Universitat Autònoma de Barcelona

Elucidation of the RecA-mediated mechanisms governing swarming motility in Salmonella enterica

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The most beautiful thing we can experience is the mysterious.
It is the source of all true art and science.

Albert Einstein

GURASOEI,
Bihotzez mila esker.

Acknowledgments

Esker hauek zuei idazten ari banaiz, azken urteotan modu batean edo bestean, momentu on edo txarretan, negar malkotan ala ardoa eskutan, nirekin bizi, eta (gehienbat) sufritu nauzuelako da.

Ehundaka hitz idatziko nituzke baina badakizue nortzuk zareten, esker aunitz

4 anys donen per molt...
Escriuria centenars de paraules però ja sabeu qui sou, moltes gràcies

Abstract

RecA is a multi-functional protein that aside from being the main recombinase involved in the central steps of homologous recombination and recombinational DNA repair mechanisms, is the activator of the SOS response. RecA acts as a DNA damage sensor by binding to single-stranded DNA, that activates the protein (RecA*) and prompts the auto-cleavage of the LexA repressor, inducing the expression of SOS response genes. Further, it has been described that RecA is directly associated with swarming motility.

Swarming is defined as a rapid, organized multicellular translocation of bacteria across a moist surface powered by rotating flagella that is widely distributed in the *Bacteria* domain. In fact, through-output screening studies associated RecA protein with CheW, a key component of the chemotaxis machinery deeply involved in chemotaxis, signaling array assembly and swarming behavior.

The results presented herein unequivocally demonstrated RecA-CheW interaction.

We characterized the RecA-CheW protein complex, that allowed the identification of the critical interfaces implied in the interaction and its role in the signaling array assembly. RecA residues Gln20, Arg222, Arg176 and Lys250 that are located in the multi-functional N-terminal and central structural domains of the protein, were described as essential for the interaction. In the case of CheW protein, residues Phe21, Lys55, Asp83 and Phe121 were involved in the RecA-binding, that do not seem to interfere with any other CheW-biding targets.

Further, the obtained results demonstrate that the loss of swarming ability when there is an increase of RecA concentration was the consequence of chemosensing array assembly disruption, that previous works have established as essential for swarming in temperate swarmers. Using high resolution microscopy assays we were able to track CheW and RecA protein distribution within the cell during SOS response induction, elucidating the role of the RecA protein in the distribution of CheW and the assembly of chemoreceptor signaling arrays.

The obtained results head to the proposal of a model that explains how bacterial cells adapt their surface motility in response to the presence of DNA-damaging agents by sensing them via SOS system induction. During surface colonization, bacterial cells will likely be exposed to a wide range of injurious, and potentially lethal, compounds that are avoided through SOS response induction and consequent swarming ability impairment. When DNA injuries are generated, RecA activates the SOS machinery, and its concentration rises swiftly since *recA* is one of the first genes to be induced in the hierarchy of SOS activation. The increase of intracellular RecA concentration during SOS-response disturbs

the equilibrium between this protein and CheW, causing the cessation of swarming. RecA prompts the titration of CheW protein, preventing polar signaling array assembly during SOS response, and thereby inhibiting motility. By this mechanism, bacteria avoid exposure to higher concentrations of the DNA damaging agent, and so, cell death. Following DNA damage repair, RecA concentration returns to its basal level, releasing CheW, that restores chemosensory array assembly, returning the cell to a non-DNA damage motile condition.

Therefore, the present work characterizes the molecular mechanisms that govern RecA-mediated swarming modulation, by which using RecA as a sensor, *Salmonella* cells can adapt their surface motility in response to adverse environmental conditions.

Contents

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Introduction

1.1 Bacterial motility

1.1.1 Motility types

Motility is the ability of an organism to move spontaneously that allows bacteria to colonize many different environments. Bacteria can interact dynamically with the surroundings to detect environmental signals as well as the presence of other bacteria[1].

As a group, spatially organized living bacteria present advantages to optimize growth and survival, access to nutrients or to improve defense mechanism for protection. Accordingly, some bacteria have developed multiple mechanisms in order to modulate their motility and to adapt to the environment. Thus, motility types are not mutually exclusive. Depending on the genera, bacteria may switch motility on and off as required, or be able to oscillate between sessile and motile lifestyle during colonization depending on the growth conditions.

H. Henrichsen[2] identified six different motility categories almost 50 years ago: swimming, twitching, gliding, sliding, darting and swarming. Figure 1.1 presents a scheme of each motility mechanism while Table 1.1 highlights actual differences between them.

In the next sections, the main characteristics of each motility type are described.

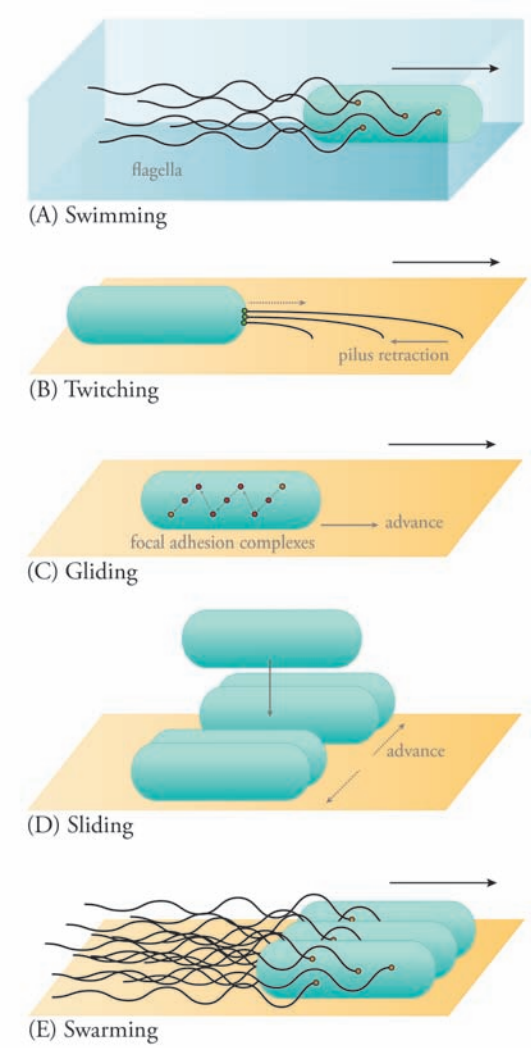

Figure 1.1: Mechanisms of bacterial motility. Schematic representation of the bacterial motility mechanisms: (A) swimming, (B) twitching, (C) gliding, (D) sliding and (E) swarming. The direction of cell movement is indicated by black arrows, and the motors that power the movement are indicated by colored circles. Modified from Kearns *et al.*, 2010 [3].

Table 1.1: Main features of motility types.

Type	Motive Organelles	Cell diff.	μm/s	Environment	Movement
Swimming	Flagella	No	25-160	Liquid	Active
Twitching	Type IV pili	No	0.06-0.3	Surface	Active
Gliding	Unknown	No	0.025-10	Surface	Active
Sliding	None	No	0.03-6	Surface	Passive
Swarming	Flagella	Yes	2-10	Surface	Active

Modified from Harshey *et al.,* 2003 [1].

1.1.1.1 Swimming

Swimming motility is a mode of bacterial movement powered by rotating flagella that take place as individual cells moving in aqueous environments [3] (Figure1.1A). Swimming is widely spread within *Bacteria* domain, but speeds can vary between species: *Escherichia coli* swims at a rate of 25-35 μm per second, whereas *Bdellovibrio bacteriovorus* can reach 160 μm per second (Table 1.1). The flagellum presents a complex structure, assembly mechanism, and regulation. Each flagellum is composed of a long helical filament, a short curved structure called the hook, and a basal body consisting of a central rod and several rings [1,4]. The basal body is embedded in the cell surface, whereas the hook and filament are external to the cell. The flagellar filament is normally a left-handed helix of variable length (typically 5 to 10 μm), with a diameter of 20 nm. In fact, the ability to swim involves over 60 structural and regulatory proteins that are required for processing sensory cues and the function and assembly of operating flagella [5].

Bacteria swim by rotating these flagella. The rotating helical filament creates a viscous slip down the long axis of the helix, displacing the water. When the flagellar motor is turning counterclockwise (CCW), the mechanical and hydrodynamic forces cause the helical filaments to assemble into a bundle along the cell body, resulting in pushing the cell longways linear trajectories known as the "running" movement [1,4] (Figure 1.2). Periodically the flagellar motors switch to a clockwise(CW) rotation, inducing the disassembly of the bundle that redirects the cell. This transition results in a "tumbling" movement, causing the cell to reorient its travel direction randomly. The alternation of runs and tumbles makes the bacteria to random walk. During the normal CCW rotation, the flagellum structure is a left-handed helix. Upon rotatory sense change to CW, the filament undergoes a discrete structural transition to a right-handed curly helix where the structural tensions are higher not only within the filament structure but also at the flagellum basis and motor [6]. In a constant environment, a cell typically moves in random runs of approximately 1 s interspersed by tumbles of 0.1 s.

How the CCW/CW states of individual flagellar motors collectively determine the run-tumble swimming behavior of the whole, multi-flagellated cell remains poorly understood [8]. Depending on the genera, the position and number of flagella on an individual swimming cell can vary widely from one to more than ten [9], yet very few is known about how flagellar quantity affects swimming behavior.

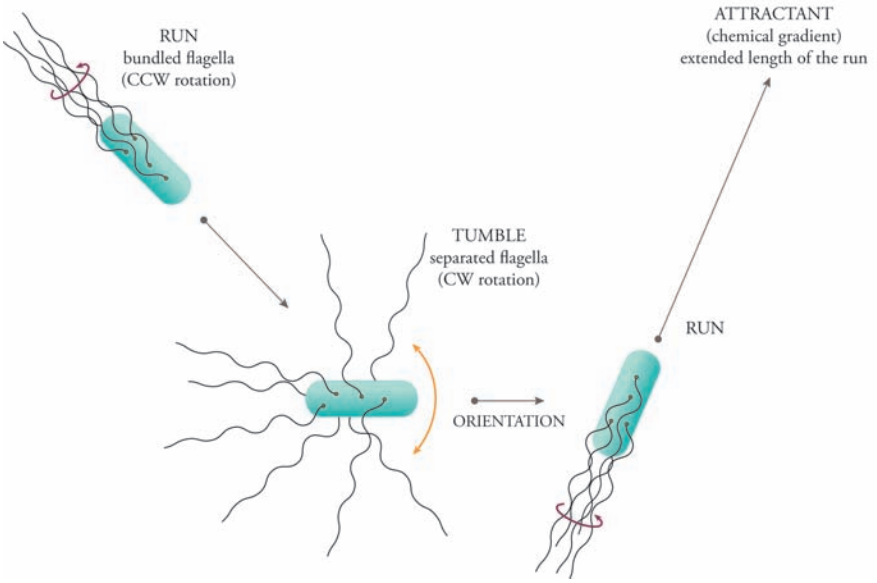

Figure 1.2: Flagellar behavior during swimming motility. When a cell is running, all its flagellar filaments are rotating counter clockwise (CCW), thus forming a bundle that pushes the cell forward. Periodically, one or more filaments start rotating clockwise (CW), provoking the cell to tumble. These running/tumbling periods allows the bacteria to sense and response to environmental stimuli. In the absence of a chemical gradient, flagellar rotation cycles between running and tumbling with no overall directional movement. However, when a chemical gradient of an attractant exists, the length of the runs is extended, while the length of tumbles is decreased. Modified from OpenStax[7].

1.1.1.2 Twitching

Twitching, also called retractile motility or social gliding, is a slow surface movement of bacteria powered by the repetitive extension-tethering-retraction of Type IV pili (TFP) that results in the translocation of the cell body[3,10–12] (Figure1.1B). Twitching cells are able to move at 0.06-0.3 μm/s (Table 1.1). These repetitive movements of extension and retraction are observed on solid surfaces, interfaces or media with moderate viscosities (1% agar) and have been implicated in virulence, host colonization and other forms of complex colonial behavior, including biofilm development and fruiting bodies[10,13]. Twitching motility has been shown to occur in a wide range of bacteria such as *Pseudomonas aeruginosa*, *Neisseria gonorrhoeae* or *Acinetobacter baumannii*[1,12]. At the macroscopic level, the manifestation of twitching motility can be variable depending on the species and culture conditions.

Although twitching motility is primarily a social activity that involves the formation of organized and compact rafts, it has also been demonstrated that individual cells can move by twitching[1,10]. It is remarkable that it can be employed either to bring cells together into complex structures under conditions of nutrient depletion or to promote the colonization of new surfaces by bacterial communities under high nutrient availability. Complex signal transduction systems regulate these processes. As previously mentioned, twitching motility is mediated by TFP located at one or both cell poles[14,15]. TFP are typically 5-7 nm in diameter and can extend to several μm in length[10]. To date, little is known about the adhesion and nanomechanics of TFP, but the key role played in controlling bacterial attachment to both biotic or abiotic surfaces is well confirmed[16,17].

TFP mediated bacterial attachment to hydrophobic surfaces can sustain forces up to 250 pN[18]. This force-induce conformation reveals hidden epitopes previously buried in the pilus, which maintain cell attachment to the surfaces. Hidden epitopes form a tip at pilus end that is exposed due to quaternary structure changes during surface sensing and is capable of promoting adhesion. Also, it has been suggested that the pilus structure could act as a mechanical signal for the cell to recognize the firm attachment to a surface[16].

After pili adhere to the surface, the mechanisms of retraction is triggered, pulling the cell towards the point of attachment[19]. Once adhered, the retractile force is generated by filament disassembly of the helical array of PilA (main pilin) subunits into the inner membrane[20], mediated by PilT motor protein[21,22], which promotes pilus disassembly, pilin degradation or both. Retraction is usually terminated with the release, or breakage of the pilus tether. After retraction occurs, and through the assembly proteins (PilB, among others) the pilus is assembled and extended again until the epitope-tip sends a new signal and retraction commences again (Figure1.1B).

1.1.1.3 Gliding

Gliding, also known as adventurous gliding, is an active smooth movement along the long axis of the cell that involves focal adhesion complexes instead of depending on flagella or pili[1,3,12] (Figure1.1C). Many different models have been proposed to explain gliding motility, but there is not a clear evidence supporting any specific one. Unlike twitching, gliding motility usually functions on drier surfaces. Because of the motors for gliding were unknown and gliding bacteria have no apparent external structures associated with

motility, gliding has been traditionally defined by the motors that were not used (flagella and pili) rather than by the motors that were used[23].

Lately, advanced techniques revealed that gliding in different bacteria involves diverse variety of motors and a broad spectrum of mechanisms[23], and has been particularly described in three broad bacterial groups: the Myxobacteria, the Cyanobacteria and the Flavobacteria[24–27]. Gliding motility appears to be unlike other characterized prokaryotic motility systems, various motors and a broad spectrum of mechanism are implied in this kind of motility. Accordingly, gliding bacteria present a great variability in velocity depending on the species that can be up to 0.1 μm/s for *Myxococcus xanthus*, 2 μm/s for *Flavobacterium johnsonae* or 2-4.5 μm/s for *Mycoplasma mobile*[23] (Table 1.1). It is believed that bacteria might glide using a variety of motors, mostly unknown, but that all generate a propulsive force by very similar mechanisms[23]. It has been recently suggested that there is an intriguing possibility that motors moving in helical patterns cause the rolling of cell bodies, and thus, propel glide movement, which could constitute a universal formula for this type of motility[23,28].

1.1.1.4 Sliding

Sliding, also known as spreading, is a passive surface translocation mechanism independent of appendices that does not involve motors but requires surfactants or other molecules capable of reducing surface tension[3] (Figure1.1D). During sliding, sheets of cells packed in different directions can spread outward as a unit, suggesting that this is not an active form of movement.

It is powered by expansive forces that are transmitted through the growing colony starting from the center to the edges, but the mechanism underlying sliding has not been characterized in detail. Examples of sliding bacteria include members of the genera *Escherichia*, *Pseudomonas*, *Bacillus*, *Serratia*, *Vibrio*, *Alcaligenes*, *Flavobacterium*, *Acinetobacter*, *Streptococcus* and *Corynebacterium*, among others[1,29–31]. The expansion rate of the colony edge can range from relatively slow (0.03 μm/s for *Mycobacterium smegmatis*) to moderately fast (2-6 μm/s for *Serratia marcescens*) and it is group-dependent, as observed for gliding motility[1] (Table 1.1).

There is a strong correlation between the production of surfactants such as lipopeptides, lipopolysaccharides (LPS) and glycolipids, and the sliding phenomenon[1]. Although passive, this mode of translocation likely plays a significant role in bacterial surface colonization and has been described as essential for *Mycobacteria* to colonize environments and hosts[32].

1.1.1.5 Darting

Darting is a kind of surface translocation produced by the expansive forces developed in an aggregate of cells inside a common capsule and resulting in the ejection of cells from the conglomeration[2]. The micro-morphological pattern is composed of cells and aggregates of cells distributed randomly with empty areas of agar in between. Neither cell pairs nor aggregates move except during the ejection which is observed as a flickering under the microscope. Nothing is known about darting motility regulation and it is not commonly used to describe bacterial motility[33].

1.1.1.6 Swarming

Swarming is a specialized form of flagellar-driven multicellular surface translocation movement by which bacteria can rapidly advance in moist surfaces in a coordinated manner[1,34,35] (Figure1.1E). This type of motility has been found in many members of Gram-negative and Gram-positive flagellated bacteria, as *Proteus*, *Vibrio*, *Bacillus*, *Clostridium*, *Chromobacterium*, *Escherichia*, *Salmonella*, *Azospirillum*, *Aeromonas*, *Yersinia*, *Serratia*, *Burkholderia*, *Pseudomonas* or *Sinorhizobium*[36–42].

As most of the collective behaviors, swarming requires reaching a certain cell number before the process could be initiated. During swarming, motile cells move in groups (also called rafts) organized in parallel to their long axis to maximize cell-to-cell contact, colonizing the entire available surface. Swarm cells can reach up to 10 μm/s[1,13], and is the most rapid surface motility known so far (Table1.1). The migration front is preceded by a visible layer that embeds cells in a matrix of slime-like extracellular material that allows the right movement of the flagella[38,43].

Regarding their ability to displace over moist surfaces, swarming bacteria can be divided into two categories: (i) robust swarmers, that can swarm across hard surfaces (1.5% agar and above), and (ii) temperate swarmers, that can only swarm over soft surfaces (0.4% to 0.8% agar)[1,43,44].

1.1.1.6.1 Robust swarmers

When a bacterial colony is grown under swarming conditions, upon contact with a surface and the inhibition of flagellar rotation, cell differentiation process is induced from short, motile vegetative cells to a multinucleate aseptate swarmer cells. In robust swarmers, such as *Proteus*, *Vibrio*, *Rhodospirillum* or *Clostridum*, swarmer cell morphology is clearly distinguishable from their vegetative counterparts by a prominent elongation of cell bodies (up to 20-40 times vegetative cell length). This phenotype is usually caused by the inhibition of cell division and by the acquisition of an hyperflagellated (50-fold higher surface density of flagella) and polynucleated state[1,38,43]. Some genera, including *Vibrio* and *Aeromonas*, however, encode a separate flagellar system for motility on surfaces and in viscous fluids[45]. These bacteria use a sheathed polar flagellum for translating through bulk fluids and express unsheathed lateral flagella for motility on surfaces[46].

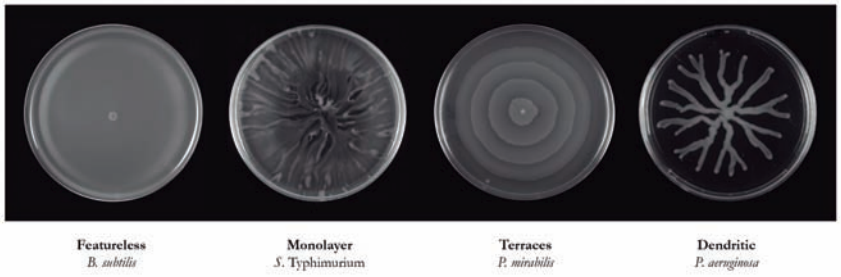

Figure 1.3: Common colony patterns during swarming. Variety of colony patterns observed in *Bacillus subtilis*, *Salmonella enterica* sv. Typhimurium, *Proteus mirabilis* and *Pseudomonas aeruginosa*. The image for dendritic colony pattern is adapted from de Vargas Roditi *et al.*, 2013[47] and featureless pattern image is a courtesy of Sánchez-Osuna M (unpublished).

There are three well-defined stages of swarming: (i) differentiation of vegetative cells into swarm cells, (ii) migration of swarm cell population and (iii) consolidation[38]. When cells sense a surface, first, regular colonies will be formed. After the initial growth, cells at the periphery undergo differentiation, elongating to develop into a multinucleate, aseptate, hyperflagellated swarm cells due to septation repression and the induction of flagella synthesis.

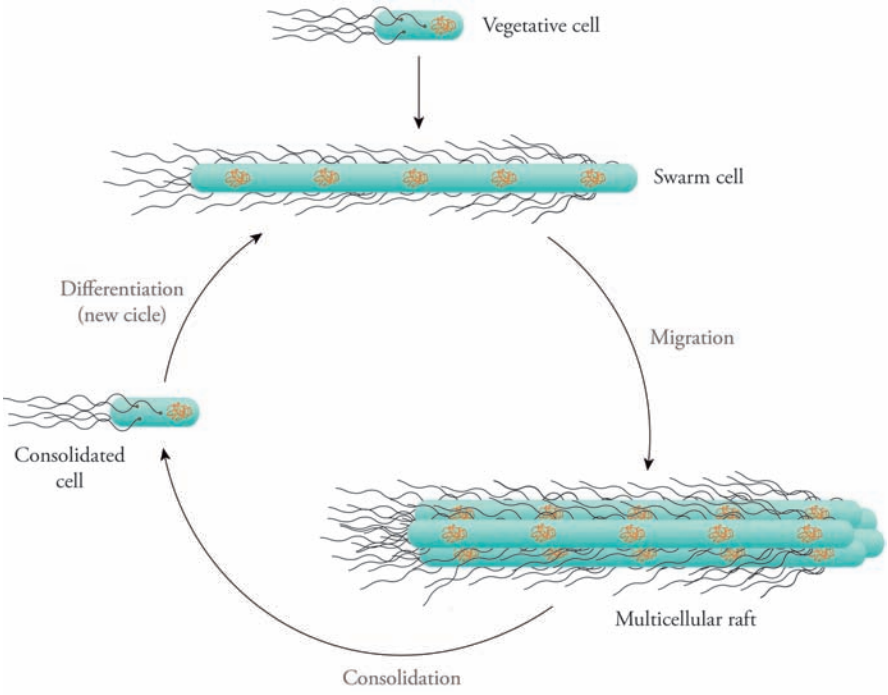

Figure 1.4: General differentiation process of robust swarmer bacteria on surfaces. Schematic representation of robust swarmer cell differentiation process from vegetative to swarm cells. The length and the number of flagella per swarmer cell has been reduced for clarity and the orange spots depict the bacterial chromosome. Modified from Copeland *et al.*, 2009[46].

Migration occurs when swarm cells form rafts and move over the surface. Finally, after a period of migration, swarm cells undergo consolidation, *i.e.* reversion to normally flagellated, short vegetative cells. These vegetative cells are capable of initiating further cycles of swarming differentiation and migration. Figure 1.4 represents the mentioned stages and the differentiation process during surface migration.

Different organisms show several consolidation models which lead to a variety of colony patterns that are species-specific under certain environmental conditions (Figure1.3). Macroscopically, *Proteus mirabilis* colonies alternate between periodic phases of migration and consolidation, that due to cyclic repetition results in regularly spaced concentric terraces that are geometrically resembling a bulls-eye pattern[38,43,46]. Similar patterns can be observed in *Vibrio parahaemolyticus*, another robust swarmer[34,43,48].

1.1.1.6.2 Temperate swarmers

Temperate swarmers as *Escherichia*, *Salmonella*, *Pseudomonas*, *Yersinia*, *Serratia* or *Bacillus* do not display a significant swarmer cell differentiation[44], except for a modest increase in cell length in *Salmonella*[35]. This slightly elongated phenotype of *Salmonella* is the results of a slow down and not due to a complete inhibition of the cell division process, as occurs in robust swarmers. The absence of a swarming-specific program is in concordance with the lack of swarming-associated regulation of flagellar synthesis genes observed in some of the mentioned species[49,50].

Temperate swarmers tend to move continuously without showing differentiation and consolidation periods, not forming terraces[38,43](Figure1.3). Even though differences can be found when comparing, for example, some species like *Clostridium sporogenes* or *P. aeruginosa* that present a dendritic fractal consolidation pattern, to *Salmonella*, which usually creates a monolayer pattern (Figure 1.3). In the case of *Bacillus* species, it is worthy to mention that some of them display a variety of colony patterns, ranging from featureless to dendritic consolidation stages, in a poorly understood fashion that has been related to media and environmental conditions[3].

However, there are many other changes in gene expression of temperate swarmer bacteria during surface growth, mainly linked to the induction of virulence pathways[13].

1.1.1.6.3 Environmental factors that affect swarming

Swarming present high sensitivity to moist conditions that can be explained by its requirement for water, nutrient availability, and temperature[1]. Three critical elements will allow bacteria to swarm within a surface where there is no free water: (i) to attract sufficient water to the surface to fully immerse cells, (ii) to surpass surface friction, and (iii) to reduce surface tension[44]. Bacteria overcome these challenges by secreting polysaccharides by a variety of mechanisms including altering surface charge/lubrication with surface-attached polymers such as lipopolysaccharide (LPS) or enterobacterial common antigen (ECA), increasing flagella numbers (increased thrust), employing unique stators or stator-associated proteins to increase motor power, and/or by secreting powerful surfactants.

1. **Surface moisture**. Swarmer cells move within the extracellular slime, a complex mixture of polysaccharides, surfactants, proteins, peptides, etc., that surrounds the colony, which along with the morphology of swarmer cells improves surface wettability. In temperate *Enterobacteriaceae* swarmers, LPS, ECA, surface glycolipids (ubiquitous among enteric bacteria), and the *flhE* gene (that belongs to the flagellar regulon) could be involved in colony hydration functions by extracting water from the surface[51–53]. The FlhE protein, the last protein of the flagellar regulon whose function is unknown, is likely involved in changing the outer membrane properties as much as increasing the expression of genes activated by the Rcs signaling pathways[53–55].

   Even if the LPS, ECA or FlhE are considered crucial for swarming motility, it is not plausible to include them as part of the swarming-specific program. LPS genes appear to be up-regulated in a surface-specific manner instead of in a swarming-specific one[49,56], as its overproduction is a characteristic used to prompt swarming and not part of the swarming-specific gene expression pattern[44].

2. **Frictional forces.** Swarmer cells must overcome frictional forces to translocate over a surface, generated by surface-cell charge interactions and the viscosity of the fluid between the surface and the cell wall[44]. Surface friction is defined as an electrostatic force between two surfaces sliding past each other. The magnitude of this force depends on the material properties of the surfaces and whether they are dry or coated in viscous or in lubricated fluid[57]. Due to swarm over surfaces, bacteria have to overcome frictional resistance, by lubricating the cell-surface interface, reducing charge, or generating more thrust by increasing flagellar motor power.

Surfactants or lubricating molecules are usually attached to the bacterial surface or secreted into the medium, altering the interactive surface between the bacteria and the agar and possibly contributing to lowering friction during swarming. Despite that, the mechanical approach based on an increase of the flagellar motor force that moves a bacterium through water seems the most common mechanism found so far[44](Figure 1.6). Again, some differences between robust and temperate swarmers arise at this point.

Robust swarmers are known to present an hyperflagellated and elongated phenotype when swarming, as reviewed in previous sections. Increasing the cell length have been hypothesized to be a strategy, among others, to accommodate the growing number of flagella that will provide motor-power to overcome the surface friction even in hard surfaces[44]. Robust swarmers encounter frictional resistance during swarming increasing the number of flagella (hence more motors) or by specific stator-associated proteins (FliL) that likely provides more power to existing motors. Nevertheless, the overproduction of flagella is not universally conserved in all swarming bacteria, thus supporting the idea that there must be multiple mechanisms to beaten frictional forces[58].

As for temperate swarmers, strategies against frictional forces can not be explained through the swarmer phenotype, some other mechanism to overcome this challenge must exist. *Salmonella* neither overproduce flagella nor significantly increase their cell length[35], but it has been suggested that this modest increase in cell length could be *per se* a mechanism to reduce cell friction as rods are more proficient reducing friction resistance[35]. *P. aeruginosa* is reported to require two sets of stator proteins

(MotAB and MotCD) that likely transmit more power to flagellar motors allowing the bacteria to move across surfaces or swim in more viscous mediums[59,60]. Stator proteins are also required by *E. coli*, *Vibrio* and *Salmonella* to properly swarm[61–64]. Mot proteins form a complex that acts as the stator and use membrane potential to conduct ions and supply the torque for rotation. A single transmembrane protein named FliL is associated with torque generation in the flagellar motor. FliL is thought to function as a structural reinforcement of the flagellar basal body, protecting the motor from the torsional stress suffered when motor power is increased by a higher proton motive force during swarming[35,61].

3. **Surface tension.** The phenomenon of surface tension occurs due to the cohesive forces existing between liquid molecules that interact more strongly with each other than with any other associated with the surface[44]. Breaking through this tension is difficult for a moving object such a swarmer cell. So, it is reasonable that swarmer cells would benefit from a lowering of surface tension. Biosurfactants are amphipathic molecules that reduce the surface tension between the cell and the surface while swarming, thus facilitating movement[3]. The role of surfactant agents is not attracting water to hydrate the colony, but they are involved in facilitating the spreading of the colony over the surface[44].

   In robust swarmers, there is no evidence of surfactants secretion and LPS is assumed to play this role[44]. On the contrary, several temperate swarmers specifically synthesize and secrete a variety of surfactant compounds during swarming motility. Among the best studied ones are the surfactin, the serawettin and the rhamnolipids, three potent surfactants produced by *Bacillus*, *Serratia* and

Pseudomonas, respectively[3,65]. Secreted surfactants reduce surface tension, allowing the water (and hence the colony) to expand readily.

4. **Available nutrients.** The supply of nutrients is an important factor in determining the success of a given colony. The underlying mechanisms that allow bacterial communities to translocate and expand across surfaces, and thus, to access to a larger supply of nutrients, are of fundamental importance. Several well-studied bacteria are known to exhibit swarming motility on surfaces of varying hardness and nutrient availability, not only in *Salmonella* but in members of the genera *Bacillus*, *Chromobacterium*, *Clostridium*, *Escherichia*, *Proteus* or *Vibrio*[1,66,67]. Even though it is possible to observe swarming motility in minimal media, a carbon source supplementation is usually required, typically glucose or casamino acids[1].

5. **Temperature.** It is a crucial factor affecting swarming motility. Since changes in temperature cause modifications in growth rate, it might be expected that the occurrence of swarming would be readily affected by variations in temperature, but this has been reported to be species-specific. While species like *Salmonella* are able to swarm in a wide range of temperature, usually between 30 and 37°C[68], others, like *Serratia marcescens*, are temperature-sensitive[69]. Swimming and swarming are inhibited in this bacteria if the temperature slightly surpasses 30°C, which correlates with the serrawettin synthesis in a temperature dependent fashion[1,29]. Furthermore, temperature changes may affect tumbling frequency and subsequently swarming speed; a drop in temperature increments tumbling frequency while a quick rise results in a temporary suppression of tumbling[70,71], which affects the chemotactic response of the cell and so swarming motility.

1.1.1.6.4 Signaling pathways involved in swarming

Apart from the environmental conditions needed for motile bacteria to success, there are three signaling pathways known so far that also have influence over swarming in motility.

1. **The chemotaxis system.** The ability to sense of swarmer cells is reported to play a variable role within the swarming-capable bacteria. In species like *E. coli* or *Salmonella*, it has been reported that the chemotaxis machinery but not chemosensing ability is required for swarming motility[36,72]. Indeed, it is the chemotaxis pathway what constituents the key element controlling swarming behavior and not chemotactic ability itself[73]. Furthermore, it is not clear if all the structural and signaling components of the chemotaxis system are implied in swarming motility.

   The flagellar motor has been established as an important factor affecting swarming motility, as the primary role of the chemotaxis system is to control the flagellar motor bias between CW and CCW states[73]. This modulation allows the cell to change the movement direction and to generate an appropriate response to external stimuli[74]. Thus, *che* mutants presented a broad range of affectations in the motor bias, fewer and shorter flagella and down-regulated "late" motility genes when propagated on a surface[75]. The *cheA*, *cheY*, *cheR* and *cheW* knockout mutants present a CCW rotation of the motor whereas *cheB* and *cheZ* mutations cause the bias to be displaced towards CW rotation[73,76]. The aberrant flagellar phenotype is related to a lack of adequate hydration and subsequently loss of motility in the mutant colonies[73].

In fact, the inability to swarm due to an extreme motor bias phenotype is reversible when the surface moisture is sufficiently high in spite of the motor rotation direction, suggesting that the flagellar motor switch is an influential factor for swarming only in a dry surface situation[73]. It is believed that when all the flagellar motors are rotating CCW, the helical flagellar filaments form a left-handed bundle that facilitates propulsion for running, whereas when one or more motors switches to CW rotation, the bundle is disrupted and a transient conversion towards right-handed filaments occurs, which undergoes tumbling movement[77,78]. This right-handed phenotype is related to a hypothesis that sustains that CW biased mutants can swarm due to a phenomenon known as inverse motility, that consist on swarming only over moist surfaces using the right-handed bundle flagella and CW rotation[73].

2. **Quorum sensing (QS).** It is a mechanism of bacterial cell-to-cell communication that regulates gene expression and coordinates behavior in agreement with cell population density[79,80]. QS allows bacteria to synchronize their social activity by creating, releasing and detecting hormone-like small signaling molecules called autoinducers (AIs) in response to fluctuations in the surrounding environment[81]. When a particular concentration of autoinducers is reached, bacteria detects the signal and triggers the activation of transcription factors that regulates gene expression and finally enables the population to adapt[82].

   QS systems regulate a large number of physiological processes in bacteria including biofilm formation, virulence factor production, bioluminescence, sporulation, motility, and antibiotic production[13]. The extracellular concentration of autoinducers is related to the population density of the producing organisms, and

in fact, cell density is believed to be a key factor controlling the swarming behavior due to the QS, coupling swarming and social communication in bacteria[13].

Gram-positive and Gram-negative bacteria present different mechanisms of QS, mainly differing in signal molecules secreted to measure their population. Gram-negative bacteria utilize N-acyl-homoserine lactone (AHL), homoserine lactone ring with an additional fatty acid side chain which length is species-specific. In contrasts, Gram-positive bacteria secrete peptides as signal molecules. In general, AI molecules produced by Gram-negative bacteria diffuse passively in and out of cells, whereas AIs synthesized by Gram-positive bacteria are actively transported[83,84].

Therefore, the molecular components of QS systems have been suggested as promising targets for developing new anti-infective compounds, as the inactivation of the QS system of a pathogen can result in a significant decrease in its virulence[85]. Recent studies demonstrated that punicalagin, the main active compound in pomegranate peel, presents anti-QS properties and is able to reduce motility in *Salmonella* down-regulating flagellum associated genes[86] and also by disrupting the QS signaling system due to *sdiA* down-regulation[86].

3. **Secondary messengers.** Motility and cellulose biosynthesis are inversely controlled by the secondary messenger c-di-GMP, unique to bacteria[87,88] that it is considered as the most important secondary messenger known to affect the state of mobility[89,90]. This nucleotide-based secondary messenger is an important signaling molecule in the transition between motile and sessile forms of bacteria, high levels of c-di-GMP inhibit motility and promote biofilm formation, whereas low concentrations stimulate motile behaviors[91,92].

