanisms [114], [124], [200].

Bacteriophage T5

Bacteriophage T5 is a caudal virus belonging to the Siphoviridae family. This bacteriophage specifically infects *E. coli* bacterial cells and follows a lytic life cycle. The T5 structure includes a 90 nanometers icosahedral capsid and a 250 nanometer-long flexible, non-contractile tail. The capsid contains the phage's 121,750 base pair double-stranded DNA genome [201]. T5 infects *E. coli* upon binding of its receptor-binding protein, Pb5, to the outer membrane ferrichrome transporter, FhuA [202]. This leads to a rearrangement of the tail that trigers DNA injection [203]. T5 has a peculiar way of infection in two steps. During the first step, only 8% of the genome is injected, leading to take-over of the host. During the second step, T5 DNA injection resumes and the lytic particles are formed [204].

FhuA: Ferrichrome outer membrane transporter/phage receptor

FhuA is an *E. coli* outer membrane protein which transports the ferric siderophore ferrichrome and is the receptor for phage T5, φ80 and T1 and for colicin M [205]. Cristal structures of FhuA are similar to other porin proteins, FhuA consists of a β-barrel (composed 22 instead of 16 antiparallel β strands for porins), but unlike the porin proteins, it contains a plug (or cork or hatch) which fills the lumen of the barrel. The plug is a globular N-terminal domain that enters the β-barrel from the periplasmic side and tightly closes the pore in the β-barrel. The β-barrel extends well above the lipid bilayer. The β-strands are connected by short periplasmic turns and extracellular loops of up to 31 residues [206], [207].

Bacteriophage T5-encoded lipoprotein

Bacteriophage T5-encoded lipoprotein (*llp* gene) is synthesized upon *E. coli* infection, and prevents superinfection of the host cell by the same virus. The molecular basis of its ability to inactivate the receptor of phage T5, the FhuA protein, has been investigated *in vitro* [208]. In the early stage of infection of *E. coli* by bacteriophage T5, the phage-encoded *llp* gene is expressed. Synthesis of Llp not only prevents superinfection but also protects progeny phages from being inactivated by the receptor molecules present in envelope fragments of lysed host cells [209].

Phage therapy

The first attempts to eradicate infections with phage therapy involved treatment of dysenteric humans [210]. The use of the bacteriophage to treat bacterial infection initially yielded impressive results. However, many problems soon appeared, such as the lack of reproducibility and the impossibility of achieving quality controls [211]. In addition, the discovery of antibiotics further decreased interest in phage therapy research in Western countries. In contrast, phage therapy continued in the Soviet Union and Eastern Europe. In the 80s, due to the emergence of resistance to antibiotics, some research groups focused on phage therapy as an alternative to the use of these antibiotics. [212].

Advantages of phage therapy over use of antibiotics include that method phages isolation is fast, simple and inexpensive, and that resistance to phage develops about ten times more slowly than antibiotic resistance [213]. Furthermore, the fact that phages continue replicating as long as host bacteria are present [214] means that a small amount of phages could be used to carry out a treatment. Moreover, most phages have high host specificity, which may constitute an advantage in the treatment of chronic diseases where the microbiota is affected by the use of broad-spectrum antibiotics. On the other hand, phage therapy is harmless for humans since phage do not infect eukaryotic cells [213], However, more studies are needed to understand interactions between phage and the human immune system [215].

Recent investigations using animal models have explored phage treatment against a range of clinically significant pathogens. When challenged with gut-derived sepsis due to *Pseudomona aeruginosa*, oral administration of phage saved 66.7% of mice from mortality compared to 0% in the control group [216]. A single dose of phages used as prophylaxis in a Hamster model of *Clostridium difficile*-induced ileocecitis was sufficient prophylaxis against infection, saving 11 of 12 mice whereas control animals receiving *C. difficile* and clindamycin died within 96 h [217].

The Eliava Institute of Bacteriophage, Tbilisi, Georgia, and the Institute of Immunology and Experimental therapy in Wroclaw, Poland, have extensively used phage in preclinical and clinical treatment of common bacterial pathogens such as

Staphylococcus aureus, *Escherichia coli*, *Streptococcus* spp., *P. aeruginosa*, *Proteus* spp., *Shigella dysenteriae*, *Salmonella* spp., and *Enterococcus* spp. [218]. In a 1938 clinical trial, 219 patients with bacterial dysentery were treated solely with a phage cocktail consisting of a variety of phages targeting *Shigella spp., E. coli, Proteus* spp., *P. aeruginosa*, *Salmonella typhi*, *Salmonella paratyphi* A and B, *Staphylococcus* spp., *Streptococcus* spp. and *Enterococcus* spp.; cocktails were administered both orally and rectally. Overall 74% of the 219 patients showed improvement or were completely relieved of symptoms [219], [220]. More recently, six patients with antibiotic unresponsive diabetic foot ulcers were treated in the USA by topical administration with *S. aureus*-specific phage. One application of this phage was enough for recovery in all individuals [221].

Currently, in Europe some phage preparations have approved by local authorities from Belgium, Poland and France for compassionate usage. In the U. S. A., several commercial phage preparations used for biocontrol of bacterial pathogens in the food industry have been approved by the FDA under the classification of “generally considered as safe”. Evidence suggests that phage biocontrol can be an effective method for improving food safety at numerous stages in meat production and processing. For example, Atterbury and colleagues [222] used phages to control *C. jejuni* contamination on the surface of chicken skin. After 24 hours post-administration, the treatment with phages resulted in a 1-1.3 log reduction in *C. jejuni*. In another trial, Hungaro et al. [223] used a mixture composed of a phage in combination with chemical agents (dichloroisocyanurate, peroxyacetic acid and lactic acid) to treat *S. enteritidis* on chicken skin. A reduction of 1-log CFU / cm^2 was shown. Phages have been also used to reduce bacterial contamination in fruits, vegetables, and dairy products. For example, Levenrentz et al. [224] attempted phage therapy against *Salmonella* on fresh fruit. The literature on phage therapy has also reported applications in sheep [225], cattle [226], swine [227] and poultry [228]–[230].

DNA methylation, a potential target for novel antibiotics

The involvement of Dam methylation in bacterial virulence was initially shown in the mouse model of typhoid: the "lethal dose 50" (LD_{50}) of a *dam* mutant of *Salmonella*

enterica serovar Typhimurium is 10,000-fold higher than that of the wild type upon oral ingestion [100]. This extreme attenuation reflects the pleiotropic defects in *dam* mutants: (i) reduced colonization capacity due to inefficient activation of genes in pathogenicity islands 1 (SPI-1) and SPI-5 [100], [231], [232]; (ii) envelope instability, with release of outer membrane vesicles and leakage of proteins [233]; (iv) ectopic expression of fimbriae [231]; and (v) sensitivity to bile salts [233]. In the related serovar Enteritidis, altered O-antigen chain length in the lipopolysaccharide may contribute to attenuation [101].

An intriguing connection between DNA adenine methylation and virulence is found in the grampositive pathogen *Mycobacterium tuberculosis*. Strains of the Euro-American lineage of *M. tuberculosis* harbor a DNA adenine methyltransferase called MamA, which is absent from strains of the Beijing lineage [234]. MamA methylation appears to control survival in hypoxia, a stress condition typical of human infections, and may affect expression of a number of *M. tuberculosis* genes. A different DNA adenine methyltransferase is found in the Beijing lineage, suggesting that strain-specific differences in DNA methylation may control certain lineage-specific features [234].

Virulence-related defects associated with loss of DNA methylation have been reported in other pathogens. Certain strains of *Haemophilus influenzae* require Dam methylation for efficient invasion of both endothelial and epithelial cell lines [235]. Reduced invasion of epithelial cells by *dam* mutants is likewise observed in the periodontal disease agent *Aggregatibacter actinomycetemcomitans* [236] and perhaps in the intestinal pathogen *Campylobacter* [237]. A potential role of Dam methylation in virulence has been also described in the oral pathogen *Streptococcus mutans* [238]. Other virulence-related phenotypes of *dam* mutants are more difficult to interpret, and relevant examples are increased pedestal formation [239] and control of Shiga toxin 2 production in *dam* mutants of enterohemorrhagic *Escherichia coli OH157:O7* [240].

In bacterial pathogens where DNA adenine methylation is essential, the involvement of Dam methylation in pathogenesis has been tested by Dam methylase overproduction. Among Gamma-proteobacteria, overproducers of Dam methylase are attenuated in *Vibrio cholerae, Aeromonas hydrophyla, Yersinia* spp., and *Pasteurella multocida* [241]. Dam overproducers of *Yersinia enterocolitica* show enhanced invasion capacity, probably associated to transcriptional alterations in invasion genes and to changes in the composition of lipopolysaccharide (LPS) O-antigen. Furthermore, *Y. enterocolitica* Dam overproducing-strains show enhanced motility and impaired secretion of the

pathogenicity factors called "*Yersinia* outer proteins" [85], [242]. Among Alpha-proteobacteria, overproduction of CcrM methylase decreases proliferation of *Brucella abortus* inside murine macrophages, suggesting that CcrM methylation may control intracellular replication, which is a hallmark of *Brucella* infections [243].

The involvement of Dam methylation in the virulence of *Salmonella* and other Gamma-proteobacterial pathogens has raised the possibility of using strains lacking DNA adenine methylation as live vaccines. Such vaccines have been assayed indeed against *S. enterica*, *Haemophilus influenzae,* and *Yersinia pseudotuberculosis* [244]. Hypermutation, however, is a negative trait that may hamper the use of *dam* mutants as live vaccines, especially in humans.

Another potential application of DNA methylation research is the use of DNA methylase inhibitors as antibacterial drugs [245], [246]. Because adenine methylation is rare or absent in mammalian cells, DNA adenine methylase inhibitors should be harmless for the host. In pathogens in which DNA methylation is not essential, DNA adenine methylase inhibitors would attenuate virulence by transforming wild-type bacteria into phenocopies of *dam* mutants. Such drugs might have broad spectrum as DNA methylation is essential for virulence in many bacterial pathogens. Because DNA methylation is dispensable in many bacterial taxa and absent in others, inhibitors of DNA adenine methylases should be harmless for the normal microbiota. Hence, DNA adenine methylation inhibitors might approach the holy grial of antibacterial therapy: they would be harmless for the host and efficient against the pathogen without impairment of the pathogen's fitness.

A well known inhibitor of DNA methylation is sinefungin, a naturally occurring nucleoside isolated from cultures of *Streptomyces griseolus* and *S. incarnatus*. Sinefungin is structurally related to S-adenosyl-methionine (SAM) and S-adenosyl-L-homocysteine (SAH) [247], and exerts a potent, competitive inhibition of eukaryotic DNA methyltransferases [248].

Appendix 1

Lipopolysaccharide (LPS)

The cell envelope of Gram-negative bacteria consists of three layers: the cytoplasmic or inner membrane, the peptidoglycan cell wall, and the outer membrane. The outer membrane is highly asymmetrical: its inner leaflet consists mainly of phospholipids, while the outer leaflet is almost entirely composed of a particular kind of glycolipid known as lipopolysaccharide (LPS) [249].

The LPS is essential for many aspects of the lifestyle of *Salmonella*, including swarming motility [250]; intestinal colonization [251]; invasion and intracellular replication [252]–[254]; and resistance to serum [255], [256], bile [257], and cationic peptides [254]. LPS is also a common receptor for bacteriophages [120]. The LPS can be divided in three structural regions: lipid A (endotoxin), a highly conserved hydrophobic molecule which serves as an anchor to the membrane; the core saccharide, a genus-conserved short oligosaccharide; and the O-antigen, an immunogenic molecule made up by a number of repeats of the same saccharide unit composed of three to five sugars [258], [259] (**Figure I9**). Most of the structural diversity of LPS is found in the O-antigen. Altered sugar composition, linkage, and number of O-antigen repeats lead to the production of many different kinds of O-antigen molecules both between and within bacterial strains.

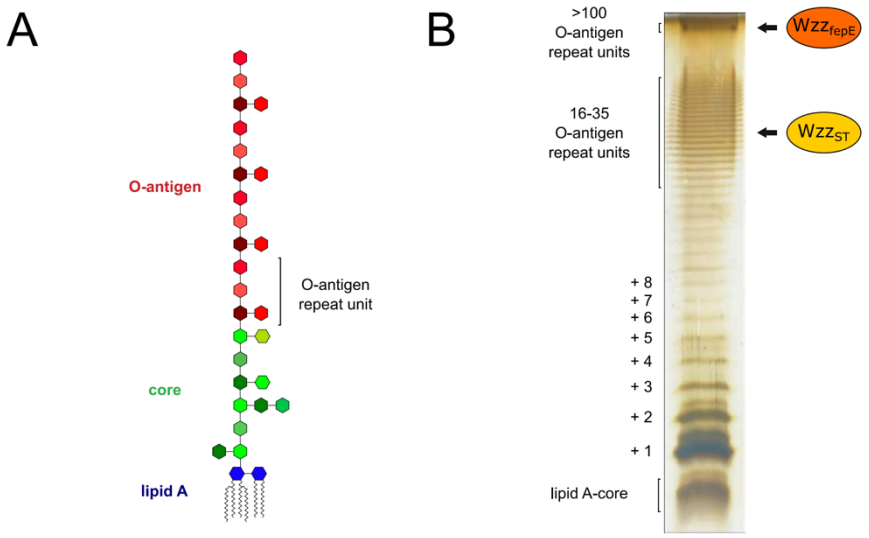


Figure I9. A. Idealized structure of an LPS molecule. For simplicity, only four O-antigen repeat units are represented, but individual LPS molecules can have more than 100 O-antigen repeat units. B. Visualization in SDS-PAGE gel of a typical LPS structure from *Salmonella enterica*. The O-antigen chain modal lengths imposed by the regulators Wzz_{ST} and Wzz_{fepE} are indicated. Adapted from [122].

Synthesis of the O-antigen is complex and involves a large number of inner membrane proteins [260]. First, synthesis of a single O-antigen repeat unit linked to the lipid carrier undecaprenyl pyrophosphate takes place in the cytoplasmic leaflet of the inner membrane. The lipid-linked O-antigen unit is then flipped to the periplasmic leaflet of the inner membrane by the O-antigen flippase Wzx. In the periplasm, the O-antigen polymerase Wzy (also called Rfc in *Salmonella*) combines O-antigen repeat units in a growing O-antigen chain until preferred modal lengths conferred by O-antigen chain length regulators (also known as polysaccharide copolymerases or PCPs and commonly designated Wzz) are produced. Once the O-antigen chain is complete, it is ligated to the lipid A-core by the O-antigen ligase WaaL and tranported to the outer membrane (**Figure I10**)

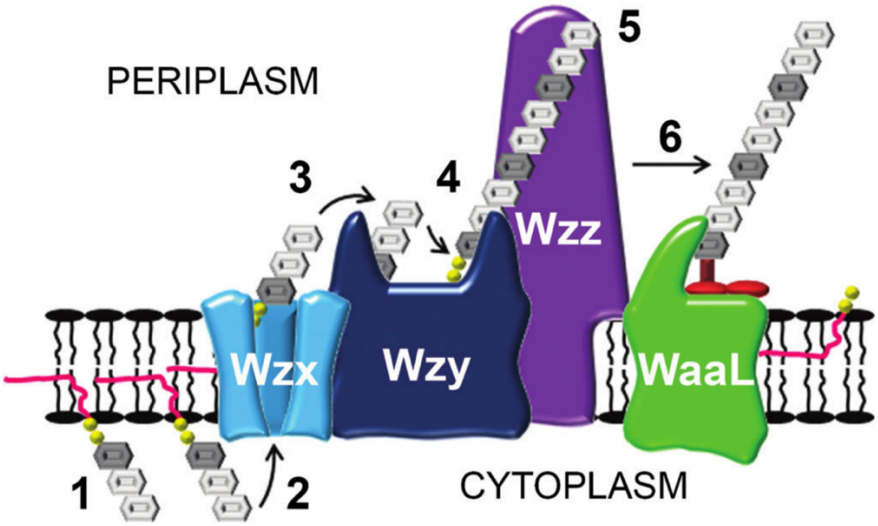


Figure I10. **Diagram of the synthesis of O-antigen**. 1. O-antigen repeat unit synthesis on the undecaprenyl pyrophosphate-lipid carrier in the cytoplasmic leaflet of the inner membrane. 2. Flipping of the lipid-linked O-antigen subunit to the perisplasmic leaflet by the O-antigen flippase Wzx. 3. Transfer to the O-antigen polymerase Wzy. 4. Addition of single lipid-linked O-antigen subunits to the growing O-antigen chain. 5. Length regulation of the O-antigen chain by O-antigen chain regulators Wzz. 6. Ligation of the polymerized O-antigen chain to a lipid A-core molecule by the O-antigen ligase WaaL. Reproduced from [261].

Visualization of LPS in SDS-PAGE gels results in a typical "ladder" structure in which every step reflects the addition of a single O-antigen repeat unit (**Figure I9B**). Different O-antigen chain modal lengths are easily identified within the same bacterial strain by increased intensity of bands of a particular size range. As already stated, O-antigen chain modal lengths depend on O-antigen chain length regulators. As many other species in the Enterobacteriaceae family [262], *Salmonella enterica* displays two modal lengths (**Figure I9B**): long O-antigen (16-35 repeat units) is conferred by Wzz_{ST} (also known as Rol, Cld or WzzB) [263], and very long O-antigen (>100 repeat units) by Wzz_{fepE} (FepE) [262]. In the absence of both Wzz regulators, *Salmonella* LPS displays a stochastic distribution, with bands decreasing in intensity as the O-antigen chain grows longer. Albeit with remarkable variations in amino acid sequence, Wzz regulators share several structural properties: nearly all harbor transmembrane segments near the N and C termini and a hydrophilic α-helical periplasmic domain containing a coiled-coil region [264, p. 2] and display a particular set of conserved amino acid residues near the

N terminus [265]. Wzz regulators are known to form oligomers in a characteristic bell shape [266]. The mechanism for regulation of O-antigen chain length by Wzz regulators is not established and has been the subject to considerable debate. Depending on the model, Wzz regulators have been proposed to act as molecular timers that modulate the ability of Wzy to elongate the O-antigen chain [267], as chaperones that assemble O-antigen synthesis machinery proteins in particular ratios [268], as scaffolds in which O-antigen chain lengths are conferred by the number of Wzz subunits in a given oligomer [265] or as rulers that set O-antigen chain length by direct interaction to the O-antigen chain [269]. Later on, the role of changes in the structure of the growing O-antigen in the interaction with the Wzz regulators has been incorporated into a chain-feedback model [270]. A hybrid model combining ruler and chain-feedback elements has been recently proposed, and is supported by a large body of evidence [260]. According to this model, the interaction between Wzy and Wzz favors the formation of a longer O-antigen chain by direct binding of the growing O-antigen chain to the Wzz protein. However, when the O-antigen chain attains a particular length, higher-order structures begin to destabilize the interaction with Wzz. When the O-antigen chain reaches the tip of the bell-shaped Wzz oligomer, the lipid-linked O-antigen is freed from the O-antigen synthesis complex rendering it susceptible to ligation by WaaL to form a mature LPS molecule. The LPS molecule is subsequently exported to the outer membrane at a limited number of sites, in a ribbon-like shape that is largely immobile [271]–[273], probably due to strong lateral interactions between LPS molecules [274], [275]. These interactions induce the assembly of a mechanically stable network in which the structure of the lipid A-core is rigid and well-defined [276] but the O-antigen is flexible and can adopt many conformations [277]. Ribbon-like dispositions have also been described for many inner and outer membrane proteins, suggesting that the whole cell envelope might be organized in a helical fashion [273], [278].

Appendix 2

Antibiotic heteroresistance

Heteroresistance is a phenomenon where subpopulations of a bacterial isolate exhibit a range of susceptibilities to a particular antibiotic. The first report that described heteroresistance was published in 1947, and described the phenomenon in *Haemophilus influenza* [279]. The term 'heteroresistance' was not used until 1970 [280]. Heteroresistance complicates the global crisis of resistance to antibiotics. Frequently, the clinical diagnostic tests are not effective in detecting a heteroresistant subpopulation, which goes unnoticed and conbtributing to treatment failure [281]. Heteroresistance in many ha been detected in both Gram-negative and Gram-positive bacteria [282], [283].

A relevant example is colistin heteroresistance. Polymyxin E, usually known as colistin, is a polycationic antibiotic that acts by replacing Ca^{2+} and Mg^{2+} in the LPS, causing loss of membrane integrity and cell death [284]. Colistin heteroresistance was first detected in 2006 in clinical isolates of *Acinetobacter baumanii.* Since then the number of publications which are reporter colistin heteroresistence in clinical isolates of different Gran-negatives is increasing [285]–[288]. Resistance towards polymyxins in gran negative bacteria can be caused by: (i) alterations in the LPS that decrease its net negative charge [284], [289], [290]; (ii) loss or modification of lipid A [291]; (iii) activation of efflux pumps [292]; and (iv) capsule shedding [293]. Heteroresistance can be caused by mutation [294], [295] or by gene amplification [296]. For instance, variations in the copy number of the *pmrD* gene cause colistin resistance in *Salmonella enterica* [282].

OBJECTIVES

The overall objective of this Thesis is the exploitation of the *opvAB* phase variation system of *Salmonella enterica* for the design of biological sensors. The background information necessary to address this goal has been provided by previous studies of our laboratory. The specific objectives addressed in the Thesis are as follows:

1. Engineering of genetic constructs for bistable expression of heterologous genes under *opvAB* control.

2. Tests of bistable expression under *opvAB* control in a heterologous host, *Escherichia coli*.

3. Design and experimental testing of a sensor of inhibition of DNA methylation.

4. Design and optimisation of *opvAB*-based biosensors capable of bacteriophage detection.

5. Design of biosensors for bacteriophage receptor discrimination.

MATERIALS AND METHODS

Bacterial strains

Strains of *Salmonella enterica* and *Escherichia coli* used in this study are listed in Table 1. *S. enterica* strains belong to serovar Typhimurium, and derive from the mouse-virulent strain ATCC 14028. For simplicity, *S. enterica* serovar Typhimurium is routinely abbreviated as *S. enterica*. Unless indicated otherwise, *E. coli* strains derive from K12 MG1655 (*E. coli* Genetic Stock Center).

Strain construction by targeted gene disruption was achieved using plasmids pKD3, pKD4 or pKD13 as templates to generate PCR products for homologous recombination [297]. Antibiotic resistance cassettes introduced during strain construction were excised by recombination with plasmid pCP20 [297]. Primers used in strain construction are shown in Table 2. For the construction of translational *lac* fusions on the *S. enterica* chromosome, FRT sites generated by excision of Km^R cassettes were used to integrate plasmid pCE40 [298]. Transductional crosses using phage P22 HT 105/1 *int201* were used for transfer of chromosomal markers between *S. enterica* strains [152]. To obtain phage-free isolates, transductants were purified by streaking on green plates [299]. Phage sensitivity was tested by cross-streaking with the clear-plaque mutant P22 H5. Bacteriophage P22 H5 is a virulent derivative of bacteriophage P22 that carries a mutation in the *c2* gene [189], and was kindly provided by John R. Roth, University of California, Davis. Directed construction of point mutations was achieved using the QuikChange® Site-Directed Mutagenesis Kit (Stratagene) using the suicide plasmid pDMS197 [247] and propagated in *E. coli* CC118 λ *pir*. Plasmids derived from pMDS197 were transformed into *E. coli* S17-1 λ *pir*. The resulting strains were used as donors in matings with *S. enterica*.

Restoration of O-antigen

E.coli K12 MG1655 is unable to synthesize O-antigen due to a disruption of the *wbbL* gene, which encodes rhamnose transferase. The activity of this enzyme is necessary for the correct composition of the O-antigen [300], [301]. For restoration of the O-antigen, a DNA fragment was amplified using oligos SacI-wbbl1 and XbaI-wbbl2. The resulting PCR fragment was cloned onto plasmid pDMS197 [302] to obtain plasmid pDMS::*wbbL*. This plasmid was propagated in *E.coli* CC118 λ pir. Plasmids derived

from pDMS197 were transformed into *E.coli* S17 λ pir. The resulting strain was used as donor in matings with DR40 harboring a Km^R cassette from pKD4. Tc^R transconjugants were selected on E plates supplemented with tetracycline. Several Tc^R transconjugants were grown in nutrient broth (beef extract, 5 g/l; peptone, 5 g/l) and plated on nutrient broth supplemented with 5% sucrose. Individual tetracycline-sensitive segregants were then screened for kanamycin sensitivity and characterized by DNA sequencing using external oligonucleotides of the fragment. The resulting strain, DR28, is a derivative of DR3.

Table M1. List of strains of *S. enterica* and *E.coli* constructed in this study.

Strain	Genotype
S. enterica serovar Typhimurium	
ATCC 14028	Wild type
SV6727	*opvAB:gfp*
SV6729	*opvAB:gfp* GATC-less
SV8499	P_{opvAB}*::lacZY*
SV8578	*opvAB::gfp* $GATC_{1,2}$
SV9700	P_{opvAB}*::lacZY::gfp*
SV9701	P_{opvAB} $GATC_{1,2}$*::lacZY::gfp*
SV9702	P_{opvAB} GATC-less *::lacZY::gfp*
SV9703	P_{opvAB}::BI-*aac6::gfp*
SV9704	P_{opvAB}::D25-*aac6::gfp*
SV9705	P_{opvAB} GATC-less::BI-*aac6::gfp*
SV9706	P_{opvAB}::BI-*ctxm::gfp*
SV9707	P_{opvAB} GATC-less::BI-*ctxm::gfp*
SV9716	P_{opvAB}::*lacZY*
SV9776	P_{opvAB}::BI-*aac3::gfp*
SV9777	P_{opvAB}*::gfp*
SV9792	P_{opvAB}::D25-*aac3::gfp*
SV10048	P_{opvAB} $GATC_{1,3}$::BI-*aac6::gfp*
SV10049	P_{opvAB} $GATC_4$::BI-*aac6::gfp*
E. coli	

MG1655	Wild type
CC118 λ *pir*	*phoA20 thi-1 rspE rpoB argE(Am) recA1* (lambda *pir*)
S17 λ *pir*	*recA pro hsdR* RP4-2-Tc::Mu-Km::Tn*7* (lambda *pir*)
DR3	Δ*lacZY*
DR22	Δ*lacZY* P_{opvAB}::*lacZY::gfp*
DR23	Δ*lacZY* P_{opvAB} $GATC_{1,2}$::*lacZY::gfp*
DR24	Δ*lacZY* P_{opvAB} GATC-less::*lacZY*::*gfp*
DR28	Δ*lacZY* LPS^+
DR29	Δ*lacZY opvAB::gfp* LPS^+
DR30	Δ*lacZY* GATC-less *opvAB::gfp* LPS^+
DR40	Δ*IS5::km*
DR41	*E. coli* MG1655 P_{opvAB}::*llp*::*gfp*
DR42	*E. coli* MG1655 Δ*fhuA*

Bacteriophages

Bacteriophages 9NA [303], [118], Det7 [38] and HK620 [304] were kindly provided by Sherwood Casjens, University of Utah, Salt Lake City. Bacteriophage P22_H5 is a virulent derivative of bacteriophage P22 that carries a mutation in the *c2* gene [189], and was kindly provided by John R. Roth, University of California, Davis. Bacteriophages Se_F1, Se_F2, Se_F3 and Se_F6 infecting *S. enterica* were isolated and purified from waste water obtained at various locations around Seville. Se_AO1 was isolated from a sheep farm. Se_ML1 bacteriophage was isolated and purified from samples taken at ponds of Maria Luisa Park in Seville (37°22'28.0"N 5°59'19.1"W).

Additional bacteriophages were isolated using strain DR28 as host (*E.coli* MG1655 Δ*lacZY* LPS^+). Ec_Unk_EM1, Ec_Unk_EM1.1, Ec_Unk_EM2 were isolated from waste water from Seville, Ec_Unk_AO1 was isolated in a water sample from a sheep farm, and Ec_Unk_ML, Ec_Unk_ML1 and Ec_Unk_MLB were isolated from water samples from Maria Luisa Park, Seville. Ec_Unk_PO1, Ec_Unk_PO2 and Ec_Unk_PO3 were isolated from a chicken farm. A full list of phages is presented in table M2.

Table M2. List of bacteriophages used in this work.

Bacteriophage	Host	Origin
P22	*S. enterica*	Sherwood Casjens lab
Det7	*S. enterica*	Sherwood Casjens lab
9NA	*S. enterica*	Sherwood Casjens lab
T5	*E.coli* MG1655	Pascale Boulanger
Se_F1	*S. enterica*	Seville, Spain (waste water)
Se_F2	*S. enterica*	Seville, Spain (waste water)
Se_F3	*S. enterica*	Seville, Spain (waste water)
Se_F6	*S. enterica*	Seville, Spain (waste water)
Se_ML1	*S. enterica*	Seville, Spain (Maria Luisa Park)
Se_AO1	*S. enterica*	Seville, Spain (Sheep farm)
Ec_Unk_EM	DR28	Seville, Spain (waste water)
Ec_Unk_EM1	DR28	Seville, Spain (waste water)
Ec_Unk_EM2	DR28	Seville, Spain (waste water)
Ec_Unk_AO1	DR28	Seville, Spain (sheep farm)
Ec_Unk_ML	DR28	Seville, Spain (Maria Luisa Park)
Ec_Unk_ML1	DR28	Seville, Spain (Maria Luisa Park)
Ec_Unk_MLB	DR28	Seville, Spain (Maria Luisa Park)
Ec_Unk_PO1	MG1655	Cáceres, Spain (chicken farm)
Ec_Unk_PO2	MG1655	Cáceres, Spain (chicken farm)
Ec_Unk_PO3	MG1655	Cáceres, Spain (chicken farm)

Culture media

Bertani's lysogeny broth (LB) was used as standard liquid medium for *S. enterica* and *E. coli*. Solid LB contained agar at 1.5% final concentration.

	g/l
Tryptone	10
Yeast extract	5
NaCl	10

EBU: rich medium used to discard the presence of lysogenic isolates after transduction. EBU is LB medium supplemented with the following components:

	ml/l
K_2HPO_4 25%	10
Glucose 50%	5
Fluorescein 1%	2.5
Evans Blue 1%	1.25

SOB: rich medium used to grow competent cells during recovery:

	g/l
Tryptone	20
Yeast extract	5
NaCl	0.5
KCl	0.19
pH 7	Adjusted with NaOH
After autoclaving, 5 ml of $MgCl_2$ 2M is added	

NCE: No-carbon essential (NCE) medium was supplemented with glucose (0.2%) or lactose (0.2%) after autoclaving.

	ml/l
50× NCE salts	20
$MgSO_4$ 1M	1

Antibiotics

Antibiotic	Stock concentration (mg/ml)	Final concentration (μg/ml)
Ampicillin	100	100
Chloramphenicol	20	20
Kanamycin	50	50
Tetracycline	12	12
Cefotaxime	40	40

Growth conditions

S. enterica cultures were routinely grown at 37ºC, and exceptionally at 30ºC due to plasmid thermosensitivity. For microaerophilic conditions, 5 ml of bacteria were incubated at 37 ºC without shaking in 10 ml plastic tubes.

Solutions

PBS	
	mM
NaCl	1,370
KCl	27
$Na_2HPO_4 \cdot 7H_2O$	43
KH_2PO_4	14
pH 7.3	

NCE salts 50×	
	g
KH_2PO_4	197
$K_2HPO_4 \cdot 3H_2O$	323
$NaNH_4HPO_4 \cdot 4H_2O$	175

Bacterial transduction

Lysates

To prepare a bacteriophage lysate, 4 ml of bacteriophage broth was mixed with 1 ml of an overnight culture of the host strain. The mixture was incubated at 37 ºC with shaking (200 rpm) for 18 h. Bacterial debris was removed by centrifugation for 20 min at 4,500 rpm. The supernatant was filtered with a filter 0.22 μm pore size and recovered in a fresh tube. 250 μl of chloroform were added, and the mix was vortexed. Lysates were stored at 4 ºC.

Bacteriophage stock (Broth)	
	ml
NB	100
E50x	2
Glucose 20%	1
Bacteriophage	0.1 [10^9 phages/ml]

NB: nutrient broth	
	g/l
Yeast extract	3
Peptone	5

E 50×	
	g/l
$H_3C_6H_5O_7 \cdot H_2O$	300
$MgSO_4$	14
$K_2HPO_4 \cdot 3H_2O$	1,965
$NaNH_4HPO_4 \cdot H_2O$	525

Chemicals were added to 1 l of warm H_2O following the order indicated in the table. Water was added to reach a total volume of 3 l. The medium was cooled and sterilized with chloroform.

Transduction in liquid medium

To carry out transductions in liquid medium, 100 µl of an overnight culture of the recipient strain and 10 µl of the donor strain lysate were mixed in a sterile 1.5 ml tube. This mix was incubated at 37°C and 200 rpm for 30-45 minutes (depending on the marker to be scored). The mix was then spread on selective plates that were incubated at 37°C until colonies appeared.

Detection of lysogenic transductants

Transductant colonies were isolated in EBU plates (with antibiotic if necessary). On these plates, pseudolysogens are dark-colored and P22-free colonies are light colored. This color difference is due to cell lysis in the pseudolysogenic colony, which causes acidification of the medium and turning of the pH indicator, darkening the agar. A transductant was considered P22-free when streaking did not give rise to any dark colony.

P22 sensitivity assay

In EBU plates, isolates that form light color colonies could be lysogens that do not undergo visible lysis. These isolates are P22-resistant and can be mistaken by real P22-free isolates. To avoid this situation, an assay to detect P22-sensitive strains is advisable. A streak with a P22_H5 Lysate is done on an LB or EBU plate, and air-dried. The test strain is then streaked in a perpendicular way to the H5 streak. P22-sensitive strains grow until they reach the H5 streak, while P22-resistant strains grow over the streak.

DNA manipulation and transfer

Plasmids

Plasmids	Characteristics	Reference
pKD46	Lambda Red recombinase, Amp^R	[297]
pKD4	Kanamycin resistance gene flanked by FRT sites	[297]
pCP20	FLP recombinase, temperature sensitive replicon at 37°C, Amp^R, Cm^R	[297]
pET20b	T7 promoter, Amp^R	
pET20b-*wbbl*	T7 promoter, Amp^R. *wbbl* gene under constitutive expression	[305]
pDMS197	Contains lambda pir-dependent R6K replication origin; requires lambda pir-containing bacteria strain. SacB (sucrose sensitivity), Tc^R	[247]
pDMS197-*wbbl*	Tc^R. Containing the *wbbl* gene for chromosome integration	This work
pXG1	P_{LtetO} promoter , gfp fusion cloning vector	[306]
pXG1::*llp*	*Llp* gene under constitutive expression	This work
pIZ2224	pDMS197::P_{opvAB} $GATC_{1,2}$	[123]
pIZ2225	pDMS197::P_{opvAB} $GATC_{1,3}$	[123]
pIZ2226	pDMS197::P_{opvAB} $GATC_{1,4}$	[123]
pIZ2234	pDMS197::P_{opvAB} GATC-less	[123]

Extraction of plasmid DNA

For the extraction of plasmid DNA the Gen Elute™ Plasmid Miniprep Kit was used (Sigma-Aldrich Co., St. Louis, Missouri, USA).

Extraction of genomic DNA

For the extraction of genomic DNA, 5 ml of cells were collected from an exponential culture and re-suspended in 0.4 ml lysis buffer. Four µl of RNAse (10 mg/ml) were added and the mixture was incubated at 37°C for 30 min. After that, 20 µl of proteinase K (20 mg/ml) was added and the sample was incubated for 2 h at 65°C. Finally, 3 or 4 extractions were performed with phenol:chloroform-isoamyl alcohol in a 2:1 proportion. DNA was precipitated at -20°C by adding 1/10 volume of sodium acetate 3 M and 2.5 volumes of ethanol. After precipitation, genomic DNA was washed with 70% ethanol and re-suspended in 20 µl of TER buffer.

Buffer lysis	
	Concentration
Tris-HCl	50 mM pH 8
EDTA	10 mM
NaCl	100 mM
SDS	0.2 %

TER buffer	
	Concentration
Tris-HCl	10 mM pH 7.5
EDTA	1 mM pH 8
RNAse	20 µl/ml

Extraction of bacteriophage DNA

For the extraction of bacteriophage DNA, the Phage DNA Isolation Kit produced by Norgen Biotek Corp. (Schmon Parkway, Thorold, ON, Canada) was used.

Digestion, modification and ligation DNA fragments

Restriction endonucleases were purchased from Roche Diagnostics GmbH (Indianapolis, Indiana, USA), New England Biolabs (Beverly, Massachusetts, USA) and Promega Biotech (Madison, Wisconsin, USA).

For ligation of DNA fragments, 1U of T4 DNA ligase (1 U/ml, Roche Diagnostics) was used in the buffer supplied by the manufacturer. Routinely, the mixture was incubated at 16°C for 12 h or at room temperature 24 h.

For building plasmids with 2 or more inserts, the commercial Gibson Assembly® Master Mix was used following the manufacture's instructions.

Agarose DNA gel electrophoresis

Electrophoresis in an agarose gel was used to test the quality of DNA extraction, to separate DNA fragments after plasmid restriction, to estimate the efficiency of endonuclease restriction, to confirm PCR amplification, etc. The agarose gel was submerged in TAE 1× buffer. Low electro endosmosis agarose (Pronadisa, Conda, Spain) was employed. Its concentration varied between 0.8 and 1.5% depending on the size of the fragments to be separated. The loading buffer used was a solution of bromophenol blue (0.125%) and Ficoll 400 (12.5%).

The 1 Kb ladder (GIBCO BRL, Life Technologies, New York, USA) was used as molecular weight marker. Samples were mixed with 1/10 of loading buffer. SYBR™ Safe DNA Gel Stain (Invitrogen) was added to the gels to make bands visible. Gels were illuminated and pictures were taken with a ChemiDoc™ (Bio Rad).

TAE	
	mM
Tris-acetate	40 mM
EDTA	10 mM pH 7.7

Isolation of DNA fragments from agarose gels

For the isolation of DNA fragments from agarose gels the commercial system Wizard® SV Gel and PCR Clean-Up System was used (Promega Co.).

Bacterial transformation

High efficiency *E. coli* transformation

Competent cells were prepared using a variation of the Inoue method (Inoue et al. 1990), which guarantees high transformation efficiency (5×10^7 to 5×10^8 transformants per μg of plasmid DNA). An overnight culture of *E. coli* DH5α was diluted 100-1000 times in 200 ml of SOB and incubated at 22°C and 200 rpm to reach an OD_{600} around 0.5. The culture was then chilled quickly on ice and kept on it for 10 min. Cells were harvested by centrifugation at 2,500 g and 4°C for 10 min, the pellet was re-suspended in 20 ml of cold TB, and 1.5 ml of DMSO was added. After a 10 min incubation on ice, aliquots of 0.2 ml or 0.5 ml were prepared, frozen in liquid nitrogen, and stored at -80°C.

For transformation, an aliquot of competent cells was slowly thawed on ice and was mixed with the plasmid. The mixture was incubated on ice for 30 min subjected to heat shock (42°C, 45 s), and cooled on ice for 1 min. One ml of LB was then added. The mixture was incubated at 37°C for 1 h; finally, the cells were concentrated in 100 μl and spread on selective media.

TB	
	mM
PIPES	10
$CaCl_2$	15
KCl	250
pH 6.7	Adjust with KOH
$MnCl_2$	55
This solution was sterilized by filtration	

E. coli and *Salmonella* electroporation

The method was the same for *E. coli* and *S. enterica*: an overnight culture was diluted 100 times in LB medium and was incubated at 37°C or 30°C depending on the strain to reach an optical density of 0.6-0.8 approx. The culture was then incubated in ice for 5 minutes. Fifty ml were transferred to a new tube, and the cells were harvested by centrifugation at 4500 rpm for 10 min at 4°C. The supernatant was discarded, and the bacterial pellet was softly re-suspended in 5 ml of cold glycerol 10% prepared in dd H_2O. Afterwards, 45 ml of cold glycerol were added. A second wash step was repeated in the same conditions. Finally, the cells were harvested and re-suspended in 250 µl of glycerol 10%.

Electroporation was done by mixing approx. 600 µg of DNA with 60 µl of competent cells. The mixture was transferred to a cooled cuvette with a 2 mm distance between plates. The mixture was subjected to an electric discharge in the electroporator (2.5 KV, 200 Ω and 25 µF). The electroporator employed was a BTX Electrocell Manipulator 600 (Harvard Apparatus, Holliston, Massachusetts, USA). After the discharge, 1 ml of LB was added to the cells, which were then transferred to a 10 ml plastic tube and incubated at 37°C with shaking (200 rpm) for 1 h. Finally, the cells were concentrated in 100 µl and spread on selective media.