In *Salmonella*, c-di-GMP inhibits flagellar motility at the level of either gene expression, flagellar assembly, or function[93]. It is not known what keeps c-di-GMP levels low during swarming, but there are several putative c-di-GMP metabolizing protein domains, such as EAL (phosphodiesterase) and GGDEF (di-guanylate cyclase) which act as phosphodiesterases and that are known to play a determinative role in the expression level of multicellular behavior in this pathogen[92]. Environmental signals that increase c-di-GMP levels are also unknown, but when c-di-GMP binds YhjH or YcgR receptors[94–96], the complex interacts directly with the flagellar motor components, inducing a CCW motor bias, and thus, inhibiting chemotaxis and putting a brake on motor rotation. Knockout mutants of *yhjH* gene are reported to be unable to swarm in both *Salmonella* and *E. coli*[94,97]. Also, the c-di-GMP signaling network is involved in the formation of RDAR (Red, Dry, and Rough) morphotype of *Salmonella*, a multicellular behavior characterized by the expression of the extracellular matrix components cellulose and curli fimbriae, controlled by c-di-GMP, which allows bacteria to persist in nutrient-limited environments[91,92,98].

Indeed, motility and RDAR biofilm formation are interconnected by the motility-specific phosphodiesterase YhjH, which down-regulates expression of the transcriptional regulator CsgD, the central RDAR biofilm activator[92,99]. This system is independent of the previously explained flagellum-driven surface sensing mechanism[44].

1.1.1.6.5 Environment sensing and signaling pathways

The exact mechanisms that allow bacteria to sense the surface conditions are still poorly understood, and even the existence of such a program remains controversial. Although, for cells to start swarming, there has to be an initial surface-sensing signal that triggers the required regulation pathway in preparation for moving. Lately, a new mechanism that translates environmental stimuli into biological responses has been studied. The detection of mechanical stimuli through surface contact, termed mechanosensing, is responsible for multiple cellular responses that result in surface-associated behaviors, such as attachment, movement across a surface or cellular differentiation.

No universal surface-sensing mechanism has emerged for bacteria that regulate gene expression in response to a surface attachment; neither has been demonstrated the existence of an apparent gene regulation program. Little is known about how bacteria make use of mechanosensing to regulate surface-associated behaviors, including movement, biofilm formation, and virulence. These mechanisms are sure to be varied, but there are at least two of them implied in surface sensing through mechanical stimulation; the flagellum and the cell envelope[35,100,101].

1. **The flagellum as a sensor.** There is evidence from several bacteria where conditions implicated in slowing or arresting flagellar rotation lead to cellular responses that aid a particular bacterial lifestyle. A standard model for signaling pathway has been recently proposed[102], suggesting a unifying model where the ion-conducting conformation of the motor plays a key role in regulating the response.

In swarmers as *Proteus*, *Vibrio*, *Bacillus* and *Caulobacter* the induction of a specific cellular response occurs due to a motor stalling by creating a condition where the flagellar motor is stopped[102]. *V. parahaemolyticus*, which swims using a single Na^+-driven polar flagellum, synthesizes hundreds of lateral flagella during swarming to override surface tension by increasing propulsive force[103], besides inhibiting cell division and increasing the synthesis of virulence factors associated with signal transduction[104]. The affection of the flagellar motor, leading to a decrease in the rotatory speed of the polar flagella triggers the swarmer cell differentiation[1], mainly due to a stalled motor or the absence of ion-conducting stators[102]. In biofilm former bacteria such as *B. subtilis*, the inhibition of flagellar rotation results from the increased transcription and secretion of poly-γ-glutamate (PGA), a polymer that forms an external slime layer and promotes adhesion to surfaces[105–107]. The genetic disruption of flagellar components or the physiological disruption of the rotor-stator interface are the responsible for the activation of the PGA synthesis, that is additionally dependent on the two-component system DegS-DegU, which is activated by flagellar disruptions[107].

In the case of the freshwater bacterium *Caulobacter crescentus*, that presents a motile cycle enabled by H^+-driven polar flagella, and a sedentary life-cycle due to the presence of a thin cylindrical cell membrane extension with an adhesive tip that allows surface attachment[108]. Pilus-dependent contact stimulates immediate attachment and polysaccharide "holdfast" secretion, *C. crescentus* cells are tethered to the surface, also pinning the flagellum and rapidly arresting flagellar rotation.

Finally, a sensing mechanism involving the FliL protein has been proposed for *P. mirabilis*[35], that is not polarly flagellated but presents several H^+-driven peritrichous flagella, whose synthesis is up-regulated while swarming[109]. However, it remains unclear if it is a mechanosensor mechanism or the sensing ability is dependent on any other pathway[35,102].

Besides the importance of rotating flagella for colony hydration, it may play one more active role, as it has been speculated that flagellar filaments normally stick to swarm agar and thus get tethered or restricted, while is the motor switch which unsticks or liberates them[75]. In the case of *Salmonella*, flagella are thought to be a surface sensor for unfavorable environmental conditions. The mechanical impairment of the flagellar rotation, due to adverse moist conditions or the interference of flagellin polymerization, sends a signal to Type III secretion system to avoid flagellin and FlgM protein synthesis, which is the inhibitor of flagellar Class-3 genes transcription and their translocation across cell membrane. In this scenario, FlgM has a role sensing external environmental conditions during motility[75].

2. **The cell envelope as a sensor.** In *P. mirabilis*, the Up-regulator of the master operon (Umo) proteins that are associated with the cell envelope, are thought to participate in surface sensing and signal transduction of the FlhDC master regulator[110]. In *E. coli* and *Salmonella* the *flhDC* master regulator is the primary site for the integration of signals coming from the species two-component *rscBC* system that is activated in response to outer membrane perturbations[43,58,111]. This system is quite similar in *P. mirabilis*, whose sensing pathways have been more profoundly studied. Umo proteins activate *flhDC* expression and are themselves

up-regulated during swarming, while defective mutants either abolish or reduce the *flhDC* transcript[109,110,112]. The Rcs and Cpx signaling pathways, which sense cell envelope stress[113,114], also play a role in transducing external signals to the FlhDC master regulator[115]. It has been hypothesized that Rcs and Cpx pathways might serve as a model for how dependent fashion proteins might communicate surface information via response regulator to activate *flhDC*[109]. Both surface sensors sense perturbation of the membrane(s), cell wall or periplasmic space, triggering the canonical stress systems and thereby also alerting the bacteria about cell-to-surface contact[114,115].

1.1.2 Bacterial motility and pathogenicity

Motility plays a significant role in the life cycle of bacteria. Motility benefits bacteria through the capacity to move toward favorable environments and to avoid detrimental conditions, thus enabling successful competition. Motile bacteria spend a considerable part of their metabolic energy to colonize an environmental reservoir or any animal/human host, in cooperation with their gene expression in response to external stimuli[116].

Apart from the involvement of the flagellum in aspects related to virulence, motility is also necessary for the infective capacity of pathogenic bacteria. There are several examples of pathogens in which motility plays a role during initial phases of infections, including *Salmonella*, *E. coli*, *Bordetella bronchiseptica* or *Bordetella pertussis*[117–119]. The flagellum has some other biological functions apart from being the motility organelle, as it plays a significant role in bacterial pathogenicity during invasion of host cells[120].

Besides, the flagella also promote bacterial biofilm formation supporting pathogen survival *in vivo*[121], translocates virulence proteins into host cells via special Type III secretion systems[122] and trigger host pro-inflammatory responses[123].

All along the first phases of colonization, flagella have been reported to function as adhesins[124], and also, the flagellin subunits play a role in innate immunity as a dominant antigen of the adaptative immune response. Also, flagellar tethering serves as a mechanical signal to the cell and triggers the regulation program for host contact[116,124]. Other bacterial pathogens need to continuously maintain motility during infections and cannot survive without being motile and competent for chemotaxis. *Borrelia burgdorferi* and *Treponema* spirochetes, *Helicobacter*, *Campylobacter*, *P. aeruginosa* and *Proteus* are some examples[125,126].

Recently, it has been shown that *P. aeruginosa* can regulate virulence on surface contact using TFP as a mechanosensor that carries the signal through the Chp chemosensory-like system to up-regulate cAMP/Vfr-dependent virulence gene expression[101]. The stimulation of the Chp chemosensory-like system that is linked to twitching motility simultaneously modulates the transcription of more than 200 genes including key virulence factors such as the Type II and Type III secretion systems, QS, and the pilus itself[127]. Furthermore, the TFP is believed to be a major virulence-associated adhesin[18], as is critical for surface mechanosensing and consequent up-regulation of virulence factors and the delivery of toxins[16,17,128].

Motility profiles have been associated not only with virulence but also to elevated resistance to antibiotics[56,129–131]. Some bacterial colonies exhibit a greater resistance to multiple antibiotics when moving, as has been described for flagellated species *S. enterica*,

E. coli and *P. aeruginosa*[68,130]. This phenomenon is known as Adaptative antibiotic resistance (AdR), albeit the mechanisms underlying this transient resistance is poorly understood. This phenotype is known to emerge as a consequence of concentration gradients, as well as contact with sub-inhibitory concentrations of antibiotics, both known to occur in human patients and livestock[132]. Moreover, AdR has repeatedly been correlated to the appearance of multi-drug resistance, although the biological processes behind its emergence and evolution are not well understood.

At least in the species mentioned above, motility-based resistance is non-genetically conferred, since it ceases when cells are grown under non-swarming conditions[68,130,131]. The mechanisms that can confer this AdR have been related to physiological attributes, as cells may present an altered outer membrane composition[129] or a decrease in the membrane permeability[56] acquired in response to growth on moist surfaces. Furthermore, AdR seems to be a function of the bacterial cell density coupled with the swarming velocity of the bacterial colony[68].

1.2 Swarming motility in *Salmonella*

1.2.1 General characteristics of *Salmonella*

For the purpose of this study, *Salmonella enterica* sv. Typhimurium (for now on *S.* Typhimurium) was chosen as temperate swarmers model organism for the study of swarming behavior. Thus, the following sections are referred to this pathogen, except when specifically indicated.

1.2.1.1 Classification

The genus *Salmonella*, which is closely related to the genus *Escherichia*, is classified inside the γ-proteobacteria class and belongs to the *Enterobacteriaceae* family. It is composed of Gram-negative, non-lactose-fermenting, non-spore forming, rod-shaped bacteria which are facultative anaerobes and mainly show peritrichous flagellation[133]. This genus refers to primary intracellular pathogens leading to different clinical manifestations.

Salmonella are disseminated in the natural environment through human or animal excretion, as are not able to multiply significantly out of the digestive tracts. Anyhow, cells might survive several weeks in water and several years in soil if conditions of temperature, humidity, and pH are favorable. *Salmonella* are mesophilic and neutrophilic bacteria which show optimal growth at 37°C and pH 7, reaching a cellular size of 0.7-1.5 μm diameter and 2-5 μm length.

According to the World Health Organization (WHO) Collaborating Centre for Reference and Research on *Salmonella* (Pasteur Institute, Paris), nowadays the genus *Salmonella* consists of two species, *S. enterica* and *S. bongori*, due to the difference in 16S rRNA sequence analysis. Kauffmann-White-Le Minor serotyping scheme is accepted worldwide as a "gold-standard" for the classification of *Salmonellae* below the subspecies level. It classifies *Salmonella* according to three major antigenic determinants found in the cell wall of different *Salmonella* strains, composed of flagellar H antigens, somatic O antigens, and virulence (Vi) capsular K antigens[134].

The O antigen reflects variation in the exposed part of the lipopolysaccharide (LPS) as the H antigen reflects variation in flagellin, the major protein of the flagellum. Most *Salmonella* carry two flagellin genes that code for *fliC* (phase 1 H antigen, H1) and *fljB* (phase 2 H antigen, H2). Isolates are identified by serotyping, each having an unique combination of O, H1 and H2 antigens[134,135], and in turn, *S. enterica* can be divided into six subspecies[133,134] (Table 1.2).

Table 1.2: *Salmonella* serotypes classification.

Species	Subspecies code	Subspecies name	No. of Serotypes
Salmonella enterica	I	*enterica*	1531
	II	*salamae*	505
	IIIa	*arizonae*	99
	IIIb	*diarizonae*	336
	IV	*houtenae*	73
	VI	*indica*	13
Salmonella bongori	V		22
Total			2579

Reference: WHO (2007)[134]

1.2.1.2 Pathogeny

In the case of humans and other mammalians, host infection by *S. enterica* spp. usually occurs by ingestion of contaminated food or water, or by the capacity of the bacteria to pass the stomach and colonize the gastrointestinal track, primarily the terminal ileum and colon[136–139]. As the majority of *Salmonella* infection occurs via the fecal-oral route, the pathogen needs to overcome the stomach low pH to proceed with the infective process,

which implies a minimum cell number to trigger the disease. This concentration is dependent on the serovariety and the host health, the minimum infective dose being 10^5-10^6 CFU/mL. This dose is variable and can be decreased down to 10^3 CFU/mL if the host is immunocompromised[140,141].

Salmonellosis in humans and animals is characterized by fever, acute intestinal inflammation, and diarrhea within 24 h after infection. Once ingested, *Salmonella* enters into the intestine and encounters host responses targeted toward the clearance of the pathogen. The first defense line that must cross is the acidic condition of the stomach[139,142]. *Salmonella* overcomes low pH by triggering the acid tolerance response, an adaptative mechanism that enhances the survival at lethal pH conditions found in the stomach[143]. Once in the small intestine, the pathogenic cells need to compete against the commensal anaerobic bacteria by employing multiple virulence factors to overcome the colonization resistance.

Also, *Salmonella* has to use various mechanisms to survive the harsh conditions presents in the gut, such as bile salts presence, osmotic-adverse conditions, low oxygen tension conditions or nutrient availability, which allow bacteria to efficiently traverse the intestinal mucous layer and infect intestinal epithelial cells[136,139,143,144]. Recent insights into the nutrient-niche occupied by *Salmonella* in the inflamed gut suggested that virulence factors enable the pathogen to solicit help from its host to compete with resident hydrogenotrophic microbes over metabolites produced by saccharolytic bacteria and proteolytic bacteria[144,145]. Through this mechanism, virulence factors enable the pathogen to establish a replicative niche in the intestinal lumen to ensure its transmission by the fecal-oral route.

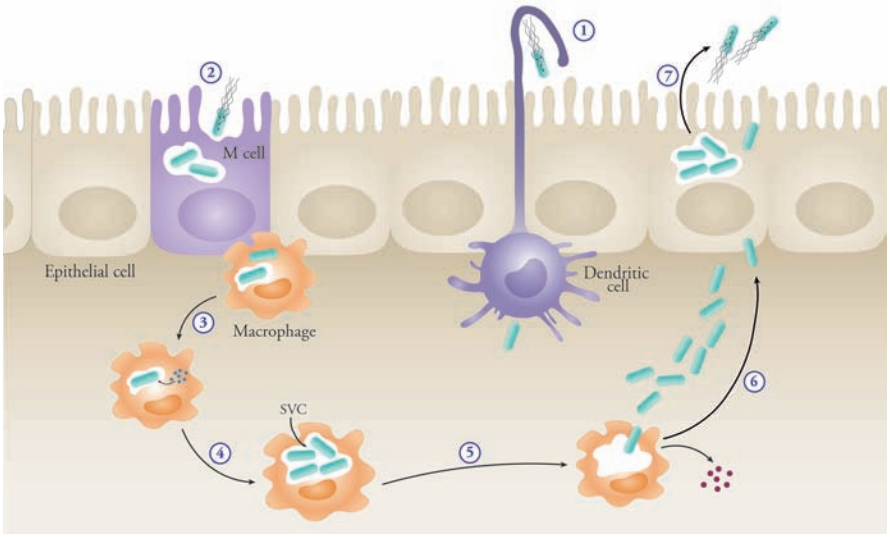

Figure 1.5: Invasion mechanisms of *Salmonella*. Disease caused by *Salmonella* spp. occurs after ingestion of food or beverage contaminated with the bacteria. Following gaining access to the gut lumen, *Salmonella* can cross the epithelial barrier either by passive mechanism (1) or by active invasion, mainly, via the M cells of the Peyer's patches (2). Active crossing of epithelial cells requires the injection of various effector proteins into host cells by T3SS-2 that enables the cells to avoid host-cell killing mechanisms by generating SCV, which allows its survival and replication inside the cell (3-4), triggering gut inflammation through cytokine secretion (5). *Salmonella* may also reseed into the gut by basolateral invasion (6-7) and excrete into the feces, ensuring fecal-oral transmission. Modified from Pérez-López *et al.*, 2016 [146].

To achieve the intestinal epithelium invasion (Figure 1.5) *Salmonella* serovars encode Type III secretion system 1 (T3SS-1), a virulence factor encoded by *Salmonella* pathogenicity island 1 (SPI1)[147]. After entering the intestinal lumen, *Salmonella* uses flagella to move to the proximity of the intestinal epithelial cells, or to aid cells escape from macrophages[136,148,149], and uses fimbriae for intimate cell attachment. Fimbriae bind the extracellular matrix glycoprotein laminin and mediate adhesion to the host cell.

Then, *Salmonella* can invade the host in two ways: by host-mediated uptake pathways or by bacterial cell invasion routes. The host-uptake pathway is mediated by immune surveillance via M cells on the luminal surface of the intestines. Bacteria that enter host cells by this mechanism are then transferred to dendritic cells, macrophages, or T cells with the goal of being processed to produce antibodies[138,144]. The second mechanism of invasion is pathogen-specific. Through the T3SS-1, *Salmonella* injects a series of effector molecules into target cells where they cause several internal modifications interfering with the host cell signaling pathways, as cytoskeletal rearrangement, bacterial engulfment, and formation of *Salmonella*-containing vacuoles (SCV). This orchestrates the active invasion of the pathogen and allows the bacterial cells to resist the intracellular killing mechanisms of the host. *Salmonella* can survive and replicate inside the host cells while trapped in this compartment due to proteins secreted through Type III secretion system 2 (T3SS-2) encoded on *Salmonella* pathogenicity island 2 (SPI2), that prevents reactive oxygen species production and enables cells to survive inside either macrophages or nonphagocytic epithelial cells[150]. This mechanism also prevents the SCV fusion to secondary lysosomes that contain enzymes which would kill the cells[133,136].

The presence of *Salmonella* cells in the epithelium triggers the innate immune response that promotes acute inflammation and neutrophil infiltration[145]. Finally, a fraction of the SCV migrate to the basolateral side of the enterocytes and, through an exocytosis process, bacteria are released into the interstitial space of the lamina propia, where are randomly phagocyted by either neutrophils, macrophages or dendritic cells.

The migration of the infected cells facilitates the rapid dissemination of *Salmonella* either to lymph nodes and finally to the blood stream, or directly to the blood stream, either way, rendering a systemic infection[133,138,151].

Swarming is thought to play a role in initiating the interaction between the pathogen and host cell during the invasion and may be the primary virulence form of the organism[152]. Even if there is no evidence of swarming motility occurring *in vivo* yet, multiple studies suggested that swarm cells present an advantage for pathogens to colonize their host, being an adaptative response stimulated by the competitive environment and may represent a sophisticated survival strategy within the gastrointestinal environment[49]. Furthermore, flagella and not motility has been suggested to be necessary for cell adhesion and invasion.

1.2.1.3 Epidemiology and clinical relevance

Among the enteric pathogens, *Salmonella* is of particular clinical relevance in both developed and developing countries, where this pathogen is one of the most common causes of foodborne illness[153]. Two major clinical syndromes caused by the *Salmonella* infection in humans are enteric fever (also called typhoid fever) and diarrheal disease.

Enteric fever is a systemic invasive illness caused by the exclusively human pathogens serovar Typhi and serovar Paratyphi A and B [154]. On the contrary, diarrheal disease is mainly caused by the broad range, nontyphoidal Enteritidis and Typhimurium serovars [138,155].

The global human health impact of nontyphoidal *Salmonella* is high, with an estimated 93.8 million illnesses, of which an estimated 80.3 million are food-borne, and causes 155.000 deaths each year [155]. Worldwide, mass production and distribution of food disseminate pathogens rapidly, and this, combined with the challenge of multi-drug resistance related to antibiotic use, creates new challenges for controlling and preventing *Salmonella* infection. But the concern for salmonellosis goes far beyond the human health as many serovars can infect the domestic livestock, also becoming a cause of major economic concern [133,138].

S. enterica has been ranked as the leading cause of foodborne disease as measured by the combined cost of illness and quality-adjusted-life-year [156], causing economic losses of $3.5 billion per year only in the US. There has been a statistically significant decreasing trend of salmonellosis worldwide (9% in 2013) due to the increasing implementation of control measures against *Salmonella*, particularly within the poultry industry. But for now, it continued to be the cause of many foodborne outbreaks at international, national and subnational levels (ECDC; 2016 [consulted March 2017]). All this data highlights not only the clinical relevance of *Salmonella* but also the economic impact that this pathogen causes worldwide in the food industry.

1.2.2 Swarming motility control

S. Typhimurium is able to swarm over moist surfaces. The prevalence of swarming behavior in the *Salmonella* genus was confirmed by Kim and Surette (2005) pointing out that the high conservation of the swarming ability is an indicative of its important role in survival and persistence[157]. Taking into account that flagellar motility is of great importance for biofilm formation, adherence, and virulence, it is presumed that it should be tightly regulated.

The many challenges that swarming bacteria have to overcome in order to initiate surface colonization have been resumed in previous sections, but it is worth highlighting that the requirements might vary and could be genera-specific, likely reflecting specific adaptations for a surface niche that the bacteria naturally inhabit. Understanding the environmental requirements and how bacteria cope with these challenges is essential to comprehend how is swarming behavior regulated in temperate swarmers, and specifically in *S.* Typhimurium, that presents high sensitivity towards moist conditions needed to swarm.

1.2.2.1 Swarming requirements

The importance of surface moisture on swarming is easily observed in temperate swarmers due to their inability to swarm when agar concentration is near or above 1%, under laboratory conditions[1]. *S.* Typhimurium is swarming-proficient only on 0.5-0.7% agar, being the water requirement the cause of this sensitivity[35]. Thus, the principal challenge is to attract sufficient water to the surface to fully immerse the cells on the surface.

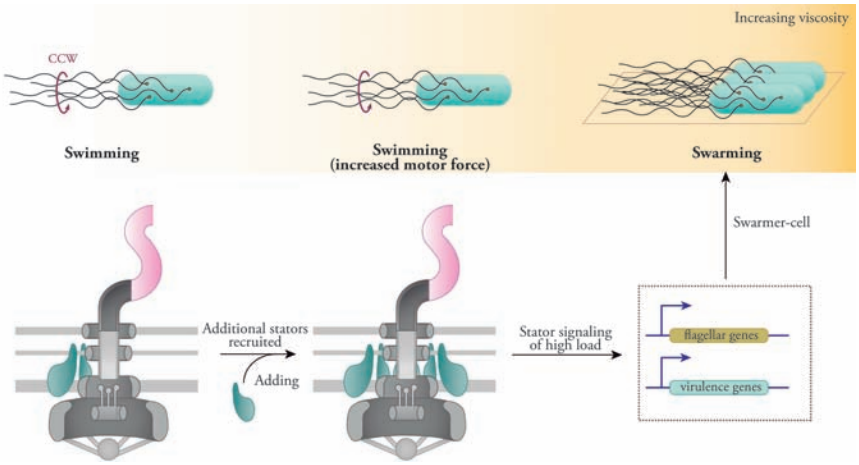

Figure 1.6: The role of flagellar motor in surface motility. *S.* Typhimurium can sense when they have reached a desirable location and trigger the changes that help them to colonize the environment. When sensing an increase in viscosity, *Salmonella* cells recruit additional stator complexes to its flagellar motors, increasing the flagellar propulsion and overcoming frictional forces and water requirement, which allows them to organize and swarm. Modified from Chaban *et al.*, 2005[158].

On the one hand, the theory that *S.* Typhimurium is able to beat the frictional resistance while increasing the current flagellar motor force is supported by the reported overproduction of stator proteins MotAB alongside with FliL, being equivalent to the strategy of inducing swarmer cell hyperflagellation in robust swarmers[35]. Figure 1.6 resumes how bacteria make use of the flagella to overcome surface friction and so colonize surfaces. On the other hand, *S.* Typhimurium is not known to secrete any surfactant molecule[159,160], and in fact, the wetting agent required to allow swarming motility is not a surfactant but LPS, which may play here a central role[52,159]. Taking into account that a swarming colony is dense and multilayered in the interior, and generally monolayered at its edges, a recent study suggested an osmolarity gradient within the swarming colony, osmolytes thought to be LPS[161].

Mutants impaired in chemotaxis (*che*) that present an anchored flagellar rotation, either CW or CCW, were observed to be defective for swarming. Motility inhibition occurs due to a less hydration phenotype observed for Che mutants[44,73,75], that were seen to be defective in hydrating their surface-grown colonies, in exporting FlgM under these conditions, and in assembling normal-length filaments[75], which highlights the role of the flagellum in proper colony hydration. Furthermore, *S.* Typhimurium presents an explicit dependency to rich media supplemented with a carbon source as glucose, galactose, mannose, fructose or glycerol to swarm under laboratory conditions[56,157]. *Salmonella* cells are able to swarm in a range of temperatures, 37°C being optimal for both cell growth and surface colonization[1,68].

Salmonella present advantages over non-flagellated bacteria due to their ability to actively search the environment instead of relying on Brownian motion. Moreover, bacteria have evolved chemoreceptor systems in conjunction with their flagella to sense their surroundings and move in favorable directions (chemosensing and directed swimming), as has been previously mentioned. These mechanisms allow bacteria to stay swimming at surfaces where receptors or promising niches are more likely to be encountered (near surface swimming), and to sense when they have reached a desirable location and trigger changes to remain there (mechanosensing).

Apart from the environmental conditions that allow *Salmonella* to swarm, the flagellar machinery and the chemotaxis system are two pathways that are essential for motility.

1.2.2.2 The flagellar system

1.2.2.2.1 The flagellar motor: Structure and function

The flagellum is a rotating, rigid and helical motility organelle which propels cell body, used by many bacteria for targeted movement towards the infection site in a chemotactic process and efficient colonization of the environment[162–165]. Each flagellum is attached to a rotary motor embedded in the cell envelope that uses the proton motive force to generate the thrust required to enable cell motility[166,167].

Salmonella presents a peritrichous flagellation, being 4 to 6 appendices randomly positioned along the cell body. The *Salmonella* flagellum structure and assembly is a cellular process composed of around 30 distinct structural proteins. The flagellar structure is usually described in three parts (Figure1.7): (i) the basal body, which contains the reversible motor that anchors the structure to the membrane, (ii) the hook, which extends out from the top of the basal body and acts as a universal joint, and (iii) the filament that extends many cell body lengths from the hook and, when rotated, forms the helical propeller[166,168].

1. **The basal body.** The basal body is spanned from the inner to the outer cytoplasmic membrane in Gram-negative bacteria and contains the flagellar protein export apparatus, the MS-, C-rings (reversible rotor), the rod (drive shaft), and the LP-rings (brushing). The basal body of the flagellum includes the motor that powers rotation. The transmembrane protein complexes, known as stator complexes, transduce energy from the flow of ions (H^+ in *Salmonella*) across the inner

membrane to induce conformational changes that exert a torque on the cytoplasmic C-ring, which is in turn coupled to the rod, hook, and filament. The propulsive force generated by this rotation is variable and results at a range of speeds that is most likely based on many factors, including cell shape, motor energy source and a broad structural diversity in flagellar motors across the bacteria.

The conserved MS-ring consists of approximately 26 copies of FliF protein and is connected to the stator proteins MotA and MotB via the C-ring that form a proton channel through the membrane. MotB has a membrane spanning domain, but the majority of the protein is found in the periplasmatic space bound to the peptidoglycan. MotB is the responsible to anchor MotA to the rigid cell wall structure in order to permit the torque to be transmitted to the flagellar filament probably through the interaction with FliG, a component of the cytoplasmatic face of the rotor system[166].

The C-ring is located at the cytoplasmic face of the MS-ring and is composed of three proteins: FliM, FliN, and FliG. The C-ring is necessary for transmitting chemosensory signals into torque generation. The FliG protein is the connection between the C-ring and the stators and is the change in this C-ring/stator interface what severely impacts torque so-called.

Finally, the rod consists of a central core part and two rings, the P-, and L-ring, which connect the basal body to peptidoglycan layer and outer membrane LPS, respectively. In *S.* Typhimurium, the rod is built up by proteins FlgB, FlgC, FlgF, FlgG, FlgH, and the adaptor protein FliE[168]. Several copies of FlgH and FlgL make the L-ring and P-ring, respectively.

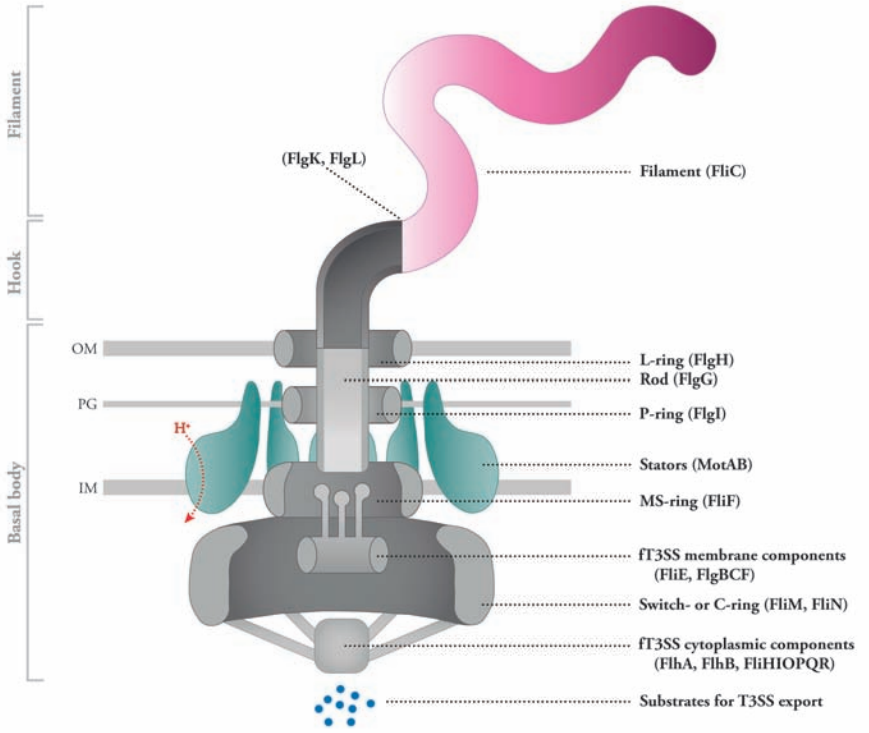

Figure 1.7: Schematic structure of the bacterial flagellum. Flagellum assembly model of *Salmonella*. During assembly, basal body T3SS components unfold and export subunits of the basal body, hook and filament for incorporation at the cell-distal tip of the growing structure. Energy harvesting stator complexes in the basal body interact with the torque-generating C-ring to bring about rotation of the extracellular filament for motility. OM: outer membrane; PG: peptidoglycan layer; IN: inner membrane. Reprinted with permission from Chaban *et al.*, 2015 [158].

2. **The hook.** It is an helical tubular assembly of a single protein, approximately 55 nm in length that acts as universal joint connecting the rod and the filament[166,169]. In *S.* Typhimurium it is composed of the structural protein FlgE, and the cap protein FlgD, that is required for assembly. Two proteins, FlhB and FliK, seem to control the hook length and change export sensitivity upon hook completion. The unique packing interactions of FlgE subunits in the hook realize the bending flexibility and twisting rigidity at the same time for the hook to work as a universal joint, allowing the motor to drive the off-axis rotation of the filament[169–171]. To increase the flexibility and strengthen the connection of hook and filament, the hook-filament junction proteins, FlgK and FlgL, form two zones on the distal end of the hook[168]. The cap protein FliD that later mediates the assembly of flagellin monomers into the filament is also associated with FlgL at the beginning of filament formation.

3. **The filament.** It is about 10-15 nm long, and it is built of up to 20.000 subunits of a single protein termed flagellin, which is assembled in an helical pattern of 11 subunits per turn with the assistance of the pentameric filament cap protein FliD. Flagellin comprises four linearly connected domains, two filament core domains (D0, D1) and two hypervariable-region domains (D2, D3). The N- and C-terminal chains of flagellin compose packed α-helical structures, which constitute the D0 and D1 domains, positioned in the filament core. The variable region of flagellin is exposed as a folded β-sheet structure (D2 and D3 domains) on the outer surface of the filament[172]. In *S.* Typhimurium two antigenically distinct flagellins are alternately expressed, FliC and FljB, in a process termed flagellar phase variation[173].

While the molecular mechanism behind this switch is well understood, its biological function remains unclear. Even though, multiple studies suggest that *Salmonella* exploits this phenotypic heterogeneity as a strategy to prime a subpopulation of FliC expressing bacteria for productive invasion of the host epithelium, and a FljB-expressing subpopulation that might have a role for efficient motility in other environmental niches[174,175].

The flagellar motor can operate in both the counterclockwise (CCW) and clockwise (CW) bias, and the stator complex is the responsible for this torque generation. Torque is produced by sequential rotor-stator interactions coupled with downhill H^+ translocation along the electrochemical gradient across the cytoplasmic membrane through the two channels formed by MotAB proteins[166,167,176,177]. Indeed, the speed of the flagellar motor increases linearly with the proton motive force generated by the MotAB stator complex[167].

Furthermore, the range of cap protein is also dependent on the cell shape[178], the motor energy source[179] and a broad structural diversity in flagellar motors across the bacteria[158,180]. The protonation and deprotonation of a specific aspartate residue of MotB are believed to modulate the MotA cytoplasmatic domain conformational change and, in turn, its interaction with FliG, generating the required mechanical movement[177,181]. FliG, FliM, and FliN, as they constitute the core of the motor regulatory proteins, are responsible for the motor rotation switching.

During the chemotactic response, CheY∼P (phosphorylated-CheY) signaling protein binds to FliM and FliN, inducing a cooperative conformational change in the FliG ring that makes the motor to spin CW instead of CCW[169,182,183]. This biases change allows bacteria

to sense the environment while tumbling and consequently, to response accordingly.

Recent studies proposed that the C-ring is a highly dynamic structure even when the motor is in action, and that is precisely this dynamic nature the responsible for the ultrasensitivity of the motor to chemotactic signals[184]. Despite the motor switch from CCW to CW rotation appears to be approximately a random phenomenon, relieving the torsional stress generated when the flagella filaments change their structure it is likely to be one of the main forces involved in recovering the normal state, thus originating the running/tumbling cycles[77].

1.2.2.2.2 Gene Expression and Regulation

Swarming motility ultimately depends on flagella function. The expression of the flagellar biosynthesis genes are organized in a hierarchy, the top of which is governed by FlhDC master regulatory transcription factor also called master regulator. Master regulators serve as an integration point for environmental signaling, to activate flagellar gene expression, and to govern the production of flagellar basal bodies. Flagellar assembly is complex, and there are species-specific mechanisms of transcriptional and post-transcriptional regulation[58]. More than 50 genes are required for flagellar formation and function in *S.* Typhimurium.

According to the cascade model of the flagellar regulon, the flagellar operons are divided into three classes (1 to 3) concerning their relative positions in the transcriptional hierarchy. This sequential transcription is coupled to the assembly process of the flagellar structure. Indeed, genes involved in the formation of the hook-basal body complex belong to the Class-2 operons, whereas those involved in the filament belong to the Class-3.

Figure 1.8 resumes the regulatory cascade governing flagellar assembly in *Salmonella*.