Construction of bacterial strains

Oligonucleotides

Name	Sequence (5'-3')
Amplification of the Km^R gene from pKD4	
Operonlac-PS1	ATGATAGCGCCCGGAAGAGAGTCAATTCAGGGTGGTGAATGTGTAGGCTGGAGATGCTTC
Operonlac-PS2	TAGGCCTGATAAGCGCAGCGTATCAGGCAATTTTTATAATCATATGAATAT
Amplification of the *opvAB* operon (wild type and variants) for integration in *E. coli*	
MG1655-opvA	ATGATAGCGCCCGGAAGAGAGTCAATTCAGGGTGGTGAATTCATTTGGTTATAAATAGAG
MG1655-opvB	TAGGCCTGATAAGCGCAGCGTATCAGGCAATTTTTATAATGAGTTTATCTCTGCGCAATGT
Amplification of *gfp* and Cm^R genes from pZEP07	
pCE40lacY-gfp-5	GCTTTCCCTGCTGCGTCGTCAGGTGAATGAAGTCGCTTAATAAGAAGGAGATATACATATGAG
pCE40-km-3	AAACTGTCTGCTTACATAAACAGTAATACAAGGGGTGTTTTATCACTTATTACAGGCGTA
Amplification of the *aac6´1b-cr* gene	
opvA-BI-aac6-F	TCTTATGAAGAAATATACGTTCGCTAAGGAGGTTTTCTAATGAGCAACGCAAAAACAAAG
opvA-D25-acc6	TTATGTGTGGGTTTTATCTTATGAAGAAATATACGTTCGCTCGGGAGGGTTTCTAATGAGCAACGCAAAAACAAA
aac6-gfp-R	AAAGTTCTTCTCCTTTACTCATATGTATATCTCCTTCTTATTAGGCATGACTGCGTGTTC
Amplification of the $bla_{CTX\text{-}M\text{-}15}$ gene	
opvA-ctxm-F	TCTTATGAAGAAATATACGTTCGCTAAGGAGGTTTTCTAATGGTTAAAAAATCACTGCG
ctxm-gfp-R	AAAGTTCTTCTCCTTTACTCATATGTATATCTCCTTCTTATTACAAACCGTCGGTGACGA
Amplification of the *aac3* gene	

PopvA-BI-aac3-F	ATCTTATGAAGAAATATACGTTCGCTAAGGAGGTTTTCTAATGCATACGCGGAAGGCAATAAC
opvA-D25-acc3	TTATGTGTGGGTTTTATCTTATGAAGAAATATACGTTCGCTCGGGAGGGTTTCTAATGCATACGCGGAAGGCAATAACGGAGGCG
GFP-aac3-R	GAAAAGTTCTTCTCCTTTACTCATATGTATATCTCCTTCTTACTAACCGGAAGGCTCGCAAG
Deletion of the *lac* operon in *E. coli* MG1655	
Op.lac-PS1	ATGATAGCGCCCGGAAGAGAGTCAATTCAGGGTGGTGAATGTGTAGGCTGGAGCTGCTTC
Op.lac-PS2	TAGGCCTGATAAGCGCAGCGTATCAGGCAATTTTTATAATCATATGAATATCCTCCTTAG
Verification of the deletion of the *lac* operon	
Op.lac-E1	GGCGCAAACTGTTAATGCTG
Op.lac-E2	CGCAATGACCATTGAACAGG
Deletion of IS-50	
IS50-PS1	CATGAAGCATGATGATTTGCTGACATATATTAAATATGTCGTGTAGGCTGGAGCTGCTTC
IS50-PS2	GATCCTGCGCACCAATCAACAACCGTATCAGAATAGATACCATATGAATATCCTCCTTAG
Cloning of the *wbbl* gene onto plasmid pDMS197	
SacI-wbbl-F	AAAGAGCTCATGGTATATATAATAATCGTTTCCCACGGAC
XbaI-wbbl-R	AAATCTAGATTACGGGTGAAAAACTGATGAAATTCGATC
Verification of *wbbl* restoration	
Wbbl-E1	ATTCTAATAACGATGACATG
Wbbl-E2	CGCTGAATGCTCGCGGCCTG
Assembly of *llp* into pGX1 plasmid	
pXG1 RBS GFP F	TAAGAAGGAGATATACATATGGCTAGCAAAGGAGAAGAAC
pXG1- Llp-R	ATTTTTTCATGGTACCTTTCTCCTCTTTAATG
Llp-F	GAAAGGTACCATGAAAAAATTATTTTTAGCTATGGC
Llp-R	TGCTAGCCATATGTATATCTCCTTCTTATTAGAAAACTCCCTCGCATG

Construction of P_{opvAB}::*Llp*::*gfp*	
PopvAB-BI-Llp-F	TTATGTGTGGGTTTTATCTTATGAAGAAATATACGTTCGCTAAGGAGGTTTTCTAATGAAAAAATTATTTTTAGC
GFP-Llp-R	AAAGTTCTTCTCCTTTACTCATATGTATATCTCCTTCTTATTAGAAAACTCCCTCGCATG
opvAB gfp fusions	
PopvAB-GFP-F2	TCTTATGTGTGGGTTTTATCTTATGAAGAAATATACGTTCTAAGAAGGAGATATACATAT
STM2208stop-GFP-5	CGCTAACAGAATATCGTATTGAGAAAAAGACAATGAATGACCGCGCATGATAAGAAGGAGATATACATATGAG
STM2208stop-GFP-3	ACTTTTACTCTTCGACACATTTCAGCGCAGAGTTTATCTCTGCGCAATGTTTATCACTTATTCAGGCGTA
Cloning of an *amp-sacB* counterselection cassette	
opvA-kansacB F	TCTTATGTGTGGGTTTTATCTTATGAAGAAATATACGTTCGCTAAGGAGGTTTTCTAATGTAGGCTGGAGCTGCTTC
kansacB-GFP R	AAAGTTCTTCTCCTTTACTCATATGTATATCTCCTTCTTACATATGAATATCCTCCTTAGTTCC

Polymerase chain reaction (PCR)

For PCR reactions, a BIO-RAD T100™ Thermal Cycler thermocycler was used. PCR reactions were carried out with 1 ng of DNA, 100 µM of dNTPs (final concentration each), 1 µM of oligonucleotides, 1 mM of $MgCl_2$, and 1 U of Taq polymerase per reaction in a final volume of 100 µl. The polymerase used in these reactions was Taq Expand™ High Fidelity PCR System supplied by Roche Diagnostics GmbH.

To confirm clones and mutations, colony PCR was performed using Go Taq® Flexi DNA Polymerase (Promega Co.). In these cases, a mixture with 100 µM of dNTPs each one, 0.2 µM of oligonucleotides, 1 mM of $MgCl_2$ as final concentration and 1U of Taq polymerase per reaction in a final volume of 25 µl was prepared. A colony was re-suspended in this mixture and was used as DNA template.

Before using a PCR product in further experiments, oligonucleotides and dNTPs were removed using the commercial Wizard® SV Gel and PCR Clean-Up System (Promega Co).

Chromosomal gene disruption using PCR products

To obtain knockout mutants of chromosomal genes, the Datsenko and Wanner method was used [297]. This method is based in the λ Red recombination system. One of the reasons why *E. coli* and *Salmonella* are not transformable with linear DNA is due to the presence of intracellular exonucleases that degrade it. The λ Red system harbors *α, δ* and *exo* genes that encode the proteins Gam, Bet and Exo, respectively. Gam inhibits host exonuclease V, allowing the Bet and Exo proteins to carry out recombination of the DNA. The strategy consists in replacing the chromosomal sequence (**Figure M1**) by an antibiotic resistance marker that is generated by PCR using oligonucleotides that harbor 40 nucleotides of homology with the sequence to be replaced (H1 and H2 in **Figure M2**). λ Red recombination gene expression is carried out under an inducible promoter of thermosensitive low copy number plasmid (pKD46). After selection, the gene resistant marker can be removed using a different plasmid (pCP20) that harbors the FLP recombinase of the 2 μ plasmid from *Saccharomyces cerevisiae.* The FLP system acts over FRT repeats ("FLP recognition target") that flank the sequence (**Figure M1**). Plasmids that harbor Red and FLP systems are thermosensitive and can be cured by growing the cells at 37°C.

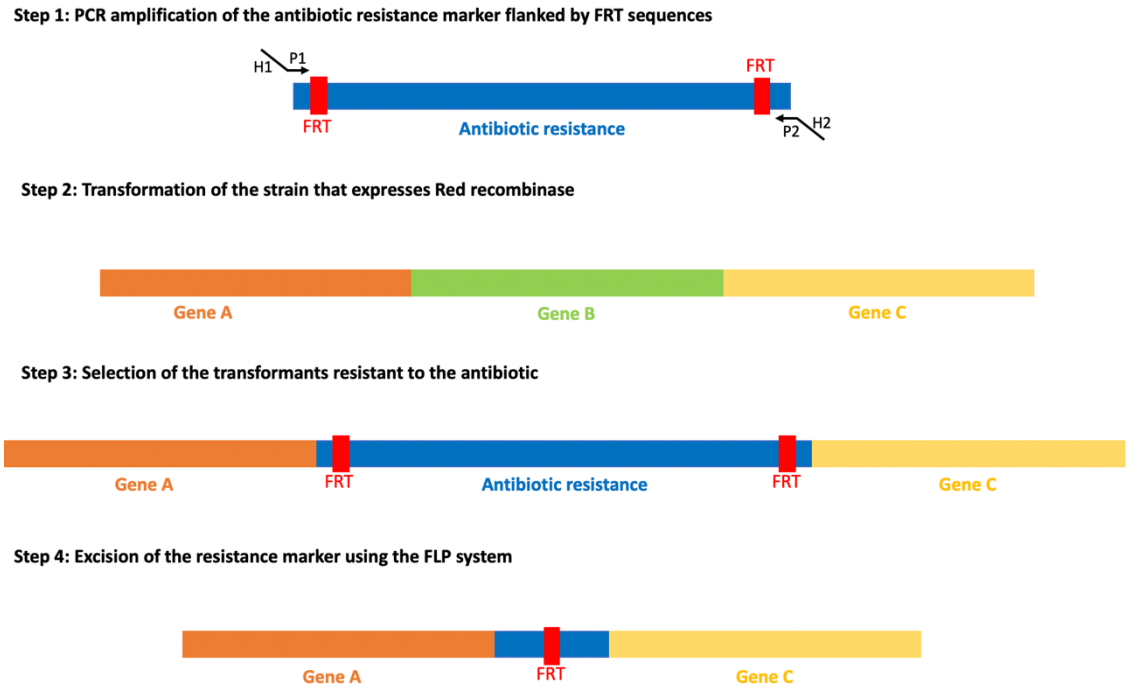


Figure M1. **Diagram of the inactivation system by PCR**. H1 and H2 are DNA sequences homologous to the target. P1 and P2 are the homology sequences that flank the antibiotic gene sequence. Figure adapted from [297].

Preparation of DNA for substitution

The plasmids used as templates in PCR reactions were pKD3 (Cm^R), pKD4 (Km^R) and pKD13 (Km^R). The oligonucleotides used had 40 nucleotides that were homologous to the genomic DNA and 20 nucleotides that were homologous to pKD3, pKD4 and pKD13 (Table M5). PCR reactions were carried out at an annealing temperature of 55°C for 2 min. For the DNA fragment extension, the enzyme employed was the Taq Expand™ High Fidelity PCR System (Roche Diagnostics GmbH). The PCR product obtained was subjected to an agarose gel electrophoresis and the amplified fragment was purified using the commercial system Wizard® SV Gel and PCR Clean-Up System (Promega Co).

Cell transformation

Competent cells of the wild type strain, which harbored the pKD46 plasmid, were prepared. This plasmid expresses the λ Red system under the control of the *araB* promoter, which is inducible by arabinose. Cultures grown in LB with ampicillin at 30°C were diluted 1:100 in LB with ampicillin and arabinose (1 mM) and were incubated in a shaker at 30°C until they reached an OD_{600} of approx. 0.5. Competent cells were prepared, and electroporation was done as described previously.

Excision of the resistance marker

After the substitution of genomic genes with antibiotic resistance cassettes (Km or Cm), mutations were transferred to appropriate genomic backgrounds by transduction with P22 and selection on appropriate media. When necessary, the resistance marker of the host was excised by transducing plasmid pCP20 with phage P22 HT. The transduction mixture was incubated at 30°C for 1 h and spread on LB with ampicillin. To eliminate the plasmid, incubation on EBU plates without antibiotic was performed at 37°C. To confirm the excision of the marker, the isolates were streaked on plates of LB ampicillin and plates of LB with chloramphenicol or kanamycin. Excision of the antibiotic marker was confirmed by colony PCR using external oligonucleotides.

Strain construction by transductional transfer of genetic markers

Genetic markers were transferred from one strain to another by transduction. The recipient strain was transduced using a P22 lysate from a strain with the desirable genetic marker. All the markers used were selected directly by spreading the transduction mixture on selective media. If necessary, acquisition of the marker by the transductant was confirmed by PCR or phenotypic analysis.

Construction of *lac* fusions

The method described by Ellermeier et al. was used to construct transcriptional and translational fusions (Ellermeier et al. 2002). This method allows the construction of *lac* fusions using the FLP/FRT recombination system. As described previously in the method developed by Datsenko and Wanner [297]), a selectable marker (e.g., conferring antibiotic resistance) can be inserted at any place in the bacterial chromosome. The insertion is flanked by two FRP sites, and the marker can be removed using the plasmid pCP20, that harbors the FLP system. As a result of the excision there is one FRT site only at the insertion place (**Figure M1**). This FRT site can be used to insert a plasmid that harbors a FRT site upstream of the *lacZY* genes, yielding a transcriptional or translational fusion, depending on the plasmid chosen. Ellermeier et al. constructed three different plasmids: pCE36 and pCE37 for transcriptional fusions, and pCE40 for translational fusions. Plasmid pCE40 lacks the ribosomal binding site of *lacZ* and has STOP codons in all the frames, except one. These plasmids harbor a Km resistant gene and the replication origin of R6K (which is active when the protein π is present). Choice of plasmid depended on the orientation of the FRT in the original mutation (**Figure M2**).

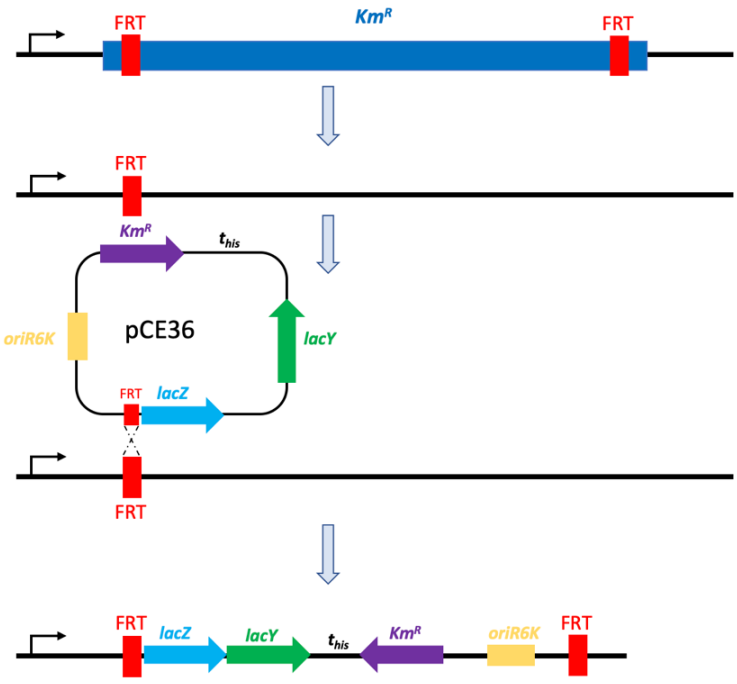


Figure M2. **Diagram of construction of a *lac* fusion on the chromosome**. As an example, construction of a translational fusion using the plasmid pCE36 is shown [298].

Calculation of phase variation rates

Phase variation rates were estimated as described by Eisenstein [307]. Briefly, a strain harboring a *lacZY* fusion was plated on LB+X-gal and colonies displaying an ON or OFF phenotype after 16 h growth at 37ºC were selected, resuspended in PBS and re-spread on fresh LB+X-gal plates. Phase variation frequencies were calculated using the formula (M/N)/g where M is the number of cells that underwent phase variation, N the total number of cells, and g the total number of generations that gave rise to the colony.

Flow cytometry

Bacterial cultures were grown in LB at 37°C until exponential phase ($O.D_{600}$ ~0.3). Cells were then diluted in PBS to a final concentration of ~10^7/ml. Data acquisition and analysis were performed using a Cytomics FC500-MPL cytometer (Beckman Coulter, Brea, CA, USA). Data were collected for 100,000 events per sample and were analyzed with CXP and FlowJo 8.7 software. Data are represented by a dot plot (forward scatter [cell size] versus fluorescence intensity).

Growth curves

Overnight cultures were diluted into a total volume of 200 µl per well. Plates were incubated at 37°C with shaking on an automated microplate reader (Synergy HTX Multi-Mode Reader, Biotek) and the absorbance at 600 nm was measured every 30 min for each well. The duration of each assay was 20 h. Assays were performed in triplicate. In P_{opvAB}*::lacZY* assays, minimal medium NCE with lactose (0.2%) or glucose (0.2%) was used. In the case of antibiotic growth curves LB medium and LB medium with antibiotics were used.

Electrophoretic visualization of LPS profiles

To investigate LPS profiles, bacterial cultures were grown in LB overnight. Bacterial cells were harvested and washed with 0.9% NaCl. The $O.D._{600}$ of the washed bacterial suspension was measured to calculate cell concentration. A bacterial mass containing about 3×10^8 cells was pelleted by centrifugation. Treatments applied to the bacterial pellet, electrophoresis of crude bacterial extracts, and silver staining procedures were performed as described by Buendia-Claveria *et al*. [308].

Phage isolation from water samples

Samples of water were filtered using a filter Millex-GS 0.22 μm filter pore. Each sample was divided into two samples, and chloroform was added to one sample. The filtered sample of water (1 ml) was added to an overnight culture of a host bacterial strain (0.5 ml). The mixture was supplemented with 3.5 ml of LB and was incubated overnight at 37ºC without shaking. The culture was then centrifuged at 5,000 rpm during 5 minutes. The supernatant was filtered using a 0.22 μm filter in order to save only phages.

A 50 μl aliquot from an overnight culture of the target bacterial strain was spread on LB plates, and lysates were dropped onto the surface. The plates were left to dry and were inspected for lysis zones after an overnight incubation at 37°C. The spot assay was used to assess the bactericidal ability of the phage lysates. Isolation of phage was done by the double agar layer method using *Salmonella* or *E. coli* as bacterial host. Isolated plaques were suspended in LB and were streaked in plates which contained a bacterial layer in order to isolate a pure preparation of phage. Phages were characterized on the basis of plaque morphology and host range. Moreover, some phage genomes were sequenced studied by electron microscopy.

Detection of phage from a crude sample of water

A tube with 3.5 ml of LB, 0.5 ml of an overnight culture of SV6727 strain (*opvAB::gfp*) and 1 ml of a 0.22 μm filtered water sample was incubated for 7 hours in static condition. The culture was diluted at a ratio of 1/200 in LB medium and incubated at 37°C under shaking (200 rpm) for 3 hours. Samples were diluted in PBS. Data acquisition and analysis were performed using a Cytomics FC500-MPL cytometer (Beckman Coulter, Brea, CA). Data were collected for 100,000 events per sample and analyzed with CXP and FlowJo 8.7 software. The existence of phages in water was checked simultaneously by plating 1 ml of water on a plate with *S. enterica* poured in soft-agar.

Electron microscopy

Electron microscopy was performed at the Mediterranean Institute of Microbiology (IMM) facility. Phage suspensions (5 µl) were dropped onto a copper grid covered with Formvar and carbon and left 3 min. at 25°C, after what the excess of liquid was removed. Phage particles were stained according to [154] with a 2% uranyl acetate solution. Dried grids were then observed using a transmission microscope FEI Tecnai 200 kV coupled to a Eagle CCS 2kx2k camera. Images were then analyzed using the ImageJ software.

Bacteriophage detection

For measurements by flow cytometry, bacterial cultures were grown at 37°C in LB (5 ml) containing phages (100 µl of phage lysate [10^8-10^{10} PFU]). Culture was diluted 1/100 in LB+phages and was incubated until exponential phase ($O.D_{600}$ ~0.3) before flow cytometry analysis.

For construction of growth curves, overnight bacterial cultures were diluted 1:100 in 200µl LB. Five µl of a bacteriophage lysate were added (10^8-10^{10} PFU). $O.D_{600}$ and fluorescence intensity were subsequently measured at 30 min intervals using a Synergy™ HTX Multi-Mode Microplate Reader from Biotek.

Epsilometer (E) tests of antibiotic resistance

Etest strips were purchased from bioMérieux. Mixes of overnight cultures of bacteria grown in MH broth were diluted 1:25 in phosphate buffered saline (PBS) to reach cell densities of 0.5 MacFarland or about 1.5×10^8 CFU/ml. Bacteria were plated onto MH agar plates using sterile cotton swabs dipped in the cell suspensions, and an Etest strip was applied on top. Plates were incubated 18 h at 37°C before reading the results and taking pictures.

Population analysis profile (PAP) tests

PAP tests were performed on MH agar plates supplemented with increasing amounts of kanamycin (Sigma Aldrich) as described elsewhere [309]. Five µl of overnight cultures in MH broth (containing approx. 3×10^9 cells/ml) and serial dilutions (down to 10^{-6}) were spread on MH plates containing no antibiotics (for total CFU determination) or

different concentrations of kanamycin. The plates were incubated overnight and the colonies were counted. Colony numbers were plotted in a graph to determine if the PAP fulfilled the criteria for heteroresistance (at least 8-fold difference in antibiotic concentration between the highest non-inhibitory concentration and the highest inhibitory concentration).

To prepare mixtures of resistant and susceptible cells, three isolated colonies of SV9776 (P_{opvAB}*::aac3::gfp*, kanamycin resistant in the ON state) and SV9777 (P_{opvAB}*::gfp*, kanamycin sensitive) were grown overnight in 2 ml MH broth at 37°C under shaking. Pure cultures of each overnight or three independent sets of SV9776:SV9777 mixtures at proportions ranging from 1:10 to 1:10,000 were used for PAP tests.

Secuencing of bacteriophage DNA

Nextera-XT kit from Illumina was used to construct the libraries. The input DNA was diluted in water at a final concentration of 0.2 ng/µl, verified by Qubit dsDNA-HS assays. A MiSeq v2 flow cell was used to generate pair-end reads (2 x 150 bp). *De novo* assembly was achieved with SPADES using the the FASTQ files generated by the MiSeq sequencer. Phage genomes were then annotated with RAST (http://rast.theseed.org/FIG/rast.cgi).

RESULTS

CHAPTER 1: A portable epigenetic switch for reversible formation of bacterial subpopulation

Bistable expression of lacZY under opvAB transcriptional control

The ability of the *opvAB* epigenetic switch to confer bistable expression to a heterologous locus was tested by engineering a strain that harbored the *E. coli lacZY* operon downstream of the *opvAB* promoter and its upstream regulatory region (**Figures C1.1A** and **C1.1B**). To avoid cell-to-cell heterogeneity associated with variations in plasmid copy numbers, the construct was engineered in the *S. enterica* chromosome. Construction involved replacement of the *opvAB* coding region with a promoterless *lacZY* operon, leaving the *opvAB* promoter and upstream regulatory region intact. The construct harbored the *opvA* ribosome binding site (RBS). Plating of the engineered strain on LB containing X-gal yielded Lac^+ (blue) and Lac^- (white) colonies, thus revealing bistable expression of the heterologous *lacZY* operon (**Figure C1.1C**). Streaking of either Lac^+ or Lac^- colonies on X-gal agar yielded a mixture of Lac^+ and Lac^- colonies, thus indicating the occurrence of reversible bistability ("phase variation") as previously described for the native *opvAB* locus [309].

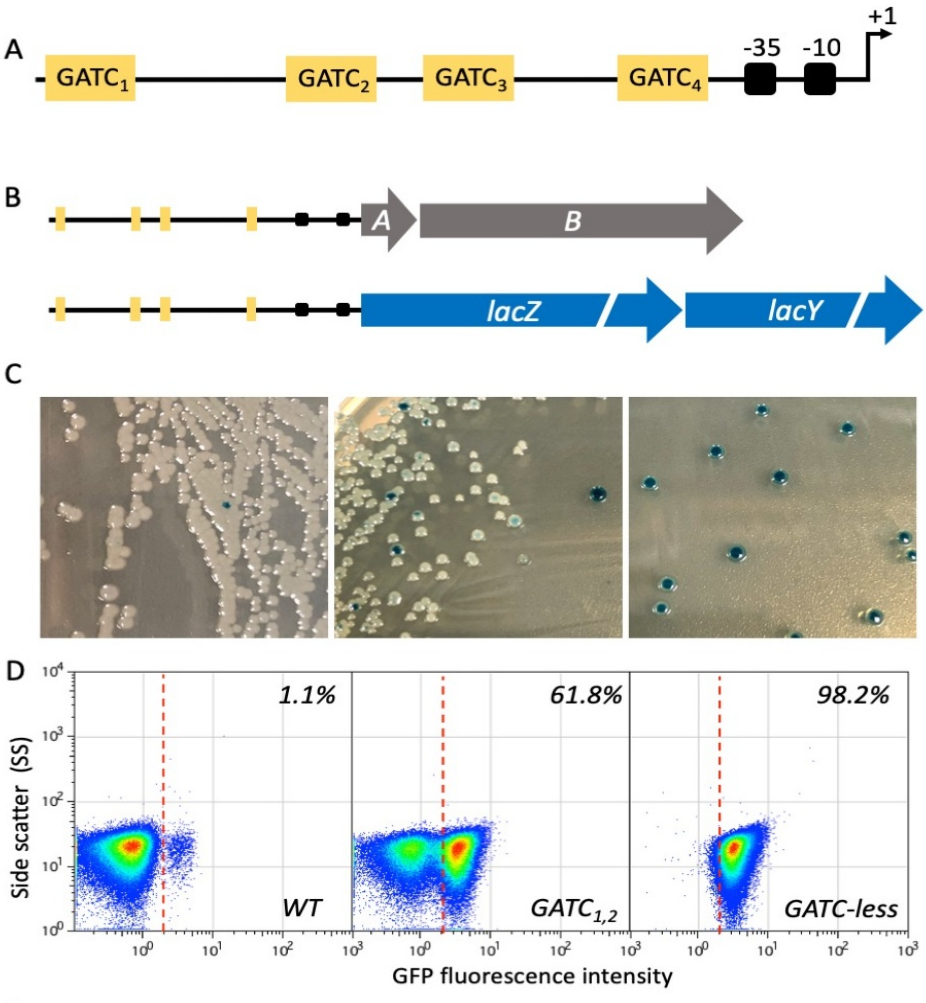
Calculation of phase variation frequencies indicated a frequency of $1.1 \times 10^{-4} \pm 0.3$ per cell and generation for the OFF→ON transition, and $3.4 \pm 0.1 \times 10^{-2}$ per cell and generation for the ON→OFF transition. The 300-fold difference between switching rates was two fold lower than in the native *opvAB* locus (OFF → ON, $6.1 \pm 1.7 \times 10^{-5}$; ON → OFF $3.7 \pm 0.1 \times 10^{-2}$; 600-fold difference between switching rates)[115]. Increased size of the Lac^{ON} subpopulation may result from multiple factors including potential differences in mRNA stability and codon usage constraints.

Variants of the *opvAB::lacZY* construct were engineered to further explore the ability of *opvAB*-driven transcription to confer bistable expression to a heterologous locus. One such variant involved the use of a mutant *opvAB* regulatory region lacking GATC sites 1 and 2 ($GATC_{1,2}$), previously shown to increase the size of the $OpvAB^{ON}$ subopulation[310]. As expected, a higher proportion of Lac^+ colonies were detected (**Figure C1.1C**). Another variant, used as control, lacked all *opvAB* GATC sites (GATC-less) and locked *lacZY* transcription in the ON state (**Figure C1.1C**) as previously described for the native *opvAB* operon [115].

Variants carrying a green fluorescent protein gene (*gfp*) downstream of the *lacZY* operon were also engineered, and assessment of subpopulation sizes by flow cytometry confirmed that the Lac^{ON} subpopulation formed by the wild type *opvAB* switch was smaller than that

formed by the $GATC_{1,2}$ variant (**Figure C1.1D**). Furthermore, only cells in the OpvAB::LacZY^{ON} state were detected in the strain that harbored the GATC-less construct, and subpopulation formation was abolished as above (**Figure C1.1D**).

The ability of the *opvAB* switch to permit selection of one of the subpopulations was examined by testing the ability of strains carrying *opvAB::lacZY::gfp* and *opvAB* $GATC_{1,2}$*::lacZY::gfp* constructs to grow in minimal medium with lactose as sole carbon source. As above, a strain carrying the GATC-less *opvAB::lacZY::gfp* construct was included as a control. Assessment of the growth patterns of the strains under study revealed that the time required for culture saturation was dependent on the size of the OpvAB::LacZY^{ON} subpopulation present at the start of the culture (**Figure C1.1E**). Reversibility of the OpvAB::LacZY^{ON} state was confirmed by growth on NCE-glucose (**Figure C1.1F**).



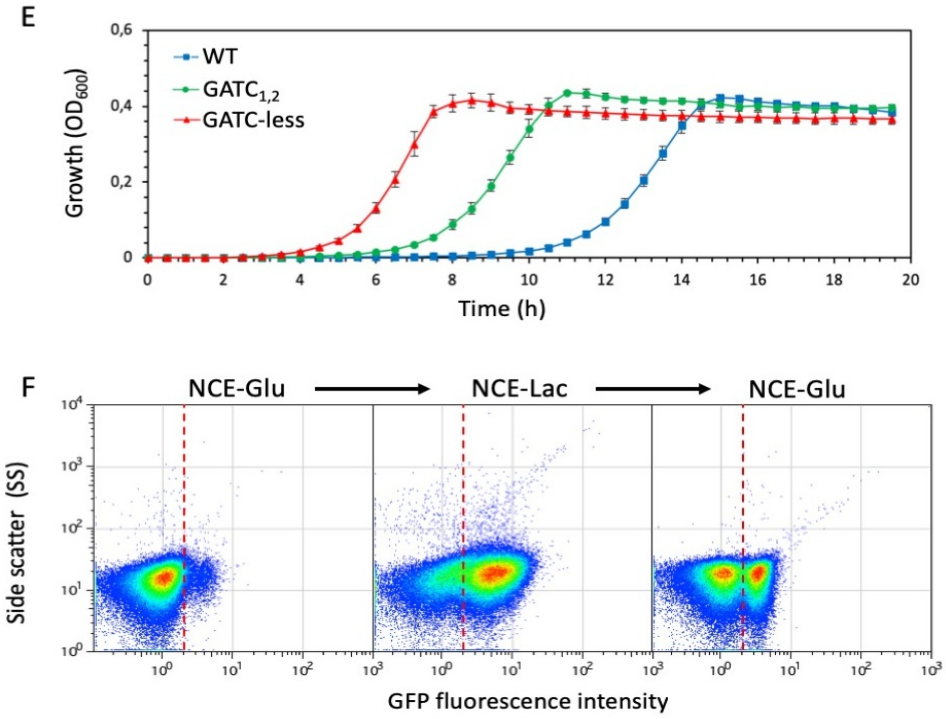


Figure C1.1. Formation of LacZY^{OFF} and LacZY^{ON} subpopulations under *opvAB* control. A. Diagram of the *opvAB* promoter and regulatory region, with the GATC sites outlined. **B.** Diagram of the wild type *opvAB* operon and the P_{opvAB}*::lacZY* construct. **C.** Colonies formed on LB + X-gal by a *S. enterica* strain carrying the *lacZY* operon under the control of the wild type *opvAB* control region (SV9700, left), and by *S. enterica* strains carrying the *lacZY* operon under the control of mutant *opvAB* control regions (SV9701, P_{opvAB} $GATC_{1,2}$*::lacZY::gfp,* center; SV9702, P_{opvAB} GATC-less*::lacZY::gfp,* right). **D**. Flow cytometry analysis of P_{opvAB}*::lacZY* expression in strains SV9700, SV9701 and SV9702. The sizes of LacZY^{ON} subpopulations are indicated. **E**. Growth of strains SV9700, SV9701 and SV9702 in NCE-lactose. **F.** Reversible formation of LacZY^{OFF} and LacZY^{ON} subpopulations under *opvAB* control in strain SV9700.

Bistable expression of the chimaeric *opvAB::lacZY* operon in a heterologous host, *E. coli*

The ability of the *opvAB* switch to operate in a heterologous host was tested in *E. coli*. For this purpose, the *opvAB::lacZY::gfp* construct and its $GATC_{1,2}$ and GATC-less variants were introduced into the chromosome of *E. coli* DR3 (Δ*lacZY*). Strains carrying the *opvAB::lacZY::gfp* and *opvAB* $GATC_{1,2}$*::lacZY::gfp* constructs (DR22 and DR23, respectively) formed Lac$^+$ and Lac$^-$ colonies on X-gal agar plates, and the number of Lac$^+$

colonies was higher in the strain carrying the *opvAB* GATC$_{1,2}$*::lacZY::gfp* construct. The strain carrying the GATC-less construct (DR24) formed Lac^{+} colonies only (**Figure C1.2A**). Flow cytometry assessment of *gfp* expression upon growth in LB confirmed the occurrence of subpopulations of Lac^{OFF} and Lac^{ON} cells in the strains carrying the *opvAB::lacZY::gfp* and *opvAB* GATC$_{1,2}$*::lacZY::gfp* constructs but not in the strain carrying the GATC-less construct (**Figure C1.2B**). Altogether, these observations indicated that the *opvAB* switch is functional in *E. coli.*

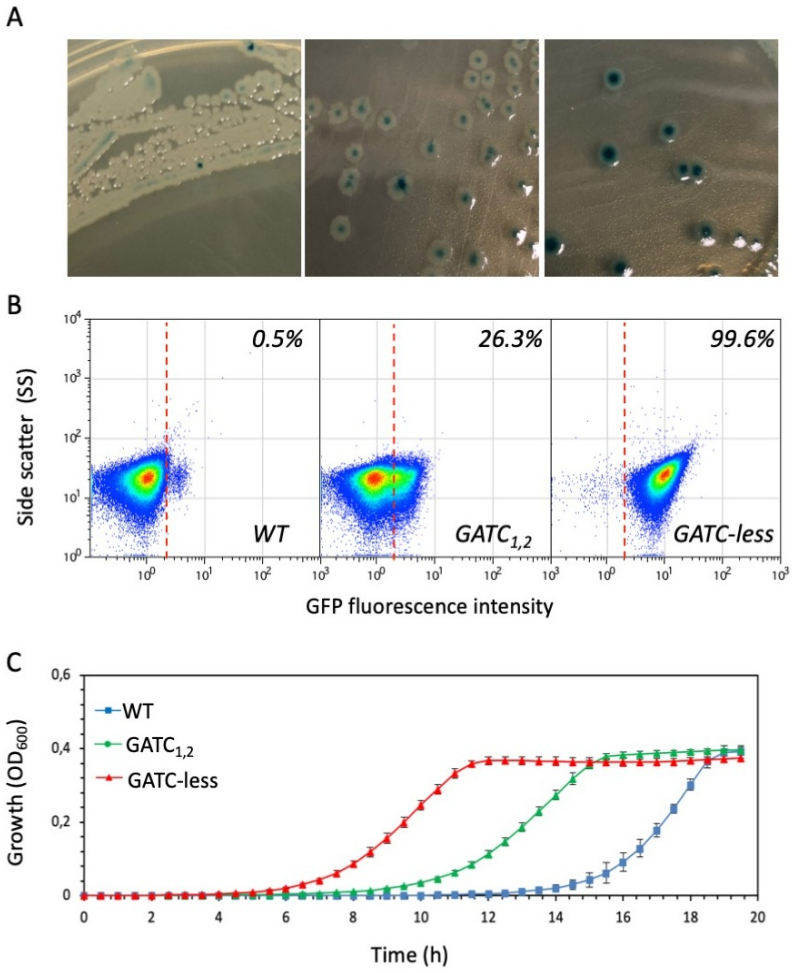


Figure C1.2. Formation of LacZY^{OFF} and LacZY^{ON} subpopulations under *opvAB* control in *E. coli*. **A.** Left: Colonies formed on LB + X-gal by an *E. coli* strain carrying the *lacZY* operon under the control of the wild type *opvAB* control region (strain DR22). Center and right: Colonies formed on LB + X-gal by *E. coli* strains carrying the *lacZY* operon under the control of mutant *opvAB* control regions (DR23 and DR24). **B**. Flow cytometry analysis of *opvAB::lacZY* expression in strains DR22, DR23 and DR24. The sizes of LacZY^{ON} subpopulations are indicated. **C**. Growth of strains DR22, DR23 and DR24 in NCE-lactose.

CHAPTER 2: Design and optimization of an epigenetic switch for the study of antibiotic heteroresistance

Bistable expression of antibiotic resistance genes under *opvAB* control

An additional test of the ability of the *opvAB* bistable switch to generate bacterial subpopulations was performed by cloning antibiotic resistance genes downstream of the *opvAB* promoter in the *S. enterica* chromosome. The antibiotic resistance genes chosen for these experiments were *aac3-Ib* (henceforth, *aac3)* and *aac(6')-Ib-cr* (henceforth, *aac6*), which encode aminoglycoside acetyl transferases [311], and $bla_{\text{CTX-M-15}}$ (henceforth, *ctxM)*, which encodes an extended-spectrum β-lactamase [312]. In these constructs, the native ribosome binding sites were replaced with a stronger RBS, named BI [313] to adjust the sensitivity of the switch to a level that could permit unambiguous detection of the antibiotic resistance phenotype under study, thus facilitating discrimination between OFF and ON cells. Experiments with strains carrying P_{opvAB}::*aac6*::*gfp* and P_{opvAB}::*ctxM*::*gfp* fusions (strains SV9703 and SV9706, respectively) yielded bacterial subpopulations resistant to kanamycin and to cefotaxime, respectively (**Figure C2.3A**). Controls using strains that constitutively expressed *acc6* and *ctxM* (SV9705 and SV9707, respectively) showed that the concentrations of antibiotics used permitted growth (**Figure C2.3A**). The wild type strain ATCC 14028 failed to grow under such conditions, confirming that the concentrations of antibiotics used were bactericidal.

Flow cytometry analysis confirmed that growth in the presence of kanamycin and cefotaxime was a consequence of subpopulation selection (**Figure C2.3B**), excluding the idea that growth might result from selection of mutants present in the inoculum. This conclusion was further strengthened by the observation that growth in LB restored the initial sizes of ON and OFF subpopulations (**Figure C2.3B**).

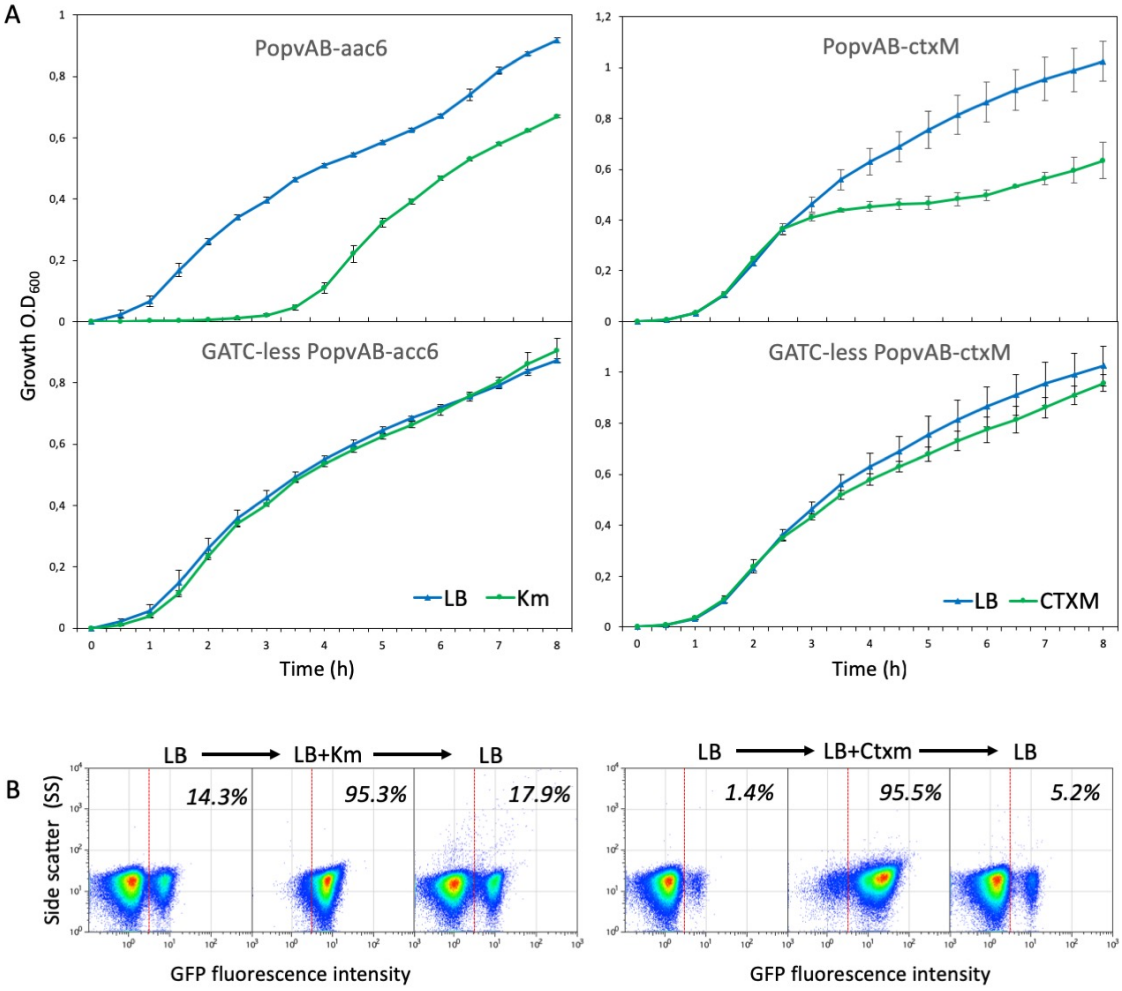


Figure C2.1 A. Growth of strains SV9703 (P_{opvAB}::*aac6::gfp*), SV9705 (P_{opvAB} GATC-less::*aac6::gfp*) SV9706 (P_{opvAB}::*ctxM::gfp*) and SV9707 (P_{opvAB} GATC-less::*ctxM::gfp*) in LB and in LB + antibiotic (kanamycin and cefotaxime, respectively). **B.** Left: reversibility of formation of Km^S and Km^R subpopulations under *opvAB* control. Right: reversibility of formation of $CtxM^S$ and $CtxM^R$ subpopulations.

Use of OpvAB synthetic switches for modulation of exponential growth

A strain carrying the construction P_{opvAB}*::aac6* showed phase variation as described above. In this strain, the *opvAB* promoter drives the expression of *aac6*, which encodes an aminoglycoside acetyl transferase. Thus, the $Aac6^{ON}$ subpopulation is able to grow in

presence of kanamycin. Because mutations in GATC sites of the *opvAB* promoter alter OFF and ON subpopulation sizes, we tested whether strains carrying these mutations showed different growth patterns in the presence of kanamycin. Relevant observations were as follows:

(i) A strain carrying the construction P_{opvAB}*::aac6* reached a density of 0.6 in LB + kanamycin after 524 min, while strains carrying $GATC_{1,3}$, $GATC_4$ and $GATC_{1,2,3,4}$ mutations reached the same absorbance after 428, 356 and 300 min, respectively. An interpretation is that the growth rate is higher when the ON subpopulation is larger.

Because the conversion rate from the ON state to the OFF state is much higher than the conversion ratio from OFF to ON, during growth in the presence of kanamycin most of the offspring switch to the OFF state and therefore die by the antibiotic effect. The slowdown of growth is thus explained by death of cells in OFF state. In other words, growth in the presence of kanamycin is slower or faster depending on the ON subpopulation size.