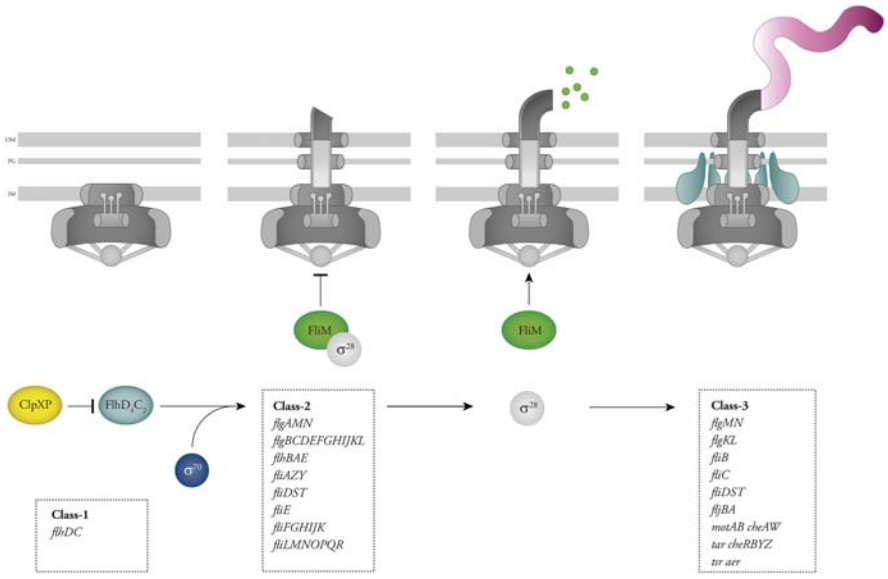

Figure 1.8: Transcriptional regulatory cascade of the flagellar operon. The flagellar regulon of *S.* Typhimurium is organized into a transcriptional hierarchy of 3 operon classes. Class-1 operon encodes the transcriptional-activating FlhDC master complex, which regulates Class-2 gene transduction. The Class-2 genes encode for the hook-basal body structure and the regulatory genes (*fliA* and *flgM*). *fliA* encodes the σ28 transcriptional factor, that is required for transcription of Class-3 promoters. When the basal-body is semi-functional, FlgM protein is accumulated in the cytoplasm, leading to Class-3 genes inactivation due to its interaction with σ28. Once the basal structure is totally assembled, FlgM is secreted as a Class-3 flagellar protein, thus releasing σ28 and allowing to initiate transcription of the late genes. Class-3 genes in turn lead to completion of the biiosynthesis of the flagella. Modified from references Chaban *et al.*, 2015 [158] and Karlinsey *et al.*, 2000 [185].

The hierarchic system ensures the earlier required constituents availability before the late ones, thus avoiding over-synthesis or competition between proteins for processes such as secretion to periplasmic space. Overall, this genetic expression mechanism constitutes an efficient way to save the cell resources [186].

1.2.2.3 The chemotaxis system

The chemosensing machinery is a two-component signal transduction system used by bacteria to sense the environment for the presence of attractants or repellents and to generate an adequate response[187,188]. The small size of a bacterial cell would make direct spatial detection of gradients inefficient, so chemotaxis strategies have evolved to rely on temporal comparisons of stimuli concentrations rather than spatial ones[6].

In order to overcome that, the sensory pathway has to perform several tasks for the cell to response correctly to the environment: (i) react to changes in chemoeffector concentration on a sub-second time scale; (ii) compare the level of stimulation at a given time point with that of 1-2 s earlier, which requires a short-term memory and (iii) gradually refresh the memory as the cell moves up or down the chemical gradient[189].

In *Salmonella*, as described for *E. coli*, the general chemotaxis strategy depends on controlling the relative frequency of running/tumbling patterns. Thus, regulating CCW rotation of the flagellar motor that propels the cell forward and the re-orienting tumbles, produced by the change toward CW motor rotation. As a result, the cell performs a random walk that allows it to efficiently explore its environment until a gradient is sensed when cells bias their random walk by suppressing tumbles, whereas swimming in a favorable direction, which results in an efficient net movement of a cell up to the gradient[189] (Figure 1.2).

The assembled chemosensing pathway, together with motility, is also associated with virulence and pathogenesis, as it is required for efficient colonization of the intestinal tract, host cell invasion and symbiotic associations of bacteria[163,164,190,191].

Furthermore, not only the functional chemotaxis system but the presence of polar chemosignaling arrays is essential for swarming motility in temperate swarmers[192,193]. Considering that the primary interest of this dissertation is the modulation of swarming motility, both chemotaxis system, and signaling arrays are profoundly described in the next sections.

1.2.2.3.1 Genetic organization of flagella-mediated chemotaxis pathways

The genetic operons that encode the chemotaxis system are integrated within the flagellar regulon that encodes and regulates the expression of the flagellar motility apparatus, as previously mentioned. In *S.* Typhimurium, flagellar genes are organized in five main clusters, chemotaxis genes being encoded within cluster II (Figure 1.9A). According to the hierarchical regulation of the flagellar operon, *che* genes are classified as Class-3, thus, their transcription is initiated once the flagellar motors are almost completely assembled (Figure 1.8)[194,195]. In the flagellar system, the regulation of gene expression and protein function are intimately associated[196].

1.2.2.3.2 Components of the system

The canonical flagella-mediated chemotaxis pathway has been characterized in detail in *E. coli* and serves as a model of chemotaxis signal transduction of *S.* Typhimurium. Chemotaxis is based on the concerted action of excitatory and adaptive mechanisms. Chemosensory cascades are formed by some core proteins as well as different auxiliary proteins[197], that function to convert sensory information into a signal that controls the flagellar motor switching and so, motility[198].

The chemotaxis pathway consists of four modules[199]:

1. **Sensor module.** It is formed by Methyl-accepting Chemotaxis Proteins (MCPs) that recognize environmental signals and are involved in the adaptive response through methylation and demethylation. *S.* Typhimurium has five types of receptors, four to sense a range of amino acids, sugars and dipeptides, pH and temperature (Tar, Tsr, Tap and Trg, respectively) and a fifth one to sense redox potential (Aer). Binding of chemoeffectors to the periplasmic domain of receptors modulates the conformation of their cytoplasmic parts, and consequently, also the autophosphorylation activity of the receptor-associated kinase[189]; whereby attractant binding decreases kinase activity, and repellent binding enhances it[74].

2. **Transduction module.** The core of the transduction module is a ternary complex composed of MCPs, the CheA histidine kinase, and the CheW coupling protein that modulates CheA activity in response to environmental sensing and ligand binding (Figure 1.9). The molecular stimuli arising from ligand binding in the periplasm is transmitted to the cytoplasmic side of the MCPs, where it modulates CheA autophosphorylation activity. Changes in the CheA phosphorylation state affect the transphosphorylation of the CheY response regulator that controls flagellar rotation[200].

3. **Actuator module.** The chemotaxis signal transduction network modulates the flagellar switch between CCW and CW modes through CheY∼P and FliM protein interaction.

4. **Integral feedback module.** The purpose of the feedback module is to integrate signals, giving the bacteria a primitive type of memory that is used for temporal comparisons when moving in a chemical gradient[201]. Changes in the rate of methylation occur slower than the phosphorylation-dephosphorylation steps. Thus, this differential timing confers the memory required for temporal comparisons of attractant concentrations. The canonical adaptation pathway is comprised of the receptor-specific methylesterase CheB and the receptor-specific methyltransferase CheR[202,203].

1.2.2.3.3 Architecture and regulation

As previously explained, external stimuli are detected by transmembrane MCPs that interact to form trimers of dimers (TODs)[204,205] associated with CheA kinase through CheW coupling protein. The MCPs are proteins composed of a variable ligand binding domain and a conserved cytoplasmic adaptive and signaling domain[206]. The recognition of the ligand by the MCPs could be achieved by the direct binding of the molecule to the MCP scaffold, or through other coupling proteins, depending on the chemoeffector. A schematic chemotaxis pathway of *S.* Typhimurium is shown in Figure 1.9B.

The signal recognition at the chemoreceptor level generates conformational changes that modulate the CheA autophosphorylation activity[187]. The signal is transmitted through a phosphorylation cascade to CheY response regulator that modulates the flagellar motor rotation (Figure 1.9). The motor has a baseline stochastic switching frequency in the absence of any stimulation that is increased after CheY∼P binding[191], allowing the cells to adapt to the environment rapidly.

With the aid of chemotaxis, bacteria are able to response to an attractant or a repellent given by the modulation of the CheY∼P levels present in the cell that lead to changes in the CCW/CW motor bias, re-orientating the cell during motility[207] (Figure 1.2).

The adaptative pathways comprise the mechanisms to restore the basal state of the system or to achieve a tolerance state towards a stimulus. To avoid saturation of the sensory system, the chemoreceptor signal is reset by the activity of a methyltransferase (CheR) and a methylesterase (CheB). Both proteins are located in the vicinity of the chemoreceptors to restore pre-stimulus activity through reversible covalent methylation of the MCPs[208](Figure 1.9B). This feedback mechanism is called adaptation. The methylation state has been reported to influence the affinity of the receptor for the signaling molecule[74,209,210]. Additionally, a secondary negative feedback controls the phosphorylation of CheB in a CheA-dependent manner. CheA phosphoryl transfer to CheY occurs faster than to CheB, ensuring that the chemotactic response takes place before the adaptative response.

The kinetics of methylation and demethylation are relatively slow compared to the CheY-phosphorylation response. Thus, the adaptation response presents a delay. This lag confers the short-term memory required by the chemosensing machinery for temporal comparisons of attractant concentrations that are essential for chemotaxis and motility[201,211].

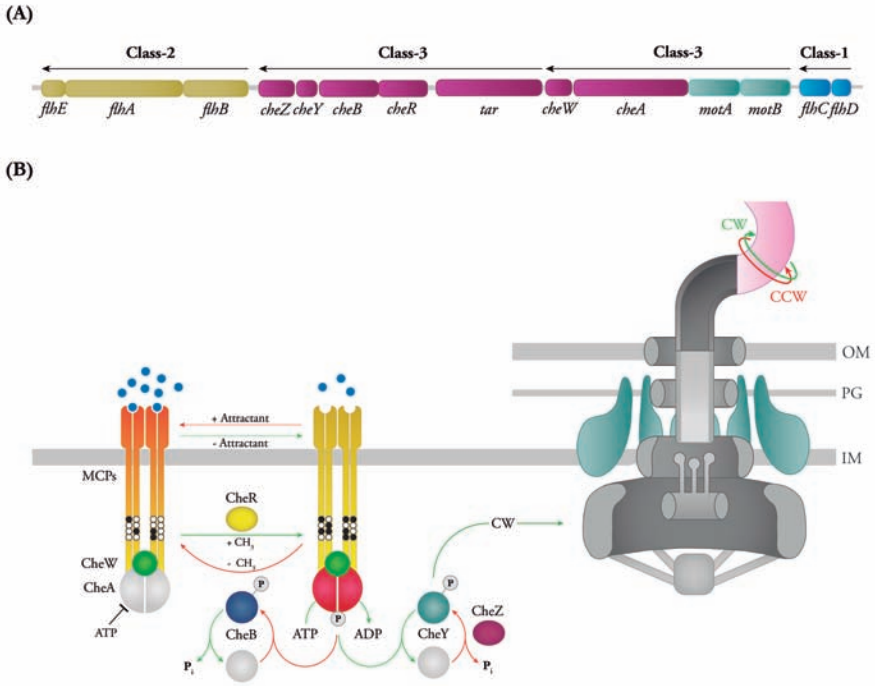

Figure 1.9: Structural organization of the chemosensory machinery. (A) Transcriptional organization of chemotaxis genes of *S.* Typhimurium. The arrows indicate the extent and orientation of the flagellar operons. The numbers are the classification within the hierarchy of the flagellar regulon. (B) Schematic representation of the chemotaxis pathway. When MCPs are highly methylated (yellow), CheA kinase is activated by phosphorylation. CheA∼P phosphorylates CheY, that interacts with the flagellar motor FliM, increasing the frequency of CW rotation, causing the cell to tumble. When MCPs bind to a ligand or are less methylated (orange), CheA is maintained in a non-phosphorylated inactive state. As a consequence, CheY∼P levels are reduced, leading to an increase in CCW motor rotation and causing the cell to run. Arrows indicate an additive effect, whereas cut line arrows mean a subtractive effect. Components and reactions in red promote counter clockwise (CCW) flagellar rotation; those in green promote clockwise (CW) flagellar rotation. OM: outer membrane; PG: peptidoglycan layer; IN: inner membrane. Modified from Hazelbauer *et al.*, 2012 [201].

1.2.2.3.4 Assembly and spatial organization of signaling arrays

MCPs, CheA and CheW proteins form the primary complex whose role is to initiate the signaling cascade leading to an appropriate response to each situation. Two receptor TODs, one CheA homodimer, and two molecules of the coupling protein CheW form the so-called basal core signaling unit[212], that is the smallest structure capable of chemosensory functions (Figure 1.11). This assembly, [TODs-CheA-CheW], is an independent signaling unit capable of receptor-mediated kinase regulation in response to attractant and adaptation signals[213–215]. Despite the fact that CheA is the leading recipient of the receptor signaling stimulus, CheW has been hypothesized to play a complex role *in vivo* than only serve as a coupling protein to tether CheA and MCPs[216,217]. CheW, and the CheA-P5 domains are paralogs: each presenting two structurally similar subdomain, that allows each CheA protomer to bind one CheW molecule through the P5 domain[218].

The folded CheW protein has an SH3-like regulatory domain and two intertwined five-stranded β-barrels sandwiching a hydrophobic core, designated subdomains 1 and 2, respectively[218] (Figure 1.10). Strands β1, β2, and β8-β10 form the first subdomain while strands β3-β7 form the second subdomain[219]. The central groove between the two subdomains has been implicated in the interaction with chemoreceptors[217,220–223].

Basal core signaling complexes assemble and organize into a supra-molecular complex through hexagonal CheA-P5/CheW rings[224], present in bacterial cells in amounts ranging from a few to thousand copies (Figure 1.11A). This signaling complexes are located at cell poles, where they form large signaling arrays[225–228] (Figure 1.11B).

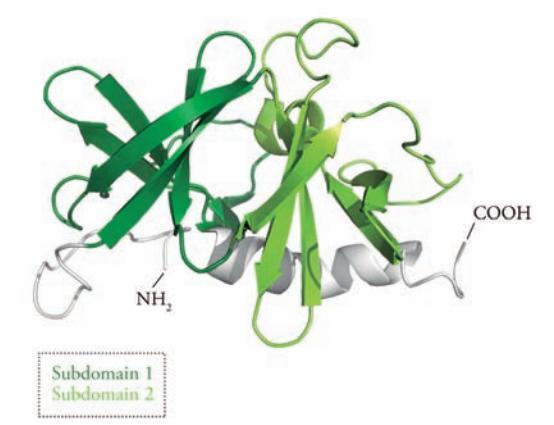

Figure 1.10: Structure of the CheW protein. Ternary structure of *E. coli* CheW protein (PDB: 2HO9). Subdomains 1 and 2 are colored in dark-and light-green, respectively.

In fact, these signaling arrays are one of the largest structures known in bacterial cells that can be easily observed at *Salmonella* cell poles[226,228–231]. Following the assembly of the signaling complexes in hexagonal arrays, not only [MCPs-CheA-CheW] units, but the rest of the cytoplasmic proteins implicated in the chemosensing pathway also colocalize at cell poles, indicating that a stable protein complex is formed surrounding the arrays at this physical region[229].

The signaling array structure has been deeply characterized[189,224,232,233]. Stabilization of these clusters is a function of both the membrane curvature at cell poles[234] and the presence of CheA and CheW proteins[235]. These proteins are directly involved in the stabilization of these arrays, as they interact to form structural linkers [CheW-CheA-CheW] across the cytoplasmic domain of MCPs, packing core signaling units in a two-facing-two fashion forming CheA filled and CheA-empty rings[232,236,237] (Figure 1.11A). The contacts between MCPs, CheA, and CheW proteins make the core complex a stable entity that contributes to the ultra stability of higher-order complex assemblies[238–240].

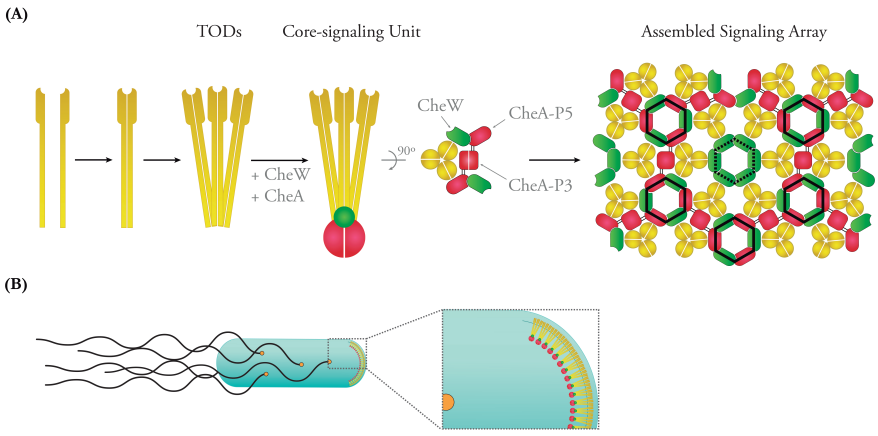

Figure 1.11: Schematic representation of the signaling array assembly and location. (A) Molecular model for the signaling array assembly and its location within the cell. MCP homodimers assemble to form trimers of dimers (TODs) that aggregates in a hexagonal-way to form packaged signaling arrays upon recruitment of CheA and CheW proteins, whose function is to stabilized and network the assembled TODs. In addition to hexagonal CheA-P5/CheW rings (unbroken black line), hexagonal CheW-rings are also formed (broken black line). (B) Location of the assembled core-signaling complexes forming large arrays at the cell poles. Modified from Parkinson *et al.*, 2015[241].

Even though, it is well determined that a proper CheA and CheW stoichiometry is critical for the formation of the ternary signaling complex. The reported stoichiometry for [MCPs-CheA-CheW] is of 6:1:1 although it may vary depending on the strain and the signaling state of the cell[236]. Recently, the so-called CheA "empty-hexagon" had been elucidated not to be empty but filled by individual CheW monomers forming rings that are in fact the structural foundation responsible for the ultra-stability of the chemosensory array[224] (Figure 1.11A). Furthermore, CheW protein has the capacity of titrating TODs formation, as its intracellular availability is essential for the correct assembly of the functional core signaling [TODs-$CheA_2$-CheW] unit, either absence or overexpression of this protein leads to an inhibition of array assembly[192].

By acting as antennae, the arrays amplify the signal generated in response to slight changes in the concentrations of attractants or repellents and reinforces the cooperative behavior of the chemotaxis chemoreceptors[208,224,224,232,233,236]. The basal signaling unit is, therefore, stable on time scale of response and adaptation, and consequently, neither the assembly nor the disassembly of signaling arrays is involved in the signal transduction. Also, the physical position of the arrays within the cell is not essential for efficient signaling and there is no need to be close to the flagellum, due to temporal and not spatial comparisons of ligand concentrations[242]. It has been estimated that the time taken for CheY∼P to diffuse along the length of an average cell is only 100 ms, approximately[228].

Nevertheless, the assembly and spatial organization of signaling arrays have been the focus of several studies[228,243,244]. As being motile and chemotactic is a survival advantage for *Salmonella*, both the number and positioning of the signaling arrays must be controlled.

The newly synthesized signaling complexes are distributed in an helical fashion at the cell membrane via their association with cytoskeletal proteins such as MreB or the Sec secretion system[234,243]. Then, by stochastic self-assembly[245] or by an active process[243], these complexes form large clusters by joining existing arrays or by the formation of new nucleation centers. Indeed, the size of the polar signaling array appears to increase as the cells grow. Nonetheless of the preferred polar location of the bigger clusters, lateral smaller ones might also be observed[229,242,245].

Altogether, the flagellar switch modulation by the chemosensing machinery promotes the proper lubrication of the cell-surface interface, which is needed to overcome surface friction, both of which are critical requirements for swarming motility in temperate

swarmers such as *S.* Typhimurium[35]. It is remarkable that mutants with defects in the chemotaxis pathway, flagellar biosynthesis, or polar chemoreceptor cluster assembly give rise to non-swarming colonies[36,192,193].

1.2.3 Implication of SOS response in swarming modulation

1.2.3.1 General characteristics of SOS response

The bacterial SOS response is a global regulatory network targeted at addressing DNA damage. Following a model of autogenous negative regulation by induction, SOS response is regulated by the *lexA* and *recA* gene products, which act, respectively, as inducer and repressor of the system, being both members of the SOS regulatory network[246–248]. Traditionally, the SOS network has been defined as a system that involves, in *E. coli*, nearly 40 genes directly regulated by LexA and RecA[249]. Nowadays, the SOS response is considered as a universal adaptation system of bacteria to DNA damage as it is widely distributed among a large number of phyla, with a few exceptions[247,250].

1.2.3.2 Architecture and Regulation

In a steady-state situation with no DNA damage, the LexA protein represses a set of genes that encode for proteins involved in many different cellular processes, such as inhibition of cell division, error-prone replication or excision repair[247,251]. Repression occurs by the specific binding of LexA to a motif in the promoter region of the so-called SOS genes.

These motifs (also known as SOS boxes) present a phylogenetic group-specific consensus sequence and are typically located near or inside the RNA-polymerase binding site[247]. In the case of *Salmonella*, as for γ-proteobacteria, the motif is the 16 bp-long CTG-N_{10}-CAG sequence[247,252]. Consequently, the binding of a LexA dimer to the SOS box physically interferes with the RNA polymerase, effectively blocking transcription initiation and repressing gene expression (Figure 1.12)[247,253].

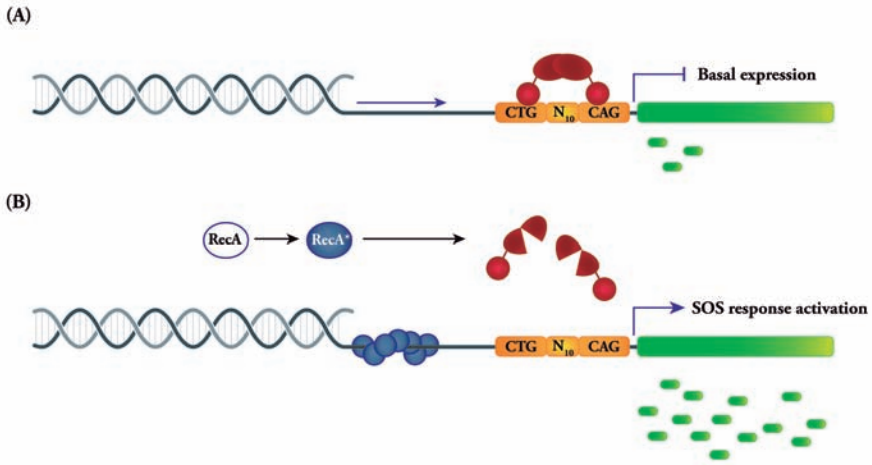

Figure 1.12: SOS response mechanism. In a non-DNA-damaging state, LexA repressor inhibits SOS genes transcription by binding SOS-boxes. When DNA damage occurs, and following ssDNA appearance within the cell, the RecA turns into its active form (RecA*) by binding to ssDNA. RecA* triggers the auto-cleavage of the SOS machinery represor LexA, thus, increasing the expression of the SOS genes. Modified from Andersson *et al.*, 2014[254] and Erill *et al.*, 2007[247].

On the other hand, and following a DNA-damaging-event, that generates single-stranded DNA (ssDNA) or double-stranded DNA (dsDNA) breaks, RecA protein acts as a sensor of the SOS system. Sensing is mediated by unspecific binding of RecA protein to ssDNA fragments, generated either by DNA-damage-mediated interruption of replication or by enzymatic processing of broken DNA ends[255].

After ssDNA binding, RecA acquires an active state (RecA*) and thereby its co-protease activity, which promotes the autocatalytic cleavage of LexA repressor[252]. The cleavage of the LexA protein prevents the SOS-box binding, therewith relieving the repression of the SOS regulon and inducing the programmed expression of SOS genes, aimed at dealing with DNA damage and its repercussion inside the cell[256,257].

Important features of the SOS response are its temporary control and hierarchical expression, as not all the genes are induced at the same time and to the same level. This expression occurs in an ordered mode depending on the existing stimuli that modulates the affinity of LexA for the SOS box present in the promoter. Once SOS induction is triggered (Figure 1.12), several SOS genes, such as *recA*, are rapidly induced to protect and stabilize the damaged DNA, while a second set of genes is expressed to deal with the lesions through nucleotide excision (*uvrABC*, *uvrD*) or recombination repair mechanism (*recN*, *recA*). To repair those lesions that are not easily patched, SOS system also induces genes that encode several DNA polymerases, such as *dinA*, *dinB* and *umuDC* (pol II, pol IV and pol V, respectively), that are able to perform translesion DNA synthesis[258].

Lastly, the SOS response also acts on cell division by regulating several genes interfering with septation, such as *sulA*, that inhibits septum formation by interacting with *ftsZ* leading to a filamentation and delaying cell division until DNA damage has been adequately addressed[249]. It is worth highlighting that among the genes regulated by LexA, it is included *lexA* itself, which generates a negative-feedback loop to re-establish repression after injuries are repaired. Once DNA lesions have been repaired, newly synthesize RecA ceases its activation by increasingly scarce ssDNA fragments. As both *lexA* and *recA* are

induced during the SOS response, levels of non-cleaved LexA protein rapidly increases as non-activated RecA levels raise, returning the system to its repressed state[247] (Figure 1.12A).

Induction of the SOS response takes place under a variety of physiological stress states of bacteria in response to changes in pH, oxidative stress, DNA damage distress, the presence of antimicrobials, bacteriophages, the transition from exponential to stationary growth or starvation of the cells, among others[247,259]. Apart from the genes implied in DNA repair, there are other genes regulated by SOS response that makes the system a global response to stress and not only an activator for the reparation genes.

1.2.3.3 RecA protein

Aside from being the activator of the SOS response[256], RecA is multi-functional DNA-dependent ATPase[260,261] involved in the central steps of homologous recombination and recombinational DNA repair mechanisms[262,263]. Further, it has been described that RecA is associated with the cell membrane forming foci often located at cell poles that are redistributed along the cell in response to DNA damage[264–266].

Activated RecA protein also serves as co-protease for UmuD and some bacteriophage repressors, such as λ or P22 phages that infect *E. coli* or *S.* Typhimurium, respectively[267]. Through the SOS response, RecA is involved in several other cellular processes such as the control of integron dynamics[268,269], the appearance and horizontal transfer of antibiotic resistances[270], the induction and horizontal transfer of pathogenicity islands and other virulence factors[271–273], and finally to the control of bacterial motility[274].

1.2.3.3.1 Structure and functions

RecA is a member of an ATPase family, an oligomeric protein with a molecular mass of 36 KDa in *Salmonella*. Structurally, the RecA monomer has three domains, a major domain surrounded by relatively smaller amino and carboxyl domains[275–278] (Figure1.13).

1. The small **N-terminal** is a 30 residue domain that contains a large α-helix and a short β-strand that are mainly implicated in the formation of the RecA multimers.

2. The **core domain**, consisting primarily of a twisted β-sheet with eight β-strands bound by eight α-helices, contains about 240 residues and is involved in DNA binding, ATP binding and hydrolysis and the formation of the RecA polymer. This core domain is shared by a wide range of proteins such as F1 ATPase, many helicases and DNA transport proteins[277,279]. ATP is required for the RecA activation and LexA autocleavage[260,280–282], and thus, for the correct binding and stabilization of ssDNA.

3. The **C-terminal** domain is 83 residue-long, and it is made up of three α-helices and three β-strands that facilitate interfilament associations[275]. This carboxyl domain contains two disordered loops (L1 and L2 motifs) that are highly conserved in all RecA proteins that binds ssDNA and are responsible for the ssDNA-stimulated ATPase activity of the protein.

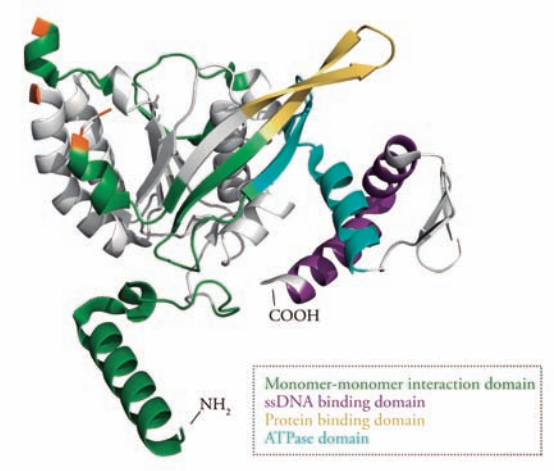

Figure 1.13: Structure of the RecA protein. Ternary structure of *E. coli* RecA protein (PDB: REB2). Major functional domains are shown.

It is worth to mention that as a multi-functional protein that combines several roles assigned to different regions of the polypeptide chain, the three domains of RecA exhibit functional overlap[283–285], indicating that functional domains do not exactly match structural ones.

In general, RecA protein is substantially conserved among *Bacteria* domain[262,286,287], the extent of similarity among RecA sequences ranging from 43-100% at amino acid level[276]. Actual structural and functional homologs of RecA have been found in bacteriophage and eukaryotic cells.

For RecA to function, monomers interacting with ATP form a flexible helical filament also known as the presynaptic complex[275], wrapped around ssDNA at a stoichiometry of three nucleotides per monomer and about six monomers per turn[288]. This function is evolutionarily conserved in other members of the RecA family, not only in bacteria but also in homologous archaeal RadA and the eukaryotic Rad51 and Dmc1 proteins[278,289].

A broad outline of the mechanism by which these proteins align homologous sequences and promote DNA strand exchange is well known. Furthermore, RecA protein is associated with the inner-membrane anionic phospholipids[265],an essential interaction for RecA activity during damage repair. Also, RecA protein forms foci that associate with DNA under certain cell states[266]. DNA-less protein foci, referred to as RecA storage structures, are often located at cell poles that are redistributed along the cell in response to DNA damage[264–266].

1.2.3.4 RecA protein and motility

Increasing its activity profile, in the past few years, a new role for RecA protein has been added to its functional catalog[274,290]. Even though the relation between RecA and swarming is well established, little is known about the molecular mechanism governing RecA-mediated motility modulation.

In *Salmonella*, it has been reported that an alteration in the balance of RecA/CheW impairs swarming motility, as does the absence or the overexpression of either RecA or CheW proteins[192,290]. Likewise, a RecA-overexpressing mutant of *S.* Typhimurium presents not only a non-swarming phenotype but also a significantly reduced capacity to cross the intestinal epithelium, apparently affecting the virulence of those cells[290].

The first report suggesting an interplay between the SOS response mechanism and motility systems pointed towards RecA-CheW pair formation[291], the late being a key component of the chemotaxis signaling array assembly and indeed, deeply involved in bacterial chemosensing and motility.

Despite the non-canonical role played by the RecA protein, swarming modulation activity is exclusively dependent on this protein and not the SOS machinery itself. Even more, none of the yet known RecA activities, *i.e.*, SOS response activation, recombinational DNA repair, or genetic recombination, seem to be necessary for the control of swarming motility[274]. Further work was needed to elucidate the role of RecA and SOS response in modulating swarming motility.

2

Objectives

Motility over a surface is an essential property shown by the majority of bacterial pathogens, and it has been associated with virulence and elevated resistance to antibiotics. Although previous work in our laboratory suggested that RecA protein is involved in swarming motility through its association with CheW protein, how the SOS system modulates motility is not well understood.

Elucidating how swarming modulation mechanisms work under stress conditions would deepen the understanding of how bacterial cells adapt to a surface niche and respond to external stimuli. Accordingly, the primary aim of the present study is to shed light on the particular role of the SOS-response in the RecA-mediated mechanisms governing the swarming motility of flagellated bacterial pathogens, specifically in *Salmonella enterica* sv. Typhimurium.

Subsequently, derived objectives are:

1. To characterize RecA-CheW protein interaction and determine the essential residues for the protein pair formation.
2. To locate RecA-CheW complex within the cell and elucidate the molecular mechanism by which RecA protein modulates motility.
3. To determine the effect of SOS-response induction in chemoreceptor assembly and swarming motility.

3

Results

3.1 Role of the RecA protein in chemotaxis and swarming motility (Article 1)

RecA protein plays a role in the chemotactic response and chemoreceptor clustering of *Salmonella enterica*

Mayola A, Irazoki O, Martínez IA, Petrov D, Menolascina F, Stocker R, Reyes JA,
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In *Escherichia coli* and *Salmonella enterica* sv. Typhimurium, RecA is also essential for swarming, a flagellar-driven surface translocation mechanism widespread among bacteria. The RecA protein is the main recombinase and the activator of the SOS system, and lately, a new role controlling motility has been added to its functional repertory. In this work, the direct interaction between RecA and CheW coupling protein was confirmed, unequivocally relating the SOS response, the chemotactic machinery and swarming motility. In order to describe this association, the motility and chemotactic phenotype of a *S.* Typhimurium Δ*recA* mutant was characterized through microfluidics, optical trapping, and quantitative capillary assays. The herein obtained results demonstrate the strong association between RecA protein and the chemosensing pathway and also its involvement in polar chemoreceptor cluster formation. While further work is needed to determine the exact role of RecA in the chemotaxis pathway, our results clearly reveal previously unknown functions of RecA: (i) its involvement in the modulation of flagellar rotation switching, not only in swarming motility but also in the chemotactic response adaptation, and (ii) its role in the architecture and assembly of polar chemoreceptor arrays.

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RecA Protein Plays a Role in the Chemotactic Response and Chemoreceptor Clustering of *Salmonella enterica*

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Abstract

The RecA protein is the main bacterial recombinase and the activator of the SOS system. In *Escherichia coli* and *Salmonella enterica* sv. Typhimurium, RecA is also essential for swarming, a flagellar-driven surface translocation mechanism widespread among bacteria. In this work, the direct interaction between RecA and the CheW coupling protein was confirmed, and the motility and chemotactic phenotype of a *S.* Typhimurium *ΔrecA* mutant was characterized through microfluidics, optical trapping, and quantitative capillary assays. The results demonstrate the tight association of RecA with the chemotaxis pathway and also its involvement in polar chemoreceptor cluster formation. RecA is therefore necessary for standard flagellar rotation switching, implying its essential role not only in swarming motility but also in the normal chemotactic response of *S.* Typhimurium.

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Introduction

RecA is a DNA-dependent ATPase [1,2] present in almost all members of the Domain Bacteria [3,4]. As a protein that is highly conserved among bacterial species, it is commonly used in phylogenetic studies [5]. In addition, RecA is the main bacterial recombinase involved in the central steps of homologous recombination and recombinational DNA repair [6–8]. It is also the activator of the DNA damage response known as the SOS system [9]. In this system, RecA acts as a DNA damage sensor by binding to single-stranded DNA, which activates the protein and thereby its co-protease activity. Activated RecA (RecA*) prompts autocleavage of the LexA repressor, thus inducing its expression and that of other SOS genes, mostly those involved in DNA recombination and repair [9]. RecA* is also able to induce the autocleavage of other serine proteases such as UmuD [10] and several repressors of the bacteriophage lytic cycle [11–13]. Furthermore, RecA is directly associated with other repair pathways such as the activated error-prone DNA polymerase V [10,14], excision base repair [15], and RecN, involved in the repair of double-stranded DNA breaks [16].

Increasing its activity profile, a new role for RecA in swarming motility, has been added to its functional catalog [17,18]. Swarming is a specialized and highly coordinated form of flagellar-driven multicellular surface translocation [19,20] and the fastest mode of bacterial surface navigation [21]. In the absence of RecA, the swarming ability of both *Escherichia coli* and *Salmonella enterica* sv. Typhimurium is impaired [17,18]; interestingly, the same phenotype is observed in *S.* Typhimurium strains overexpressing RecA protein [18]. Thus, none of the as yet known RecA activities, i.e., SOS response activation, recombinational DNA repair, or genetic recombination, seem to be necessary for the control of swarming motility [17]. Nevertheless, a possible link between RecA and the chemotaxis pathway through the CheW protein has been suggested. The CheW coupling protein is essential for the formation of the ternary signaling complex that also contains the CheA autokinase and MCPs (methyl-accepting chemotaxis proteins) [22]. The *in vitro* interaction of RecA and CheW was shown in a large-scale genome-wide screen assay [23] and a balance between the intracellular concentrations of these two proteins was shown to be essential for swarming [18].

Swarming is not governed by chemotaxis, nevertheless it has been described that the chemosensory pathway is essential for the motility on solid surfaces [24]. Mutants in the chemotaxis (*che*) pathway fail to swarm because of defective colony hydration [25], which in turn is associated with the flagellar rotation bias present in *che* mutants. Thus, in the wild-type strain flagellar rotation switches from clockwise (CW) to counterclockwise (CCW), whereas *che* mutants have CW or CCW biases depending on the specific mutation [26]. During CCW rotation, all flagella of the bacterium form a bundle that falls apart when one or more

flagellar motors turn in the CW direction. This switch promotes lubrication of the cell–surface interface, which is needed to overcome surface friction, both of which are critical requirements for swarming motility in temperate swarmers such as *S*. Typhimurium and *E. coli* [27]. The impaired swarming phenotype of *che* mutants can be restored by adding water or osmolytes to the semi-solid surface [25,27] or by restoring the normal flagellar rotation bias [28].