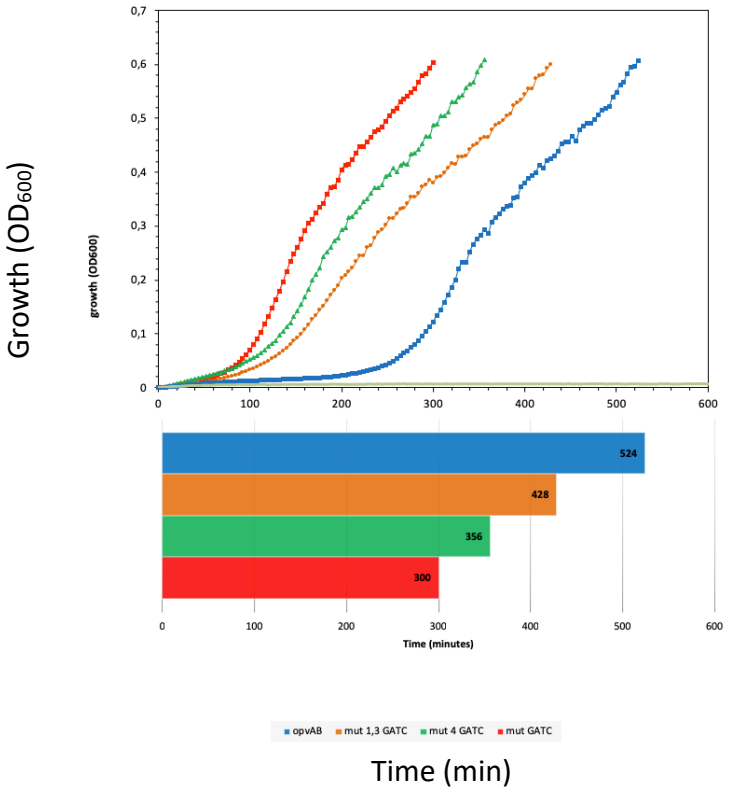


Figure C2.2. Growth of strains SV9703 (P_{opvAB}::BI-*acc6::gfp*), SV10048 (P_{opvAB} $GATC_{1,3}$::BI-*aac6::gfp*) SV10049 (P_{opvAB} $GATC_4$::BI-*aac6::gfp*) and SV9705 (P_{opvAB} GATC-less::*acc6::gfp*) in LB + kanamycin. Time each strain takes to reach an optical density of 0.6 in the presence of kanamycin represented in a bar chart.

Use of different ribosome binding sites (RBS) to optimize gene expression

The *opvAB* operon in OFF state has a basal expression that is difficult to detect in the native *opvAB* operon (in other words, OFF cells are truly OFF). However, basal expression can be detected in other constructs: for instance, when the *opvA* and *opvB* genes are replaced with other genes such as the aminoglycoside resistance gene *aac6* (**Figure C2.3**), OFF cells show some degree of kanamycin resistance which may blur the difference between OFF and ON cells. This problem was overcome by modification of the ribosome binding site. In an initial trial, we cloned the *aac6* gene downstream of the *opvAB* promoter, the BI RBS and the first (5') 20 nucleotides of *opvA*. For replacement of the *aac6* ribosome binding site, we used information described by Mutalik et al. on RBS optimization [313]. Because the BI ribosome binding site is strong and the Etest allows to discriminate small differences in antibiotic resistance levels, basal expression of *aac6* gene in the OFF population was high enough to change the resistance level of the OFF subpopulation by increasing its MIC over that of the wild type lineage. This construct was therefore useless.

In order to reduce the translation rate of *aac6* RNAm, a second construction was designed using a weaker RBS named D25 [314]. Because D25 is not a strong RBS, translation is decreased and the basal expression of *aac6* in the OFF subpopulation is not enough to produce the protein concentrations that permit phenotypic change. The adjustment of RNAm translation in order to obtain the desired OFF phenotype counteracting the effects of the basal expression of the OFF state might help to adjust the phenotype depending on the use. In the tests shown in **Figure C2.3**, the wild type strain ACC14028 showed a MIC about 1.5 µg/ml. The strain carrying the construction P_{opvAB}*::*BI-*aac6* showed phase variation where the OFF subpopulation presents a MIC up to 8 µg/ml. In the case of P_{opvAB}*::*D25-*aac6,* the strain showed two resistance levels, confirming the existence of phase variation: an OFF subpopulation with MIC about 1.5 µg/ml, like in the case of the wild type strain, and an ON subpopulation with a MIC of about 16 µg/ml. Note that in a strain carrying the P_{opvAB}*::*BI-*aac6* construct the MIC of the ON subpopulation exceeds the upper limit of detection of the Etest.

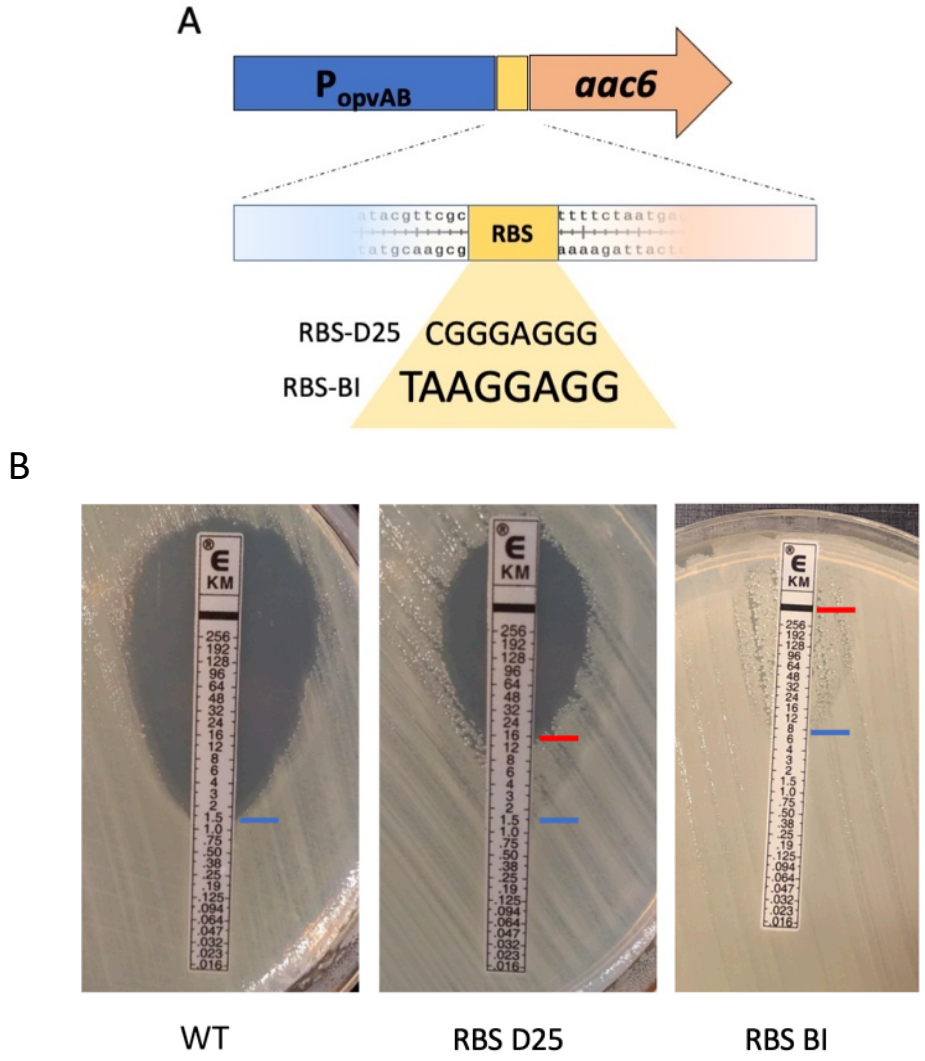


Figure C2.3. A. Diagram of the P_{opvAB}*::aac6* construct with RBS variants (BI, D25). B. Epsilometer tests (Etests) from a wild type strain and SV9704 (D25) and SV9703 (BI).

Use of the OpvAB synthetic switch in generating antibiotic heteroresistance

As a proof of concept, we made a preliminary, reductionist examination of the utility of the OpvAB switch to address the study of antibiotic heteroresistance. Specifically, we tested whether the OpvAB switch could generate, in a susceptible main population, defined subpopulations of cells with increased antibiotic resistance. For this purpose, we used a *S. enterica* strain harboring a P_{opvAB}::BI-*aac3*::*gfp* construct (SV9776). Expression of *aac3* ($Aac3^{ON}$) leads to kanamycin resistance (Km^R). The frequency of Km^R cells formed by a pure culture of SV9776 was $1x10^{-2}$ (**Figure C2.4A**), similar to the frequency of ON cells

detected when *gfp* was cloned behind the *opvAB* promoter (1.1% and $1.1x10^{-2}$, respectively: **Figure C2.1D**). To obtain smaller subpopulation sizes without altering other phenotypic traits of the strain, SV9776 was mixed with an isogenic strain that expressed P_{opvAB}::*gfp* (SV9777) and did not produce any Km^R resistant subpopulation. Mixtures of cells were prepared from overnight cultures in Mueller-Hinton (MH) broth at proportions 1:10, 1:100, 1:1000, 1:10,000 and 0:1. Population analysis profile (PAP) tests were then performed by plating on MH agar containing increasing concentrations of kanamycin. After overnight incubation, the number of resistant cells and total number of cells were determined to allow calculation of the fraction of resistant cells. The numbers of Km^R colonies detected in the PAP tests were proportional to the amounts of the $Aac3^{ON}$ subpopulations present in each mixture, and ranged from $1x10^{-2}$ to $1x10^{-6}$ (**Figure C2.4A**). Epsilometer tests (Etests) further confirmed that the size of the Km^R subpopulation decreased in a manner proportional to dilution (**Figure C2.4B**).

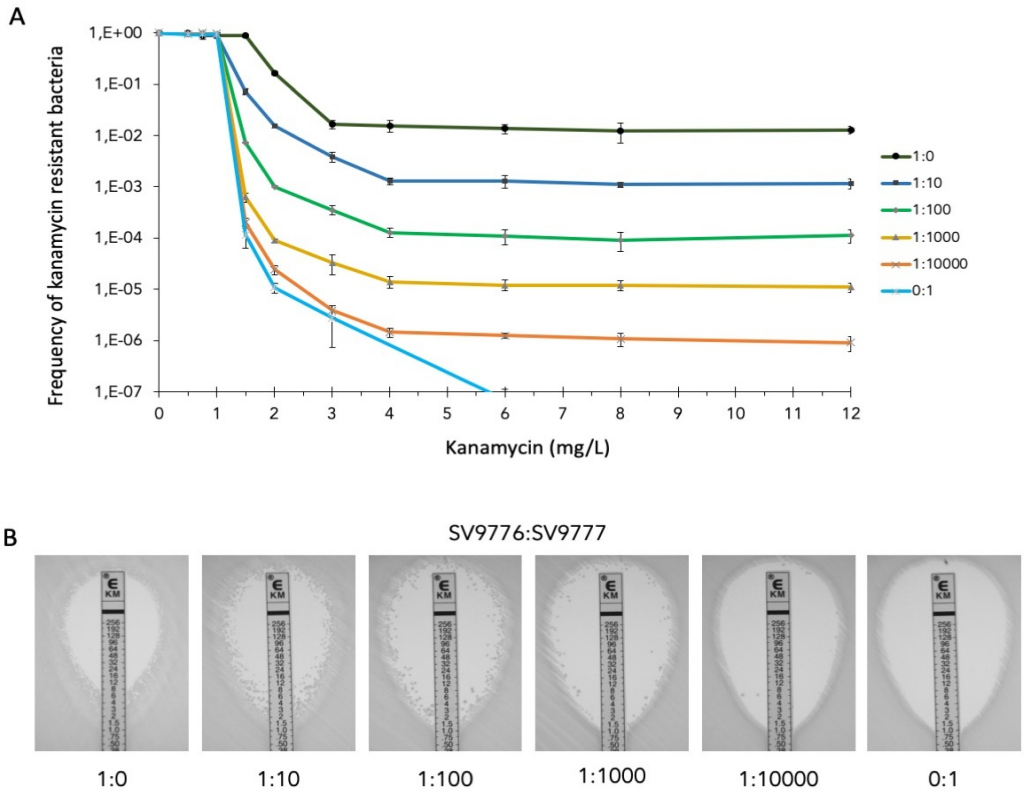


Figure C2.4. The *opvAB* epigenetic switch as a tool for the study of antibiotic heteroresistance. **A**. Population analysis profile (PAP) tests of kanamycin resistance in strains SV9776 and SV9777 (carrying *opvAB::*BI-*acc3::gfp* and *opvAB::gfp* constructs, respectively). The proportions indicated are those of the mixtures of SV9776:SV9777. Error bars represent standard deviations of three independent mixes. **B**. Epsilometer tests (Etests) performed on the same mixes of SV9776 and SV9777.

Design and study of synthetic switches with clinical antibiotics

Aminoglycoside (AG) antibiotics are used to treat many Gram-negative and some Gram-positive infections and, importantly, multidrug-resistant tuberculosis. Among various bacterial species, resistance to AGs arises through a variety of intrinsic and acquired mechanisms [315]. Two new synthetic switches were designed in order to test them against different aminoglycosides. The two RBS previously described (BI, D25) were cloned upstream of the *acc3* gene yielding strains SV9776 and SV9792, respectively. The aminoglycosides assayed were kanamycin, streptomycin, gentamicin, amikacin, netilmicin and tobramycin. MICs for different strains and antibiotics, shown in **Table C2.1**, reveal the cases in which the OFF subpopulation presents a MIC similar to the wild type and the ON subpopulation presents a significant increase in resistance to the antibiotic.

Strain	Kanamycin	Streptomycin	Tobramycin	Gentamicin	Amikacin	Netilmicin
Sty LT2	1.5	6	0.75	0.5-0.75	1.5	0.19-0.25
SV9777	1.5	6	0.75	0.5-0.75	1.5	0.25-0.5
SV9776	1.5 H 16	6	1 H 24	4 H >256	1.5	1 H 32
SV9792	1.5 H 12	6	1 H 12-16	4 H >256	1.5 -2	0.5 H 12

Table C2.1. Etest values on MH (1:10 dilution) for wild type *S.enterica*, SV9777, SV9776 and SV9792.

Further study was focused on those antibiotics for which an antibiotic resistant subpopulation was detected: kanamycin, tobramycin and netilmicin. Initially, the resistance to these three antibiotics was monitored by Etests which permitted determination of the MIC and detection of the presence of an ON subpopulation more resistant to the antibiotic. Additional assays were performed by PAP tests (**Figure C2.5**).

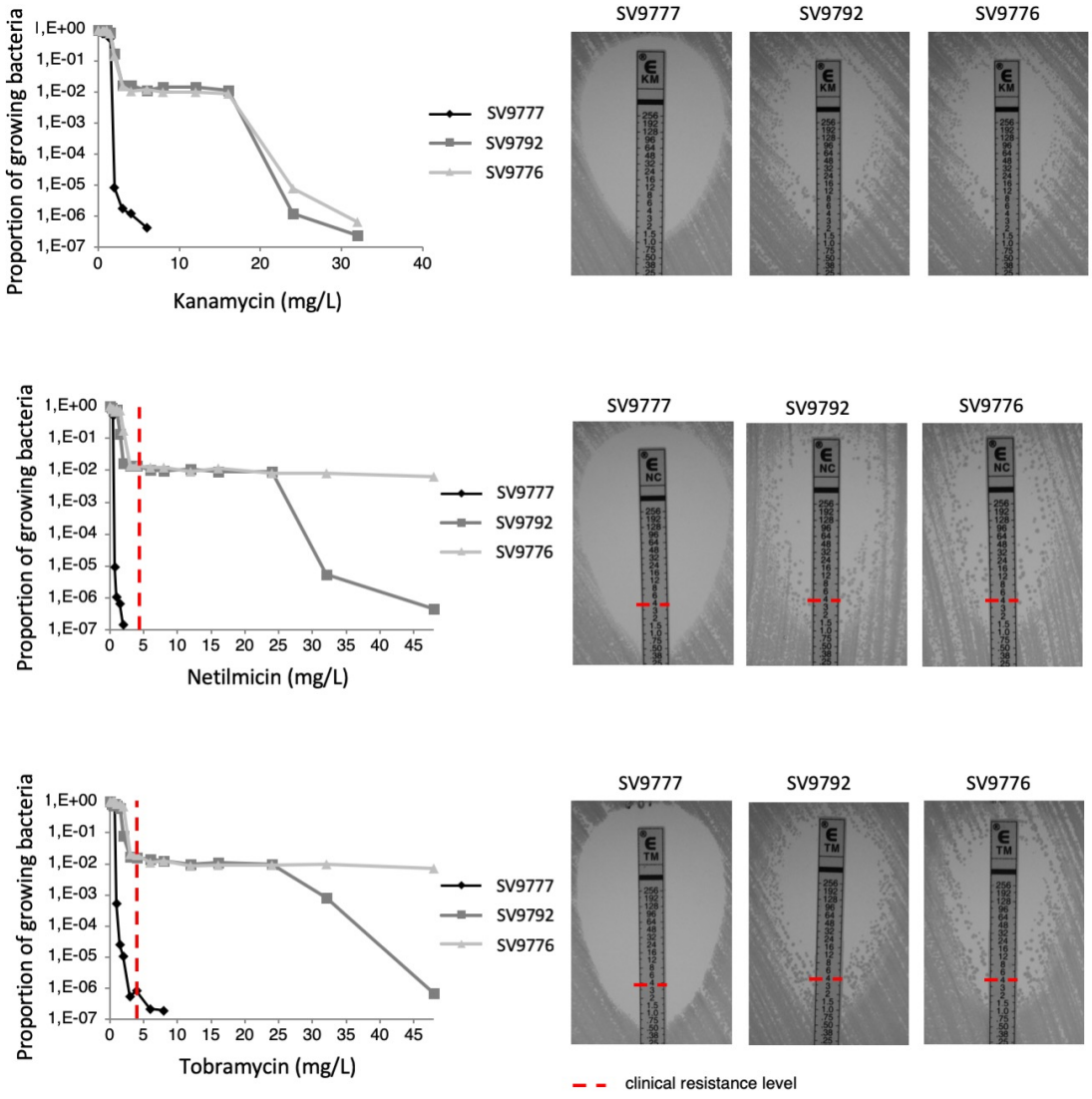


Figure C2.5: Population analysis profile (PAP) tests and Etests of kanamycin (A), tobramycin (B) and netilmicin (C) resistance in strains SV9776, SV9792 and SV9777 (carrying P*opvAB::BI-acc3::gfp,* P*opvAB::D25-aac3::gfp* constructs and P*opvAB::gfp*, respectively).

CHAPTER 3: Design and optimization of bacterial biosensors able to detect bacteriophages in raw environmental samples

Proof of concept using characterized bacteriophages

Based on results published by Cota and collaborators [114], we foresaw that the *opvAB* phase variation operon could be used as a bacteriophage biosensor. Indeed, the $OpvAB^{OFF}$ subpopulation was shown to be killed by bacteriophages that belonged to different families and used LPS for infection, leading to enrichment of the $OpvAB^{ON}$ subpopulation. This population is insensitive to such infections due to the shortening of its LPS by the *opvAB* gene products. Thus, if an *opvAB::gfp* fusion was used, enrichment of $OpvAB^{ON}$ cells could be detected by increased fluorescence intensity [114]. To start with, we wanted to compare the efficiency of two different methods of fluorescence detection, flow cytometry and plate reader monitoring, to detect 3 different bacteriophages known to use LPS for infection. As shown in **Figure C3.1A**, flow cytometry turned out to be not only very sensitive but also descriptive of the bacterial population with the quantification of its heterogeneity. In contrast, fluorescence detection using a plate reader only provided a rough assessment of the population structure (**Figure C3.1B**). On the other hand, fluorescence detection using a plate reader does not necessitate a high-cost machine such as a flow cytometer and has the advantage to give a measure according to the OD_{600} of the culture. Our standard protocol for phage detection by flow cytometry includes 8 h of incubation to ensure that phages kill the vast majority of $OpvAB^{OFF}$cells thus leading to the enrichment in the $OpvAB^{ON}$ population. However, thanks to the sensitivity of this technique, measurements can be done earlier than 8 h. As depicted in **Figure C3.1C**, incubation for 1 or 2 h after dilution into fresh medium in the presence of P22_H5 led to 2.6% and 11.3% of positive cells respectively. Since flow cytometry is highly reproducible, these levels are sufficient to discriminate a $OpvAB^{ON}$ subpopulation from the background level of cells that passed the fluorescence threshold in the absence of phage (0.23%) (**Figure C3.1C**). These experiments thus validated the proof-of-concept of a fluorescent biosensor for the detection of LPS-using bacteriophages based on a modified version of the *opvAB* operon.

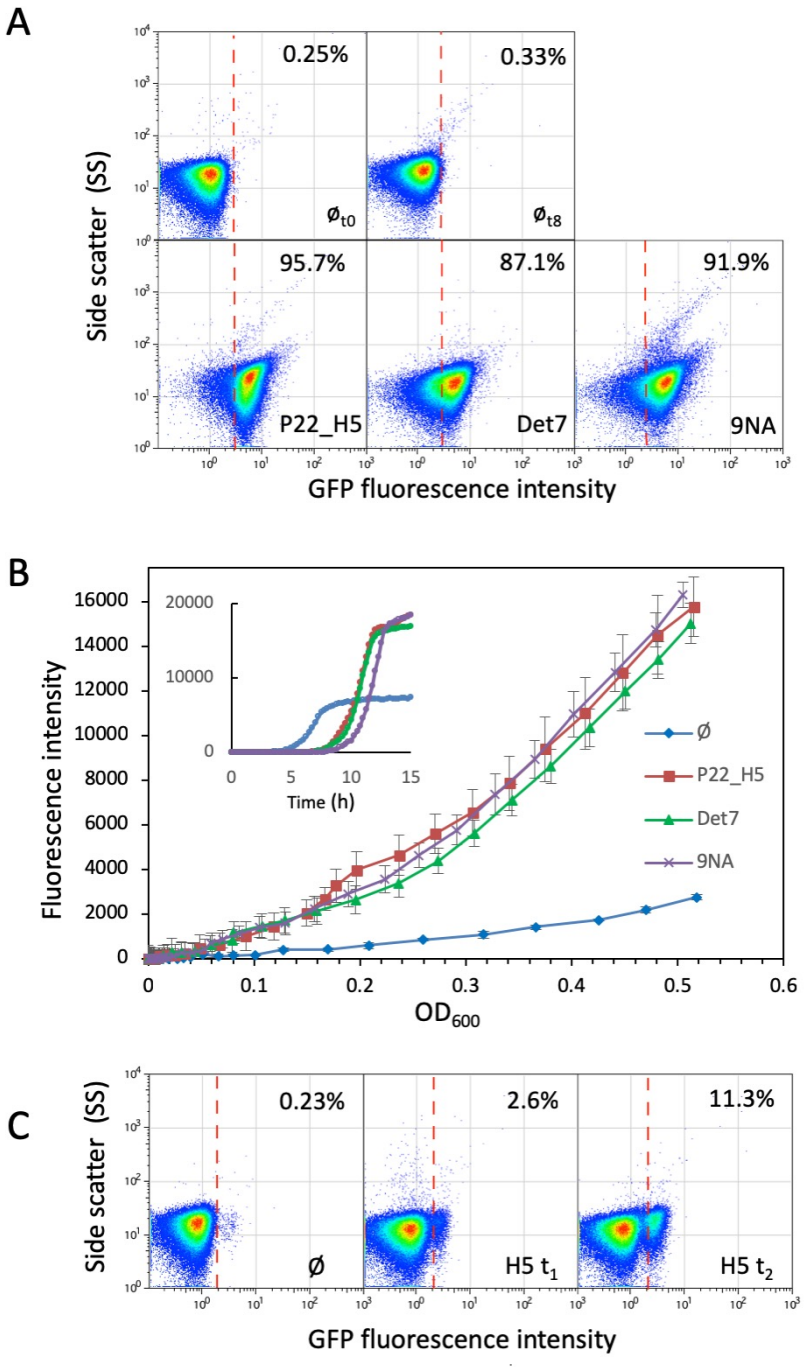


Figure C3.1. Detection of the increased of fluorescence intensity when ON-subpopulation is selected by bacteriophages. A. GFP fluorescence distribution in a ATCC 14028 derivative strain (SV6727) carrying an *opvAB::gfp* fusion (SV6727) before (t = 0 h) and after growth in LB without phage, or in the presence of either P22_H5, 9NA, or Det7 (t = 8 h). Data are represented by a dot plot (side scatter versus fluorescence intensity [ON subpopulation size]). All data were collected for 100,000 events per sample. **B.** Growth curves of SV6727 strain in contact with P22_H5, Det7 or 9NA phage. Data are represented by growth curves [GFP fluorescence intensity] versus growth [OD_{600}]. **C.** GFP fluorescence distribution of SV6727 strain before (t = 0 h) and after growth in LB containing Det7 (t = 1 h or 2 h).

Detection of uncharacterized bacteriophages

To go further with the phage detection tool, we decided to isolate and purify various *S. enterica*-infecting bacteriophages and test whether they could be detected without previous characterization of the phage receptor. Indeed, a phage that would need a functional and extended LPS should kill the OpvAB^{OFF} subpopulation as the known phages did. In contrast, *S. enterica* OpvAB^{OFF} cells should resist to the killing by a phage that would not need such LPS. Various phages were enriched, isolated and partially characterized from different environments in the Seville area as described in the Methods section. Without going deeper into the characterization of those phages, the plaque morphologies (not shown), and even more the images obtained after negative staining by TEM indicated that the 6 isolated phages were different from each other (**Figure C3.2A**). The 6 of them were assayed by flow cytometry for detection using the *opvAB::gfp* fusion. Interestingly, all 6 were perfectly detected 8 hours post-infection with more than 60% of the cells in the ON state (**Figure C3.2B**). This proportion reached up to 90% for three individual phages (Se_F1, Se_F6 and Se_AO). In contrast, Se_F3 and Se_ML killed the OpvAB^{OFF} subpopulation only partially. A conclusion from these experiments was that the *opvAB::gfp* fusion provides an efficient tool to detect unknown LPS-using bacteriophages of *S. enterica*. The variations observed between the different flow cytometry profiles could be due to a difference in the usage of the LPS as a receptor for those phages as discussed below.

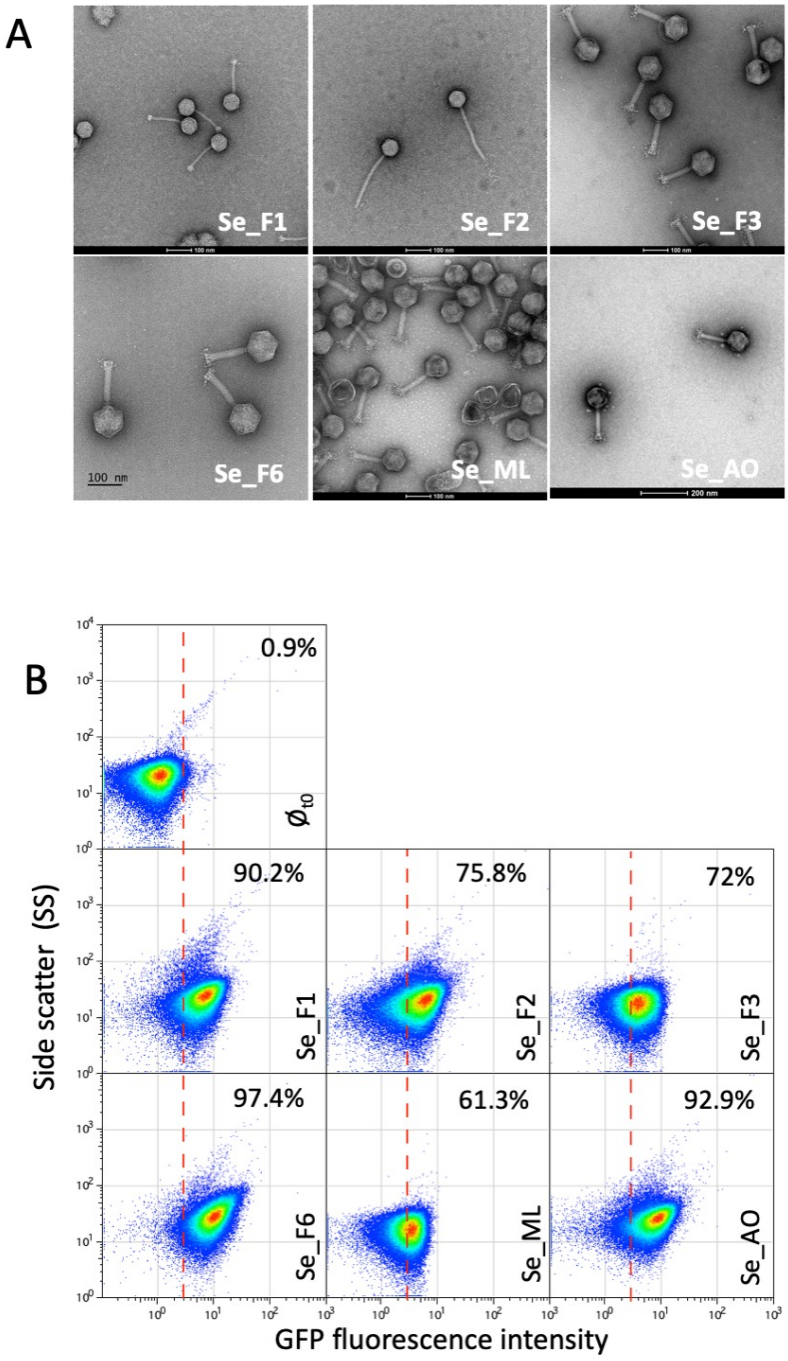


Figure C3.2. Detection of new bacteriophages. A. GFP fluorescence distribution in strain SV6727 (*opvAB::gfp*) before (t = 0) and after growth in LB containing new purified bacteriophages (Se_F1, Se_F2, Se_F3, Se_F6, Se_ML and Se_AO) after 8 h. Data are represented by a dot plot (side scatter versus fluorescence intensity [ON subpopulation size]). The percentage of ON cells in each sample is indicated. All data were collected for 100,000 events per sample. **B**. Negative staining of bacteriophages visualized by electron microscopy.

An especially appealing objective of the present tool is to use *opvAB::gfp* fusion to detect bacteriophages in crude samples and before any enrichment step (in other words, before processing). Different crude water samples collected in the Seville area were assayed for the presence of phages in the presence of the biosensor strain for 10 h before analyzing the resulting populations by flow cytometry. Among the four samples tested, three (EM1, EM2 and EM3) displayed a significant increase in GFP activity, indicating that indeed these samples contained bacteriophages using LPS for infection (**Figure C3.3**). This conclusion was confirmed by plaque assay (not shown). One sample (AO) did not show increase of the OpvAB^{ON} subpopulation, which suggested that the sample did not contain any LPS-using phage active against *S. enterica*. Alternative explanations were that the sample might contain a phage inhibitor or that phage present in the sample infected both OpvAB^{ON} and OpvAB^{OFF} *S. enterica*.

Taken together these results prove that our *opvAB*-based fluorescent biosensor can efficiently detect the presence of LPS-using bacteriophages in environmental samples without any pre-processing other than incubation with the biosensor.

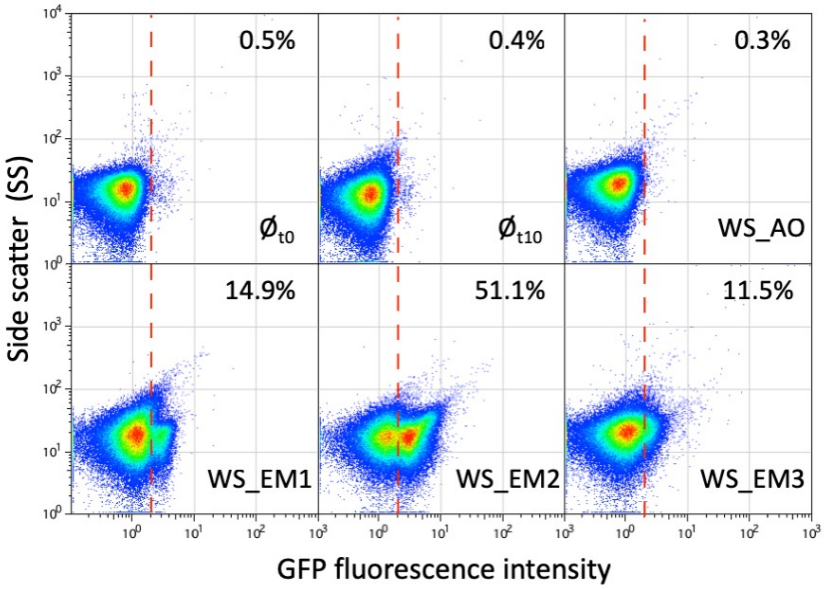


Figure C3.3. Detection of bacteriophages from crude water samples. GFP fluorescence distribution in SV6727 (*opvAB::gfp*) before and after growth in LB for 10 hours in the presence of crude water previously filtered. Data are represented by a dot plot (side scatter versus fluorescence intensity [ON subpopulation size]). The ON subpopulation percentage is shown for each sample. Data were collected for 100,000 events per sample. The presence of bacteriophages was previously verified by a plaque assay (20-30 PFU /ml) on a *S. enterica* lawn.

Sensitivity of the phage detection tool

To characterize the limits of the phage detection tool, 9NA was used (**Figure C3.1**). We first determined the detection limit in a fluorescence plate reader by using serial dilutions of 9NA. As shown in **Figure C3.4A**, only the most diluted sample, equaling to 10 PFU/ml, could not be distinguished from the control experiment with no phage. Therefore, using a fluorescence plate reader, as little as 100 PFU/ml could be detected. Interestingly, the curves obtained in the presence of effective amounts of phage show an interesting profile. In the first five hours, the global tendency was an increase of the whole population ($OpvAB^{ON}$ and $OpvAB^{OFF}$), followed by a rapid decrease (**Figure C3.4A, Inset**). Five hours post-infection, the bacterial growth resumed more or less rapidly depending on the amount of phage initially present in the culture. As expected, the fluorescence intensity correlated with growth only when samples were initially infected with 9NA (≥100 PFU/ml) (**Figure C3.4A**), indicating that only the ON subpopulation grew under these conditions. This was confirmed by the fact that the highest fluorescence intensity was reached in the presence of the highest amount of phage, which killed the $OpvAB^{OFF}$ subpopulation more efficiently. As expected, without initial phage infection (or with phages ≥ 10 PFU /ml) essentially no increase of fluorescent was correlated to bacterial growth, indicating that mainly the $OpvAB^{OFF}$ population accounts for bacterial growth. Based on this experiment, we tentatively concluded that strong, dose-dependent fluorescence intensity was reached 10 hours post infection (**Figure C3.4A, Inset**). In order to test those parameters using flow cytometry, the same serial dilutions of phages were applied for 10 h and enrichment of the $OpvAB^{ON}$ subpopulation was monitored. At variance with the plate reader experiment, except the 1 PFU/ml, all the phage concentrations tested were able to enrich the ON subpopulation (**Figure C3.4B**). Moreover, above a concentration 10^2 PFU, a linear correlation was observed between the phage concentration and the $OpvAB^{ON}$ subpopulation size, which could be used as a calibration curve to determine phage concentration in a given sample. Hence, the phage biosensor appears to be highly sensitive and can detect as little as 10 PFU/ml.

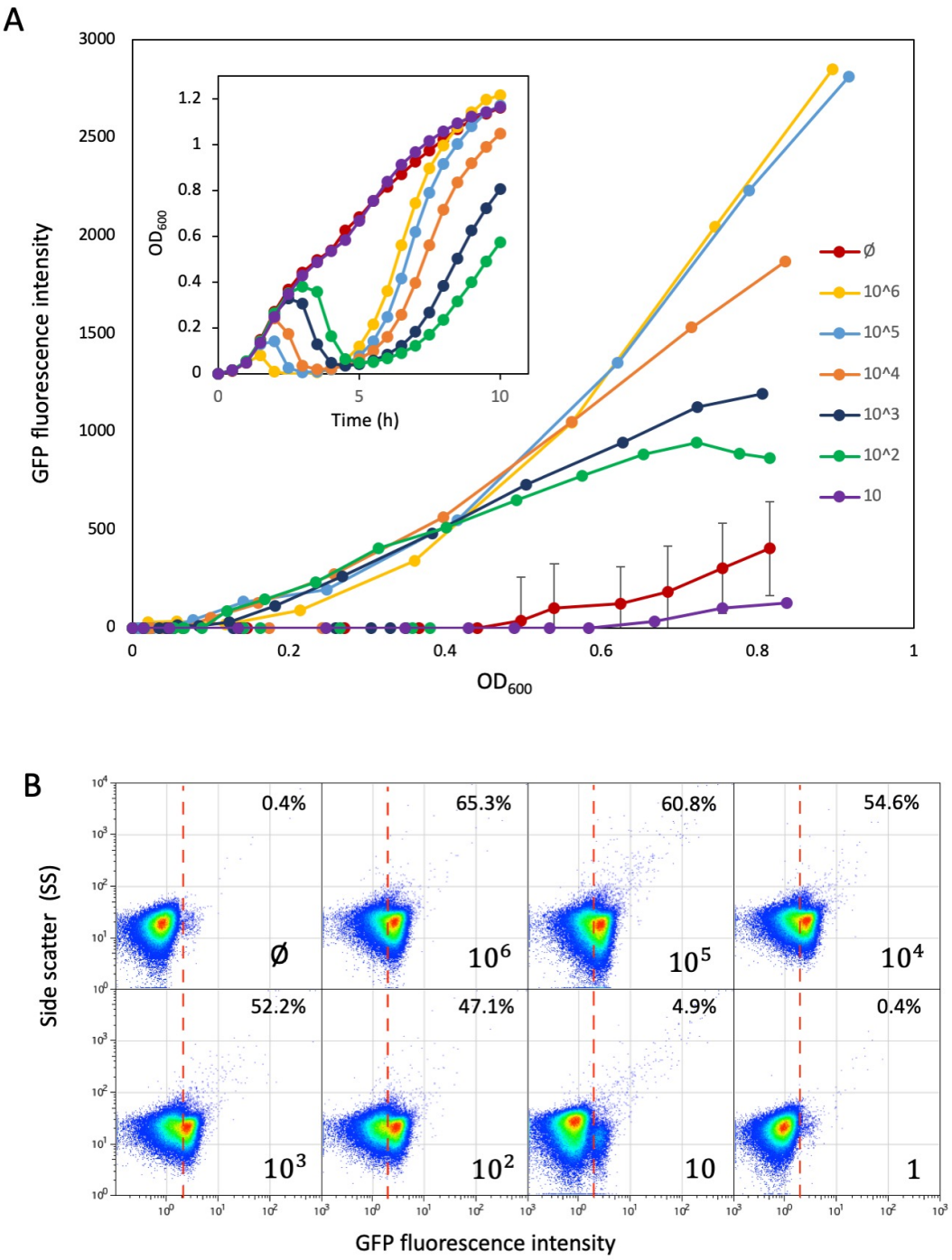


Figure C3.4. Sensitivity of the OpvAB phage biosensor. A. Growth curves of SV2767 strain (*opvAB::gfp*) in contact with serial dilutions of phage 9NA starting from 10^6 up to 10^1 PFU/ml. Data are represented by growth curves showing OD_{600} versus time [hours], and fluorescence intensity [ON subpopulation size] versus growth [OD_{600}]. **B.** GFP fluorescence distribution in SV6727 before (t = 0) and after growth (t = 10 h) in LB containing serial dilutions of 9NA phage starting from 10^6 up to 10^0 PFU/ml. Data are represented by a dot plot (side scatter versus fluorescence intensity [ON subpopulation size]). All data were collected for 100,000 events per sample.

Optimization of the detection tool

Our next objective was to improve the detection limits of our biosensor. As we noticed that killing of the OpvAB^{OFF} subpopulation (and thus selection of the OpvAB^{ON} subpopulation) was more efficient in the presence of a high number of phages 9NA (**Figure C3.4A**), we aimed at improving the method by adding less cells to increase the ratio phage / biosensor. Indeed, the more diluted were the cells, the smaller number of phages that could be detected (**Figure C3.5A**). It is remarkable that for the smallest dilution of the overnight culture, the concentration of phages detected using a microplate reader was around 4.10^5 PFU/ml, whereas as little as 4 PFU/ml were detected using a higher dilution of the biosensor (equivalent to 8.8×10^4 cells/ml, corresponding to about 17,600 ON cells), thus increasing 5-log the detection limit when the number of cells decreased 3-log only.

Another improvement originated from earlier work showing that *opvAB* expression increased when specific GATC sites present in the promoter region were mutated [114], [115]. Indeed, when the *opvAB::gfp* GATC$_{1,2}$ construct was used as a biosensor for phage 9NA, the fluorescence increased faster than with the construct bearing the wild-type promoter and reached a plateau in about 10 h (**Figure C3.5B, Inset**). Interestingly, the largest difference was observed around 7 h post-infection.

We then combined both improvements (low cell density and mutations in GATC sites) to reach an even lower detection limit. The experiment described in **Figure C3.5C** was performed in two steps. First, we used as little as eight 9NA phages diluted into 500 ml of LB containing a dilution (1/10) from an overnight culture of the biosensor strain and let the phage enrichment proceed for 15 h. Then the mixture was diluted again into fresh medium to let the OpvAB^{ON} subpopulation enrich for 5 h before the flow cytometry assay. It is remarkable that this extremely small initial number of phages was able to enrich the ON subpopulation up to 86.5% under the conditions described (**Figure C3.5C**), thus lowering the detection level to about 1.6 phages per 100 ml. As a reminder, before optimization the detection limit with flow cytometry was 1000 PFU/100 ml.

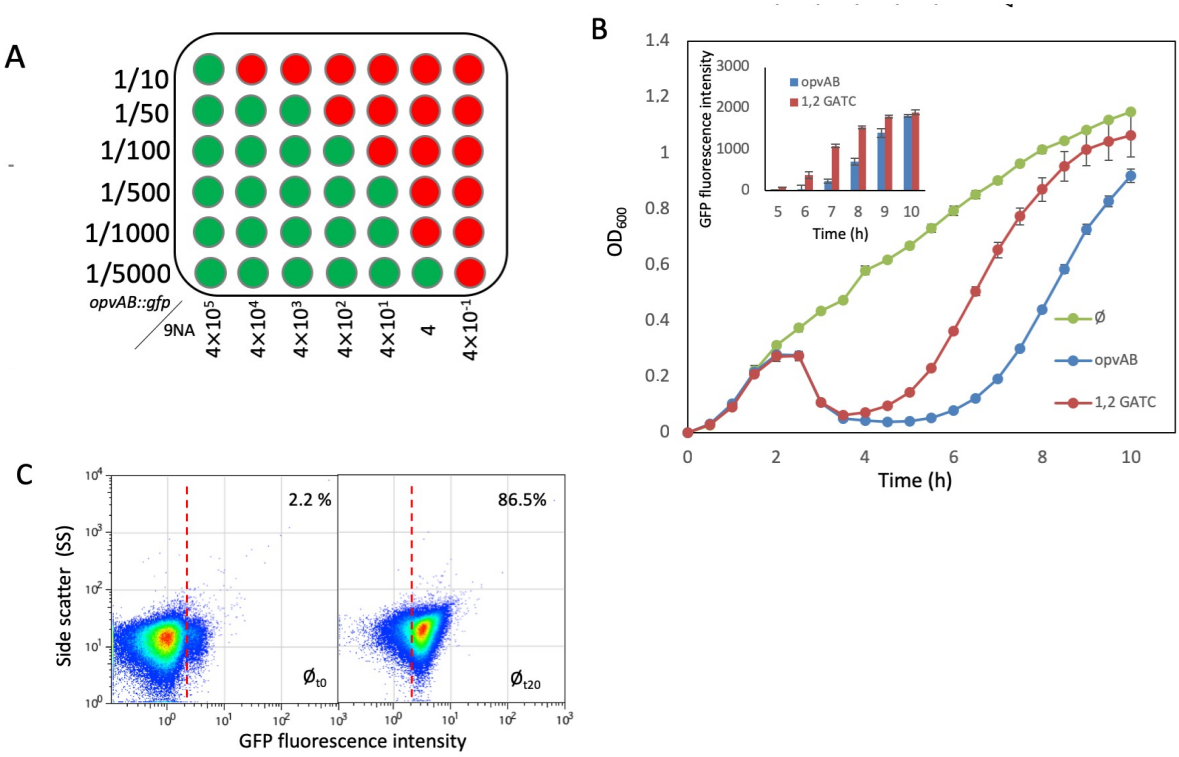


Figure C3.5. Improving the *opvAB* biosensor sensitivity.