To further investigate the role of RecA in the control of swarming motility, in this work we examined the relationship between RecA and CheW in two-hybrid experiments and on co-immunoprecipitation assays, which confirmed the interaction of these two proteins. In addition, we explored the possibility that RecA affects the bacterial flagellar rotation pattern, by studying the swimming profile, flagellar motor rotation, and the chemotactic response of a *recA* knockout *S*. Typhimurium mutant (*ΔrecA*). The results confirmed a role for RecA in polar chemoreceptor cluster formation and therefore in flagellar rotation switching.

Materials and Methods

Bacterial strains, plasmids, and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. Except when indicated, all strains of bacteria were grown at 37°C in Luria–Bertani (LB) broth or on LB plates. When necessary, ampicillin (100 μg/ml) or chloramphenicol (34 μg/ml) was added to the culture. The same cell growth conditions were used for the microfluidics and optical trap assays (described below). The bacteria were grown overnight in 2 ml of LB broth supplemented, when needed, with the appropriate antibiotic. Each culture was diluted 1:10 into LB broth without antibiotics but containing an oxygen scavenging system consisting of 100 μg glucose oxidase/ml and 20 μg catalase/ml (final concentrations) [29]. The added glucose is a substrate for the oxygen scavenging system and provides the energy needed for swimming under anaerobic conditions [29]. The cells were incubated at 37°C for 1 h and then diluted 100-fold in measuring medium (1% Bacto Tryptone, 0.8% NaCl, 2% glucose, 100 mM Tris-Cl, pH 7.5) containing the oxygen scavenging system. In all cases the scavenging system was added at least 2 h before the medium was used, to ensure a stable low level of oxygen.

Dead cells used for optical trap assays were prepared by the addition of 2% formaldehyde to the culture, with subsequent dilution steps carried out following the same protocol used for live bacterial cultures.

For chemotactic assays, overnight cultures of *S*. Typhimurium were grown in tryptone broth (1% Bacto Tryptone, 0.5% NaCl) supplemented, when required, with the appropriate antibiotics [30]. All strains used in the chemotactic assays (LT2, UA1928 (*ΔcheB*), UA1931 (*ΔrecA*) and UA1931/pUA1130) had similar growth kinetics in tryptone broth medium (data not shown). The cultures were then diluted 1:100 in the same medium but without antibiotics and incubated at 30°C with constant shaking until an OD_{600} of approximately 0.5 was reached. The culture was then harvested by centrifugation at 4500 g for 10 min at room temperature. The obtained cell pellet was washed twice in 1 ml of tempered tethering buffer (10 mM potassium-phosphate pH 7, 67 mM NaCl, 10 mM Na-lactate, 0.1 mM EDTA, and 0.001 mM L-methionine) and the resuspended cells were diluted to approximately 6×10^7 colony-forming units (cfu)/ml.

Construction of the *S*. Typhimurium mutant strains

The *S*. Typhimurium mutants were constructed using the one-step PCR-based gene replacement method [31], except when indicated. All DNA techniques were performed as described elsewhere [32]. The chloramphenicol resistance cassette from the plasmid pKD3 was amplified using suitable oligonucleotides containing 80-nucleotide stretches homologous to each of the insertion sites and the corresponding P1 and P2 sites of pKD3 (Table S1). The PCR products were digested with *Dpn*I and used to transform *S*. Typhimurium electrocompetent cells containing the pKOBEGA plasmid [33]. Following selection of the transformant clones, the latter plasmid was eliminated by taking advantage of its temperature sensitivity, incubating the clones at 42°C. Gene substitution was confirmed by PCR and sequencing using the appropriate primers.

To construct the *ΔcheRΔcheW* and *ΔcheRΔrecA* mutant strains, the chloramphenicol resistance cassette present in the *ΔcheR* strain was eliminated as previously described using the pCP20 plasmid [31]. Afterwards, either *ΔrecA* or *ΔcheW* mutations from UA1927 and UA1907 were transferred to the *ΔcheR* chloramphenicol-sensitive strain (UA1910) by transduction, using the P22 HT bacteriophage [34]. The same procedure was used to construct the *ΔrecA ΔcheW* strain. In this case, the chloramphenicol resistance cassette present in *ΔcheW* strain was removed and the *ΔrecA* mutation was transducted from the UA1927 strain. In all cases, the absence of the prophage in the selected clones was determined by streaking them onto green plates as previously described [35]. All of the resulting strains were verified by PCR and sequencing.

β-Galactosidase-based two-hybrid system

The two-hybrid assay was performed as described [36]. The *recA* and *cheW* genes were PCR-amplified using suitable oligonucleotides (Table S1) that included *Sph*I and *Bam*HI restriction sites in the amplicon. After their release from the plasmids by endonuclease digestion, the amplified genes were cloned in both pB2H*Δ*α and pB2H*Δ*ω vectors. The same procedure was used to clone the *amyA* and *dnaE* genes into pB2H*Δ*α and pB2H*Δ*ω, respectively. These constructs served as the non-interaction assay controls, as previously described [36]. All of the constructs were confirmed by sequencing.

To simultaneously express the two fusion proteins within a cell, electrocompetent *E. coli* MC1061 cells were co-transformed with the two plasmids of interest and the transformants were selected by adding chloramphenicol and ampicillin to the solid medium. The presence of both fusions was confirmed by PCR and sequencing.

For β-galactosidase assays, the selected clones were grown in LB supplemented with ampicillin and chloramphenicol at 37°C until an OD_{550} of 0.2 was reached. IPTG was then added to the culture to a final concentration of 20 nM and the cultures were incubated at 37°C. Samples were taken 5 h after IPTG addition, and β-galactosidase activity was assayed as described by Miller (1991) [37]. The relative expression of β-galactosidase in each strain was calculated as the enzyme's activity with respect to that of the non-interaction control strain, which expressed the *Δ*αAmyA and *Δ*ωDnaE proteins [36]. The reported results are the means of at least three independent assays, each performed in triplicate.

Construction of RecA and CheW tagged proteins

Co-immunoprecipitation assays were carried out using RecA-6×His and CheW-FLAG tagged proteins. The *recA* and *cheW* genes were PCR-amplified using the appropriate oligonucleotide pair (Table S1). In both cases, the corresponding tag sequence was included at the 5′ end of the suitable oligonucleotides that also contained a 3× Gly linker between the tag and the corresponding

Table 1. Bacterial strains and plasmids used in this work.

Strain or plasmid	Relevant characteristic(s)	Source or reference
Strains		
DH5α	*E. coli supE4 ΔlacU169 (φ80 ΔlacZ ΔM15) hsdR17, recA1, endA1, gyrA96, thi-1, relA1*	Clontech
MC1061	*E. coli* F^- *Δ(ara-leu)7697 [araD139] B/r Δ(codB-lacI)3 galK16 galE15* λ^- *e14*$^-$ *mcrA0 relA1 rpsL150* (Str^R) *spoT1 mcrB1 hsdR2*(r^-m^+)	CGSC
BL21 (DE3) pLysS	*E. coli* F^- *dcm ompT lon hsdS*($r_B^-m_B^-$) *galλ(DE3)* carring pLysS plasmid, Cm^R	Stratagene
LT2	*S.* Typhimurium wild type strain	ATCC
UA1907	*S.* Typhimurium *ΔcheWΩcat.* Cm^R	This work
UA1908	*S.* Typhimurium *ΔcheW*	This work
UA1910	*S.* Typhimurium *ΔcheR*	This work
UA1915	*S.* Typhimurium *ΔcheR ΔcheW*	This work
UA1913	*S.* Typhimurium *ΔcheR ΔrecA*	This work
UA1927	*S.* Typhimurium *ΔrecAΩ,cat.* Cm^R	This study
UA1928	*S.* Typhimurium *ΔcheB*	[29]
UA1929	*S.* Typhimurium *ΔcheY*	[29]
UA1930	*S.* Typhimurium *ΔcheW ΔrecA*	This work
UA1931	*S.* Typhimurium *ΔrecA*	This work
Plasmids		
pKOBEGA	Vector containing the λ Red recombinase system, Amp^r, temperature sensitive	Generous gift of Prof. G. M. Ghigo, [33]
pKD3	Vector carrying FRT-Cm construction, Amp^R, Cm^R	[31]
pCP20	Vector carrying FLP system, $OriV_{ts}$, Amp^R	[31]
pGEM-T	Cloning Vector; Amp^R	Promega
pGEX 4T-1	Expression vector carrying the Ptac IPTG - inducible promoter and the *lacI*^{q} gene; GST fusion tag, Amp^R	Amersham Biosciences
pUA1108	pGEX 4T-1 derivative plasmid carrying without the GST fusion tag, carrying only the Ptac promoter and the lacIq gene; used as overexpression vector, Amp^R	This work
pUA1109	pUA1108 derivative containing the native *S.* Typhimurium *recA* gene under the control of the Ptac promoter, Amp^R.	This work
pUA1127	pUA1108 derivative vector carrying the eYFP::*cheR* fusion, Amp^R	This work
pUA1130	pUA1108 derivative containing the native *S.* Typhimurium *recA* gene under the control of the Ptac promoter, Amp^R.	This work
pUA1131	pUA1108 derivative overexpression vector carrying the *cheW*-FLAG gene	This work
pB2H*Δα*	pACYCDuet-1 derivative vector with the *E. coli Δα* β-galactosidase fragment under the control of the Ptac promoter	BCCM/LMBP, [36]
pB2H*Δω*	pACYCDuet-1 derivative vector with the *E. coli Δω* β-galactosidase fragment under the control of the Ptac promoter	BCCM/LMBP, [36]
pUA1114	pB2H*Δα* derivative vector contaning the *Δα-recA* fusion	This work
pUA1115	pB2H*Δω* derivative plasmid contaning the *Δω-recA* fusion	This work
pUA1116	pB2H*Δα* derivative vector contaning the *Δα-cheW* fusion	This work
pUA1117	pB2H*Δω* derivative plasmid contaning the *Δω-cheW* fusion	This work
pUA1118	pB2H*Δα* derivative plasmid contaning the *Δα-amyA* fusion, used as a control in two hybrid assays.	This work
pUA1119	pB2H*Δω* derivative plasmid contaning the *Δω-dnaE* fusion, used as a control in two hybrid assays.	This work

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gene sequence (Table S1). The PCR products were digested with *Nde*I and *Bam*HI and cloned into pUA1108, with each tagged protein under the control of the Ptac promoter. The plasmids were transformed into *E. coli* DH5α and confirmed by sequencing. The confirmed plasmids were used in the electrotransformation of the *S.* Typhimurium *ΔrecA ΔcheW* strain, thereby ensuring that every RecA and CheW protein produced by that strain carried the specific tag. The selected transformants were confirmed again by PCR and sequencing.

Co-immunoprecipitation assays

The assays were performed as described by D'Ulisse and others [38] with modifications. Briefly, cultures of *S.* Typhimurium *ΔrecA ΔcheW* harboring the plasmids with constructs encoding the tagged proteins were grown in LB broth supplemented with

ampicillin to an OD_{550} of 0.2. Expression of the tagged genes was induced by the addition of 1 mM IPTG. After 3 h of growth, the cultures were centrifuged and the resulting pellet was washed in TBS 1× buffer (1.5 M NaCl, 250 mM Tris, pH 7.3) and then resuspended in cold IP lysis buffer (1× TBS, 15% glycerol, and 1% Triton X-100). The samples were incubated at 4°C for 40 min, with vortexing every 5 min. Finally, the samples were centrifuged at 4°C and the supernatant was collected. The protein concentration was determined by the Bradford method. As a control, cell lysates of *S.* Typhimurium *ΔrecA ΔcheW* containing the empty pUA1108 were also obtained following the same procedure.

Pure Proteome Protein A magnetic beads (Millipore) were used for immunoseparation. Either anti-6×His mouse IgG (Roche) or anti-FLAG mouse IgG (Acris) antibody was attached to the beads according to the manufacturer's instructions.

For co-immunoprecipitation the corresponding cell lysates were mixed at a ratio of 1:1 and incubated at 30°C for 1 h without shaking, to allow interaction of the proteins. The appropriate antibody-coated magnetic beads (either anti-6×His or anti-FLAG IgGs) were added to the mixture and the samples were incubated overnight at 4°C with gentle mixing on a shaker. The beads were recovered and washed three times with wash buffer (1×TBS, 15% glycerol, and 1% Triton X-100) and finally resuspended in 45 µl of Laemmli sample buffer and heated for 10 min at 90°C. The samples were separated by SDS-PAGE on a 15% polyacrylamide gel and analyzed by western blotting using as primary antibody either anti-6×His or anti-FLAG mouse IgG and as secondary antibody horseradish peroxidase (HRP)-coupled anti-mouse IgG goat IgG antibody (Acris) together with Luminata western HRP chemiluminescence substrates (Millipore). HRP-coated Precision Plus protein western C standard (BioRad) was used as the molecular mass marker.

Microfluidics assays

Microfluidics experiments were performed for each bacterial strain as previously described by Ahmed *et al.* [39]. After overnight incubation, cultures in log phase were diluted 1:50, to a final volume of 2 mL, in fresh medium and grown to an optical density $OD_{600} = 0.45 \pm 0.02$. In order to optimize trajectory identification, cells were then diluted 1:4 before injecting them in a 60 µm thick microfluidic channel hosting a microwell (∅ 2 mm) at its center. The focal plane was set to 30 µm above the glass bottom, at channel mid-depth, to minimize the interaction of bacteria with surfaces. Cells were imaged with a 20× objective (Nikon Pan Fluor ELWD, NA 0.45, WD 7.4 mm) in phase contrast at 25 frames per second, for a total of 20 s per experiment. Three biological replicates, each with 5 technical replicates, were carried out for each strain. All frames were segmented to obtain the cells' coordinates and cells were then tracked using in-house developed tracking routines based on the nearest neighbor method, implemented in MATLAB (The Mathworks, Natick, MA). Manual selection of high-quality tracks followed trajectory identification and allowed 300–500 trajectories per strain to be used for the analysis of motility.

Optical trapping assays

Optical trapping was carried out as previously described using a 1064-nm optical beam from a laser coupled to a single-mode fiber (Avanex) expanded up to 10 mm and then highly focused by an immersion oil objective (Nikon, CFI PL FL 100× NA 1.30 WD 0.16 mm) [29]. The oxygen scavenging system guaranteed a constant low level of oxygen and hence cell survival during the measurements [40].

As previously described [29], data for each bacterial strain were obtained from ten different randomly chosen cells of four distinct biological replicates; thus 40 cells per strain were analyzed.

The forward scattered light is collected by a 40X objective and projected into a quadrant photo diode (New Focus 2911). By this technique, the position of each trapped cell was acquired for 1000 s at 2 kHz of acquisition rate. The entire set of acquired data (1000 s) was then divided into 1-s-blocks. For each data block the angular velocity of the cell around the optical axis (Θ value) was calculated as described [29]. About 80% of the histograms showed very similar patterns. All plots shown below for the wild-type, mutant strains, and dead bacteria present the Θ histogram of one trapped cell either from the corresponding bacterial strain or from a dead cell control. In all cases, the selected histograms were within the above-mentioned 80%. During the experiments, videos were recorded using a CCD camera at the beginning (capture) and end (liberation) of the measurements.

Chemotaxis capillary assays

Chemotaxis assays were conducted as described by Adler [30], with some modifications. The chemotaxis chamber set was formed by placing three V-shaped bent needles (40 mm 18 G needle, Nipro) on the surface of an aseptic 140-mm Petri dish (Deltalab) and then covering them with a 24×65 mm microscope cover slip (Menzel-Glässer).

One-ml capillary tubes, 3 cm long (Microcaps, Drummond Scientific Co.), were used. One end of each tube was heat-sealed in a flame. After autoclaving, the sealed capillaries were filled with either tethering buffer or 10 mM L-aspartate dissolved in tethering buffer [41].

Approximately 2 ml of each cell suspension was placed in the chemotaxis chambers, which were then incubated for 1 h at 30°C. After the incubation, the exterior of the capillaries was rinsed under a stream of sterile double-distilled water. The sealed end of the capillaries was then broken off and the contents of the tube were emptied into a 1.5-ml microcentrifuge tube containing 0.9% NaCl. Suitable dilutions were plated on LB plates; after an overnight incubation, the cfu/ml were calculated.

Construction of the eYFP::*cheR* fusion

The enhanced yellow fluorescent protein (eYFP)::*cheR* fusion was constructed by the overlap extension procedure as follows. The *cheW* and *eYFP* genes (Clontech) were amplified using CheRstmF/CheRstmBamHI and eYFPNdeI/eYFPR oligonucleotide pairs, respectively, with eYFPR and cheRstmF containing complementary overhangs and a 3× Gly linker (Table S1). The resulting DNA fragments were annealed and amplified in a second round of PCR using eYFPNdeI and CheRstmBamHI to form the corresponding eYFP::*cheR* fusion. These outer primers contained *Nde*I and *Bam*HI restriction sites that were used to clone the fragments into the IPTG-inducible pUA1108 expression vector, giving rise to plasmid pUA1127, in which the eYFP::*cheR* fusion is under Plac promoter control. The fusion was confirmed by sequencing and the pUA1127 plasmid was transformed into several genetic backgrounds (*ΔcheR*, *ΔcheRΔrecA*, and *ΔcheRΔcheW*) to obtain the bacterial strains used in the chemoreceptor clustering assays.

Chemoreceptor clustering assay

Receptor clustering experiments were performed as described [22,42,43] with modifications. Briefly, overnight cultures of *S.* Typhimurium strains carrying the pUA1127 (eYFP::*cheR*) plasmid were grown at 30°C in tryptone broth supplemented with ampicillin under constant agitation. After 24 h of incubation, the

cultures were diluted 1:100 in tryptone broth supplemented with ampicillin and 25 mM IPTG to induce eYFP::*cheR* fusion expression. The cultures were then incubated at 30°C until an OD_{600} of 0.5 was reached. The cells were harvested by low speed centrifugation (5300 g) for 15 min, washed once in cold tethering buffer, and finally resuspended in 100 ml of ice-cold tethering buffer. The cells were maintained on ice throughout the assay.

For fluorescence microscopy assays, the cells were immobilized and fixed at the same focal plane using thin 1% agarose pads in tethering buffer. Three µl of cells were applied on the pad, which was then covered with a clean cover slip. Fluorescence microscopy was performed using a Zeiss AxioImager M2 microscope (Carl Zeiss Microscopy) equipped with a Zeiss AxioCam MRm monochrome camera (Carl Zeiss Microscopy) and a filter set for eYFP (excitation BP 500/25; beam splitter FT 515; emission BP535/30). Cell fields were photographed and at least 250 cells were inspected by eye to determine the presence and type of clusters. All fluorescence images were obtained at 1000× magnification and were acquired under identical conditions. Each experiment was performed in triplicate using independent cultures. The images presented in the corresponding figure are representative of the entire images that are included as (Files S1, S2 and S3). ImageJ software (National Institutes of Health) was used to either quantify the number of clusters or to prepare images for publication.

Statistical analysis

The chemotaxis capillary and chemotaxis clustering assays were statistically evaluated using, respectively, a two-way or one-way analysis of variance (ANOVA) with Prism (GraphPad), as previously described [44,45]. In all cases, the analyses were followed by the Bonferroni multiple comparison post-hoc test, with $p<0.05$ defined as statistically significant. In all of the figures, the error bars indicate the standard deviation.

Results

The interaction of RecA and CheW proteins

Large-scale protein-protein *in vitro* interaction studies had previously identified RecA as a prey protein when CheW was used as bait, but not vice versa [23]. Thus, to ascertain the RecA and CheW interaction two-hybrid assays and co-immunoprecipitation experiments were carried out.

In the two-hybrid assay, the previously described pB2H$\Delta\alpha$/pB2H$\Delta\omega$ system [36] was used. RecA and CheW proteins were fused to the two non-functional but complementary β-galactosidase truncations ($\Delta\alpha$ and $\Delta\omega$) in the system. In the reporter strain, β-galactosidase activity is driven by protein-protein recognition between both non-β-galactosidase parts of the chimeras. As shown in Fig. 1A, relative β-galactosidase expression by strains co-expressing either the $\Delta\alpha$RecA/$\Delta\omega$CheW or the $\Delta\alpha$CheW/$\Delta\omega$RecA chimera pair was >7, indicating significantly higher β-galactosidase activity in these strains than in the non-interaction assay control strain [36].

To further confirm the two-hybrid assay results and thus obtain additional evidence for the interaction between RecA and CheW, co-immunoprecipitation assays were carried out using *S*. Typhimurium *ΔrecA ΔcheW* strains carrying the corresponding plasmids that overexpress either the RecA-6×His or the CheW-FLAG tagged proteins (Table 1). The immunoprecipitation was performed by using magnetic beads coated with either anti-6×His or anti-FLAG mouse IgG antibodies that specifically interact with the corresponding tagged protein and the recovered proteins were detected by Western blot. As seen in Fig. 1B, when both recombinant proteins were present in the protein mixture, and anti-6×His antibody coated beads were used, CheW-FLAG proteins were observed in the recovered supernatants. The same results were observed when anti-FLAG antibody coated beads were added to the mixture, then RecA-6×His proteins were recovered. All together, these data indicate that RecA-6×His was able to pull down CheW-FLAG and vice versa. Thus, the results of the two assays together confirm RecA–CheW pair formation and suggest the association of RecA with the chemotaxis pathway through its interaction with CheW.

The absence of RecA causes a decrease in swimming speed

To further understand the role of RecA in motility, the swimming speed of a *S*. Typhimurium Δ*recA* mutant was evaluated through microfluidics assays and compared to that of the wild-type strain. In this assay, swimming speed computed over the whole set of identified trajectories was measured. As shown in Fig. 2, which depicts the relative frequency of swimming speeds for each strain, the absence of RecA prompted a change in the swimming profile. Specifically, at the highest relative frequency, the velocity of the mutant was lower than that of the wild-type strain under the same experimental conditions.

The *ΔrecA* mutant present a CW-bias of flagella rotation

To determine whether the slower swimming speed prompted by the absence of RecA was due to a bias in flagellar rotation, the flagellar rotation patterns of the *ΔrecA* mutant was studied using a single optical trap. Optical trapping is an excellent tool for analyzing the dynamic properties of bacteria [46–48]. It is based on the ability of an optical beam to trap a single cell because the refractive index of cells (and their constituents) is higher than that of the surrounding medium. Once trapped, the movement of that cell is measured, yielding information on its momentary position. In the case of rod shaped *S*. Typhimurium, a cell trapped in the single optical beam aligns itself along the optical axis. Thus, besides Brownian motion, the torque produced by flagellar rotation alters the dynamics of the cell [29]. The measurement of the rotation profile for each strain is expressed as the distribution of the change in the mean value of Θ, which is the angular velocity of the cell around the optical axis [29]. Using this technique we were able to distinguish CW to CCW switching of the flagella.

Figure 3 shows the flagellar rotation profile of both the *S*. Typhimurium *ΔrecA* mutant and the wild-type strain. Dead wild-type cells and *ΔcheB* and *ΔcheY* mutants were used as controls. There is no change in the angular velocity of dead cells, which exhibit only Brownian motion; thus, their Θ distribution pattern is centered at zero. Among the mutants, Δ*cheB* cells, described by their tumbling motility because of their CW flagellar rotation bias [49], displayed a Θ distribution pattern centered near zero. Thus, there was no change in the average angular velocity of the mutant cells but, as would be expected, the histogram was broader than that of the dead cells. Conversely, the flagella rotation profile of *ΔcheY* cells had a mean Θ that was not zero and was highly positive, consistent with the smooth swimming ability characteristic of this strain because of its CCW-biased flagellar rotation pattern [50,51]. In the living wild-type cells, the Θ histogram showed the anticipated two peaks, reflecting normal switching between CCW and CW flagellar rotations. The peak centered at zero along the *x* axis corresponded to the tumbling state (CW rotation) whereas the peak located around 7.5 was due to the running state (CCW rotation) (Fig. 3). By contrast, but similar to the *ΔcheB* mutant, *ΔrecA* cells showed only one peak, centered

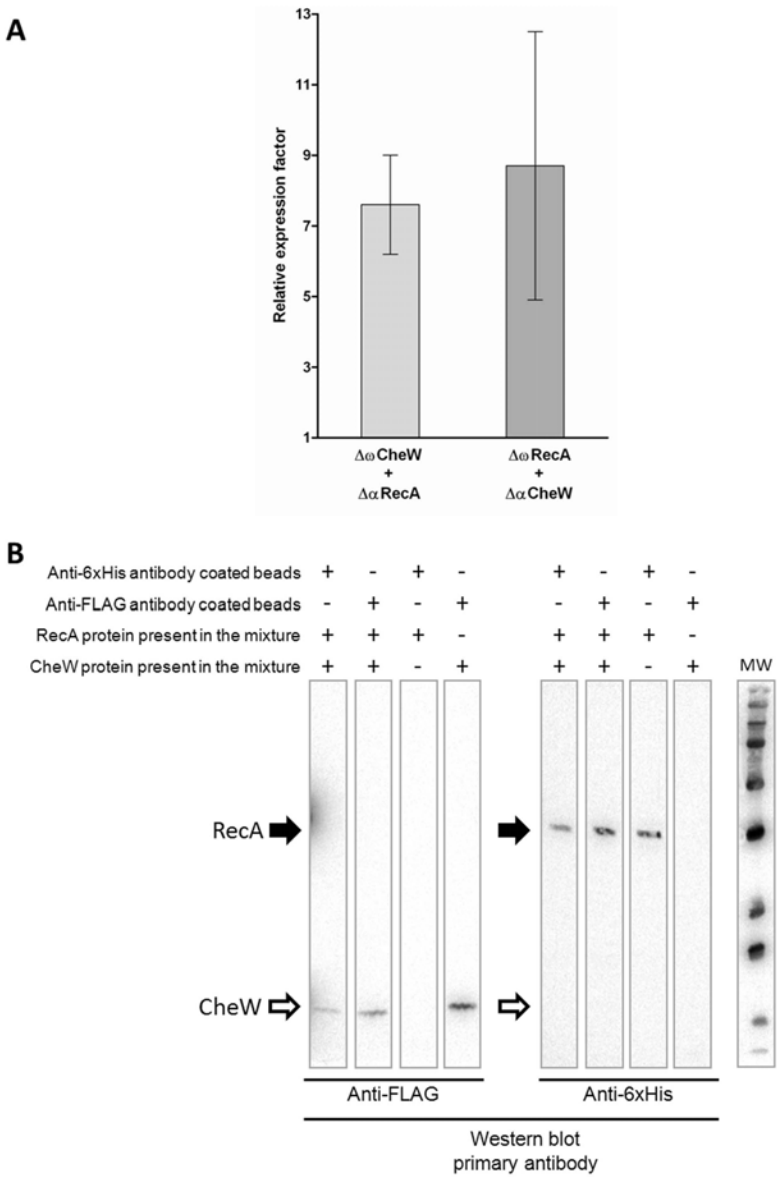

Figure 1. RecA protein directly interacts with CheW. A) Two-hybrid assay. Measurement of the β-galactosidase activity of strains co-expressing the chimera protein pairs *Δ*αRecA/*Δ*ωCheW or *Δ*αCheW/*Δ*ωRecA. The results are expressed relative to those obtained with the non-interacting control strain expressing *Δ*αAmyA and *Δ*ωDnaE [36]. Measurements were made 5 h after the addition of 20 nM IPTG to the culture. In

each case the mean value from three independent experiments (performed in triplicate) is shown. Error bars indicate the standard deviation. **B) Co-immunoprecipitation assay.** Lysates prepared from cells overexpressing RecA-6×His and CheW-FLAG tagged proteins were mixed to allow the proteins to interact. Immunoprecipitation (IP) was performed by adding magnetic beads coated with either anti-6×His or anti-FLAG antibodies to the mixture and the attached proteins were recovered and separated by SDS-PAGE electrophoresis. The presence of the recombinant protein in the supernatants was assessed by western blotting (WB). As a control, co-IP assays were conducted using lysates from a *ΔrecAΔcheW S.* Typhimurium strain carrying an empty overexpression plasmid, thus expressing neither RecA-6×His nor CheW-FLAG proteins. The presence (+) or absence (−) of RecA, CheW, or both tagged proteins in the corresponding lysate mixture is indicated. Black and white arrows show the position of RecA-6×His and CheW-FLAG, respectively. IP indicates the antibody attached to the beads and WB the primary antibody used in western blotting. MW indicate the molecular mass marker.
doi:10.1371/journal.pone.0105578.g001

near zero, in their Θ distribution pattern (Fig. 3). The absence of the second peak was indicative of the CW-biased rotation of Δ*recA* cells.

Chemotaxis response of *recA* mutants

To further confirm the switching defect of the *ΔrecA* mutant, its ability to move towards an L-aspartate source was evaluated using a classical capillary assay. The results are shown in Fig. 4. As expected, and in concordance with observations in other tumbling strains such as the *ΔcheB* mutant [49], the capillary assays clearly demonstrated that cells lacking RecA are unable to respond to the presence of L-aspartate. Furthermore, chemotaxis by the *ΔrecA* mutant was restored when the RecA deficiency was complemented by the presence of a plasmid containing the *recA* gene under the control of an IPTG- inducible promoter (Fig. 4) but not by the presence of the empty plasmid (data not shown). These results unequivocally showed that the presence of RecA is essential for a normal chemotactic response.

Chemotaxis receptor clustering

Based on our results, the phenotype of the *ΔrecA* mutant is similar to that of other *che* mutants, since in all cases the absence of RecA impairs not only swarming [17,18] but also the switching of flagellar rotation (Fig. 3) and the chemotactic response (Fig. 4). To elucidate the role of RecA in chemotaxis, and taking into consideration the direct interaction of RecA with the CheW coupling protein, which bridges the MCPs to histidine kinase CheA [43,52], we asked whether, like CheW, RecA was involved in chemoreceptor clustering. To investigate this possibility fluorescently tagged CheR (eYFP-CheR) was used as a specific reporter for chemoreceptor localization [43].

The eYFP-CheR fusion was constructed and cloned into pUA1108 vector under the control of an IPTG-inducible promoter (Table 1). For correct chemoreceptor localization, native CheR had to be removed; accordingly, the plasmid was included in the *S.* Typhimurium *ΔcheR ΔrecA* transformant. Additionally, it was also used to obtain the *ΔcheR* and *ΔcheR ΔcheW* mutants. The *ΔcheR* mutant served as the positive control strain since it exhibited normal polar clusters. The *ΔcheR ΔcheW* strain was used as the negative control strain since the absence of CheW inhibits polar cluster formation [22]. As expected, and in agreement with previous reports [43], in the positive control (the *ΔcheR* strain) single tight polar spots were seen in ~70% of the observed cells. These spots corresponded to the clustering of thousands of chemoreceptors at the cell pole (Fig. 5). However, in agreement with previous data [43], in the absence of CheW compact polar clusters were formed in only ~10% of the cells; instead, the presence of diffuse clusters (known as caps) was observed (Fig. 5A). Thus, according to our findings, the absence of RecA significantly impairs normal polar cluster formation, which occurred in only ~50% of the cells, and increases the presence of caps. Nonetheless, neither the reduction in polar spot formation nor the increase in caps was as high in the absence of RecA as in the absence of CheW.

Discussion

A role for RecA in controlling the swarming motility of both *E. coli* and *S.* Typhimurium was clearly shown in previous studies [17,18]. However, besides its possible connection to CheW [18,23], nothing was known about the mechanisms that link RecA to motility. To determine whether RecA is associated with the chemotaxis pathway and, specifically, with flagellar function, we examined its putative direct interaction with CheW, its role in swimming motility and in chemotaxis, as well as the flagellar switching pattern of cells lacking RecA.

Our results support a tight relationship between RecA and the chemotactic response. First, our results unequivocally confirmed the interaction of RecA with CheW through two widely used techniques. The results of the two-hybrid assays were in concordance with those previously obtained in a large-scale genome-wide screening assay [23] and suggested an interaction between RecA and CheW (Fig. 1A), which was definitively shown in the co-immunoprecipitation assays (Fig. 1B). Second, the motility phenotype of cells lacking RecA, as determined herein, was similar to that of some *che* mutants. The latter finding was supported by microfluidics assays, in which the average swimming speed of *S.* Typhimurium *ΔrecA* was lower than that of the wild-type strain (Fig. 2), and by the observed differences in the flagellar rotation patterns of these two strains (Fig. 3). Thus, the absence of

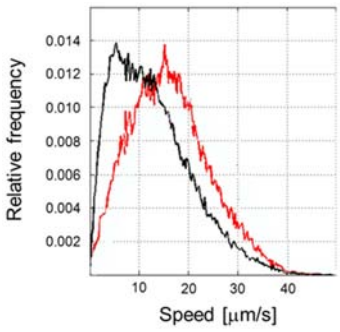

Figure 2. The lack of *recA* reduces the swimming speed of *S.* Typhimurium. The experimentally observed probability distribution of the swimming speeds within a population of wild-type *S.* Typhimurium (red line) and of the *ΔrecA* mutant (black line), assessed using a microfluidics assay [39]. Values are expressed as the relative frequency of a given speed within a cell population. For each strain, the results were obtained from three independent experiments supported by five technical replicates each, for a total of 300–500 cells tracked per strain.
doi:10.1371/journal.pone.0105578.g002

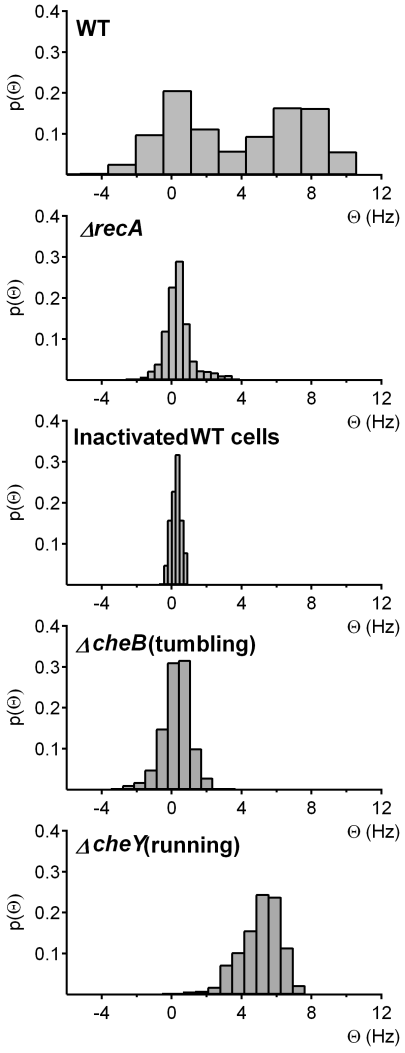

Figure 3. Flagellar rotation is CW-biased in the *S.* Typhimurium Δ*recA* mutant. The flagellar switching profiles of *S.* Typhimurium LT2 wild-type (WT), dead wild-type (dead), *ΔrecA*, *ΔcheB* (tumbling), and *ΔcheY* (running) cells were evaluated. The resulting histograms show the distribution of the change in the mean cellular angular velocity around the optical axis (Θ). A zero-centered peak, as displayed by dead cells and the *ΔcheB* tumbling mutant, is indicative of CW-biased flagellar rotation, and a peak with positive values, as displayed by the *ΔcheY* running mutant, CCW-biased rotation. The presence of two peaks, one zero-centered and the other centered at positive values, indicates a mixed population displaying both CW and CCW rotational patterns and thus a non-biased flagellar rotational pattern. For each strain, the results were obtained from four independent experiments of ten cells each.
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RecA impaired flagellar switching, leading to a CW bias similar to that of other tumbling strains, like the *ΔcheB* mutants [49]. Furthermore, consistent with the tumbling phenotype of the *ΔrecA* mutant, our results demonstrate that RecA is essential for a normal chemotactic response. Specifically, in quantitative chemotaxis assays the *ΔrecA* mutant was unable to detect the presence of L-aspartate, a well-known chemoattractant (Fig. 4); instead, chemotaxis was restored only when the *recA* deficiency was complemented by a plasmid carrying a copy of the *recA* gene (Fig. 4). The slower-moving phenotype of the RecA-deficient mutants can be explained by the CW bias displayed by these cells. By being anchored in a tumbling state, without normal running, the *ΔrecA* mutant was slower than the wild-type strain. In a previous study, the inability to switch the direction of flagellar rotation was linked to defects in chemotaxis and to improper colony hydration, leading to an inability to swarm [25,27,28]. It was previously reported that an *E. coli recA1* mutant did not exhibit any apparent alterations in chemotaxis [53]. Nevertheless, this *recA1* strain was not a knockout mutant, as was the *ΔrecA* mutant used in this work, in which the *recA* gene was completely removed. Furthermore, the *recA1* allele is a single amino acid missense mutation that prevents RecA recombinatorial activity [54] but still allows normal binding to ssDNA as well as ATP-independent renaturation of comple-

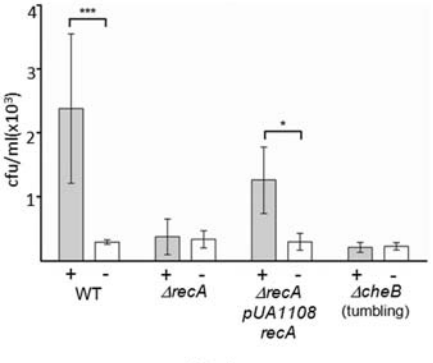

Figure 4. The chemotactic response of the *S.* Typhimurium Δ*recA* mutant is impaired. The chemotactic responses of *S.* Typhimurium wild-type (WT), Δ*recA*, Δ*recA* complemented (Δ*recA* pUA1109), and Δ*cheB* (tumbling) cells were assessed using Adler's capillary assay [30] with the modifications described in Materials and Methods. Values are expressed as the number of viable cells (in cfu/ml) in a capillary tube containing either 10 mM aspartate (+) or tethering buffer alone (−). The results are the mean of five independent experiments of three capillaries each. Error bars indicate the standard deviation. ***$p<0.001$ and *$p<0.05$ as determined by two-way ANOVA with Bonferroni correction.
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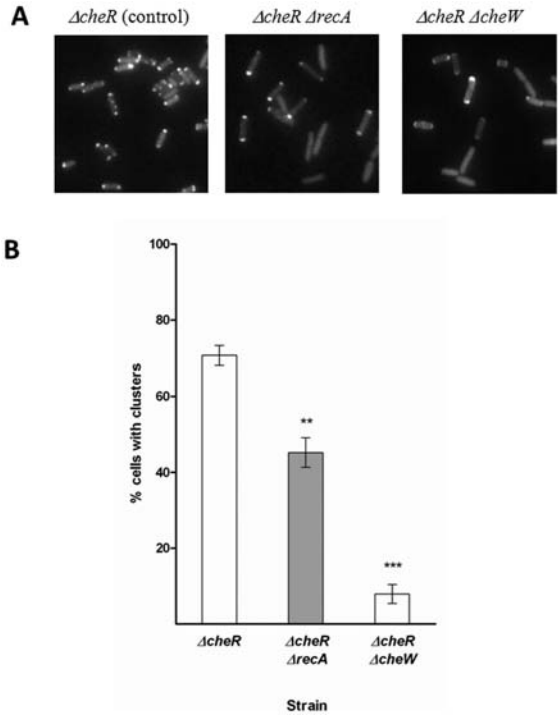

Figure 5. The formation of polar chemoreceptor clusters is altered in the absence of RecA protein. A) A representative fluorescence microscopy image of the *ΔcheR ΔrecA* strain harboring plasmid pUA1127, containing the inducible eYFP::*cheR* fusion. Images of the *ΔcheR* and *ΔcheRΔcheW* strains containing the gene fusion were also included as positive and negative controls of polar chemoreceptor cluster structuring, respectively. **B**) The fraction of cells with well-structured polar chemoreceptor clusters. The percentage of cells showing polar, round, and diffraction-limited spots (previously referred to as clusters; [43]) was quantified in each strain. The results are the mean of three independent imaging experiments. Error bars represent standard deviation. ***$p<0.001$ and *$p<0.01$ as determined by one-way ANOVA with Bonferroni correction.
doi:10.1371/journal.pone.0105578.g005

mentary ssDNA molecules [55]. Thus, the results obtained with the two mutants cannot be compared.

Nevertheless, how RecA modulates flagellar rotation was unclear. In an earlier study, the absence of RecA had no effect on the expression of genes involved in either flagellar biosynthesis or chemotaxis [17], as shown for other proteins such as H-NS, which is required not only for flagellar motor function but also for flagellar biogenesis [56]. The direct association of RecA with the CheW coupling protein led us to ask whether RecA, like CheW, plays a role in the architecture of chemoreceptor arrays. CheW tethers CheA kinase to the MCPs forming the MCP-CheW-CheA ternary complexes and chemoreceptor arrays, enabling MCPs to modulate CheA autokinase activity [22,57] which, in turn, controls the level of phosphorylated CheY (CheY-P). Once activated by the MCPs, phosphorylated CheA transfers its phosphoryl group to CheY. CheY-P then promotes a switch in the direction of flagellar rotation, from CCW to CW. According to our observations, the RecA protein is necessary for the formation of normal polar chemoreceptors arrays. Although its role may not be the same as that of CheW, the absence of RecA significantly reduces the polar clustering of chemoreceptors (Fig. 5). The absence of MCPs, CheW, or CheA is known to impair chemoreceptor array formation, leading cells to run constantly because of the CCW bias of their flagellar rotation [51]. Conversely, and in addition to the demonstrated effect of RecA on chemoreceptor array formation, our results show that the absence of this protein results in a CW bias, similar to that observed in *cheZ*, *cheR*, or *cheB* null mutants. All of these Che proteins are associated with chemotactic response adaptation: CheZ phosphorylase returns phosphorylated Che (CheY-P) to its non-phosphorylated state (CheY), and CheB methylesterase and CheR methyltransferase control the MCP methylation state, adjusting it to the presence and concentration of external stimuli. Therefore, a similar function can be hypothesized for RecA in the chemotactic response adaptation. It is worth noting that although CheZ, CheR, and CheB co-localize with MCPs-CheW-CheA

complexes at the cell poles [42], none of these proteins have an effect on polar cluster formation [43], unlike RecA. While further work is needed to determine the exact role of RecA in the chemotaxis pathway, our results clearly reveal previously unknown functions of RecA: its involvement in the control or modulation of flagellar rotation, and thus not only with swarming but also with swimming and chemotaxis, and its role in the architecture of polar chemoreceptor arrays.

Supporting Information

Table S1 Oligonucleotides used in this work.
(DOCX)

File S1 ZIP File containing the entire set of images used to determine the presence and type of clusters found in the *S.* Typhimurium UA1910 (*ΔcheR*) strain carrying the pUA1127 (eYFP::*cheR*) plasmid.
(RAR)

File S2 ZIP File containing the entire set of images used to determine the presence and type of clusters found in the *S.* Typhimurium UA1915 (*ΔcheRΔcheW*) strain carrying the pUA1127 (eYFP::*cheR*) plasmid.
(RAR)

File S3 ZIP File containing the entire set of images used to determine the presence and type of clusters found in the *S.* Typhimurium UA1913 (*ΔcheRΔrecA*) strain carrying the pUA1127 (eYFP::*cheR*) plasmid.
(RAR)

Acknowledgments

IN MEMORIAM

We mourn the loss of Dmitri Petrov (1947–2014) a great colleague and friend.

Author Contributions

Conceived and designed the experiments: SC AM JB DP RS. Performed the experiments: AM OI IAM FM SC. Analyzed the data: SC AM OI JB DP IAM FM RS. Contributed reagents/materials/analysis tools: JB DP RS TK JAR. Contributed to the writing of the manuscript: SC JB.

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3.2 The SOS response impairs chemoreceptor assembly (Article 2)

SOS system induction inhibits the assembly of chemoreceptor signaling clusters in *Salmonella enterica*

Irazoki O, Mayola A, Campoy S & Barbé J

PLoS ONE 11(1): e0146685 (2016)

In this work, we demonstrate that activation of the SOS response reversibly inhibits swarming motility by preventing the assembly of polar signaling arrays. In *S. enterica*, SOS response activation impairs chemoreceptor polar array assembly and consequently the swarming ability, this inhibition being due to the increase in the RecA concentration but not to other SOS-response-associated functions. Thus, activation of the SOS response by the presence of a DNA-injuring compound increases the RecA concentration, thereby disturbing the equilibrium between RecA and CheW, that results in the cessation of swarming. Nevertheless, when the DNA-damage decreases and the SOS response is no longer activated, basal RecA levels and thus, polar cluster assembly, are reestablished. The reversibility of polar array assembly and the swarming behavior in response to a gradient of a SOS inducer are crucial aspects underlying the biological significance of the SOS response modulating swarming motility. Taken together, these results further demonstrate the ability of bacterial cells to adapt their surface motility in response to the presence of direct or indirect DNA-damaging agents by sensing these compounds via SOS system induction.

PLOS ONE

RESEARCH ARTICLE

SOS System Induction Inhibits the Assembly of Chemoreceptor Signaling Clusters in *Salmonella enterica*

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Abstract

Swarming, a flagellar-driven multicellular form of motility, is associated with bacterial virulence and increased antibiotic resistance. In this work we demonstrate that activation of the SOS response reversibly inhibits swarming motility by preventing the assembly of chemoreceptor-signaling polar arrays. We also show that an increase in the concentration of the RecA protein, generated by SOS system activation, rather than another function of this genetic network impairs chemoreceptor polar cluster formation. Our data provide evidence that the molecular balance between RecA and CheW proteins is crucial to allow polar cluster formation in *Salmonella enterica* cells. Thus, activation of the SOS response by the presence of a DNA-injuring compound increases the RecA concentration, thereby disturbing the equilibrium between RecA and CheW and resulting in the cessation of swarming. Nevertheless, when the DNA-damage decreases and the SOS response is no longer activated, basal RecA levels and thus polar cluster assembly are reestablished. These results clearly show that bacterial populations moving over surfaces make use of specific mechanisms to avoid contact with DNA-damaging compounds.

Introduction

Swarming is the rapid, flagellar-driven, and highly coordinated translocation of a bacterial colony across a moist surface [1]. This form of motility is widely distributed throughout the Domain *Bacteria*, in which it is associated with increased antibiotic resistance [2–4] and virulence [5–9]. In fact, swarming is one of the first steps in the bacterial colonization of host surfaces [8,10,11].

Salmonella enterica, the most frequent cause of food-borne disease outbreaks worldwide [12], is able to swarm on soft agar surfaces (0.5–0.8% agar) and is thus considered a temperate swarmer [13]. During swarming, the morphology of temperate swarmers does not significantly change, and processes such as elongation, the formation of multi-nucleoid cells, and hyperflagellation are not observed, unlike in robust swarmers such as *Proteus* and *Vibrio* species [14,15]. While swarming by *Salmonella* is clearly related to bacterial invasion and the

expression of virulence factors [4,10,11,16,17], little is known about the mechanisms that control this form of motility. It is well established that the chemotaxis signaling pathway, but not chemotaxis itself, plays a key role in the swarming motility of *S. enterica* [18]. The chemotaxis pathway includes transmembrane ligand receptors, known as methyl-accepting chemotaxis proteins (MCPs), which interact with each other to form trimers of dimers that are associated, through CheW adaptor proteins, with the CheA kinase. These signaling complexes, present in bacterial cells in amounts ranging from a few to thousands of copies, normally cluster together at the cell poles, where they form signaling arrays [19–21]. During chemotaxis, signal recognition by chemoreceptors modulates CheA kinase autophosphorylation. In turn, phosphorylated CheA mediates phosphorylation of the CheY response regulator, which acts on the flagellar motor to prompt flagellar rotation switching [22,23]. Swarming, however, requires only flagellar propulsion and the related mechanical interactions; the fine control offered by the chemotaxis pathway is dispensable [24–26]. The flagellar switch promotes lubrication of the cell–surface interface, thus minimizing surface friction and allowing swarming motility by temperate swarmers [13]. Mutants with defects in the chemotaxis pathway, flagellar biosynthesis, or polar chemoreceptor cluster assembly give rise to non-swarming colonies [27–31].

The RecA protein is also related to swarming ability [32–34]. RecA is a multifunctional protein that during DNA damage stress acts as a positive regulator of the SOS system, which mediates DNA repair [35]. The SOS response comprises a genetic regulatory network that is widely distributed among *Bacteria*. When DNA damage occurs, the RecA protein acquires an active conformation (RecA*) that promotes autocleavage of the SOS system repressor (the LexA protein) and the SOS response induction [36]. After its autohydrolysis, the LexA repressor is no longer able to repress either its own expression or that of the genes it controls (including *recA*, which is also part of the SOS network), thereby inducing the SOS response [37]. Once the DNA lesions are repaired, RecA is no longer activated and LexA again represses expression of the genes directly involved in the SOS network, which restores their basal-level expression. The SOS response coordinates the expression of genes involved in DNA recombination, DNA repair, cell division inhibition, mutagenesis, pathogenesis, antibiotic resistance, biofilm formation, and mobile element distribution [38–42].

The absence of the RecA protein impairs the swarming ability of both *Escherichia coli* and *S. enterica* [33,34]. We recently reported that, at least in *S. enterica*, this defect occurs because the RecA protein is essential for standard flagellar rotation switching and for the formation of normal chemoreceptor polar arrays [32]. Moreover, not only the absence but, conversely, also the overexpression of RecA in the absence of DNA damage impedes swarming motility. We were thus able to show that a *recA*-overexpressing mutant of *S. enterica* has both a non-swarming phenotype and a significantly reduced capacity to cross the intestinal epithelium [43]. In both the absence and the overexpression of RecA, a link between the RecA protein and the chemotaxis pathway, through the CheW anchor protein, has been suggested [32,34]. In fact, the interaction between RecA and CheW was confirmed by co-immunoprecipitation assays [32]. Furthermore, it has been demonstrated that the restoration of a normal swarming phenotype in a *recA*-overxepressing strain can be achieved by increasing the concentration of intracellular CheW [34].

Despite these insights, the effect of SOS response induction on swarming and the pathways by which increased RecA levels inhibit swarming have yet to be determined. The aim of this work was to further dissect these mechanisms in order to deepen our understanding of how bacterial cells adapt to a surface niche and respond to external stimuli. Specifically, we studied swarming ability and chemoreceptor polar cluster assembly in *S. enterica* in the presence of the SOS system inducer mitomycin C and the roles played by CheW and RecA proteins. We found

that induction of the SOS response impairs swarming motility by reversibly bypassing chemoreptor polar array assembly, through a disturbance of the balance between RecA and CheW.

Materials and Methods

Bacterial strains, plasmids, and growth conditions

The bacterial strains and plasmids used in this study are listed in S1 Table. Except when indicated, all strains were grown at 37°C in Luria–Bertani (LB) broth or on LB plates. When necessary, ampicillin (100 μg/ml), kanamycin (100 μg/ml), and/or chloramphenicol (34 μg/ml) were added to the culture. The growth conditions for swarming and the polar cluster assays are described elsewhere in this section.

The vectors used in this work are also listed in S1 Table. The molecular procedures required for this work were described previously [44]. *E. coli* DH5α strain was used in vector constructions. When needed, vectors were transformed in the corresponding *S. enterica* or *E. coli* strain by electrotransformation.

Construction of the *S. enterica* mutant strains

The *S. enterica cheW* FLAG-tagged mutant was constructed as described previously [45] using the pKO3 plasmid [46]. An overlap-extension PCR-generated *cheW::FLAG* gene fusion (which adds the DYKDDDDK epitope to the CheW protein) was introduced at the *Bam*HI restriction site of pKO3, generating plasmid pUA1121. The vector was confirmed by sequencing and electroporated into *S. enterica* ATCC14028. The resulting mutants were confirmed by sequencing and western blot. One mutant (UA1916) was selected for further studies.

To construct *S. enterica recAo cheW::FLAG*, the marker *recAo6869* was introduced into UA1916 strain by transduction using the P22int7(HT) bacteriophage [47] and UA1876 as the donor strain [34]. The absence of the prophage in the transductants was determined by streaking them onto green plates as described previously [48]. The resulting strains were verified by sequencing and the deregulation of *recA* expression was confirmed by ELISA, using the anti-RecA antibody (see below). The same procedure was used for the *ΔcheR* mutant derivatives, generated using the P22 int7(HT) bacteriophage and UA1907 strain. The latter includes the *ΔcheR* construct obtained by one-step PCR-based gene replacement and the chloramphenicol resistance cassette from pKD3 instead of the native *cheR* gene [49].

The *S. enterica* ATCC14028 *ΔsulA* mutant was constructed by one-step PCR gene replacement as described previously [49,50].

Gene substitution in all constructs was confirmed by PCR using the appropriate primers followed by sequencing.

Swarming assays

Swarming motility was assayed as described previously [33,34] using the corresponding *S. enterica* strains (S1 Table). Briefly, freshly prepared LB-swarming plates (1% tryptone, 0.5% yeast extract, 0.5% NaCl, 0.5% D-(+)-glucose, and 0.5% agar) supplemented when needed with suitable antibiotics, IPTG, and/or mitomycin C (0.08 μg/mL) were point inoculated using a sterile toothpick with a single *S. enterica* colony of the corresponding strain grown on normal LB plates. Once inoculated, the plates were incubated at 37°C for 14 h, by which time the wild-type strain had grown to reach the plate borders.

The same procedure was used for the swarming assays in the presence of a mitomycin C gradient generated by the disk diffusion method, as described previously [51]. Sterile filter-paper disks (Whatman 6 mm, grade AA discs, GE) were soaked in either water or mitomycin

C (2 mg/mL), dried at room temperature for 2 h, and aseptically placed onto the LB-swarming plates. The plates were then inoculated with the corresponding strains as described above and incubated at 37°C. Bacterial migration was observed for the indicated time.

To evaluate swarming motility, the plates were photographed (ChemiDoc XRS + system, Bio-Rad) and the diameter of the swarming colony was measured using ImageJ software (National Institutes of Health). The swarming ability of each strain under each condition was determinate at least three times, each in triplicate. The images shown in the figures are representative of the entire image set.

The relative swarming motility index (RSMI) for each condition was calculated as the ratio of the colony diameter of the studied strain to that of the control strain under the same experimental conditions, as described previously [52].

Mitomycin C cell susceptibility assay

To evaluate the cell susceptibility to mitomycin C, the corresponding bacterial inoculum was applied using a sterile swab all over the surface of LB plates. Afterwards, disks soaked in mitomycin C (2 mg/mL), prepared as described above, were placed onto the middle of the inoculated plates. After 14h of incubation, the bacterial growth inhibition zone was observed.

The plates were photographed (ChemiDoc XRS + system, Bio-Rad) and the diameter of the cell growth inhibition was measured using ImageJ software (National Institutes of Health). The images shown in the figure are representative of the entire image set.

Chemoreceptor clustering assay

S. enterica ΔcheR strains carrying the pUA1127 vector containing the *eYFP::cheR* fusion under the control of a IPTG-inducible promoter (P*tac*) were used (S1 Table) to visualize the polar, round, diffraction-limited spots previously referred to as polar clusters [53]. The eYFP::CheR fusion served as a polar cluster localization reporter, as described previously [27,29,32,53]. In these strains, the *cheR* gene was removed to better visualize the chemoreceptor arrays by avoiding the presence of native CheR protein. Clustering experiments were performed as described previously [32], except that in this work the corresponding strains were grown, depending on the experiment, on LB-swarming plates or in liquid medium. In the former, samples were taken as described previously [54]. Briefly, the cells were grown on LB-swarming plates supplemented with ampicillin, 25 μM IPTG and, when needed, 0.08 or 10 μg mitomycin C/mL or 0.06 μg ciprofloxacin C/mL (final concentration). After 14 h of incubation at 37°C, the cells were suspended in 1 mL of ice-cold tethering buffer (10 mM potassium-phosphate pH 7, 67 mM NaCl, 10 mM Na-lactate, 0.1 mM EDTA, and 0.001 mM L-methionine) by gently tilting the plates back and forth and harvested by 15 min of low-speed centrifugation (5000 g). With this method, migrating cells were easily lifted off the surface, whereas the vast majority of cells in the middle of the plates remained intact on the surface. Non-swarming colonies were recovered using the same method but with 0.5 mL of cold tethering buffer.

For cells grown in liquid medium, overnight cultures of the corresponding *S. enterica* strains were grown under constant agitation at 30°C in tryptone broth (TB) supplemented with ampicillin and 25 μM IPTG. One day later, the cultures were diluted 1:100 in TB without antibiotics but with the addition of 25 μM IPTG to maintain the induction of the *eYFP::cheR* fusion construct. The cultures were incubated at 30°C until an OD_{600} of 0.08–0.1 was reached. Mitomycin C was then added to the corresponding culture to achieve a final concentration of 0.08 μg/mL or 10 μg/mL. The samples were collected at the indicated times and the cells were harvested by low-speed centrifugation for 15 min. For reversibility studies, cultures treated for 300 min were harvested by centrifugation. The supernatant was discarded and the cells were resuspended in

TB with or without the SOS inducer and incubated at 30°C. Samples were collected at the indicated times and the cells were harvested by low-speed centrifugation for 15 min.

In all experiments, the harvested cells were washed once using ice-cold tethering buffer, resuspended in 20–100 μL of the same buffer, and maintained on ice until they were applied onto thin 1% agarose pads as described previously [32].

Fluorescence microscopy was performed using a Zeiss AxioImager M2 microscope (Carl Zeiss Microscopy) equipped with a Zeiss AxioCam MRm monochrome camera (Carl Zeiss Microscopy) and a filter set for eYFP (excitation BP500/25; beam splitter FT 515; emission BP535/30). Cell fields were photographed and at least 500 cells were visually inspected to determine the presence and type of clusters in each sample. All images were acquired under identical conditions. Each experiment was performed at least in triplicate using independent cultures; a minimum of 1500 cells from each studied strain of *S. enterica* were therefore analyzed. The images presented in the figures are representative of the entire image set. ImageJ software (National Institutes of Health) was used to quantify the number of clusters and to prepare images for publication.

ELISA for CheW and RecA quantification

Samples for the ELISA were obtained either by recovering the cells directly from the colony edge of the corresponding LB-swarming plates, following the same procedure as described above, or by sampling the culture at the same time that it was used in a polar cluster assay. In both cases, cells were resuspended in sonication buffer (PBS 1×, cOmplete mini EDTA-free tablets, pH 7.3) and whole-cell lysates were obtained by sonication (2 30-s pulses and 20% amplitude, Digital sonifierR 450, Branson). After centrifugation (12000 g for 10 min), the supernatants were recovered and the total protein concentration of each sample was quantified according to the Bradford method using the protein reagent DyeR (BioRad) and a bovine serum albumin standard curve (range: 1.5–200 μg/mL).

The RecA and CheW::FLAG proteins used in the standard quantification curves were cloned in overexpression vectors, purified using *E. coli* BL21 (DE3) strain pLysS, and overexpressed by the addition of IPTG to the cultures. The *recA* gene was cloned in the pGEX-4T-1 vector, which includes a GST-tag (pUA1125), and purified by glutathione affinity chromatography using Sepharose 4BR resin (GE Healthcare) following the manufacturer's instructions. The *cheW::FLAG* gene was inserted into the pET15b overexpression vector (Novagene); the protein products were purified using the Talon kit (Clontech). The RecA and CheW::FLAG proteins were eluted by the addition of 20 μL of a solution containing 1 U thrombin/μL. The final concentrations of the two proteins were quantified using the Bradford method as described above.

RecA and CheW::FLAG proteins were quantified by ELISA as described [55]. Pre-treated 96-well microtiter plates (Nunc-Immunoplate F96 Maxisorp, Nunc) were coated with serial dilutions of the whole-cell lysates. Purified RecA and CheW::FLAG proteins were used for the standard quantification curve, and lysates from a *S. enterica ΔrecA* strain [55] and *S. enterica* wild-type as background controls for RecA and CheW::FLAG quantifications, respectively. These controls were necessary to correct for possible unspecific binding of the antibodies to other cellular components of the lysates. Anti-RecA (monoclonal antibody to ARM193 RecA clone, MBL) and anti-FLAG (monoclonal antibody to DYKDDDDK epitope Tag, Acris) mouse IgG antibodies were used in RecA and CheW::FLAG quantification. The secondary antibody was an anti-mouse-IgG horseradish-peroxidase-conjugated antibody (polyclonal antibody to mouse IgG (HEL)-HRP, Acris). The BD OptEIA TMB substrate reagent set (BD

Biosciences), prepared following the manufacturer's instructions, was used as the developing solution. Plate measurements were made at 650 nm using a multiplate reader (Sunrise, Tecan).

Statistical analysis

The results of the chemoreceptor clustering assay were statistically evaluated using a one-way analysis of variance (ANOVA) with Prism (GraphPad), as previously described [32,56,57]. The analyses were followed by the Bonferroni multiple comparison post-hoc test. A p value <0.05 was considered to indicate statistical significance. In all cases, the error bars in the figures indicate the standard deviation.

Results

S. enterica swarming ability and SOS system induction

The effects of the SOS system inducer mitomycin C on the swarming behavior of the wild-type strain and on four different SOS-network-mutants were analyzed. In the absence of mitomycin C, the wild-type, *lexA3*(Ind$^-$) (containing a non-hydrolyzable LexA repressor [58]) and *ΔsulA* (lacking the SOS-associated cell division inhibitor SulA [59]) strains were able to swarm (Fig 1). The *recAo* (carrying a point mutation in its LexA operator resulting in the constitutive expression of *recA* [60]) and *recAo lexA3*(Ind$^-$) mutants had a non-swarming phenotype either in the absence or presence of a sublethal concentration of mitomycin C (Fig 1). Swarming ability was also inhibited by the presence of mitomycin C in wild-type and *ΔsulA* strains but not in the *lexA3*(Ind$^-$) mutant (Fig 1). These results clearly indicated that SOS response activation impairs swarming ability via a SulA-independent pathway, since in the presence of mitomycin C neither the wild-type and nor the *ΔsulA* strain swarmed. Moreover, the absence of swarming motility in the wild-type, *ΔsulA*, *recAo*, and *recAo lexA3*(Ind$^-$) strains cultured in the presence of mitomycin C was not due to any substantial decrease in cell viability since swarming was exhibited by the *lexA3*(Ind$^-$) mutant cultured under the same conditions (Fig 1). Finally, the *recAo lexA3*(Ind$^-$) strain (which is incapable of SOS response induction but expresses high levels of RecA) is unable to swarm, either in the absence or presence of mitomycin C indicating that the activation of RecA is not necessary for swarming inhibition. Taken together, these results show that among all the cellular-associated phenomena that make up the SOS response, only the amplification of RecA impairs swarming motility.

Effect of SOS induction on chemoreceptor polar cluster assembly

The increase in RecA mediated by the SOS response generates the same non-swarming phenotype (Fig 1) exhibited by CheW-overexpressing strains [29]. High levels of CheW interfere with the assembly of trimers of chemoreceptor dimers, which prevents the formation of the polar chemoreceptor clusters by cells growing in liquid medium [27]. We therefore asked whether the increase in intracellular RecA levels that occurs during SOS system induction gives rise to the same defect in chemoreceptor polar cluster formation in swarming cells. To examine this possibility, we constructed *ΔcheR* mutant derivatives of the wild-type, *ΔsulA*, *recAo*, *lexA3* (Ind$^-$), and *recAo lexA3*(Ind$^-$) strains carrying the pUA1127 plasmid containing an *eYFP*::*cheR* fusion (S1 Table) and then analyzed the dynamics of chemoreceptor polar cluster assembly in swarmer cells in the presence of mitomycin C. The eYFP::CheR fusion was previously used as a reporter for polar cluster localization [27,29,32,53]. Note that neither *cheR* deletion nor the presence of the pUA1127 plasmid affected the swarming phenotype of the parental strains shown in Fig 1 (data not shown).

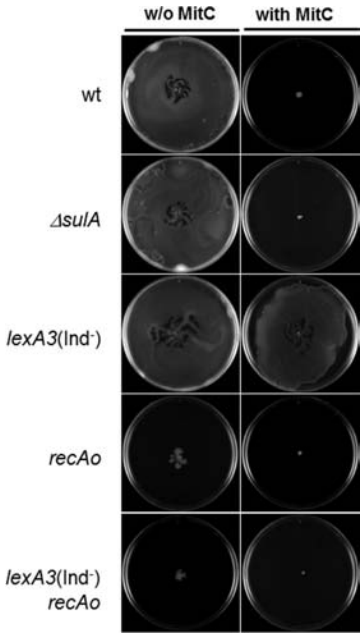

Fig 1. Swarming ability of the *S. enterica* wild-type and the *ΔsulA*, *recAo*, *lexA3*(Ind⁻), and *lexA3*(Ind⁻) *recAo* mutant derivatives in the absence or presence of mitomycin C. Representative images of a bacterial colony swarming on a semisolid agar surface following incubation of the culture for 14 h at 37°C. When indicated, 0.08 μg mitomycin C/mL was added to the semisolid agar plates.

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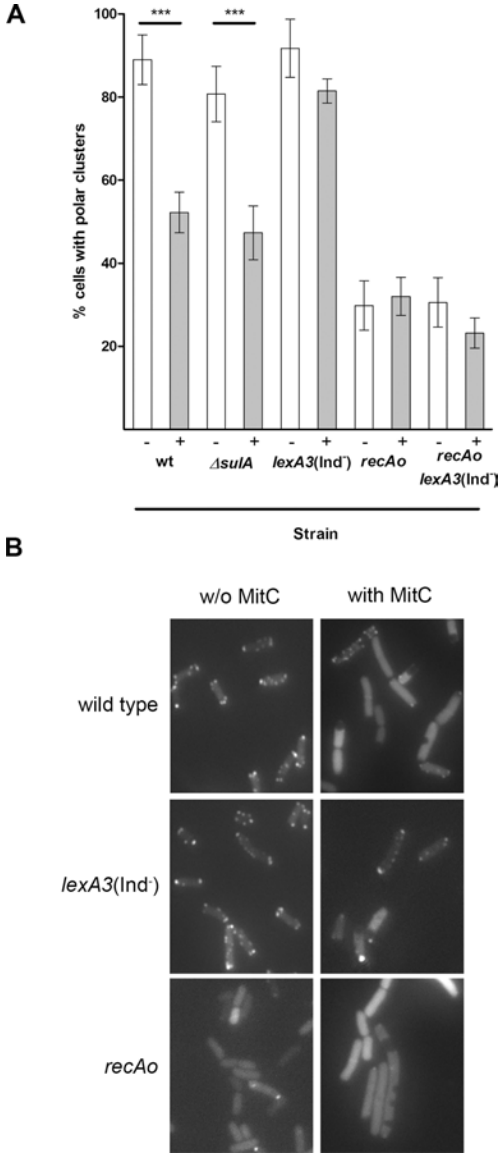
The percentage of polar-cluster-containing cells growing on swarming plates with or without mitomycin C is shown in Fig 2. Polar clusters formed in >90% of wild-type cells grown in the absence of mitomycin C but in only about 50% of the cells grown in the presence of the SOS inducer. The same results were obtained in the *ΔsulA* strain. In the *lexA3(Ind⁻)* strain, either in the absence or presence of mitomycin C, the percentage of cells with polar clusters was almost 90, i.e., the same as in wild-type cells growing without mitomycin C. In the *recAo* and *recAo lexA3*(Ind⁻) strains only about 30% of the cells contained polar clusters, regardless of the presence or absence of mitomycin C (Fig 2). Thus, the inability of the cells to form polar clusters correlated with the non-swarming phenotype (Fig 1). The same association was reported in studies associated to *cheW* and *Δtol pal E. coli* mutants [27,29].

Temporal evolution of polar chemoreceptor cluster assembly during SOS response induction

To further understand the changes in polar chemoreceptor cluster assembly originated by mitomycin C treatment, we evaluated the percentage of polar-cluster-containing cells as well as RecA protein concentrations during SOS system induction. In addition, since CheW overexpression gives rise to a decrease in polar arrays [27], we measured the CheW concentration in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wild type
lexA3(Ind-)
recAo

Fig 2. A) Percentage of cells of *S. enterica ΔcheR* harboring plasmid pUA1127 (wild type) and of *ΔsulA*, *recAo*, *lexA3*(Ind⁻) or *lexA3*(Ind⁻) *recAo* mutant derivatives that developed polar clusters while growing on swarming plates in the absence (-) or presence (+) of mitomycin C. The cells were harvested from the edge of the swarming colony growing on soft agar plates. When indicated, 0.08 μg mitomycin C/mL was added to the plates. The results are the mean of at least four independent imaging studies. Error bars represent the standard deviation. ***$p<0.001$ as determined by a one-way ANOVA with a Bonferroni correction. **B)** Representative fluorescence microscopy images of cells from wild-type, *lexA3*(Ind⁻), and *recAo* strains grown in the presence or absence mitomycin C.

doi:10.1371/journal.pone.0146685.g002

mitomycin-C-treated cells, although the *cheW* promoter does not contain a LexA operator [61,62]. As there are no commercial antibodies against CheW, a FLAG-tag was added to the *cheW* gene of the *S. enterica ΔcheR* harboring the eYFP::CheR fusion (pUA1127). The FLAG-tag did not change the swarming phenotype of this strain (data not shown) but it did allow CheW quantification during SOS induction.

Liquid cultures of *S. enterica ΔcheR cheW::FLAG*/pUA1127 were treated with two different mitomycin C concentrations. Polar cluster assembly and the concentrations of RecA and CheW during SOS response induction are shown in Fig 3 and Fig 4, respectively.

The kinetic assay of polar cluster assembly clearly indicated that the formation of chemoreceptor polar clusters was stable during bacterial population growth (Fig 3). Over time, in the absence of SOS system induction, about 65% of the cells contained polar clusters. This percentage is in concordance with data previously reported for *E. coli* cultures growing exponentially in liquid medium [53].

It should be noted that in *S. enterica* a higher percentage of cells with polar clusters are present when cells are growing on plates rather than those cultured in liquid medium (90% and 65%, respectively; Fig 2 and Fig 3). This may be due to the fact that, in plate cultures, the sampled cells were actively moving over the surface since they were those at the edge of the colony. In liquid cultures, the addition of mitomycin C caused a dose-dependent reduction in the number of cells containing chemoreceptor polar clusters (Fig 3). Specifically, after 300 min of treatment with 0.08 μg mitomycin C/mL, only about 45% of the cells contained polar clusters. This percentage continued to decrease for the next 2 h and then stabilized such that polar clusters were seen in only about 35% of the cells. In liquid cultures containing 10 μg mitomycin C/mL, the decrease in polar clusters occurred abruptly, with the minimum of 35% reached as early as after 60 min of incubation (Fig 3). Similar results were obtained when cells were treated with ciprofloxacin (Fig 3), another well-known SOS inducer [63], indicating that the polar clustering decrease is due to SOS activation and not specifically to mitomycin C treatment. Furthermore, all these results are also in agreement with the data obtained for cells swarming on plates (Fig 2). The similar decreases in polar clusters prompted by SOS response activation in both solid and liquid cultures suggests that mitomycin C reduces by half the number of polar-cluster-containing cells.

The absence of a mitomycin-C-induced variation in CheW over time was confirmed by ELISA (Fig 4). The amount of CheW in mitomycin-C-treated cells was similar to that in non-treated cells [mean = 1.17 (±0.17) × 10^{11} molecules CheW/μg total protein], which is in agreement with previous concentrations reported for *E. coli* [64]. Furthermore, the RecA concentration in non-SOS induced cells was nearly the same as that of CheW [mean = 1.16 (±0.21) × 10^{11} molecules RecA/μg total protein] and similar to that previously reported[43]. However, in the mitomycin-C-treated cultures, the RecA concentration rose quickly, increasing by about 25-fold after 300 min of treatment (Fig 2).