A. Bacterial dilutions (10^{-1} up to 2×10^{-4}) where grown in the presence of serial dilutions of bacteriophages 9NA stock as indicated. Bacterial growth was monitored according to time in a microplate reader and represented schematically. The green wells are fluorescence positive, and red wells are fluorescence negative.

B. Growth curves obtained for a wild type strain carrying the classical *gfp* fusion (SV6727) in the absence of phage (green), or in the presence of 9NA phage (blue), and for a derivative strain carrying point mutations in the first and second GATC sites located in the *opvAB* promoter (SV8578) in the presence of 9NA phage (red). Fluorescence intensity data obtained at several time points of the growth curve (inclusion).

C. GFP fluorescence distribution in SV8578 (*opvAB::gfp* $GATC_{1,2}$) before (t = 0 h) and after growth in LB containing 9NA phage (t = 20 h). Data are represented by a dot plot (side scatter versus fluorescence intensity [ON subpopulation size]). All data were collected for 100,000 events per sample.

Design of a biosensor able to detect coliphages

Up to this point, the design and improvement of the phage biodetection method was performed using *S. enterica* as a chassis. To widen our capacity to detect phages in environmental samples, we decided to adapt the biosensor to coliphages. To do so, we first had to restore a full length LPS as *E. coli* K12 MG1655 is well-known to carry an IS5-interrupted version of the *wbbL* gene encoding a rhamnosyltranferase [305]. This is clearly evidenced by the length and profile of the LPS following extraction, separation by SDS-PAGE and silver staining is affected in MG1655 (**Figure C3.6A, lane 1**). The interrupted *wbbL* gene was complemented either by adding a plasmid-based copy of *wbbL* or by integrating ectopically a single copy of the wild type gene. In both cases, the LPS was restored and showed a more complex profile (**Figure C3.6A, lanes 4 and 5**). Engineering of strains carrying a *opvAB::gfp* fusion on the chromosome allowed us to examine the consequences of *opvAB* expression in *E.coli*. A wild type *opvAB* control region caused a subtle alteration of the LPS profile, an observation consistent with the small size of the ON subpopulation (**Figure C3.6A, lane 6**). Modification of the length distribution of glycan chains in the O-antigen was however unambiguous when the LPS$^+$ *E.coli* strain harbored a GATC-less *opvAB::gfp* fusion (**Figure C3.6A, lanes 6 and 7**).

Using the *opvAB::gfp*-carrying LPS$^+$ *E. coli* strain, we isolated and purified 7 coliphages from water samples from the Seville area. As shown in **Figure C3.6B**, seven of the coliphages were able to select the OpvAB^{ON} subpopulation with an enrichment efficacy ranging from 11.2 to 58.6%, far higher than the control without phage (1.27 %). Again, this difference could substantiate the use of the LPS as a primary receptor or as a full receptor for the isolated coliphages except for Ec_unk_PO1 whose profile does not distinguish from the negative control.

Whatever the case, this experiment shows that the method works alike in *E. coli* as in *S. enterica* and that the *opvAB*::*gfp* fusion is a versatile tool that could be used with different enterobacterial chassis.

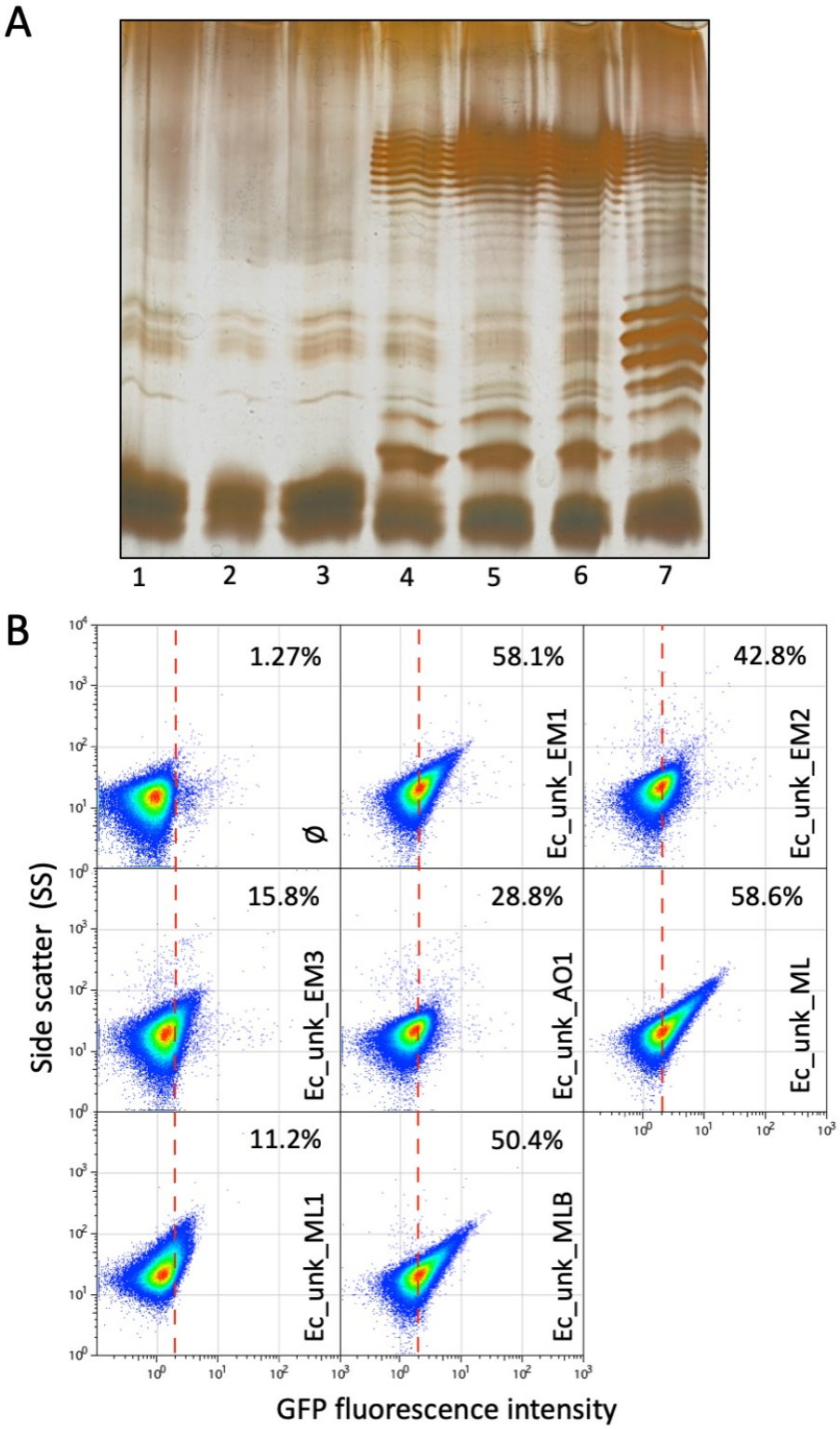


Figure C3.6. Restoration of *E. coli* MG1655 O-Antigen and detection of unknown coliphages.

A. Electrophoretic visualization of LPS profiles from different MG1655 derivatives, as follows**:**

1. Wild type *E. coli* MG1655 strain containing an IS5-inactivated version of the *wbbl* gene; **2.** *E. coli* MG1655 with a deletion of the lactose operon (DR3); **3**. *E. coli* DR3 strain carrying the empty pETb vector; **4**. *E. coli* DR3 strain carrying the pETb vector containing a copy of the wild type *wbbL* gene;

5. *E. coli E. coli* DR3 strain carrying the *wbbL* gene integrated into the genome replacing the altered IS5-*wbbl* gene (LPS$^+$ strain); **6**. *E. coli* MG1655 LPS$^+$ carrying the *opvAB* construction (DR29 strain); **7**. *E. coli* MG1655 LPS+ *opvAB* GATC-less (DR30).

B. GFP fluorescence distribution in *E. coli* DR29 derivative before (t = 0 h) and after growth in LB, or LB containing purified uncharacterized bacteriophages (Ec_Unk_EM1, Ec_Unk_EM2, Ec_Unk_EM3, Ec_Unk_AO1, Ec_Unk_ML, Ec_Unk_ML1 and Ec_Unk_MLB (t = 8 h). Data are represented by a dot plot (side scatter versus fluorescence intensity [ON subpopulation size]). ON subpopulation percentage frequency is showed for each sample. All data were collected for 100,000 events per sample.

Expanding the *opvAB::gfp* biosensor to the detection of phages using proteins as receptors: Bacteriophage T5 detection as a proof of concept

As we have seen that coliphage detection could show high variation (**Figure C3.6B**), probably depending on the receptor molecule used for recognition at the surface of the bacteria, we decided to design an *E. coli* biosensor that would specifically detect a phage known to bind a protein. Phage T5 is an appropriate model: although it uses LPS for initial adsorption, binding to the ferrichrome transporter FhuA on the outer membrane constitutes the trigger for DNA injection [316], [317]. We first thought to make a simple P_{opvAB}::*fhuA*::*gfp* fusion, but that would have provided us with a biosensor working the opposite way from the *opvAB*::*gfp* biosensor. Indeed, in this case the presence of T5 would select for the OFF (non-fluorescent) subpopulation, and it would mean we should be able to detect a slight decrease in fluorescence, which can be expected to be less reliable than monitoring an increase of fluorescence intensity. We thus looked for a gene which under the control of the *opvAB* promoter would confer resistance to T5 in the ON state. Interestingly, T5 encodes a lipoprotein, encoded by the *llp* gene, which is an inhibitor of T5 infection [318]. Llp is synthesized upon T5 infection and prevents superinfection of the host by other T5 virions by interacting with the FhuA receptor, resulting in its inactivation [319]. Moreover, the *llp* phage gene is expressed in the early stage of T5 infection, which not only prevents superinfection but also protects progeny phages from being inactivated by the receptor present in envelope fragments of lysed host cells [209]. We thus reasoned that placing the *llp* gene under the control of the *opvAB* promoter might confer resistance to T5 only on the ON state. As

expected from the literature, a Δ*fhuA* strain proved resistant to T5, but not to T4, which uses OmpC as receptor [320] (**Figure C3.7A**). In turn, ectopic expression of *llp* from a plasmid also confers resistance to the *E. coli* MG1655 WT strain as in the Δ*fhuA* mutant. We thus integrated a P_{opvAB}::*llp*::*gfp* fusion at the *lac* locus in *E. coli* MG1655 and assayed this new biosensor with T5 for 10 hours on a plate reader, periodically monitoring the OD_{600} and the florescence intensity (**Figure C3.7B**). As predicted, enrichment of the ON subpopulation was detected after a few (4) hours as GFP fluorescence intensity increased on a growth-dependent manner, indicating that this fusion performed well as a T5 biosensor. We then assayed the biosensor using flow cytometry to estimate the efficiency of ON subpopulation enrichment, which ranged from 82% to almost 90% in 10 h for 4 different phages (**Figure C3.7C**). Interestingly, addition of colicin M led to a similar enrichment of the ON subpopulation, thus confirming that production of the FhuA inhibitor Llp was also able to prevent colicin M binding. We also performed an additional control, using one of the LPS-binding phages assayed in **Figure C3.6**. As predicted based on its effect on the Ec_*opvAB*::*gfp* biosensor, this specific phage (Ec_unk_EM1) was unable to select for the P_{opvAB}::*llp*::*gfp* ON subpopulation (**Figure C3.7C**). Conversely, the Ec_unk_PO1, selected to turn on P_{opvAB}::*llp*::*gfp* fusion, and thus likely recognizing FhuA, was not detected using the Ec_*opvAB*::*gfp* biosensor (**Figure C3.6C**). Together, these experiments show that the method can accommodate not only different bacterial chassis (*Salmonella* and *E. coli*) but also different types of receptors, thus providing a primary indication on the type of receptor used by a given phage.

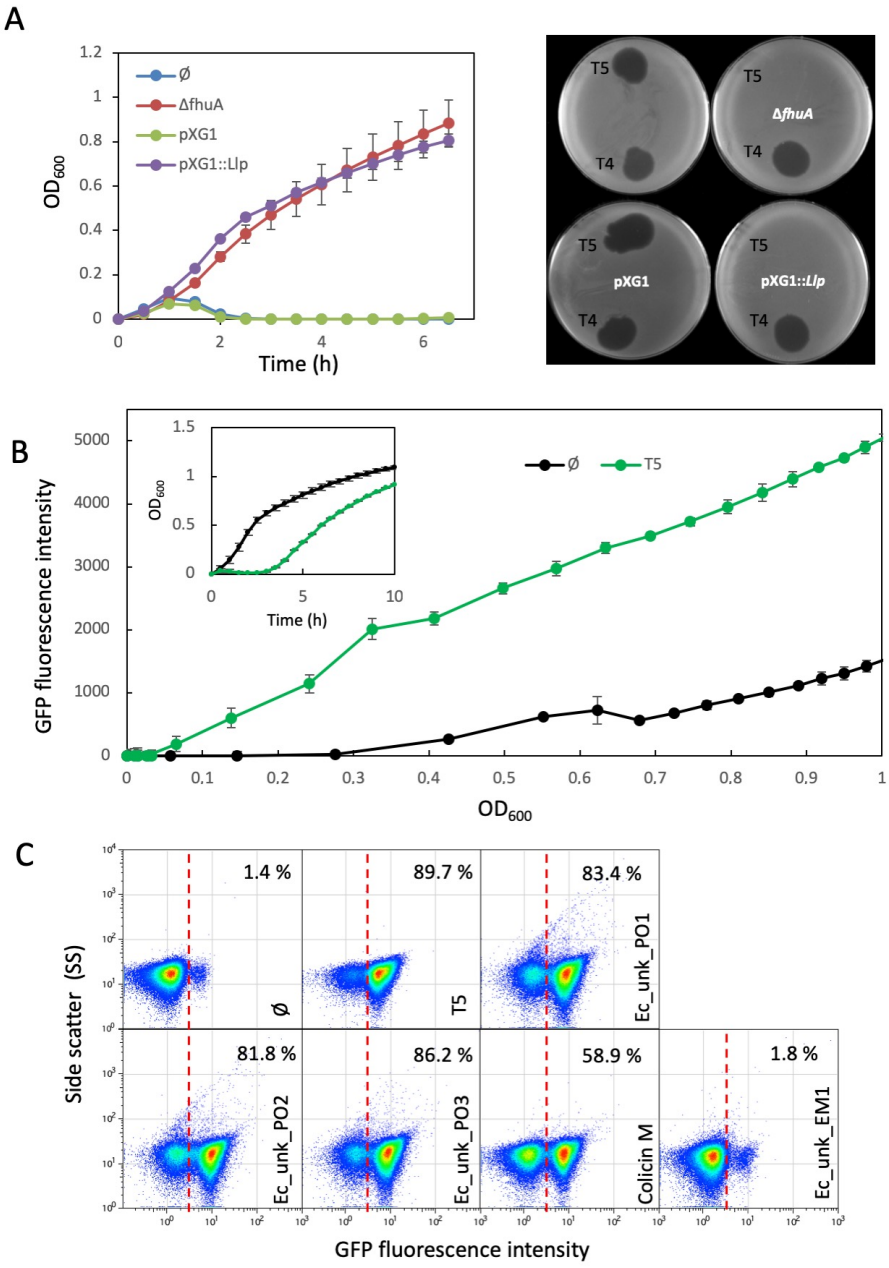


Figure C3.7. Bacteriophage T5 detection.

A. Left. Bacterial growth curves in the presence of phage T5 (2.5 x 10^7). In blue, *E. coli* MG1655 wild type. In red, a strain with a deletion of the *fhuA* gene (DR42). In green, *E. coli* carrying an empty pXG1 plasmid, used as a control. In purple, *E. coli* carrying plasmid pXG1 containing the *llp* gene from T5 phage. **Right.** Plaque assay using the same strains and bacteriophages T5 and T4.
B. Growth curves of an *E. coli* derivative strain DR41 carrying a P_{opvAB}::*llp*::*gfp* fusion in the presence or absence of T5 for 10 hours. Inlet, the same data are represented as a function of time. **C.** GFP fluorescence distribution in DR41 (P_{opvAB}::*llp*::*gfp)* fusion before (t = 0. h) and after growth in LB containing T5, unknown *E. coli* phages (Ec_Unk_PO1, Ec_Unk_PO2, Ec_Unk_PO3 isolated on *E. coli* MG1655, and Ec_Unk_EM1 isolated on DR28), or colicin M (3 µg/ml) (t = 8 h). Data are represented by a dot plot (side scatter versus fluorescence intensity [ON subpopulation size]). All data were collected for 100,000 events per sample.

CHAPTER 4: Detection of Dam methylation inhibition using an OpvAB-bases biosensor

The ability of an *opvAB*-derived biosensor to detect inhibitors of DNA methylation was tested using strain SV9700 (P_{opvAB}*::lacZY::gfp*). The DNA methylation inhibitor chosen for the trial was sinefungin, a natural nucleoside related to S-adenosyl-methionine. Sinefungin blocks base methylation in DNA and RNA upon competition with SAM [321], [322]. Analysis of bacterial cell viability in the presence of sinefungin revealed toxicity above a final concentration of 2.2 mg/ml. Based on this observation, the final concentration chosen for the inhibition trial was 1 mg/ml. The fluorescence intensity was quantified by flow cytometry. Growth of strain SV9700 in presence of sinefungin was accompanied by an increase of fluorescence intensity. An interesting observation was that the fluorescence pattern was very similar to that of a *dam* mutant strain carrying the same construction (**Figure C4.1**).

Fluorescence intensity and growth were also monitored using a Synergy HTX microplate reader. The $O.D_{600}$ of the culture was monitored to confirm that the concentration of sinefungin used in the experiment (1 mg/ml) did not inhibit growth. The curves showed differences in the presence and in the absence of sinefungin, thus confirming that sinefungin inhibits Dam methylation in *S. enterica*. Hence, the *opvAB*-based fluorescence biosensor appears to be appropriate to detect DNA methylation inhibition.