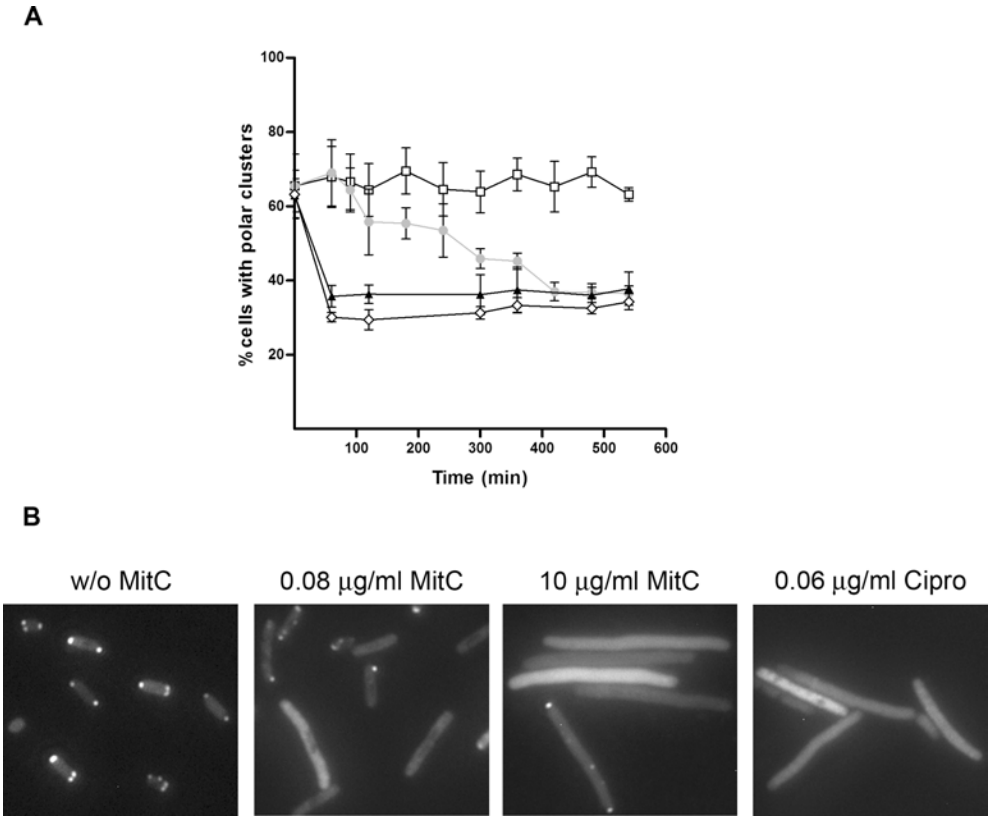

Fig 3. A) Evolution of the percentage of cells that developed polar chemoreceptor clusters during induction of the SOS system in a culture growing in liquid medium. The percentage of *S. enterica ΔcheR* cells harboring plasmid pUA1127, containing the inducible *eYFP::cheR* fusion, was quantified at several time points after the addition of either 0.08 or 10 µg mitomycin C/mL or 0.06 µg ciprofloxacin/mL concentration (● or ▲, respectively). Non-treated cells served as the control (□). The results are the mean of at least three independent imaging experiments. Error bars represent the standard deviation. B) Representative fluorescence microscopy images of cells treated for 300 min with either 0.08 or 10 µg mitomycin C/mL or 0.06 µg ciprofloxacin/mL. A control cell not treated with mitomycin C is shown as well.

doi:10.1371/journal.pone.0146685.g003

Disconnection of the SOS response allows the recovery of cluster assembly

After determining that SOS response activation impairs polar cluster assembly, we analyzed the dynamics of clustering subsequent to the removal of mitomycin C, and therefore the cessation of DNA injury, from the cultures.

After treatment with either 0.08 or 10 µg mitomycin C/mL for 300 min, bacterial liquid cultures were centrifuged and resuspended in fresh medium no longer containing the SOS inducer. As shown in Fig 5, the percentage of cells with polar clusters progressively increased

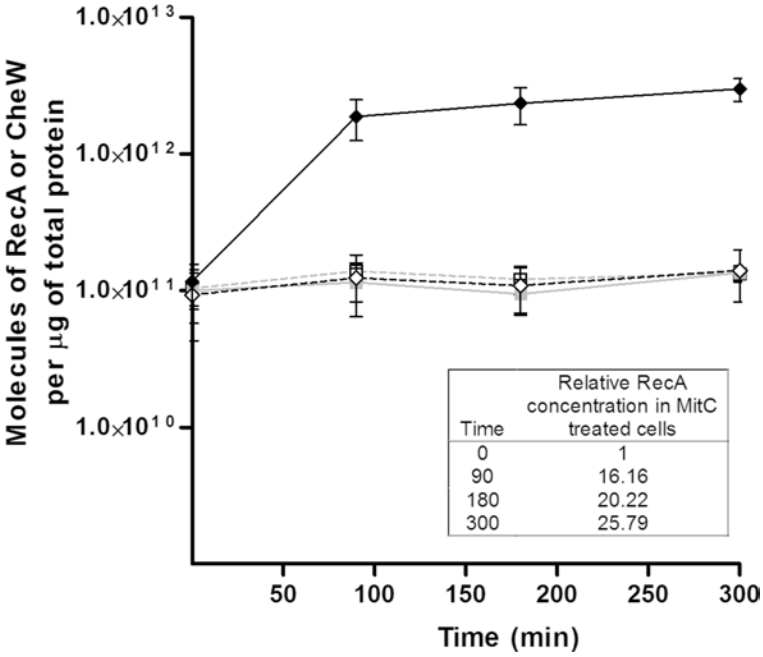

Fig 4. Concentration of RecA and CheW proteins in *S. enterica* mitomycin-C-treated cells growing in liquid medium. ELISA quantification of RecA (♦, continuous line) and CheW (■, continuous line) proteins of *S. enterica ΔcheR* cells harboring plasmid pUA1127 (eYFP::*cheR*) and treated with mitomycin C (0.08 μg/mL). The amounts of RecA (◇, discontinuous line) and CheW (□, discontinuous line) in a non-treated culture are also shown. The concentration is expressed as the number of RecA or CheW molecules per μg of total protein. The results are the mean of three independent experiments. Error bars represent the standard deviation. The relative RecA concentration (boxed) was calculated as the mean RecA concentration at each time point with respect to the mean initial RecA concentration [1.16 (±0.17) x 10^{11} molecules per μg of total protein].

doi:10.1371/journal.pone.0146685.g004

within 180 min after mitomycin C removal and after 240 min was close to the percentage in non-SOS-induced cultures regardless of the initial mitomycin C dose (Fig 5). Conversely, when mitomycin C treatment was maintained, the percentage of polar-cluster-containing cells remained at about 35% (Fig 5). At the same time, the concentration of RecA protein decreased and, like the percentage of cells containing polar clusters, gradually, returned to the basal level (Fig 5) by 300 min after mitomycin C removal (Fig 6). By contrast, mitomycin C removal had no effect on the CheW concentration, which remained the same as in non-treated cells (Fig 6). Thus, according to these observations, once SOS response activation ceases and basal RecA levels are reestablished, polar cluster assembly is restored, which implies that the SOS-mediated inhibitory effect is reversible.

Impact of changes in the RecA/CheW balance

The above-reported results also implied a relationship between the intracellular RecA concentration and polar array assembly and, thereby, an effect on swarming motility. Accordingly, swarming of the *S. enterica lexA3*(Ind$^-$) mutant was not affected by the presence of mitomycin

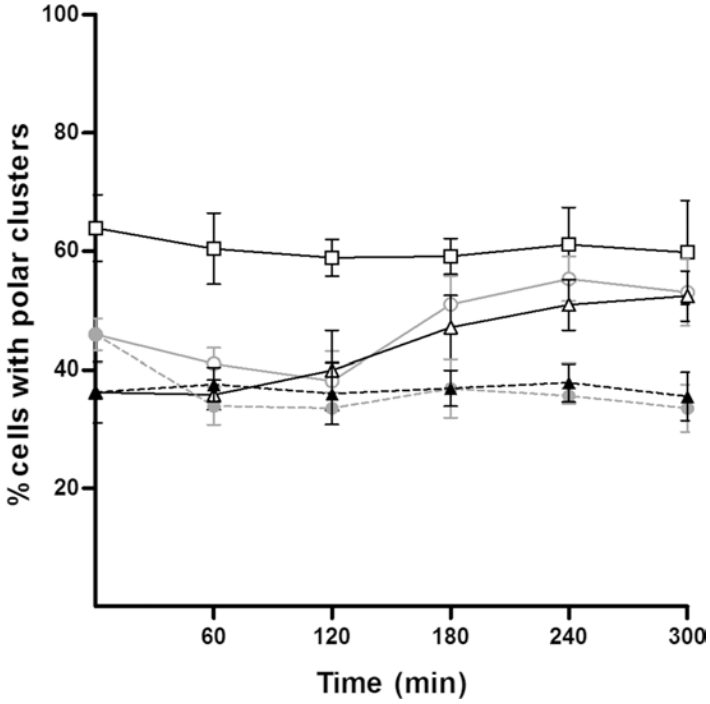

Fig 5. Evolution of the percentage of *S. enterica* cells that developed polar chemoreceptor clusters after cessation of SOS response induction in a culture growing in liquid medium. Cultures treated for 300 min with either 0.08 (○) or 10 (△) μg mitomycin C/mL were centrifuged to remove the inducer. Samples were periodically taken thereafter and the presence of polar clusters was determined. As controls, a non-treated culture (□) and two cultures treated again after centrifugation with either 0.08 (●, discontinuous line) or 10 (▲, discontinuous line) μg mitomycin C/mL are also shown. The results are the mean of three independent imaging experiments. Error bars represent the standard deviation.

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C (Fig 1). Because an increase in CheW levels was previously shown to restore the swarming ability of a *recAo* strain [43], we analyzed the swarming behavior of *S. enterica* strains expressing different concentrations of either RecA or CheW proteins.

The *recA* gene was cloned into the pUA1108 overexpression vector, yielding the plasmid pUA1130, in which *recA* is under the control of an IPTG-inducible promoter (S1 Table). After pUA1130 was introduced into the *S. enterica cheW::FLAG* strain, both the swarming ability (expressed as the RSMI) [52] and the concentrations of RecA and CheW were determined in the presence and absence of IPTG. In these experiments, the samples were consistently taken from the edge of the plates (Fig A in S1 Fig, Table 1). As a control, the same strain but carrying the pUA1108 vector was studied under the same conditions (Fig A in S1 Fig, Table 1).

The presence of IPTG had no effect on the intracellular concentrations of RecA and CheW in *S. enterica* (pUA1108) cells (Table 1); rather, the concentrations [2.03 (±0.60) × 10^{10} and

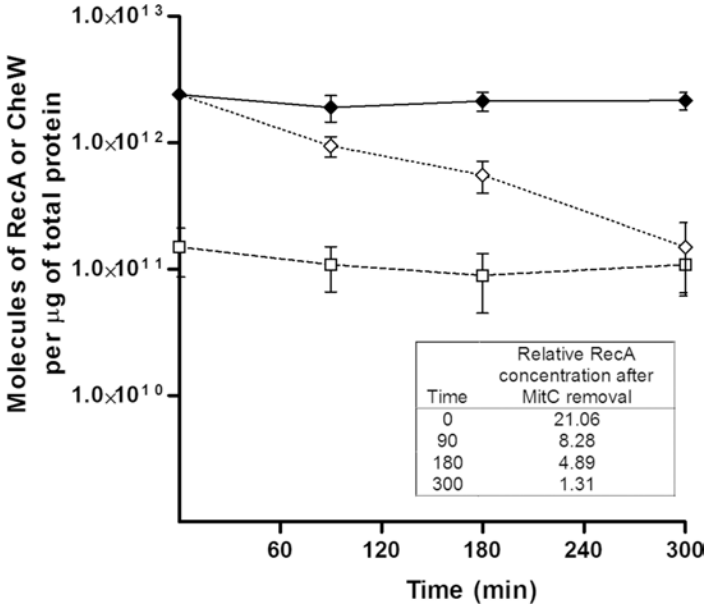

Fig 6. Concentration of RecA protein, as measured by ELISA, in *S. enterica ΔcheR* cells harboring plasmid pUA1127 (eYFP::*cheR*) and growing in liquid medium. Samples were taken periodically after the removal of mitomycin C from cultures pre-treated with 0.08 μg mitomycin C/mL for 300 min (◇, discontinuous dotted line). The amount of RecA of a non-treated culture (□, discontinuous line) and a continuously treated culture (◆, continuous line) served as controls. The RecA concentration is expressed as the number of RecA molecules per μg of total protein. The results are the mean of three independent experiments. Error bars represent the standard deviation. The relative RecA concentration after mitomycin C removal (boxed) was calculated as the mean RecA concentration at each time point with respect to the mean RecA concentration of the non-treated culture [1.13 (±0.25) x 10^{11} molecules per μg of total protein].

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2.15 (±0.21) × 10^{10} molecules of RecA and CheW/μg total protein, respectively] were proportional to those in non-treated cells grown in liquid culture (Fig 4). Nevertheless, the CheW and RecA concentrations were about 5-fold higher in cells growing in liquid cultures than on swarming plates. This is in agreement with previous reported data which described that the concentration of chemotaxis pathway proteins increases when cells are grown in nutrient-poor medium such as TB [65] herein used for visualizing chemoreceptor clusters [27]. Nevertheless this medium is not suitable for swarming assays that must be performed on LB-swarming plates [66].

The amount of RecA in cells carrying the *recA*-overexpressing plasmid (pUA1130) increased nearly 5-fold even in the absence of IPTG, as was expected because of the higher gene dosage. Nevertheless, this increase did not affect the swarming ability of these cells and the RSMI remained close to 1 (Table 1, S1 Fig). Only when *recA* expression was induced by IPTG, such that the RecA protein increased by about 8-fold with respect to the control strain, was swarming ability impaired. Moreover, swarming was totally abolished when the RecA concentration increased by >15-fold (Table 1, S1 Fig). These results are in complete agreement

Table 1. Relationship between the RecA and CheW concentrations and the swarming ability of several *S. enterica* strains.

S. enterica strain	Overexpressed gene	IPTG (μM) treatment	RecA concentration (molecules/μg of total protein)[a]	CheW concentration (molecules/μg of total protein) [a]	[RecA]/[CheW] ratio [b]	Swarming phenotype[c]	RSMI[d]
		0					
		10					
cheW::FLAG/pUA1108[e]	none	20	2.03 (±0.60)x10^{10}	2.15 (±0.21)x10^{10}	0.94	++	1.00 (±0.08)
		30					
		40					
		50					
		0	9.86 (±0.26) x 10^{10}		4.56	++	0.94 (±0.05)
cheW::FLAG/pUA1130 (P*tac*::*recA*)	*recA*	10	1.65 (±0.13) x 10^{11}	2.16 (±0.41)x10^{10}	7.64	+	0.44 (±0.08)
		20	3.39 (±0.34) x 10^{11}		15.70	-	0.23 (±0.01)
		30	4.59 (±0.17) x 10^{11}		21.25	-	0.16 (±0.03)
		0					
		10					
recAo cheW::FLAG/pUA1108[e]	*recA*	20	3.01 (±0.53)x10^{12}	2.07 (±0.49)x10^{10}	145.4	-	0.16 (±0.02)
		30					
		40					
		50					
		0		4.09 (±0.58) x 10^{11}	7.80	-	0.14 (±0.01)
		10		1.73 (±0.20) x 10^{12}	1.84	++	0.73 (±0.02)
recAo cheW::FLAG/pUA113 (P*tac*::*cheW*)	*recA* and *cheW*	20	3.19 (±0.26)x10^{12}	3.48 (±0.67) x 10^{12}	0.91	++	1.07 (±0.01)
		30		6.01 (±0.85) x 10^{12}	0.53	++	0.77 (±0.07)
		40		1.11 (±0.16) x 10^{13}	0.28	-	0.31 (±0.14)
		50		1.21 (±0.11) x 10^{13}	0.26	-	0.12 (±0.07)

[a] The mean basal concentration of a given protein measured in at least three independent experiments is indicated in those cases in which its synthesis is not under IPTG control. The standard deviation is indicated in parentheses.

[b]The [RecA]/[CheW] ratio was calculated as the ratio of their respective concentrations at the indicated time point. When there was no difference in the protein concentration, the ratios were calculated using the mean values.

[c](++) wild-type swarming ability. (+) reduced swarming ability, (-) no swarming ability.

[d] The relative swarming colony motility index was calculated as the ratio between the colony diameter of the studied strain and that of the control strain under the same experimental conditions. The mean of at least three independent experiments is shown. The standard deviation is indicated in parentheses.

[e] Expression vector that does not contain a gene fusion construct.

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with those of the kinetic cluster assembly experiments (Fig 3 and Fig 4), in which a decrease in polar-cluster-containing cells was induced by increases in RecA concentrations up to 20-fold

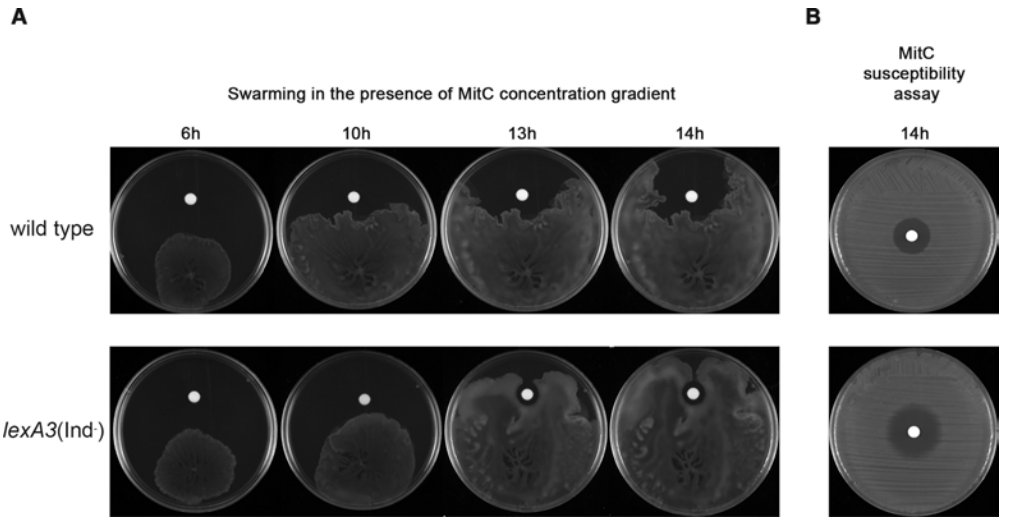

Fig 7. A) Swarming ability of either wild-type or *lexA3*(Ind⁻) strains in the presence of a mitomycin C concentration gradient. Swarming plates with a disk soaked with mitomycin C solution (2 mg/mL) were inoculated with the corresponding strain. Colony growth was followed by imaging the same representative swarming plate 6, 10, 13, and 14 h after plate inoculation. B) The susceptibility to mitomycin C of each strain was also evaluated. It must be noted that, as indicated in Material and Methods section, the swarming plates were point inoculated with the corresponding bacterial strain using a sterile toothpick while the susceptibility assays were carried out applying the bacterial inoculum using a sterile swab all over the plate surface.

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with respect to non-SOS-inducing conditions. They are also in concordance with the above-described results (Fig 5 and Fig 6) that once SOS response activation ceases, polar cluster assembly resumes when the RecA concentration is only about 5-fold higher than that of non-treated cells.

The data presented in Table 1 showed that the *recAo* strain carrying the *cheW* overexpression vector (pUA1131), in which RecA protein expression is 150-fold higher than that of the wild-type strain, is only able to swarm when the IPTG-mediated induction of CheW results in an increase of the protein to levels 80- to 280- fold with respect to wild type). The obtained results show that swarming ability is restored in the *recAo* strain when the RecA:CheW ratio is between 2 and 0.5; in all other cases, swarming is impaired (Table 1).

Effect of a DNA-damaging compound gradient on swarming motility

During surface colonization driven by swarming motility, bacterial colonies may encounter DNA-damaging compounds, which would be present along a concentration gradient generated by their surface diffusion. To evaluate swarming behavior under these conditions, swarming assays were conducted in the presence of a mitomycin C gradient.

Mitomycin C mediated-swarming inhibition was clearly observed by the wild type strain (Fig 7A). In fact, the swarming edge of wild type cells closest to the mitomycin-C-containing disk stopped but at other colony edges it proceeded, allowing colonization of the rest of the plate surface, where the mitomycin C concentration was low enough to be harmless. Nevertheless, no mitomycin C mediated-swarming inhibition was detected by the *lexA3*(Ind⁻) mutant

(Fig 7A), which is unable to activate the SOS response. As expected, swarming was not affected when the disks were instead soaked in sterile water (data not shown). Since the sensitivity to mitomycin C (measured through determination of inhibitory growth halos) is higher in *lexA3* (Ind^-) than in wild type cells (Fig 7B) the different behavior between these two strains in swarming plates (Fig 7A) must be attributed to the interference of the RecA protein increase upon this social motility during the SOS response induction. Further, the inhibition effect upon the *lexA3*(Ind^-) strain growth around the mitomycin C disk while swarming (Fig 7A) was smaller than that generated for the same cells in the mitomycin C susceptibility assay (Fig 7B). This is in concordance with previous results in which high cell density and mobility diminishes the antibiotic effect against swarming bacteria [2]. All of these results indicate that the impairment of swarming by induction of the SOS system prevents the exposure of the cells to a lethal concentration of mitomycin C.

Discussion

Activation of the SOS response in bacterial species such as *E. coli* and *S. enterica* prompts not only the error prone DNA repair pathway but also other cellular processes, such as the prevention of DNA degradation [67], the transitory inhibition of cell division [68] and respiration and sugar-related metabolic changes [69,70]. Some of these effects depend on the expression of specific chromosomal genes (e.g., the *sulA*-mediated inhibition of division [68]) whereas the basis of others is still unknown (cessation of cell respiration and the catabolism of sugars [69,70]). Other processes are the indirect consequence of SOS system induction. This is the case for the amplification of *recA* gene transcription, as the increased levels of RecA protein are able to bind to injured DNA blocking the access of DNAses such as RecBC [67]. This response together with the other functions of the SOS system contribute, both directly and indirectly, to ensuring the survival of bacteria populations in harmful environments.

The results reported herein show that the inhibition of bacterial colony motility over surfaces should be added to the pool of indirect phenomena associated with the SOS response. Our results demonstrate that, in *S. enterica*, SOS response activation impairs both chemoreceptor polar cluster assembly and consequently the swarming ability. These effects are due to the increase in the RecA concentration following SOS induction but not to other SOS-response-associated functions. The decrease of polar chemoreceptor clustering was observed in both the *recAo* and the *recAo lexA3*(Ind^-) mutants whether in the absence or presence of SOS inducer (Fig 1 and Fig 2). The same results were obtained when RecA amplification was mediated by IPTG in a *S. enterica* strain carrying a P*tac recA* gene fusion (Table 1, S1 Fig). Swarming ability was totally abolished only when the RecA concentration increased, whether in response to DNA damage or IPTG addition indicating that no RecA activation is necessary for swarming inhibition (Fig 1 and Table 1).

Although CheW concentration does not change during SOS response induction (Figs 4 and 6), our data demonstrated a role for this protein in the RecA promoted modulation of swarming. The RecA and CheW proteins were present at similar concentrations in cells growing under non-SOS-inducing conditions. An increase in CheW levels restored the swarming ability of the *recAo* strain only if the levels of this protein were balanced with those of RecA (Table 1, S1 Fig), consistent with the interaction between RecA and CheW [32]. The RecA protein participates in multiple cellular functions, as DNA recombinase, SOS activation, and co-protease [36,71,72]. Thus, RecA interacts not only with other proteins but also with DNA. For this reason, ELISA results do not indicate the proportion of RecA that actually interacts with CheW *in vivo*. Studies on the specific stoichiometry of RecA and CheW would also shed light on the exact role of RecA protein in swarming control. For example, it could be that an increase in

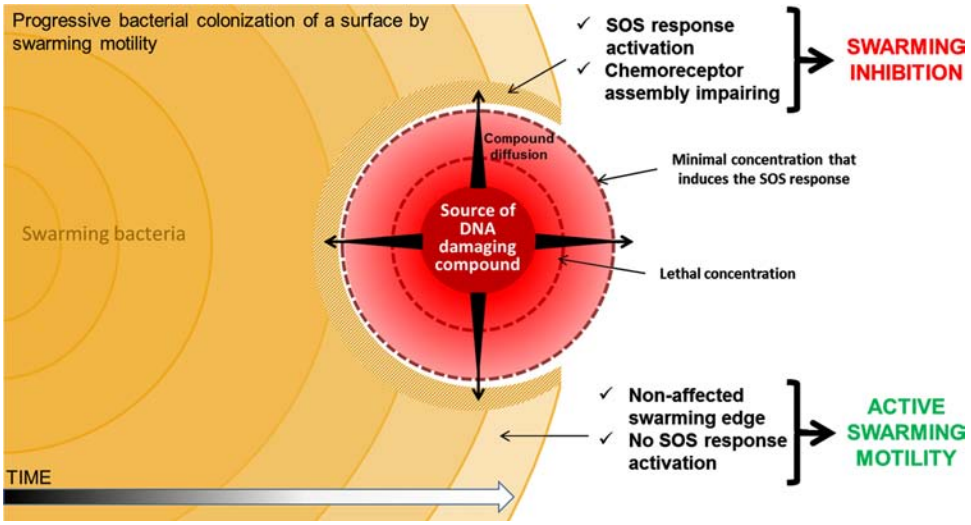

Fig 8. Proposed model for the control of swarming motility by the SOS response during bacterial surface colonization in the presence of a DNA damaging compound.

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RecA prompts the titration of CheW, thus preventing chemoreceptor assembly and therefore also polar cluster array formation. However, this scenario is unlikely since *recA* defective mutants are also unable to swarm[32]. Another possibility is that RecA is a component of the chemoreceptor cluster such that, as described for CheW [27], high levels of the RecA protein interfere directly with chemoreceptor assembly. Further work is required to discern between these possibilities.

Another relevant aspect of the present work is the demonstration of the reversibility of the SOS response effect on polar cluster assembly (Fig 5). Specifically, under the conditions tested in this study, the normal percentage of polar-cluster-containing cells was reestablished 300 min after mitomycin C removal from the liquid cultures (Fig 5), as the amount of RecA decreased to its basal level because of the removal of this DNA damaging agent (Fig 6). Taking into account that the turnover time of RecA is about 15 min [73], the 300 min needed to reestablish the polar clusters and return both the SOS system and RecA to their basal levels probably reflected residual DNA damage, which continued to induce the SOS response until repair was completed (Fig 6).

The reversibility of polar array assembly (Fig 5) and the swarming behavior in response to a concentration gradient of a SOS inducer (Fig 7A) are crucial aspects underlying the biological significance of the SOS response modulating swarming motility. Swarming control by the SOS response is summarized in Fig 8. The RecA protein, as the DNA-damage sensor, detects DNA injuries generated by the presence of SOS inducer compounds activating SOS response. Bacterial cells growing on surfaces will likely be exposed to a wide range of compounds, of either biological or chemical origin. By secreting toxic compounds such as antibiotics and bacteriocins, which diffuse though the growth surface, swarming bacterial colonies can impact neighboring

cells of other species. However, once the SOS response is activated, the RecA concentration rises up quickly since *recA* is one of the first genes to be induced in the hierarchy of SOS activation [74–76]. This increase disturbs the equilibrium between this protein and CheW, which causes the cessation of swarming. In fact, when the bacterial colony edge is exposed to SOS-inducer, the swarming ability is impaired thus avoiding the exposure to higher concentrations of the injurious, and potentially lethal, compound. At the same time, the non-exposed edges of the colony continue to swarm and thus colonize those parts of the surface that are SOS inducer-free or contain a lower, non-injurious concentration of the DNA-damaging compound (Figs 7A and 8). In the case that the DNA-damaging source decrease or disappears, the repair of the DNA damage would restore polar cluster assembly and therefore also the colony swarming ability.

The chemotaxis pathway normally includes several receptors that detect either repellent or attractant compounds; for instance in *E. coli* at least five specific receptors have been described [77]. In contrast, the swarming response against DNA-damaging compounds reported herein is driven by a single signal (the SOS-response-mediated increase in RecA) that responds to a broad range of DNA-damaging agents. This general response mechanism is an advantage for bacterial cells, as it limits the number of genes required to one (*recA*). Taken together, these results further demonstrate the ability of bacterial cells to adapt their surface motility in response to the presence of direct or indirect DNA-damaging agents, by sensing these compounds via SOS system induction.

Supporting Information

S1 Fig. A) Swarming ability of the *S. enterica cheW*::FLAG strain harboring the *recA* expression vector (pUA1130) in the presence of increasing concentration of IPTG (0, 10, 20 and 30 μM). Representative images of swarming plates supplemented with the corresponding IPTG concentration are shown. As a control, colony swarming patterns of *S. enterica cheW*::FLAG carrying the expression vector (pUA1108) is shown. B) Representative plate images showing the recovery of swarming ability by the *S. enterica recAo cheW*::FLAG strain carrying the *cheW* expression vector (pUA1131) following the addition of IPTG. The swarming phenotype of this strain carrying the expression vector (pUA1108) is shown as a control.
(TIF)

S1 Table. Bacterial strains and plasmids used in this work.
(DOCX)

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Author Contributions

Conceived and designed the experiments: SC JB. Performed the experiments: OI AM SC. Analyzed the data: OI SC JB. Contributed reagents/materials/analysis tools: JB. Wrote the paper: SC JB OI.

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3.3 RecA and CheW protein location during SOS Response (Article 3)

Molecular interaction and cellular location of RecA and CheW proteins in *Salmonella enterica* during SOS response and their implication in swarming

Irazoki O, Aranda J, Zimmermann T, Campoy S & Barbé J

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In addition to its role in DNA damage repair and recombination, the RecA protein, through its interaction with CheW, is involved in swarming motility. In order to better understand how SOS response modulates swarming, in this work the location of RecA and CheW proteins within the swarming cells has been studied by using super-resolution microscopy. Accordingly, the experiments performed herein have identified the specific RecA and CheW interfaces associated with the RecA-CheW interaction, which has also been confirmed by site-directed mutagenesis and immunoprecipitation techniques. Our results showed that the CheW distribution changes, from the cell poles to foci distributed in a helical pattern along the cell axis, when RecA protein is increased. Thus, when SOS response is activated, CheW presents the same subcellular location as that of RecA, consistent with the impairment of the chemoreceptor array assembly previously described and pointing out that RecA storage structures may also be modulators of swarming motility. Data reported herein not only confirmed that the RecA-CheW pair is essential for swarming motility but also its direct involvement in CheW distribution associated with SOS response activation. A model explaining the mechanism by which DNA damage modulates swarming and how RecA protein affects this motility is proposed.

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Molecular Interaction and Cellular Location of RecA and CheW Proteins in *Salmonella enterica* during SOS Response and Their Implication in Swarming

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In addition to its role in DNA damage repair and recombination, the RecA protein, through its interaction with CheW, is involved in swarming motility, a form of flagella-dependent movement across surfaces. In order to better understand how SOS response modulates swarming, in this work the location of RecA and CheW proteins within the swarming cells has been studied by using super-resolution microscopy. Further, and after *in silico* docking studies, the specific RecA and CheW regions associated with the RecA-CheW interaction have also been confirmed by site-directed mutagenesis and immunoprecipitation techniques. Our results point out that the CheW distribution changes, from the cell poles to foci distributed in a helical pattern along the cell axis when SOS response is activated or RecA protein is overexpressed. In this situation, the CheW presents the same subcellular location as that of RecA, pointing out that the previously described RecA storage structures may be modulators of swarming motility. Data reported herein not only confirmed that the RecA-CheW pair is essential for swarming motility but it is directly involved in the CheW distribution change associated to SOS response activation. A model explaining not only the mechanism by which DNA damage modulates swarming but also how both the lack and the excess of RecA protein impair this motility is proposed.

Keywords: SOS response, swarming, chemoreceptor polar arrays, chemosensory cluster assembly, RecA, CheW, 3D-STED

INTRODUCTION

RecA is a multifunctional protein present in almost all members of the Bacteria domain (Eisen, 1995). In the presence of single-stranded DNA (ssDNA), generated, for instance, by direct or indirect DNA damage, RecA becomes activated (RecA*) (Sassanfar and Roberts, 1990; Michel, 2005) acquiring co-protease activity that prompts auto-cleavage of the LexA repressor which governs the SOS response (Little, 1991). LexA cleavage triggers not only the expression of *recA* itself but also that of other SOS genes, mostly those involved in DNA recombination and repair (Courcelle et al., 2001). Further, it has been described that RecA is associated with the cell

membrane forming foci often located at the cell poles that are redistributed along the cell in response to DNA damage (Renzette et al., 2005; Lesterlin et al., 2014; Rajendram et al., 2015). RecA, however, aside from its role in DNA damage repair and as a DNA damage sensor, has been directly related to swarming motility (Gómez-Gómez et al., 2007; Medina-Ruiz et al., 2010), through its interaction with the CheW protein (Mayola et al., 2014; Irazoki et al., 2016).

Swarming motility is the rapid, organized multicellular translocation of bacteria across a moist surface. It is powered by rotating flagella (Henrichsen, 1972) and is widely distributed through the Bacteria Domain (Harshey, 1994). Swarming is associated with elevated resistance to multiple antibiotics (Ottemann and Miller, 1997; Kim and Surette, 2003; Kim et al., 2003; Wang et al., 2004; Overhage et al., 2008; Katribe et al., 2009) and is essential for bacterial colonization of host surfaces (Nakajima et al., 2008; Barak et al., 2009; Katribe et al., 2009). Like other components of the chemotaxis pathway, the CheW protein plays a key role in swarming ability (Burkart et al., 1998; Mariconda et al., 2006). As the chemoreceptor adaptor, CheW couples the transmembrane methyl-accepting chemoreceptor protein trimers of dimers (MCPs) to CheA, a histidine kinase that transfers the signal to the CheY response regulator, which acts on the flagellar motor by switching flagellar rotation according to the stimuli detected by the MCPs (Boukhvalova et al., 2002; Sourjik and Wingreen, 2012). To avoid saturation of the sensory system, the chemoreceptor signal is reset by the activity of a methyltransferase (CheR) and a methylesterase (CheB), both of which are located in the vicinity of the chemoreceptors and which restore pre-stimulus activity through reversible covalent modification of the MCPs (Sourjik and Wingreen, 2012).

These signaling complexes pack together to form large chemoreceptor arrays, ranging from a few to 1000s of proteins and located at the cell poles. By acting as antennae, they amplify the signal generated in response to slight changes in the concentrations of attractants or repellents detected by MCPs (Briegel et al., 2012, 2014a; Sourjik and Wingreen, 2012; Cassidy et al., 2015). The chemoreceptor array assembly has been the focus of several studies (Shiomi et al., 2006; Thiem and Sourjik, 2008; Jones and Armitage, 2015). The newly synthesized signaling complexes are distributed in a helical fashion at the cell membrane via their association with cytoskeletal proteins such as MreB or the Sec secretion system (Shiomi et al., 2006; Oh et al., 2014). Then, by stochastic self-assembly (Greenfield et al., 2009) or by an active process (Shiomi et al., 2006), these complexes form large clusters by joining existing arrays or by the formation of new nucleation centers. Stabilization of these clusters is a function of both the cell membrane curvature in the polar region (Oh et al., 2014) and the presence of CheA and CheW (Shiomi et al., 2005). These proteins are directly involved in the stabilization of these clusters, as they interact to form structural core linkers [CheW-CheA$_2$-CheW] across the cytoplasmic domain of MCPs, thereby clustering the chemoreceptors into hexagonal rings. The assembled array may thus contain dozens to 100s of hexagons (Briegel et al., 2014b; Cassidy et al., 2015). Within the hexagons is a CheW ring that couples neighboring chemoreceptors and strengthens the stability of the chemosensory array (Cassidy et al., 2015).

The presence of polar chemoreceptor arrays is essential for swarming motility in soft swarmers, such as *Escherichia coli* and *Salmonella enterica* (Cardozo et al., 2010; Santos et al., 2014). In the latter bacterium, an alteration in the balance of RecA/CheW impairs chemoreceptor cluster assembly and thus modulates bacterial swarming motility (Cardozo et al., 2010; Medina-Ruiz et al., 2010; Irazoki et al., 2016). The overexpression of RecA, without its activation, is sufficient to abolish swarming (Irazoki et al., 2016). Thus, by using RecA as a sensor mechanism, *S. enterica* cells can adapt their surface motility in response to the presence of direct or indirect DNA-damaging agents, by sensing these compounds through SOS system induction (Irazoki et al., 2016).

Although, RecA is known to interact with CheW (Mayola et al., 2014), where the interaction occurs within the cell and its nature are poorly understood. In an attempt to answer these questions and to better understand how the SOS response modulates swarming, in this work we have determined the regions involved in RecA and CheW interaction and the location of these proteins within SOS response induced-*S. enterica* swarming cells as well as RecA-CheW interaction relationship with swarming motility.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

All bacterial strains and vectors used in this work are indicated in **Table 1**. Except when indicated, all strains were grown at 37°C in Luria-Bertani (LB) broth or on LB plates, supplemented, when necessary, with ampicillin (100 μg/mL), chloramphenicol (34 μg/mL), and/or kanamycin (10 μg/mL).

Stimulated Emission Depletion (STED) Microscopy

Fluorescent immunolabeling was carried out as described (Buddelmeijer et al., 2013), with a few modifications. All samples were obtained from the edge of the corresponding swarming plates as previously described (Kim and Surette, 2005). The cells were grown, as described below for swarming assays, on LB-swarming plates [1% tryptone, 0.5% yeast extract, 0.5% NaCl, 0.5% D-(+)-glucose, and 0.5% agar] supplemented when needed with 0.08 mitomycin C/mL or 30 μM of IPTG. After 14 h of incubation at 37°C, the cells were suspended in 1 mL of ice-cold tethering buffer (10 mM potassium-phosphate pH 7, 67 mM NaCl, 10 mM Na-lactate, 0.1 mM EDTA, and 0.001 mM L-methionine) by gently tilting the plates back and forth and harvested by 15 min of low-speed centrifugation (5000 *g*). With this method, migrating cells were easily lifted off the surface, whereas the vast majority of cells in the middle of the plates remained intact on the surface. Non-swarming colonies were recovered using the same method but with 0.5 mL of cold tethering buffer.

Then, the cells were permeabilized by two subsequent treatments with 0.1% Triton X-100 and freshly prepared PBS-lysozyme-EDTA buffer (1× PBS, 100 μg lysozyme/mL and 5 mM EDTA), each for 1 h at room temperature. Then they were incubated in 0.5% blocking reagent (Sigma-Aldrich) at 37°C for 30 min with shaking. After, cells were then centrifuged at 4,500 *g* for 5 min, re-suspended in 100 μL of the appropriate primary antibody (diluted 1:20), and incubated overnight at 37°C. After three washes in wash buffer (1× PBS, 0.05% Tween20), the cells were recovered by centrifugation at 4,500 *g*, re-suspended in 100 μL of the secondary antibody (diluted 1:100), and incubated at 37°C for 2 h without shaking. Finally, after three washing steps with wash buffer, the labeled cells were resuspended in 1× PBS and placed on 35-mm poly-L-lysine pre-coated coverslips using Mowiol-DABCO mounting medium (1× PBS, 2.5% DABCO, 25% Mowiol, and 1× glycerol). The samples were air-dried and then examined under an AxioImager M2 microscope (Carl Zeiss Microscopy) equipped with the appropriate filter set (for the green channel the GFP (Zeiss filter set 38) and for the red Rhod (Zeiss filter set 20) to ensure that at least 90% of cells were correctly permeabilized and immunolabeled. Afterward, at least 300 double marked cells were visually inspected in each sample and the presence and type of clusters as well as RecA distribution were analyzed. Each experiment was performed in triplicate using independent cultures; a minimum of 900 cells from each studied *S. enterica* strain or condition were therefore examined. In all cases, at least the 70% of cells present the same RecA and CheW distribution profile.

Afterward, super-resolution images of the previously analyzed samples were taken on a Leica TCS SP8 STED3X microscope (Leica Microsystems) using a highly corrected 1.4 NA 100x Plan Apo objective specified for STED imaging. Imaging was done using the lateral resolution improvement lightpath (z-STED set to zero). The fluorophore labels were emission depleted using a 660 nm continuous wave (CW) laser for the stimulated emission effect and time-gating (rejection of early emission events) to further increase the resolution. Data were acquired in the form of two channel z-stacks for subsequent deconvolution and rendering.

For deconvolution of the z-stacks the STED module of the Huygens software package (Scientific Volume Imaging, SVI) was used. Images were analyzed using ImageJ software (National Institutes of Health). In all cases, images of 10 different randomly chosen cells were obtained for each sample. As each experiment was performed in triplicate, a total of 30 cells from each studied strain or condition were therefore examined. The images presented in **Figures 1**, **2**, and **7** are representative of the entire image set. All images shown in the Figures present the same contrast settings.

In silico Protein–Protein Interaction Docking

Simple protein–protein docking was conducted using the ClusPro server (Comeau et al., 2004a) to generate an *in silico* model for the RecA-CheW protein complex. The available resolved structures of the *E. coli* RecA (PDB: 2REB) and CheW (PDB: 2HO9) proteins were used to run the analyses. The resultant model was presumed to be reliable also for *S. enterica* as the reported BLAST identity between *E. coli* K-12 and *S. enterica* sv. Typhimurium ATCC14028 proteins is 97 and 92% for RecA and CheW, respectively.

The protein–protein docking assay was carried out in duplicate, selecting RecA as the receptor and CheW as the ligand, and *vice versa*. The protein structures and the obtained *in silico* models were visualized and analyzed using PyMOL software (Schrödinger, 2010).

Co-immunoprecipitation Assay

The cell lysates were obtained as previously described (Irazoki et al., 2016). Cultures of *S. enterica* Δ*recA* Δ*cheW* harboring the plasmids encoding the corresponding tagged proteins were used, and the gene overexpression was induced by the addition of 1 mM IPTG. As a control, cell lysates of *S. enterica* Δ*recA* Δ*cheW* containing the pUA1108 overexpression vector (Mayola et al., 2014) were processed according to the same procedure.

The immunoprecipitation assays were performed using Pure Proteome Protein A magnetic beads (Millipore) coated with either mouse anti-FLAG IgG (Sigma-Aldrich) or mouse anti-HA IgG (Sigma-Aldrich) monoclonal primary antibodies, following the manufacturer's instructions. Cell lysates were mixed at a molecular ratio of 1:1 and incubated at 30°C for 1 h without shaking to allow protein–protein interaction.

As a final step, the samples were separated by SDS-PAGE on a 15% polyacrylamide gel and analyzed by Western blotting using a horseradish-peroxidase (HRP)-coupled anti-mouse antibody (Acris). The membranes were developed using a HRP chemoluminiscent substrate (Luminata ForteTM Western HRP substrate, Millipore) following the manufacturer's instructions. The membranes were imaged using a ChemiDocTM XRS+ system (Bio-Rad).

Construction of RecA and CheW Tagged Proteins

CheW::FLAG- and RecA::HA-carrying plasmids were constructed by PCR-amplifying the *recA* and *cheW* genes using the appropriate oligonucleotide pairs (Supplementary Table S1). In both cases, the corresponding tag sequence was included at the 3′ end of the gene, such that the tag was placed at the C-terminus of the protein. A 3×Gly linker between the tag and the gene sequence was also added. The same strategy was used to obtain the *recA* and *cheW* tagged mutant derivatives. In this case, the oligonucleotides included the suitable point mutation (Supplementary Table S1).

All PCR products were digested and cloned into pUA1108 overexpression vector (Mayola et al., 2014) under the control of the IPTG-inducible Ptac promoter. These plasmids were transformed into *E. coli* DH5α and confirmed by sequencing. When needed, the plasmids containing the tagged proteins were transformed into the corresponding *S. enterica* strains with the appropriate genetic backgrounds.

The selected transformants were confirmed again by PCR and sequencing.

The *cheW*::FLAG *recA*::HA double-tagged strain was constructed as described previously (Irazoki et al., 2016), using the pKO3 plasmid (Latasa et al., 2012). The *recA*::HA construct was obtained by PCR overlap-extension (which added the epitope YPYDVPDYA to the RecA protein), cloned into the pKO3 vector, and introduced into the previously constructed *S. enterica cheW*::FLAG strain (Irazoki et al., 2016). The *S. enterica* Δ*recA cheW*::FLAG strain was constructed by one-step PCR gene replacement as described previously (Datsenko and Wanner, 2000; Irazoki et al., 2016) using the *S. enterica cheW*::FLAG strain as a recipient strain. In all cases, it was confirmed that neither the FLAG nor the HA tag insertion affected the surface motility of the tagged strains.

Swarming Motility and Biofilm Assays

Swarming assays were carried out as described previously (Gómez-Gómez et al., 2007; Mayola et al., 2014; Irazoki et al., 2016). In short, a single colony was picked from bacterial strains grown on LB plates at 37°C and inoculated in the center of a freshly prepared LB swarming plate [1% tryptone, 0.5% yeast extract, 0.5% NaCl, 0.5% D-(+)-glucose, and 0.5% agar] using a sterile toothpick and avoiding medium penetration. The plates were supplemented with IPTG (10 or 30 μM) or mitomycin C (0.08 μg/mL) as needed, incubated overnight at 37°C, and then imaged using a ChemiDocTM XRS+ system (Bio-Rad).

The phenotypic assays for biofilm formation were performed as previously described (Latasa et al., 2012). After 96 h of incubation at 25°C without agitation, the biofilm formed in standing LB broth was visualized as a floating pellicle at the air–broth interface that totally blocked the surface of the culture and could not be dispersed by shaking.

Recombinase Activity Assay

To determine the recombination efficiency of the *S. enterica* strains carrying overexpression vectors containing the *recA* tagged mutant derivatives, the P22 transduction frequency of the strain was compared with that of the same strain but carrying an overexpression vector with wild-type tagged *recA*; the method was described previously (McGrew and Knight, 2003). The transduction experiments were performed as described elsewhere (Campoy et al., 2002). The recombination efficiency was calculated as the number of transductants relative to the initial recipient cell concentration. The relative recombination frequency was the recombination efficiency (%) of each overexpressing strain with respect to the strain overexpressing wild-type *recA*.

ELISA for RecA and CheW Quantification

RecA and CheW quantification was performed by ELISA as described before (Irazoki et al., 2016). All samples recovered from the colony edge were resuspended in sonication buffer (PBS 1×, cOmplete mini EDTA-free tablets, pH 7.3) and sonicated (2x 30-s pulses and 20% amplitude, Digital sonifierR 450, Branson) obtaining the whole cell lysates. After centrifugation (12000 *g* for 10 min), the supernatants were recovered and the total protein concentration of each sample was quantified according to the Bradford method using the protein reagent DyeR (BioRad) and a bovine serum albumin standard curve (range: 1.5–200 μg/mL).

The RecA and CheW::FLAG proteins used in the standard quantification curves were obtained as previously described (Irazoki et al., 2016). The RecA::HA and CheW::FLAG proteins were quantified by ELISA as described (Mayola et al., 2014) using anti-RecA (monoclonal antibody to ARM193 RecA clone, MBL) and anti-FLAG (monoclonal antibody to DYKDDDDK epitope Tag, Acris) mouse IgG. The secondary antibody was an anti-mouse-IgG horseradish-peroxidase-conjugated antibody [polyclonal antibody to mouse IgG (HEL)-HRP, Acris]. The BD OptEIA TMB substrate reagent set (BD Biosciences), prepared following the manufacturer's instructions, was used as the developing solution. Plate measurements were made at 650 nm using a multiplate reader (Sunrise, Tecan).

RESULTS

Subcellular Localization of RecA and CheW Proteins in Swarming Cells during SOS Induction

The location of RecA and CheW proteins within SOS response activated-*S. enterica* swarming cells was analyzed by using 3D-stimulated emission depletion microscopy (3D-STED), a super-resolution fluorescence imaging technique that increases axial resolution by up to 20–40 nm in biological samples (Han and Ha, 2015). Thus, a *S. enterica cheW::FLAG recA::HA* strain was constructed and the appropriate antibodies were used to locate these proteins inside the swarming cells in the presence of a SOS response inducer.

As it is shown in **Figure 1**, besides the expected cell filamentation due to the induction of the SOS response by mitomycin C, the SOS inducer treatment gives rise to a dramatic change in the subcellular location of both RecA and CheW within cells cultured on swarming plates containing sub-lethal concentration of mitomycin C (**Figure 1A**). In agreement with *E. coli* cells grown in liquid medium under non-DNA-damaging conditions (Greenfield et al., 2009), in the non-mitomycin C-treated *S. enterica* swarming cells, the CheW protein was majorly located at the cell poles (**Figure 1B**). This CheW location is the same than that previously described for chemoreceptor polar arrays and accordingly more than 70% of cells presented chemoreceptor polar clusters in these conditions (Kentner et al., 2006; Greenfield et al., 2009; Cardozo et al., 2010; Mayola et al., 2014; Santos et al., 2014).

Nevertheless, the SOS response induction prompts a change in the CheW distribution, which instead of being at the cell poles was indeed organized in smaller foci distributed in a spiral-like configuration along the cell membrane (**Figure 1A**). Further, and in agreement with previous data about cluster assembly under SOS induction (Irazoki et al., 2016), this CheW distribution was present in more than 70% of analyzed cells. Likewise, the CheW foci resembled the distribution and organization of RecA (**Figure 1A**). Under this DNA-damaging conditions, the SOS

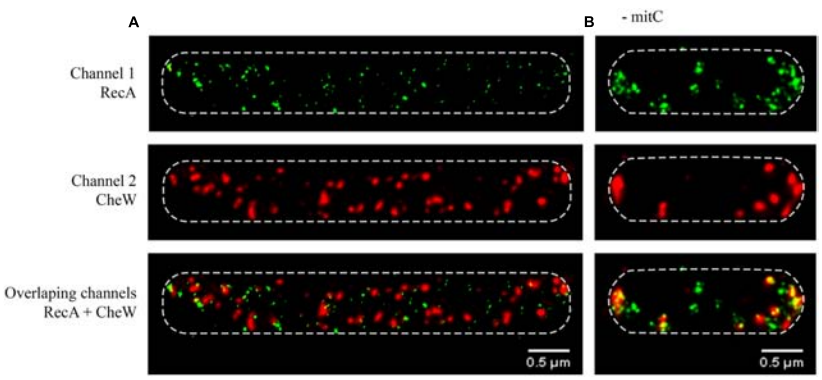

FIGURE 1 | Representative STED (Stimulated Emission Depletion) images of the subcellular locations of RecA and CheW in *Salmonella enterica* wild-type swarming cells (A) in the presence of mitomycin C (0.08 µg/mL) or (B) in the absence of this SOS inducer. The RecA and CheW proteins were labeled with Alexa Fluor® A488 (channel 1) and Alexa Fluor® A568 (channel 2), respectively. For all images, each channel is shown both individually and overlapped. In all cases, the maximum intensity projection images of the obtained z-stacks are shown.

system induction gives rise not only an increase in the RecA concentration (Irazoki et al., 2016) but also to a higher amount of cellular RecA aggregates (**Figure 1A**). After SOS induction, the RecA foci seemed to be smaller and were distributed not only at the cell poles but also along the filamented cell axis, assuming a helical configuration just underneath the bacterial wall (**Figure 1A**). These observations were in agreement with the previously reported changes in the location of RecA in liquid cultures of *E. coli* cells growing in DNA-damaging conditions and the described reduction of RecA storage structures at cell poles (Renzette et al., 2005; Lesterlin et al., 2014; Rajendram et al., 2015). Nevertheless, in our experimental settings, using sub-lethal concentration of mitomycin C, RecA was not distributed forming bundles as those described for *E. coli* (Lesterlin et al., 2014), that were only observed when *S. enterica* was grown in liquid cultures adding higher amount of the SOS inducer (data not shown).

To rule out an indirect effect of either DNA damage or SOS-dependent filamentation on the CheW distribution and to determine whether RecA activation plays a significant role in the distribution of its partner protein, *recA* was overexpressed under non-DNA-damaging conditions and the locations of the CheW and RecA tagged proteins were examined.

In this experiment, *S. enterica* ΔrecA *cheW::FLAG* strain carrying the pUA1135 vector, the pUA1108 overexpression vector containing the *recA::HA* gene under the control of an IPTG-inducible promoter was used (**Table 1**). Induction was achieved by adding IPTG to the swarming plates. The basal expression of the wild-type *recA* carried in this plasmid is enough to recover both swarming ability and the polar-clustered CheW arrangement (**Figure 2A**). In agreement, more than 70% of cells present polar chemoreceptor arrays. Following the addition of IPTG to the swarming plates, the increase in RecA was accompanied by a helical distribution of CheW along the cell axis (**Figure 2B**), as occurs following activation of the SOS response (**Figure 1B**). And, as expected, about 70% of cells did not present polar chemoreceptor clusters.

These findings indicate that neither the cell filamentation, the activated RecA protein nor DNA damage is required to modify the subcellular location of CheW.

In silico Prediction of the RecA-CheW Interaction

To further determine how does the RecA-CheW interaction occurs, the RecA and CheW residues associated with this interaction were identified by using an *in silico* model for RecA-CheW complex-formation in which simple protein–protein docking was conducted using the resolved ternary structures *E. coli* RecA (PDB: 2REB) and CheW (PDB: 2HO9).

RecA has three major functional domains. The amino domain contains a large α-helix and short β-strand that are implicated in the formation of the RecA polymer. The central domain (consisting primarily of a twisted β-sheet with eight β-strands bound by eight α-helices) is involved in DNA and ATP binding. The carboxyl domain is made up of three α-helices and three β-strands that facilitate interfilamentous associations (Story et al., 1992). On the other hand, the folded CheW has a SH3-like regulatory domain and two intertwined five-stranded β-barrels, designated subdomains 1 and 2 (Li et al., 2013).

As little is known about the forces guiding protein-complex formation, balanced-coefficient docking models were considered to be the most accurate for the analysis of the RecA-CheW interaction (Comeau et al., 2004b). Ten of the highest-scoring models were analyzed individually for each combination of RecA receptor protein and CheW ligand, and *vice versa*. Although, the spatial arrangement was not exactly the same in each paired

TABLE 1 | Bacterial strains and plasmids used in this work.

Strains	Relevant characteristic(s)	Source or reference
DH5α	*E. coli supE4 ΔlacU169 (ϕ80 ΔlacZ ΔM15) hsdR17, recA1, endA1, gyrA96, thi-1, relA1*	Clontech
ATCC14028	*S. enterica* Typhimurium wild-type	ATCC
UA1915	*S. enterica* Typhimurium Δ*recA* Δ*cheW*	Mayola et al., 2014
UA1916	*S. enterica* Typhimurium *cheW*::FLAG	Irazoki et al., 2016
UA1941	*S. enterica* Typhimurium *cheW*::FLAG Δ*recA*	This work
UA1942	*S. enterica* Typhimurium *cheW*::FLAG *recA*::HA	This work
UA1943	*S. enterica* Typhimurium *cheW*::FLAG *recA*::HA pNASΩ*cheR*::eYFP	This work
MC1061	$F^-\lambda^-$ Δ*(ara-leu)7697* [*araD139*]B/r Δ*(codB-lacI)3 galK16 galE15* e14 *mcrA0 relA1 rpsL150*(Str^R) *spoT1 mcrB1 hsdR2*(r^-m^+)	CGSC
Plasmids		
pKOBEGA	Vector containing the λ Red recombinase system, Amp^r, temperature sensitive OriV	Chaveroche et al., 2000
pKD3	Vector carrying FRT-Cm construction, Amp^R, Cm^R	Datsenko and Wanner, 2000
pCP20	Vector carrying FLP system, OriVts, Amp^R	Datsenko and Wanner, 2000
pGEX-4T-1	Expression vector carrying the P*tac* IPTG- inducible promoter and the *lacI*^{q} gene; GST fusion tag, Amp^R	Amersham Biosciences
pKO3	Vector for homologous recombination. temperature sensitive OriV, *sacB*, Cm^R	Link and Phillips, 1997
pUA1108	pGEX 4T-1 derivative plasmid carrying only the P*tac* promoter and the *lacI*^{q} gene; used as overexpression vector for *recA* and *cheW* wild-type and mutant derivative genes, Amp^R	Mayola et al., 2014
pUA1135	pUA1108 derivative containing the native *S. enterica* Typhimurium *recA::HA* gene under the control of the P*tac* promoter, Amp^R.	This work
pUA1131	pUA1108 derivative containing the native *S. enterica* Typhimurium *cheW::FLAG* gene under the control of the P*tac* promoter, Amp^R.	Mayola et al., 2014
pUA1136	pKO3 derivative carrying *recA::HA* fusion, Cm^R	This work

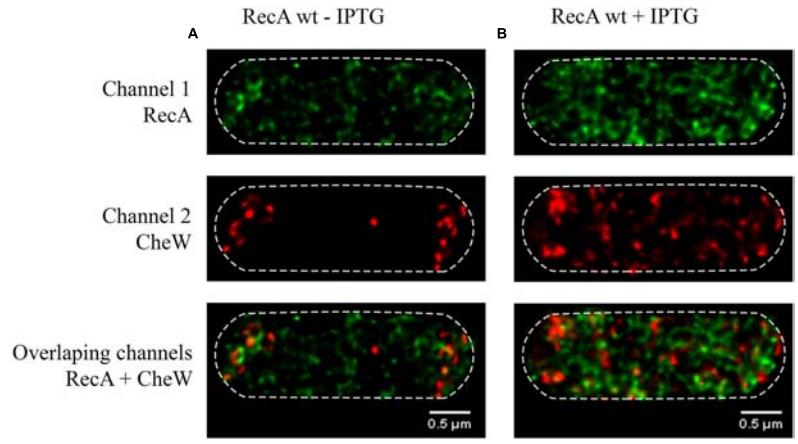

FIGURE 2 | Representative STED images of the subcellular locations of RecA and CheW in the *S. enterica* Δ*recA* overexpressing strain. (A) The effect of basal expression of wild-type RecA in the absence of IPTG. **(B)** Addition of the inducer (30 μM of IPTG) yielded the overexpression of wild-type RecA and the change of the CheW distribution within the cell. It is worth noting that the basal expression of wild-type RecA recovers swarming ability. RecA and CheW proteins were labeled with Alexa Fluor® A488 (channel 1) and Alexa Fluor® A568 (channel 2), respectively. In the images, each channel is shown individually and overlapped. In all cases, the maximum intensity projection images of the obtained z-stacks are shown.

combination, the results allowed the putative interacting regions of each protein to be identified, as they were those that were repeated in all models.

Figure 3 shows the residues of the folded RecA that putatively participate in the interaction with CheW. These were predicted to be located in the amino-terminal and central domains (in

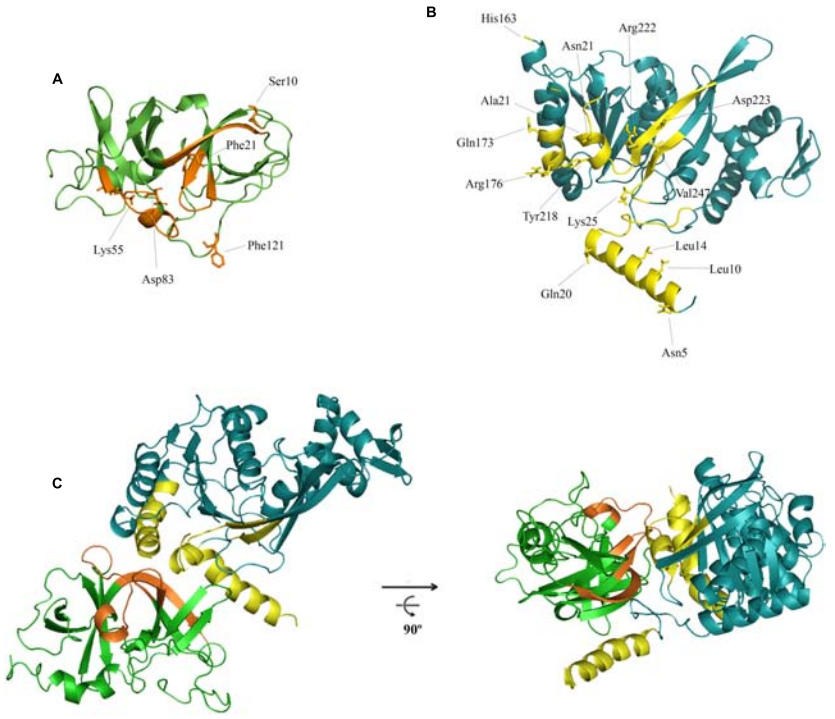

FIGURE 3 | *In silico* model for the interaction of RecA and CheW proteins. The predicted ternary structures of *S. enterica* CheW **(A)** and RecA **(B)** proteins are shown. The putative interface of RecA and CheW involved in the reciprocal interaction of the two proteins is highlighted in yellow and orange, respectively. The residues selected for site-directed mutagenesis and their locations are also indicated. **(C)** Ribbon diagrams of one of the highest-scoring models generated for RecA-CheW pair formation analyzed in this study. The two views of the interaction are rotated 90° about the *x*-axis.

α1, α10, α11, β8, and β9) whereas the presumed CheW regions were located in both subdomains, specifically, in the β1 and β4 (subdomain 1) and the T4, β8, and B6 regions (subdomain 2).

Mutational Analysis the RecA-CheW Pair Formation

To corroborate the interaction interfaces identified *in silico*, site-directed mutagenesis was used to construct several mutant derivatives for each protein in which the relevant residues were affected. In all cases, the corresponding *recA* and *cheW* gene mutants constructed *in vitro* were HA- and FLAG-tagged, respectively, and cloned into the overexpression vector (pUA1108) under the control of an IPTG-inducible promoter.

Fourteen RecA and five CheW residues were selected based on their potential roles in RecA-CheW pair formation (**Tables 2** and **3**) as well as their reactivity and exposure on the corresponding protein surface (**Figure 3**). With the exception of the A214V RecA mutant, in which the Ala residue was changed to a Val, all other selected residues were converted to an Ala (**Tables 2** and **3**), as this aliphatic amino acid is considered to be non-reactive (Cunningham and Wells, 1989). The effect of each substitution on the RecA-CheW interaction was determined *in vitro* and *in vivo* by co-immunoprecipitation and swarming inhibition assays, respectively.

For the *in vitro* co-immunoprecipitation assays, each RecA::HA mutant protein was mixed with wild-type CheW::FLAG; anti-HA-antibody coated beads were used to hijack the proteins. The CheW::FLAG mutated derivatives were mixed with the RecA::HA wild-type protein and hijacked using anti-FLAG-antibody coated beads. Previous assays confirmed the ability of RecA::HA to pull down CheW::FLAG and *vice versa* (Mayola et al., 2014). It was therefore expected that if the mutated residue altered the RecA-CheW interaction, the antibody would

TABLE 2 | *In vitro* interaction of RecA mutant derivatives with wild-type CheW protein.

RecA protein[a]	Secondary structure region containing the mutated residue	Interaction with wild-type CheW[b]	Swarming inhibition by RecA overexpression[c]
Wild-type	NA[d]	+	+
L10A	Helix α1	+	+
L14A		+	+
Q20A		–	–
H163A	NR[d]	+	+
Q173A	Helix α10	+	+
R176A		–	–
N213A	Helix α11	+	+
A214V		+	+
K216A		+	+
Y218A		+	+
R222A	Strand β8	–	–
D224A		+	+
V247A	Strand β9	+	+
K250A	NR[d]	–	–

[a]*The mutated residue and the substitution of each tagged mutant derivative are indicated.*
[b]*Results of co-immunoprecipitation assays using each RecA derivative and wild-type CheW. (+) and (-) indicate the maintenance or abolishment of the RecA-CheW complex, respectively.*
[c]*Results of the in vivo swarming assays in which each RecA derivative was overexpressed in the S. enterica wild-type strain. (+) overexpression generates a non-swarming phenotype; (-) swarming was observed despite overexpression of the mutant protein.*
[d]*NA, not applied; NR, non-resolved secondary structure.*

TABLE 3 | *In vitro* interaction of CheW mutant derivatives with wild-type RecA.

CheW protein[a]	Secondary structure region containing the mutated residue	Interaction with wild-type RecA[b]	Swarming inhibition by CheW overexpression[c]
Wild-type	NA[d]	+	+
F21A	Strand β1	–	–
K55A	Strand β4	–	–
D83A	Turn-6	–	–
S109A	Strand β8	+	+
F121A	Bend-6	–	–

[a]*The mutated residue and the substitution of each tagged mutant derivative are indicated.*
[b]*Results of the co-immunoprecipitation assays between each CheW derivative and the wild-type RecA protein. (+) and (-) indicate the maintenance or the abolishment of RecA-CheW, respectively.*
[c]*Results of the in vivo swarming assays by overexpressing each CheW derivative in the S. enterica wild-type strain. (+) overexpression generates a non-swarming phenotype; (-) swarming was observed despite overexpression of the mutant protein.*
[d]*NA, not applied.*

pull down only the corresponding tagged mutant protein and would not co-immunoprecipitate both tagged proteins (**Figure 4**). The results showed that, among the mutants tested, only four RecA (Q20A, R222A, K250A, and R176A) and four CheW (F21A, D83A, K55A, and F121A) mutants impaired the RecA-CheW interaction (**Tables 2** and **3**). These results corroborated the *in silico* docking predictions and pointed out that some residues from α1, α10, and β8 regions of RecA and from the β1, β4, T6, and B6 regions of CheW participate in the interaction between the two proteins.

To determine the contribution of these eight residues to swarming motility, *in vivo* swarming assays were carried out using the constructed mutants. As it has been previously mentioned, the overexpression of either RecA or CheW inhibits swarming (Cardozo et al., 2010; Medina-Ruiz et al., 2010; Irazoki et al., 2016; **Figure 5A**). In these swarming assays the effect on swarming of RecA and CheW mutant derivative overexpression in wild-type cells was determined. For this reason, all the vectors overexpressing the RecA and CheW mutants were transformed to *S. enterica* wild-type cells, and cultured on swarming plates containing 30 μM of IPTG. In all cases, it was confirmed by ELISA that the RecA and CheW concentration increases were at least more than 20-fold for RecA and 100-fold for CheW after IPTG induction. Representative images of *in vivo* swarming assays of *S. enterica* wild-type strains overexpressing RecA and CheW mutant derivatives that allow or impair RecA-CheW interaction are shown in **Figure 5B**. The results for all mutant derivatives are summarized in **Tables 2** and **3**. In agreement with the data obtained in the *in vitro* co-immunoprecipitation assays, only the mutant derivatives unable to interact with the corresponding wild-type protein do not inhibit swarming when overexpressed. These results confirm the importance of these residues in RecA-CheW *in vivo* interaction. Further, it is worth noting that none of the non-interacting RecA or CheW mutant derivatives are able to recover the swarming ability of the either *S. enterica* Δ*recA* or Δ*cheW* strains (data not shown).

The three domains of RecA exhibit functional overlap (Takahashi et al., 1996; McGrew and Knight, 2003; Adikesavan et al., 2011). For example, in *E. coli*, Arg176 and Lys250 RecA residues, identified in this work as essential for the RecA-CheW interaction, are involved in recombination activity (Chen et al., 2008; Adikesavan et al., 2011). To determine whether the interaction interfaces associated with RecA-CheW coupling also have other overlapping functions, the recombination ability of the obtained RecA derivatives was determined. The results showed that all of the RecA mutants causing impaired RecA-CheW coupling had also lost their recombination ability (**Figure 6**). Further, some other residues that are not involved in RecA-CheW pair formation (H163A, A214V, K216A, and D224A) also present a clear decrease in their recombinase activity (**Figure 6**). These data are not surprising since as stated above, their location matches with regions previously described to be associated with recombination (Chen et al., 2008; Adikesavan et al., 2011).

CheW Subcellular Location in a Cell Unable to Form RecA-CheW Pair

Once identified the RecA and CheW residues associated with interaction and to unequivocally associate the RecA-CheW pair formation with CheW location, the behavior of the non-CheW-interacting RecA R176A mutant was analyzed. In this case, the *recA* mutant derivative (R176A) was overexpressed in a *S. enterica* Δ*recA* strain under non-DNA-damaging conditions

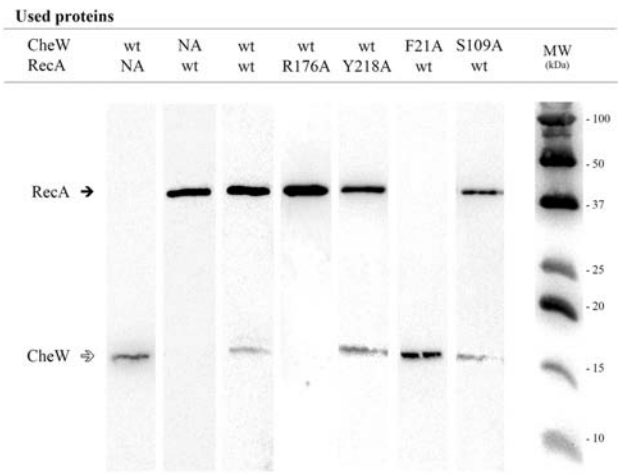

FIGURE 4 | Co-immunoprecipitation assays of RecA and CheW *S. enterica* mutant derivatives. Representative images of the co-immunoprecipitation of mutant derivatives that allow (Y218A RecA::HA or S109A CheW::FLAG) or impair (R176A RecA::HA or F21A CheW::FLAG) the interaction of RecA-CheW are shown. Each lane contains a mixture of cell lysates prepared from the overexpressed mutant derivative and the wild-type (wt) tagged protein. As controls, mixtures containing either wild-type (wt) RecA or CheW or both were included. All experiments were done at least in triplicate; in all cases, the results for all mutant derivatives were exactly the same as those shown in the figure. Black and white arrows indicate RecA and CheW protein bands, respectively. NA, non-added; MW, molecular mass marker.

and the locations of the CheW and RecA tagged proteins were examined.

As it is seen in **Figure 7**, the overexpression of the RecA R176A mutant by IPTG addition does not prompt any change in CheW distribution, as it happens when wild-type RecA is overexpressed (**Figure 2**). In the presence of RecA R176A mutant protein the CheW was never located at cell poles regardless the RecA concentration (**Figure 7**).

These results indicate that not only the concentration of RecA but also the ability of the protein to interact with CheW is required for CheW distribution and thus for chemoreceptor clustering at the cell poles, *a sine qua non* condition for bacterial colony swarming.

DISCUSSION

The experiments performed herein have identified the protein interfaces involved in the interaction between RecA and CheW in *S. enterica*. The regions of CheW specifically associated with RecA are β1, β4, T6, and B6 (**Figure 1**; **Table 3**), which are not those that interact with CheA, CheW, or MPCs (Bilwes et al., 1999; Underbakke et al., 2011; Cassidy et al., 2015). Accordingly, the interaction of RecA and CheW should not interfere with any of the three CheW-binding targets identified thus far (CheA, CheW, and MCPs). The interaction interfaces of RecA are located within the N-terminal and central domains, thus involving the α1, α10, and β8 regions of the protein (**Figure 3**; **Table 2**). These are the same regions previously reported to be involved in RecA polymer formation, ATP hydrolysis, and ssDNA and LexA interactions (Story et al., 1992; Campbell and Davis, 1999; Chen et al., 2008; Adikesavan et al., 2011), such that none of the non-CheW interacting RecA derivatives here described were able to carry out recombination (**Figure 6**). These results suggest that when a molecule of RecA is interacting with CheW, it cannot participate in DNA recombination and repair. Nevertheless, only part of the total RecA amount present in a SOS-induced cell will be associated to CheW hijack, ensuring that DNA repair and recombination take place in the DNA-damaged cells.