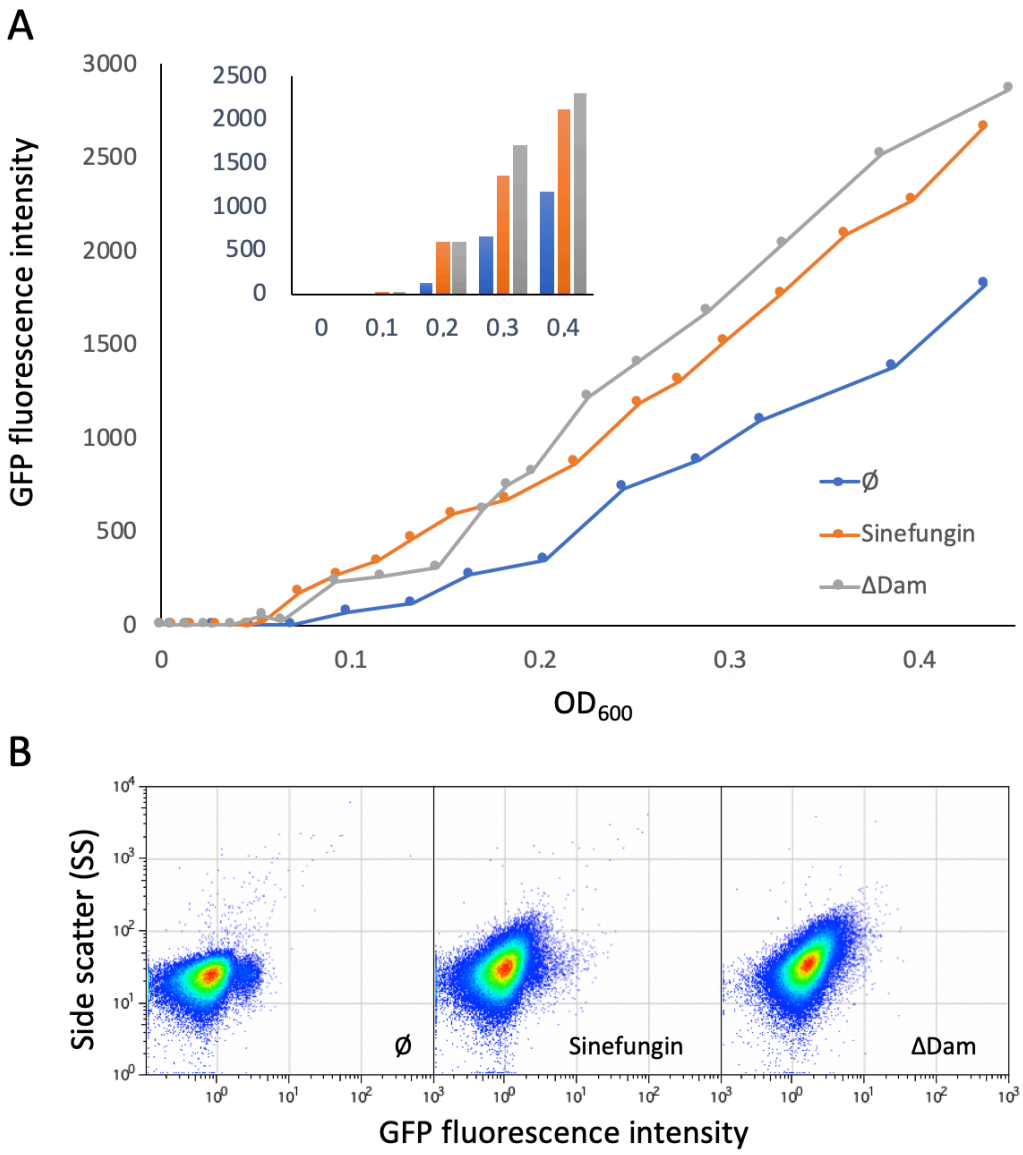


Figure C4.1. Detection of Sinefungin. A. Fluorescence intensity of the *P_{opvAB}::lacZY::gfp* fusion of strain SV9700 without sinefungin (blue) and with 1 mg/ml sinefungin (orange). In the right panel, the fluorescence intensity was plotted vs culture density. Fluorescence in the presence of sinefungin is similar to that of a *dam* mutant (grey). Fluorescence did not increase at late growth stages, probably due to exhaustion. **B**. GFP fluorescence distribution in SV9700 (*P_{opvAB}::lacZY::gfp)* in presence or in absence of Sinefungin (1 mg/ml) after 5 h. Data are represented by a dot plot (side scatter versus fluorescence intensity [ON subpopulation size]). All data were collected for 100,000 events per sample.

DISCUSSION

The work described in this Thesis is based on previous studies of the phase-variable *opvAB* operon of *Salmonella enterica,* and has used the information provided in those studies to design and develop the construction of several types of microbial biosensors able to detect environmental signals: antibiotics, phages and DNA methylation inhibitors. Such sensors may be considered a novel type of sensor that operates at the population level, unlike traditional gene circuits and whole cell sensors.

In its native host, the *opvAB* operon undergoes bistable transcription, which generates $OpvAB^{ON}$ and $OpvAB^{OFF}$ subpopulations [115]. Bistability is reversible ("phase-variable") and the switching rate is skewed to OFF in the wild type [114], [115]. In this study, we show that a 689 bp DNA fragment containing the *opvAB* promoter and the *opvAB* upstream activating sequence (UAS) confers bistability to genes cloned downstream. For instance, an engineered P_{opvAB}::*lacZY* operon produces Lac^{OFF} and Lac^{ON} subpopulations (**Figure C1.1C**), and addition of a *gfp* reporter gene permits discrimination of Lac^{OFF} and Lac^{ON} cells by flow cytometry (**Figure C1.1D**). Utilization of L-lactose sustains growth of Lac^{ON} cells (**Figure C1.1E**), thus producing increased fluorescence. However, because the *opvAB* switch is reversible, if a culture grown on L-lactose is transferred to lactose-free medium the system slowly returns to its initial state, with strong predominance of Lac^{OFF} cells (**Figure C1.1F**). The fact that the *opvAB* switch is functional in a heterologous host which is also a model organism, *E. coli* (**Figure C1.2**), might increase its potential interest for synthetic biology. The fact that the switching frequencies are slightly different in *Salmonella* and *E. coli* admits several tentative explanations and can be considered irrelevant for practical purposes. as phase variation is unambiguous in both hosts.

The versatility of the *opvAB* switch is illustrated by an additional example of subpopulation formation presented in **Figure C2.1**: *opvAB*-driven bistable expression of kanamycin and cefotaxime resistance genes permitted selection of antibiotic-resistant subpopulations in a reversible fashion.

Introduction of mutations in the upstream regulatory region of the native *opvAB* operon alters the switching rate, yielding $OpvAB^{ON}$ and $OpvAB^{OFF}$ subpopulation sizes different from those of the wild type [114], [123]. Hence, variants of the *opvAB* switch can be engineered to modulate subpopulation sizes at will. For instance, a variant ($GATC_{1,2}$) that lacks two of the four GATC sites present in the wild type increases the initial size of the ON subpopulation (**Figures C2.1 and C2.2**). Additional UAS variants that yield subpopulations

of different sizes have been described [123], and their use may allow choice of other switching frequencies. Modification of the ribosome-binding site of gene(s) under P_{opvAB} control can also contribute to adjust the sensitivity of the switch, thus facilitating detection of the phenotype under study. For instance, use of the D25 ribosome binding site (SV9704, SV9792) permitted unambiguous detection of *aac3*-mediated kanamycin resistance, thereby facilitating discrimination of Km^R cells (**Figure C2.4**).

As a proof of concept, we have used the *opvAB* switch to produce antibiotic-resistant and antibiotic-susceptible bacterial subpopulations of predetermined sizes. The aim of these experiments was to mimic bacterial heteroresistance to antibiotics under laboratory conditions, a phenotype where subpopulations of cells with higher antibiotic resistance than the main population are present [323]. Heteroresistance is difficult to detect and study in clinical samples [323], and a better understanding of the frequencies of subpopulation formation and of their antibiotic resistance levels may improve our understanding of heteroresistance as a cause of clinical treatment failure [309]. Experiments shown in **Figure C2.4** provide evidence that subpopulation formation under *opvAB* control allows accurate modulation of the number of resistant cells present in a population. In principle, the method should be applicable to any antibiotic resistance gene. This approach provides a proof of concept to study the effect that different frequencies and levels of resistance of resistant subpopulations may have on the outcome of antimicrobial treatment *in vivo* (e. g., in a murine model). A relevant advantage of the *opvAB* switch to be used *in vivo* is that it can be expected to be largely unaffected by infection bottlenecks that could eliminate very small subpopulations of resistant bacteria present in the inoculum [324]. Because of this potential advantage, the *opvAB* switch is currently being used at the D. I. Andersson's lab in Uppsala University to study antibiotic heteroresistance.

An additional application of the *opvAB* switch is the design of sensors able to detect bacteriophages in environmental samples. The challenge of accurately count bacteriophage has been around for some time as more and more researchers were interested in counting, discriminating and characterizing bacteriophages in various environments for ecological or biotechnological purposes. Besides the gold standard plaque assay method a variety of techniques, each one with pros and cons, have been developed and compared These techniques are based on different methodologies such as quantitative PCR (ddPCR or regular qPCR), FISH, electronic microscopy, and flow cytometry that can be classified into three types: culture-dependent, sequence-based, and particle-based [325]. Another way to

classify these techniques is to consider the characteristics of the enumeration and the additional infection parameters that can be obtained [326]. For example, the classical plaque assay can discriminate infectious versus non-infectious particles, provides information on the adsorption rate, is feasible without any particular equipment and does not need genome sequencing. A limitation, however, is that the assay is difficult to perform in a high-throughput manner. On the other hand, a ddPCR-based method is sequence-based and therefore necessitates the determination of the phage genome sequence. The method requires a specialized equipment, which allows high-throughput analysis. The information provided by ddPCR is also different: there is no possibility to discriminate infectious versus non-infectious particles but the method can discern between different phages in a single assay [327]. In the spectra of current methods, flow cytometry-based approaches are among the most sensitive and most published methods. Such procedures rely on the labelling of the phage genome by a fluorescent dye [328]–[330], although some label-free protocols have been developed recently [331]. In this work, we combine culture-based methods with flow cytometry, which allows to quantify with high sensitivity infectious particles only (**Figure C3.5**). Cloning of a *gfp* gene dowstream of the *opvAB* operon provides a simple an efficient sensor to monitor the increase of the $OpvAB^{ON}$ subpopulation by flow cytometry. This increase provides indirect evidence that the $OpvAB^{OFF}$ subpopulation undergoes lysis, which in turn indicates the presence of a phage that uses the O-antigen as receptor. An advantage of this type of sensor is that lysis of $OpvAB^{OFF}$ cells and concomitant increase of $OpvAB^{ON}$ cells produce amplification of the signal over time. This feature makes the sensor highly sensitive: 10^9 phages/ml can be detected in 1 h, and an amount of phage as low as 2 PFU in 100 ml can be detected in 20 h.

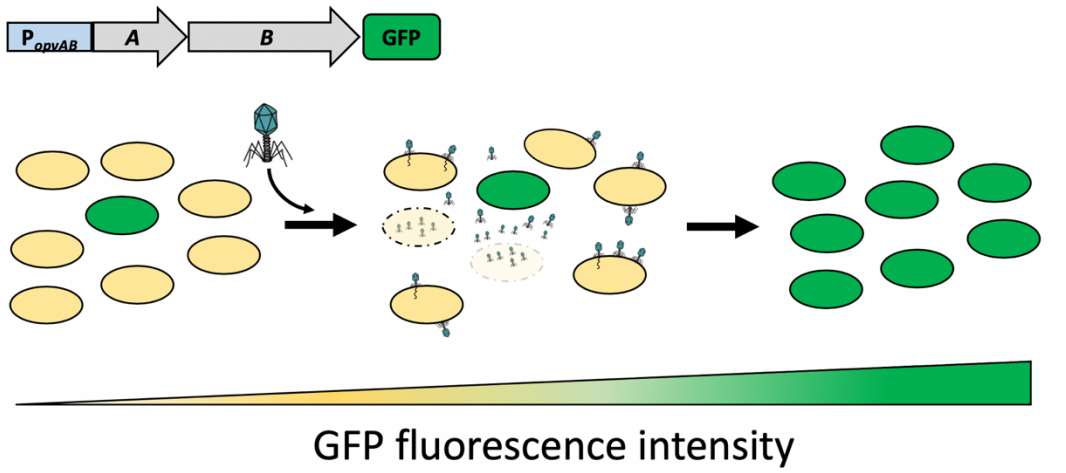


Figure D1. Diagram for the selection of the OpvAB^{ON} subpopulation in presence of a bacteriophage that uses the O-antigen as receptor. OpvAB^{OFF} cells are represented in yellow, and OpvAB^{ON} cells are represented in green. Fluorescence intensity in culture increase in the presence of bacteriophages due to an increased of the number of OpvAB^{ON} cells.

While the original biosensor constructed in *S. enterica* (**Figures C3.1 and C3.2**) proved able to detect purified or unpurified phages present in raw water samples (**Figure C3.3**), we expanded our collection of biosensors to detect coliphages. Therefore, after reconstruction of the MG1655 strain with a complete LPS, the integration at the *lac* locus of an *opvAB*::*gfp* fusion under a constitutively active promoter (OpvAB^{ON}) confers a LPS enriched with a short length fraction to *E. coli* compared to the LPS^{+} strain (**Figure C3.6**). This observation indicates that in the absence of any identified homologs, the OpvA and OpvB proteins are able to generate short-length LPS in *E. coli*. As a result, the *E. coli* strain generated that way was able to detect LPS binding phages in a way similar to the *S. enterica* strain (**Figures. C3.2, C3.6**).

Of note, the enrichment of the OpvAB^{ON} subpopulation by killing of the OFF subpopulation was not as effective with the coliphages we isolated as for the phages active against *S. enterica*. Nevertheless, as the detection by flow cytometry is very sensitive and reproducible, the enrichment levels achieved were sufficient for a proper detection although maybe not as sensitive. This result may indicate the coliphages we isolated required an additional component of the outer membrane as a receptor, rendering LPS binding less crucial for a successful infection. This hypothesis is strengthened by the fact that in the case of the FhuA binding phages detected using the P$_{opvAB}$::*llp*::*gfp* biosensor the enrichment of the ON subpopulation was much more efficient (**Figure C3.7**). Indeed, it is known that T5 relies essentially on FhuA for a

productive lytic cycle, whereas the polymannose decoration of the O-antigen is used as a primary receptor important for an efficient primary binding but dispensable for the overall cycle (**Figure C3.7**) [332], [333].

Prior to use of Llp lipoprotein, we used the membrane protein phage receptor FhuA. In this case, however, OpvAB^{ON} cells express the phage receptor in their outer membrane with consequent lysis by the phage. Phage detection thus involves a decrease in GFP fluorescence, which is a signal less reliable and less unequivocal than fluorescence increase. Hence, our second choice was a gene whose expression confers resistance instead of sensitivity (e. g., the *llp* gene in the case of phages that use the Fhua protein as a receptor, **Figure C3.7**). However, the possibility of designing sensors in which the meaningful, "positive" signal is fluorescence decrease remains open.

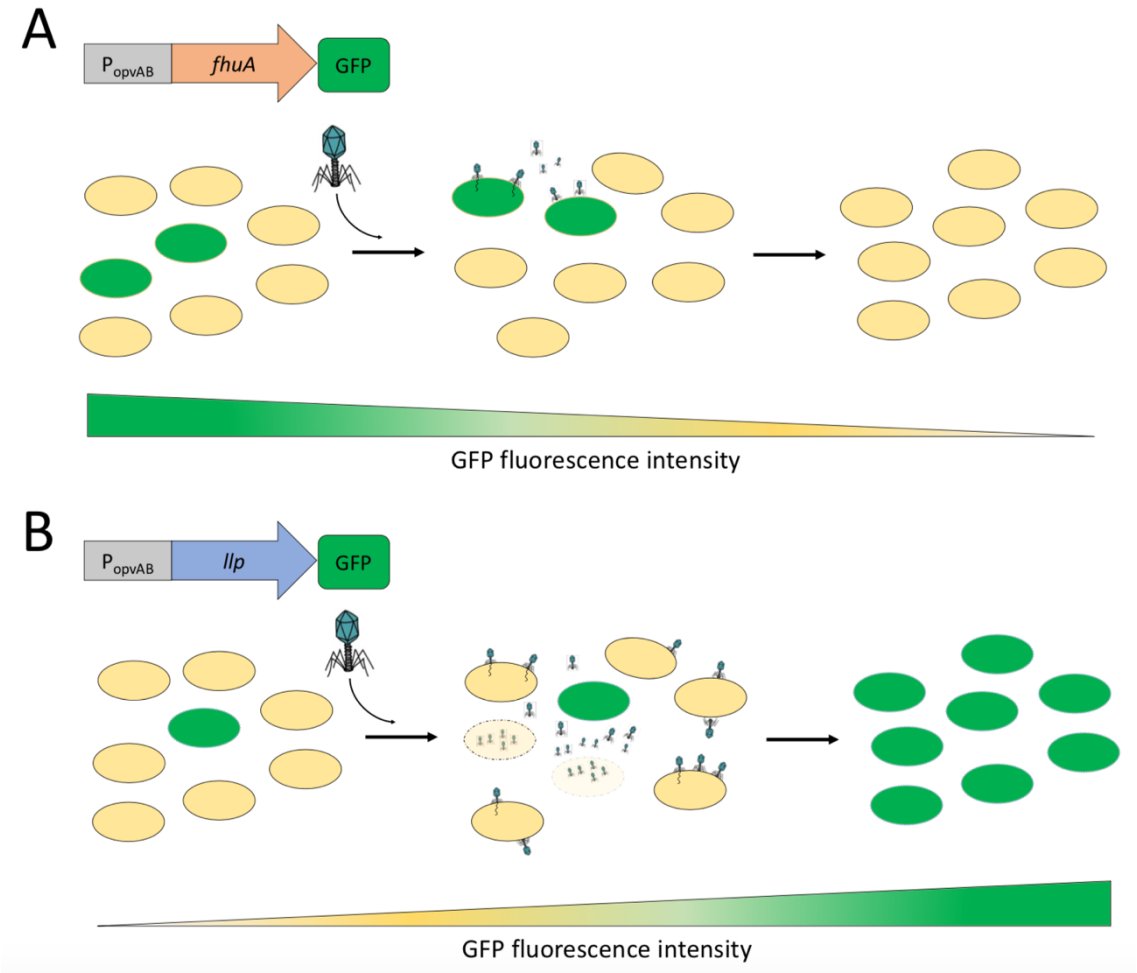


Figure D2: Selection of OpvAB^{OFF} and OpvAB^{ON} subpopulations in the presence of a bacteriophage that uses a heterologous receptor expressed under *opvAB* control. A. Cells producing FhuA are lysed by phage, thus selecting the OpvAB^{ON} subpopulation and producing a concomitant fluorescence decrease.

OpvAB^{OFF} cells are represented in yellow, and OpvAB^{ON} cells are represented in green. **B**. Cells producing Llp are resistant to bacteriophage, and lysis of OpvAB^{OFF} cells results in fluorescence increase due to growth of OpvAB^{ON} cells (green).

An important add-on value of our method is its capacity to discriminate between bacteriophages infecting the same host but using different types of receptors (**Figures C3.6, C3.7**). This property is highly relevant in the frame of phage therapy when the isolation of phages using different receptors is a must to overcome resistance due to mutations into the receptor [334]. This work, allowing a rapid discrimination between phages using LPS or FhuA as receptors, could thus be systematically used prior the assembly of phage cocktails. Moreover, this method could be implemented to other receptors used by bacteriophages as long as a specific inhibitor such as Llp is available as described for FhuA-binding phage (**Figure C3.7**). For T-even coliphages, for example, the trick would be to use the outer-membrane protein TraT, encoded by the F plasmid, that masks or modifies the conformation of outer-membrane protein A (OmpA), which is the the receptor for many T-even-like phages [335]–[337]. In case an inhibitor-encoding gene does not exist for a dedicated receptor, an alternative would be to use the receptor gene itself fused to the *gfp* and controlled by the *opvAB* promoter. However, in such a case the biosensor will be much less sensitive for phage numeration as a decrease in fluorescence is expected. Nevertheless, such an alternative should be sensitive enough to determine the host receptor of the phage under study.

Phage receptor determination is a must to achieve a complete characterization of bacteriophages [335]. The canonical method using plaque assay is of course still relevant, however it cannot be performed in a high throughput manner. A possible way to implement a fast and easy host-receptor determination would be to construct a biosensor strain carrying multiple fusions with various fluorescent protein encoding genes allowing to detect different phages in a mixture according to their receptor specificity.

Another application of the OpvAB switch described in this Thesis involves the detection of DNA methylation inhibitors. Lack of Dam methylation causes strong attenuation in *Salmonella enterica* and in other gamma- and alpha-bacterial pathogens [241], [244]. In turn, CcrM methylation has been shown to play roles in the interaction of alpha-proteobacterial pathogens with their hosts. These observations has raised the possibility of using Dam or CcrM methylase inhibitors as antibacterial drugs [245],

[246]. In alpha-proteobacteria, such drugs would be bactericidal. In *Salmonella* and other pathogens in which Dam methylation is not essential, Dam inhibitors could be expected to attenuate virulence by transforming wild-type bacteria into phenocopies of *dam* mutants. Because Dam methylation is a dispensable function in enteric bacteria, inhibitors specifically targeted at Dam methylase should be harmless for the normal intestinal flora. A drug of this kind should be also harmless for the host, because adenine methylation is rare in mammalian cells [338]. The potential problem that a Dam inhibitor might increase the spontaneous mutation rate in the intestinal flora seems minor compared with its potential benefits as an alternative to current antibiotics.

As a proof of concept, we show that a *opvAB:gfp* fusion permits dose-dependent detection of sinefungin, a well known inhibitor of DNA methylation. Previous searches for novel compounds of this kind have been improductive, and one likely reason is that the screens employed were not sensitive enough. This Thesis may contribute to overcome this problem as a highly sensitive screen can now be devised using the OpvAB switch.

Phase variation-based biosensors can have advantages over classical whole-cell sensors based on transcriptional regulators and/or inducible promoters [339], [340]. One advantage is that the sensors can be expected to be highly selective as selection of one of the subpopulations will occur under specific circumstances only. Use of fluorescence to monitor growth cells can be expected to be sensitive and highly reproducible. Furthermore, programmed switching from OFF to ON and vice versa may contribute to robustness, avoiding the problem of instability frequently found in transcription-based gene circuits [341].

Besides biosensor design, formation of phenotypic subpopulations under epigenetic control may have additional applications in synthetic biology: for instance, division in labour between subpopulations performing distinct segments of a catabolic pathway might optimize biodegradation processes [342].

CONCLUSIONS

1. A 689 bp fragment containing the *Salmonella enterica opvAB* promoter and upstream regulatory region is able to drive transcription of heterologous genes cloned downstream of the promoter.

2. Transcription of heterologous genes under *opvAB* control shows reversible bistability (phase variation), giving rise to bacterial subpopulations that differ in the expression of the cloned gene or operon.

3. Reversible bistability under *opvAB* control is reproducible in *Escherichia coli*.

4. Modification of the ribosome binding site of a gene or operon under *opvAB* control can adjust *opvAB* expression to a level appropriate to discriminate OFF and ON subpopulations.

5. The *opvAB* epigenetic switch has been used to engineer biological sensors that detect subpopulations of bacteria with specific phenotypes (e. g., utilization of lactose and resistance to antibiotics).

6. Biosensors based on the *opvAB* switch permit the detection of bacteriophages and of DNA methylation inhibitors.

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