By using super-resolution 3D-STED, we were able to show that following SOS induction the increase in the concentration of RecA, but not the activation of other SOS response associated functions, is enough to induce the redistribution of CheW from aggregates at the cell poles to foci with a helicoid configuration along the cell axis, showing the same subcellular location than RecA (**Figures 1B** and **2B**). This finding is consistent with the impairment of the chemoreceptor array assembly that occurs when the SOS response is activated (Irazoki et al., 2016). By contrast, in cells carrying a non-CheW-interacting mutant RecA, CheW is unable to cluster at the cell poles (**Figure 7**). Thus pointing out that the interaction between RecA and CheW is essential for both swarming modulation (**Figure 5**) and CheW clustering at the cell poles (**Figure 7**), and confirming the

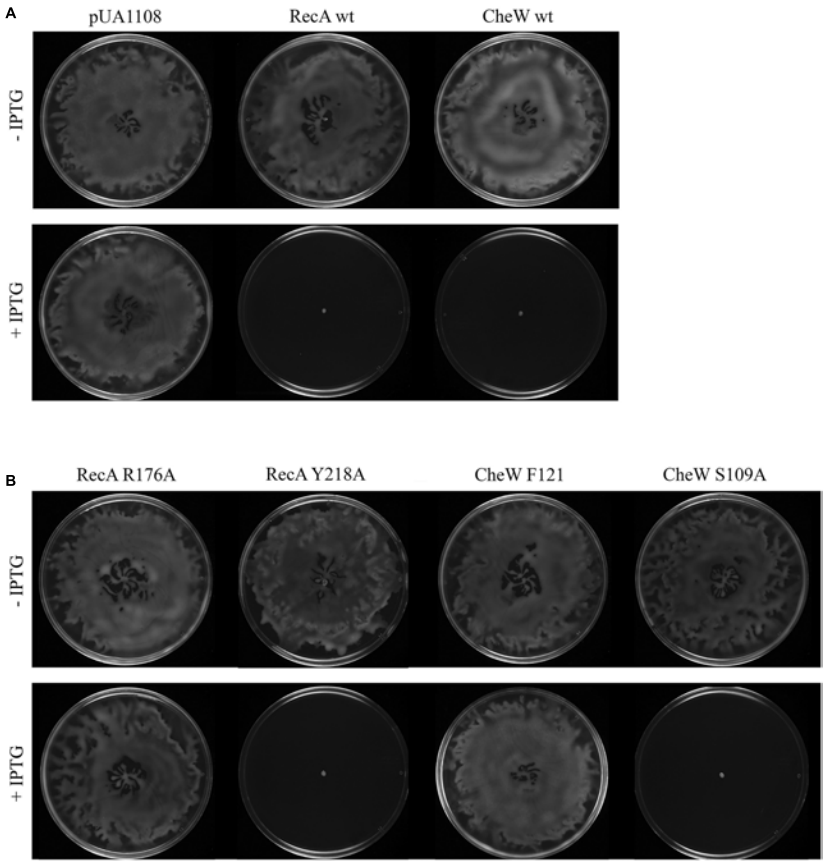

FIGURE 5 | (A) Representative images of the *in vivo* swarming ability of the *S. enterica* wild-type (wt) strain overexpressing either RecA wt or CheW wt proteins or containing only the overexpression vector (pUA1108). **(B)** *In vivo* swarming assay of RecA and CheW mutant derivatives. Representative images of the swarming ability of *S. enterica* wt strain containing the overexpression vectors encoding RecA or CheW mutant derivatives that allow or impair RecA-CheW coupling are presented. The cells were cultured in the presence (+) or absence (–) of 30 μM IPTG. Swarming was inhibited in the presence of IPTG only in mutants that maintained the RecA-CheW interaction (RecA Y218A and CheW S109A). When the interaction of the two proteins was abolished (using RecA R176A and CheW F21A mutant strains), swarming was not affected when IPTG was added. Each experiment was performed at least in triplicate. The same results were observed for all of the mutant derivatives tested.

previously described for *S. enterica* RecA-defective strains, in which chemoreceptor array assembly was inhibited (Mayola et al., 2014).

Taken together, these results suggest two different scenarios to explain the role of RecA in chemoreceptor polar cluster formation and swarming modulation. Thus, RecA may be a component of the chemoreceptor array, since either its absence or overexpression interferes directly with chemoreceptor assembly. However, this is unlikely since polar array clusters have been characterized in detail (Li et al., 2013; Briegel et al., 2014b; Cassidy et al., 2015; Eismann and Endres, 2015) and their well-organized structure does not seem to allow for RecA attachment. Alternatively, RecA could prompt the titration of CheW, thus preventing chemoreceptor assembly and therefore also polar cluster array formation during activation of the SOS response (**Figure 8**). A similar control strategy has

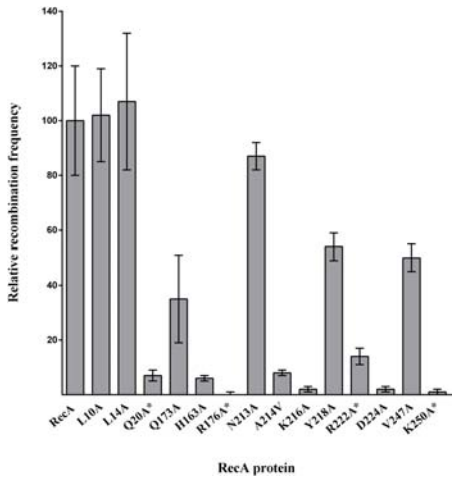

FIGURE 6 | *In vivo* recombination activity of the RecA mutant derivatives. The efficiency of strains, containing the overexpression vector carrying the corresponding RecA derivative, to recombine the selectable genetic marker transduced by bacteriophage P22intH7 was tested. The relative recombination frequency was calculated as the recombination efficiency of each mutant derivative with respect to that of the strain overexpressing wt RecA, and the recombination efficiency of each strain as the number of transductants compared to the initial recipient cell concentration. The RecA mutant derivatives unable to interact with CheW are indicated by an asterisk (*). The relative recombination frequencies were calculated as the mean of three independent experiments. Error bars indicate the standard deviation.

been described for other interacting proteins whose regulatory function relies on the availability of the protein with which they interact (Liu and Richardson, 1993; Plumbridge, 2002; Hill et al., 2013; Hernández-Rocamora et al., 2015; Paget, 2015).

The concentration of CheW is essential for chemoreceptor cluster formation and the absence or overexpression of this protein inhibits array assembly (Avram Sanders et al., 1989; Cardozo et al., 2010). In addition, a recent study showed that in these arrays, CheW not only serves as an adaptor protein anchoring MCPs to CheA but that, via ring formation, it is also responsible for chemoreceptor array stability (Cassidy et al., 2015). Therefore, in the absence of DNA damage, RecA is able to bind CheW adjusting the availability of this protein needed to allow chemosensory system assembly and thus swarming ability (**Figure 2A**).

Since activation of the SOS response increases the concentration of RecA but not of CheW (Irazoki et al., 2016), then during SOS activation the amount of RecA-CheW complex formation will be stimulated but the CheW availability will thereby be reduced (**Figure 1A**), which will affect the stability of the hexagonal receptor signaling array (Cassidy et al., 2015). Following DNA damage repair, the *recA* expression returns to its basal level restoring chemoreceptor array assembly and thus swarming ability (Irazoki et al., 2016) returning the cell the non-DNA damage condition (**Figure 1B**). This explains why only CheW overexpression can reestablish polar cluster assembly in a RecA-overexpressing strain (Irazoki et al., 2016), i.e., the increased availability of CheW restores chemosensory array assembly. Moreover, the absence of RecA (Mayola et al., 2014) or an inability of the protein to interact with CheW (**Figure 7**) will increase the available amount of CheW (**Figure 7A**), thus interfering with chemoreceptor ring structuring and cluster formation (Cardozo et al., 2010).

Besides CheW is a key protein in the *S. enterica* chemoreceptor pathway (Baker et al., 2006) and RecA seem to play a role in chemotaxis (Mayola et al., 2014), chemoreceptor arrays are not essential for chemotaxis response. It has been previously reported that despite the absence of structuration of polar clusters, the association of chemoreceptors with the chemotaxis pathway is still functional (Maki et al., 2000; Briegel et al., 2014b). Furthermore, chemotaxis and swimming are not affected when *E. coli* is treated with cephalexin (Maki et al., 2000), a beta-lactam antibiotic that induce SOS response (Bano et al., 2014). Moreover, biofilm formation is affected neither by the absence or the overexpression of RecA (data not shown).

It is also worth noting that although the absence or overexpression of RecA inhibits cluster assembly, the presence of chemoreceptor clusters is not completely abolished in either Δ*recA* or RecA overexpressing strains. It has been widely reported that chemoreceptor arrays are highly stable structures. Several models have been proposed to describe its assembly and stabilization and not only CheW but also CheA and even the cell membrane curvature seem to be involved (Shiomi et al., 2005; Thiem and Sourjik, 2008; Greenfield et al., 2009; Jones and Armitage, 2015). Then, the alteration in CheW availability would clearly affect chemoreceptor cluster assembly but not completely abolish it. Accordingly, once the SOS response is activated, more than the 70% of the cells presented CheW distributed along the cell instead of being at cell poles due to the RecA mediated – CheW titration.

As mentioned in Section "Introduction" RecA is associated to the inner-membrane anionic phospholipids (Rajendram et al., 2015). This interaction is necessary for RecA activity during DNA damage repair. However, the RecA residues that interact with anionic phospholipids do not overlap with those interacting with the CheW interface (**Table 2**). RecA proteins also form foci that may or may not be associated with DNA (Renzette et al., 2005). The DNA-less proteins, referred to as RecA storage structures, are often located at the cell poles and are redistributed along the cell in response to DNA damage (Renzette et al., 2005; Lesterlin et al., 2014; Rajendram et al., 2015). Interestingly, an *E. coli* R28A RecA mutant with an amino acid substitution in the α1 RecA region, shown in this study to be associated with RecA-CheW pair formation in *S. enterica* (**Table 2**), is

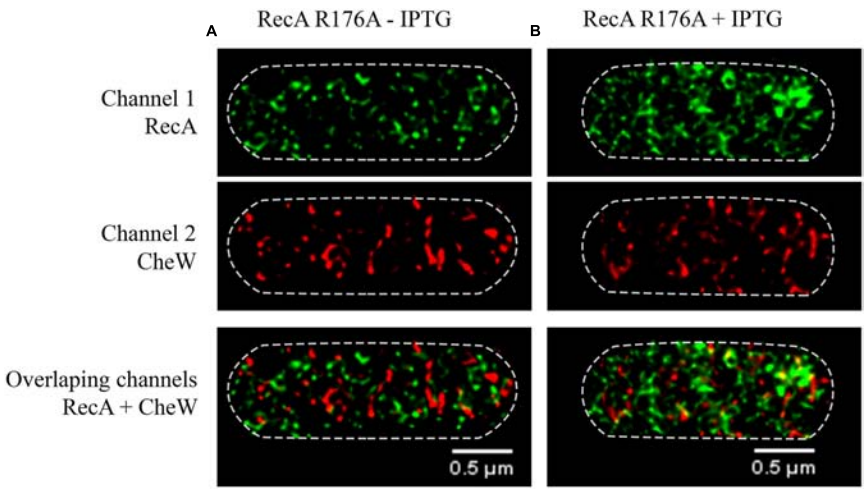

FIGURE 7 | Representative STED images of the subcellular locations of RecA and CheW in the *S. enterica* Δ*recA* strain overexpressing the non-CheW-binding RecA R176A mutant. (A) The effect of basal expression, in the absence of IPTG, of RecA R176A mutant on RecA-CheW distribution is shown. **(B)** Addition of the inducer (30 μM of IPTG) yielded the overexpression of the non-CheW-binding RecA R176A mutant. RecA and CheW proteins were labeled with Alexa Fluor® A488 (channel 1) and Alexa Fluor® A568 (channel 2), respectively. In the images, each channel is shown individually and overlapped. In all cases, the maximum intensity projection images of the obtained z-stacks are shown.

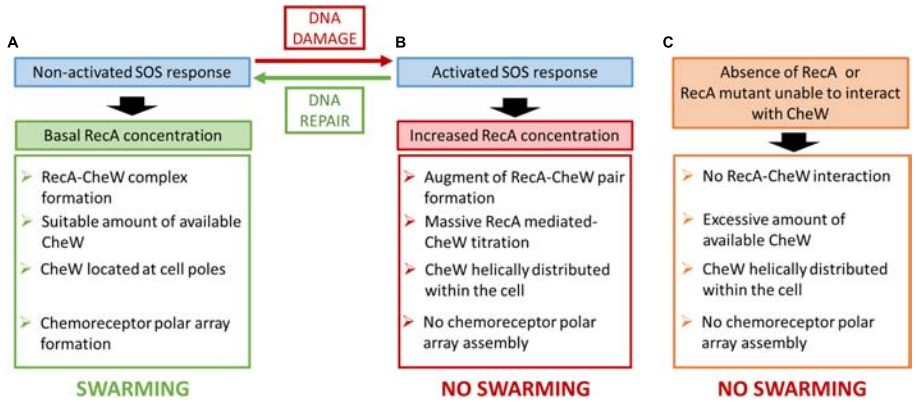

FIGURE 8 | Schematic diagram of the putative mechanism for the control of *S. enterica* swarming motility by RecA and SOS response. The effect of non-activated SOS response **(A)**, the induction of the SOS system **(B)** and the absence or the presence of a non-interacting RecA mutant **(C)** is proposed.

unable to generate DNA-less RecA foci (Renzette and Sandler, 2008). Thus, our results suggest that in addition to RecA storage, DNA-less RecA foci participate in the modulation of swarming motility.

Our data therefore shed light on a new role of RecA, the titration effect on CheW protein, which based on protein–protein interaction strategy, modulates the CheW distribution within the cell thus controlling the swarming ability.

AUTHOR CONTRIBUTIONS

OI, JA, and SC performed the *in silico* analyses and site-directed mutagenesis. OI and SC analyzed swarming phenotypes and chemoreceptor polar cluster assembly. OI, SC, and TZ performed the fluorescent immunolabeling and STED imaging. OI, SC, and JB conceived the experiment, coordinated the research, discussed the findings and interpreted the results.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2016.01560

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

4

Discussion

The results presented herein lead to the characterization of the molecular mechanisms that govern RecA-mediated swarming modulation, that allowed to determine the role of RecA protein in the flagellar rotation, chemotaxis, chemoreceptor array assembly and finally as a motility regulator. In the following sections, the obtained results will be discuss point by point.

4.1 Role of RecA protein in the chemotaxis system and the flagellar rotation

Our data pointed out that the absence of RecA not only abolished swarming motility but also impaired flagellar switching, which presented a clockwise (CW) bias anchored flagellar rotation (Figure 3, Article 1). In previous studies, the incapacity to switch the flagellar rotation has been linked to chemotaxis impairment and to improper colony hydration, which affects swarming ability[35,73,75].

Due to this flagellar motor switch bias disruption Δ*recA* cells not only lose their ability to chemosense (Figure 4, Article 1) but they also present a lower swimming speed (Figures 2, Article 1) that correlates with their inability to swarm. It has been concluded that in *E. coli* the absence of RecA did not affect swimming. Nevertheless, by analyzing *S.* Typhimurium Δ*recA* mutant more profoundly using single-cell techniques and microfluidics it was possible to determine that it presented a CW bias. The obtained results do not contradict those of *E. coli*, as in fact, *S.* Typhimurium Δ*recA* cells move more slowly than the wild-type strain due to their flagellar affectation.

However, the disrupted motor bias is the reason for chemotaxis and swarming motility inhibition (Figure 3, Article 1). By anchoring the flagellar switching, the regular tumbling/running periods that generate the correct cellular propulsion towards a chemical gradient are affected. Further, this switch promotes the lubrication of the cell–surface interface, which is needed to overcome surface friction, both of which are critical requirements for swarming motility in temperate swarmers such as *S.* Typhimurium and *E. coli*[35]. Specifically, *Salmonella* beats frictional resistance by increasing its flagellar motor force, and therefore the anchoring of the flagella in CW-bias does not generate the proper lubrication for cells to swarm. This tumbling phenotype is reversed, and chemotaxis is restored when the RecA deficiency is complemented by a plasmid carrying a wild-type copy of the *recA* gene (Figure 4, Article 1), which supports the role of RecA protein in flagellar switching.

Furthermore, Δ*recA* tumbling phenotype resembles that of Δ*cheB*, Δ*cheZ* or Δ*cheR* mutants[292]. Some *che* knockout mutants results in a flagellar CW bias, that is associated with the integral feedback module of the chemotaxis machinery: CheZ phosphorylase returns phophorilated CheY (CheY∼P) to its non-phosphorylated state (CheY), and CheB methylesterase and CheR methyltransferase control the MCPs methylation state, adjusting it to the presence and concentration of external stimuli (see Introduction, Section 1.2.2.3.2). Since Δ*recA* results in the same CW bias, a similar function can be hypothesized for RecA in the chemotactic response adaptation process regulating methylation.

The data presented herein confirms that the presence of RecA is essential for a normal chemotactic response and also that it is associated with the motor functionality.

4.2 The interaction of RecA-CheW proteins

It has been previously reported that in large-scale immunoprecipitation screenings[291], RecA protein was able to bind CheW and later, two-hybrid[293] and far-Western blot assays[294] pointed toward a direct RecA-CheW pair formation. Herein, we unequivocally confirmed the RecA-CheW interaction by co-immunoprecipitation assay, thus supporting a tight relationship between the SOS response system and chemotaxis pathway (Figure 1, Article 1).

Taking advantage of the resolved structures of the RecA and CheW proteins, a protein-protein docking assay was conducted, that combined with *in vivo* and *in vitro* assays, led to the identification of the critical interfaces for RecA-CheW pair formation (Figure 3 and Tables 2-3, Article 3).

After testing 14 residues, RecA Gln20, Arg222, Lys250, and Arg176 were unequivocally described as essential for RecA-CheW interaction. Structurally, those residues involved in CheW-binding are located within the amino and central domains of the RecA protein (Table 2, Article 3). The same regions have been previously reported to be implied in RecA polymer formation, ATP hydrolysis, ssDNA binding and LexA interactions[275,277,283,295]. For example, RecA residues Arg176 and Lys250 are involved in recombination activity in *E. coli*[283,296], that when mutated, the RecA derivatives of these residues give rise to the lost of their recombination ability and also the capacity to bind CheW protein (Figure 6, Article 3).

These results demonstrated that CheW-binding interface of RecA is also involved in other functions, reinforcing that the three domains of RecA exhibit functional overlap[283–285]. Accordingly, during swarming inhibition, only a part of the total RecA amount will be associated with CheW, ensuring that DNA repair and recombination are not blocked and might take place if DNA damage occurs.

On the other hand, CheW residues Phe21, Asp83, Lys55, and Phe121 were demonstrated to be involved in RecA-CheW pair formation (Table 3, Article 3). The interaction of RecA and CheW does not seem to interfere with any of the CheW-biding targets described so far, the signaling core unit components CheA, CheW and MCPs[224,297,298] (Figure 1 and Table 3, Article 3). In concordance with these results, it is expectable that RecA might be able to bind CheW protein even when core signaling units are structured.

4.3 Molecular mechanism governing RecA-mediated swarming regulation

4.3.1 Association of RecA protein to signaling arrays

SOS response machinery seems to be indirectly related to motility regulation through RecA protein, since is not the lack but the excess of RecA the naturally achievable condition under certain environmental stress[247,299]. One of the indirect consequence of the SOS response induction is the quick rise of the intracellular RecA concentration, as *recA* is one of the first genes activated in the hierarchical regulation of the SOS genes once the system is triggered.

The direct association between RecA and CheW suggested that like CheW, RecA might play a role in the architecture and assembly of chemosensing arrays. Our first data alluded that somehow RecA is involved in the assembly of regular polar chemoreceptor signaling arrays, for the reason that the Δ*recA* mutant presented a blurred disassembled clustering (Figure 5, Article 1). Not only the absence but also the increase of RecA protein, either by overexpression or SOS response induction, impaired the signaling array assembly, consequently abolishing swarming motility (Figures 3 and 4, Article 2).

When analyzing signaling array dynamics during SOS response activation, we found out that the intracellular RecA concentration and the number of structured polar arrays were inversely proportional. Interestingly, our results denoted that the assembly of signaling arrays is restored as the amount of RecA decreases to its basal level after the removal of the DNA injuring compound from the media and SOS response is no longer activated (Figures 5 and 6, Article 2). The delay to re-establish chemoreceptor signaling assembly and consequently swarming ability, is correlated with the time needed by the cell to repair all the DNA damage, including the remaining injuries that continue to induce the response even after the damaging compound is removed.

The reversibility of the signaling array assembly adds a dynamic component to the RecA-mediated swarming modulation mechanism. In line with our results, bacterial cells will trigger SOS response when DNA damage occurs during surface colonization, followed by the inhibition of chemosensing array assembly and motility, which will be recovered once DNA is repaired.

This reversibility endorses the usefulness of sensing DNA-damaging compounds via SOS system induction to adapt their surface motility in response to the environmental stress. Furthermore, given the wide range of SOS response inducing stimuli, this mechanism implies the power to modulate swarming motility not only in the presence of elements that directly generate DNA lesions but also to respond to other causes of stress that indirectly promote DNA injuries.

In natural environments, where SOS response is triggered by the presence of DNA injuring compounds, the non-activated and activated forms of the RecA protein are expected to coexist within the cell[248]. Our results demonstrated that along with protein increment, no RecA activation is necessary for motility modulation as the increase of non-active RecA protein generates the same non-swarming phenotype and disrupts the polar signaling array assembly (Figure 1, 2 and S1, Article 2). Overall, these results show that among all the cellular-associated phenomena controlled by SOS response is the RecA increment itself which impairs swarming motility.

4.3.2 RecA protein titrates CheW

As just discussed, the induction of the SOS response abolishes swarming due to a disruption in chemosignaling array assembly that is essential for flagellar rotation and motility. Since the link between the repair system and chemotaxis is the interaction of RecA and CheW protein, the next step of the investigation was to determine the role of this protein complex in motility regulation.

The assembly and functionality of the chemotaxis machinery are closely tied to a very specific stoichiometry between its components (see Section 1.2.2.3.3). The excess or lack of any of the constituents of the complex may lead to instability and lately to the interaction break down[192,226,242,300].

Unlike RecA, the concentration of CheW coupling protein is stable within the cell and does not change when the SOS response is induced (Figures 4 and 6, Article 2). Accordingly, the absence or increment of CheW concentration will have a remarkable effect in chemosensing array assembly[76,192] (Table 1, Article 2). The molecular concentration of the CheW is $2.15(\pm 0.21)x10^{10}$ molecules/μg of total protein, an amount similar to RecA protein when the SOS response is not induced, $2.06(\pm 0.60)x10^{10}$ (Table 1, Article 2). After SOS induction, RecA concentration can be increase up to 25-fold the basal level, reaching $3x10^{12}$ molecules/μg of total protein.

Remembering that the presence of RecA is essential for signaling array assembly, in the absence of DNA damage, the role of RecA protein might be to adjust the availability of CheW, allowing the chemosensory machinery to assemble (Figure 2A, Article 3). Furthermore, the balance between both proteins is the key for understanding the importance of RecA-CheW in modulating swarming. For example, the gradual increase of CheW can reestablish polar signaling array assembly in a strain that constitutively expresses RecA protein (*recA*o) when both proteins reach a certain balance (Tables 1 and S1, Article 2). In this context, when SOS response is triggered due to DNA injuries, part of the RecA protein will bind CheW, reducing the availability of CheW to form polar signaling arrays until DNA damage is repaired.

The absolute quantification of RecA and CheW did not allow the calculation of the real stoichiometry between both proteins, mostly due to the multi-functionality of RecA protein and the unrevealed balanced between bound and unbound protein forms of CheW.

4.3.3 RecA-CheW complex location within the cell

As mentioned in the Introduction (see Section 1.2.3.3), RecA protein is associated to membrane phospholipids forming RecA storage structures that present a polar location and are redistributed along the cell during SOS response. As a multi-functional protein (see Section 1.2.3.3.1), RecA molecules will be distributed along the cell binding DNA, RecA, CheW or phospholipids depending on the affinity and the specific demands of the cell in a determined moment. According to our data and the location of small RecA aggregates within the cell when SOS response is triggered, those DNA-less RecA foci could also participate in the modulation of swarming motility by interacting with CheW (Article 3).

The experiments carried out using stimulated emission depletion (STED) microscopy allowed us to track the dynamics of signaling array assembly during SOS response by following the location of CheW and RecA proteins. As described elsewhere, CheW protein, as the majority of the components of the signaling arrays, is mainly accumulated at cell poles forming assembled packaged structures (Figure 1B, Article 3). Following SOS response activation and the increment of RecA protein, CheW protein is redistributed from large aggregates at cell poles to smaller helical foci along the cell axis, matching the location of RecA protein.

This redistribution of CheW protein is consistent with the impairment of signaling array assembly showed when SOS response is activated (Figure 3, Article 2). When RecA was unable to bind CheW (using a point mutation RecA derivative that simulates Δ*recA* phenotype) the last is unable to assemble and cluster at cell poles, even when SOS response is not activated (Figure 7, Article 3).

Taken together, these results reinforced the idea that the regulatory role of RecA is to prompt the titration of CheW protein, preventing signaling array assembly and polar formation during SOS response, and thus inhibiting swarming motility when DNA damage occurs (Figure 8, Article 3). Moreover, the RecA-CheW complex is essential for CheW clustering at cell poles (Figure 3, Article 3), confirming the role of RecA in chemoreceptor array assembly alluded according to the inhibition present in RecA-defective cells (Figure 5, Article 1).

Besides serving as an anchor protein to MCPs and CheA protein, CheW has been described to be responsible for array stability via ring formation[224] (Figure 1.11). Even that chemosensing arrays are highly stable structures, depending on the CheW availability modulated by RecA titration, the array assembly could be clearly affected but not completely abolished. Several models have proposed that the assembly and stabilization of the arrays rest not only in CheW but also in CheA and even in the cell membrane curvature[228,235,244,245]. Furthermore, our results highlight that chemoreceptor assembly is closely linked not only to the core unit components amount but RecA protein availability, and that through titration, RecA may indirectly affect the CheW-ring formation and so signaling array stability once structured.

During SOS response, the implication of RecA binding CheW is not extended to the chemotaxis response, as occurs in the absence of the protein, and cells maintain the ability to sense normally. Even if the titration of CheW generates the disruption of the sensory arrays, it has been previously described that the presence of the structured chemoreceptor arrays are not essential for sensing ability.

In fact, despite the absence of assembled arrays, core signaling units seem to be functional, and chemotaxis pathway is still able to sense and respond to environmental stimuli[232,301]. Our results clearly showed that RecA plays a role in chemotaxis, as its absence affects the sensing ability of the cells, but the extension of its implication remains controversial. Instead, when SOS response activation triggers RecA protein increment, and signaling arrays are not able to assemble, chemotaxis ability of the cells is maintained as the core unit remains functional (unpublished results). It is remarkable that when the flagellar switch is not affected, the chemotaxis ability is also maintained. Moreover, the polar signaling array assembly is not necessary for sensing ability (unpublished results).

This data supports the previously mentioned hypothesis that according to their interaction interfaces RecA protein could bind CheW when this protein is structured in core signaling units. For the moment, whether RecA titration hijacks individual CheW monomers or the assembled core units remains to be elucidated.

4.4 Swarming control-model mediated by SOS response

The results obtained in this work lead to hypothesize how bacterial cells can adapt their surface motility in response to the presence of direct or indirect DNA-damaging

agents by sensing these compounds via SOS system induction (Figure 4.1). This modulation of swarming motility occurs via RecA-CheW protein pair formation, as has been unequivocally confirmed. Both, reversibility of polar signaling array assembly (Figure 5, Article 2) and swarming behavior in response to a gradient of SOS response triggering compound (Figure 7A, Article 2), underline the biological significance of the SOS system modulating swarming motility.

Bacterial cells growing on surfaces will likely be exposed to a wide range of compounds, of either biological or chemical origin. As mentioned, in the absence of DNA injuries, the absolute concentration of RecA and CheW proteins is quite similar (Table 1, Article 2). On the contrary, when DNA damage occurs, SOS response is triggered followed by a quick RecA concentration increase[299,302,303]. During SOS activation the formation of RecA-CheW complex will be stimulated, and as CheW concentration is not controlled by this response, CheW availability will thereby be reduced (Figure 1A and 8, Article 3). This imbalance between RecA and CheW affects the stability of the signaling arrays, causing the cessation of swarming[224]. Consequently, when the bacterial colony edge is exposed to SOS-inducer, the swarming ability is impaired thus avoiding the exposure to higher concentrations of the injurious, and potentially lethal, compound (Figure 8, Article 2). When swarming is inhibited before being in contact with a lethal concentration of the injuring elements, non-motile cells chaotically agglomerate creating a physical barrier that avoids the advance of the rest of the cells behind (Figure 4.1).

At the same time, the non-exposed edges of the colony continue to swarm colonizing surface regions that are SOS inducer-free or contain a lower, non-injurious concentration of the DNA-damaging compound (Figures 7A and 8, Article 2). Following DNA damage repair, RecA concentration returns to its basal level, increasing the availability of CheW that restores chemosensory array assembly and returning the cell to the non-DNA damage condition (Figure 8, Article 3). These data was reinforced when analyzing a strain unable to trigger the SOS response (*lexA*3(Ind)), that in contact with a low concentration of DNA damage compound was unable to stop swarming and consequently, those cells that were encountered closer to higher or lethal concentrations, died (Figure 7, Article 2). Figure 4.1 resumes the proposed model for RecA-mediated swarming modulation.

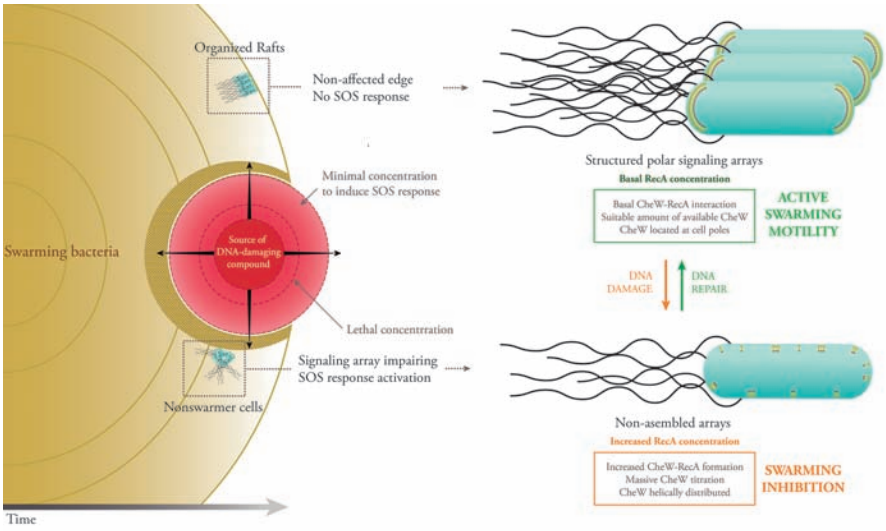

Figure 4.1: RecA-mediated swarming modulation model. During surface colonization, cells are exposed to DNA-damaging compounds. DNA injuries trigger SOS machinery, generating an increase of intracellular RecA concentration that disturbs the equilibrium between this protein and CheW, causing the disruption of chemosensory signaling arrays and inhibiting swarming. When the DNA-damaging source decreases or disappears, the repair of the DNA damage driven by the SOS-system would restore the intracellular balance between RecA and CheW, recovering polar signaling array assembly and therefore also the colony swarming ability.

This swarming response against DNA-damaging compounds reported herein is driven by a single signal that responds to a broad range of injuring agents, the SOS-response-mediated increase in RecA. Thus, using RecA as a sensor mechanism, cells can adapt their surface motility in response to the presence of direct or indirect DNA-damaging agents by sensing these compounds through SOS system induction.

Modulating swarming through RecA protein during surface colonization might be an advantage for bacterial cells, as it limits the number of genes required to one (*recA*), that casually codifies for a very conserved protein present in almost all bacterial groups. If the ability to interact between CheW and RecA protein is conserved among other bacterial groups and motility types, SOS response could be a global system for modulating surface colonization under unfavourable environmental conditions.

5

Conclusions

1. The absence of RecA protein generates a clockwise bias anchored flagellar rotation that affects both chemotaxis and swarming, which demonstrates that RecA is essential for the correct functioning of the flagellar motor.

2. The absence of RecA or the increase of its intracellular concentration disrupts chemoreceptor signaling array assembly thus abolishing swarming motility.

3. The increase of RecA protein itself, and not its activation or any of the other cellular-associated phenomena controlled by SOS response, is the responsible for signaling array assembly disruption and swarming motility impairment when SOS response is activated.

4. The regulatory role of RecA during surface colonization, once SOS response is triggered, is to prompt the titration of CheW protein, preventing signaling array assembly and thus inhibiting swarming motility.

5. The effect of SOS response activation on swarming motility inhibition is reversible. The chemoreceptor signaling array assembly and the ability to swarm are recovered once DNA is repaired and SOS response is no longer activated, thus allowing RecA concentration to return to its basal level.

6. The presence of RecA protein is essential for a normal chemosensing ability. In the absence of DNA damage, RecA might adjust the availability of CheW protein to allow chemosensory machinery assembly and swarming motility modulation.

7. The RecA-mediated swarming modulation occurs via its interaction with CheW protein. The residues Gln20, Arg222, Arg176 and Lys250 of RecA and Phe21, Lys55, Asp83 and Phe121 of CheW are directly implied in the interaction.

8. The characterized RecA-binding CheW interface does not overlap with any of CheA-, CheW- or MCPs-binding regions identified so far.

9. The RecA interfaces associated to CheW binding were described to be located within N-terminal and central domains overlapping other previously reported functional regions such as RecA polymer formation, ATP hydrolysis, ssDNA binding or LexA interaction domains.

10. Based on protein-protein interaction strategy, the disruption of the chemosensing array assembly occurs due to the re-distribution of CheW protein from polar aggregates to helically distributed small foci, following the RecA protein location within the cell.

11. Chemosensory array assembly is closely linked not only to core unit components concentration but to RecA protein availability, that through titration, may indirectly affect the CheW-ring formation and so signaling array stability once is structured.

12. Bacteria can make use of RecA-mediated mechanisms to sense direct or indirect DNA-damaging compounds via SOS system induction and adapt their surface motility in response to the environmental stress stimuli.

Appendices

A Transient multidrug resistance phenotype is abolished by sub-lethal antimicrobial concentrations (Appendix 1)

The transient multidrug resistance phenotype of *Salmonella enterica* swarming cells is abolished by sub-lethal concentrations of antimicrobial compounds

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During swarming, a rapid and coordinated multicellular migration of bacteria across a moist surfaces, bacterial cells exhibit high-level resistance to multiple antibiotics. This phenomenon is known as adaptative or transient resistance. Some antibiotic tested inhibited swarming motility (cefotaxime, ciprofloxacin, trimethoprim and chloramphenicol), while others did not. Chloramphenicol-treated cells exhibited a clear decrease in their flagella number, not exhibited by the rest of antibiotics affecting motility that disrupted the polar chemosensing array assembly. Some antimicrobial agents alone or in combination with others, prompts not only swarming inhibition but also the abolishment of transient resistance phenotype. Our results reveal the potential of targeting swarming inhibition in the development of strategies to enhance the therapeutic effectiveness of antimicrobial agents.

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The transient multidrug resistance phenotype of Salmonella enterica swarming cells is abolished by sub-lethal concentrations of antimicrobial compounds

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The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest

Author contribution statement

OI and SC analyzed swarming and multidrug resistance phenotypes and chemoreceptor polar cluster assembly. SC and JB conceived of the experiments and coordinated the research.

Keywords

swarming, Adaptative multidrug-resistance phenotype, SOS Response (Genetics), chemoreceptor polar arrays, cell flagellation

Abstract

Word count: 141

Swarming motility is the rapid and coordinated multicellular migration of bacteria across a moist surface. During swarming, bacterial cells exhibit high-level resistance to multiple antibiotics, a phenomenon described as adaptive or transient resistance. In this study we demonstrate that sub-lethal concentrations of cefotaxime, ciprofloxacin, trimethoprim, or chloramphenicol impair Salmonella enterica swarming. Chloramphenicol-treated S. enterica cells exhibited a clear decrease in their flagellar content, while treatment with all of the other antibiotics tested (cefotaxime, ciprofloxacin, and trimethoprim) inhibited polar chemoreceptor array assembly. Moreover, the increased resistance phenotype acquired by swarming cells was abolished by the presence of these antimicrobials. The same occurred in cells treated with these antimicrobial agents in combination with others that had no effect on swarming motility. Our results reveal the potential of targeting swarming inhibition in the development of strategies to enhance the therapeutic effectiveness of antimicrobial agents.

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Ethics statements

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The transient multidrug resistance phenotype of *Salmonella enterica* swarming cells is abolished by sub-lethal concentrations of antimicrobial compounds

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Abstract

Swarming motility is the rapid and coordinated multicellular migration of bacteria across a moist surface. During swarming, bacterial cells exhibit high-level resistance to multiple antibiotics, a phenomenon described as adaptive or transient resistance. In this study we demonstrate that sub-lethal concentrations of cefotaxime, ciprofloxacin, trimethoprim, or chloramphenicol impair *Salmonella enterica* swarming. Chloramphenicol-treated *S. enterica* cells exhibited a clear decrease in their flagellar content, while treatment with all of the other antibiotics tested (cefotaxime, ciprofloxacin, and trimethoprim) inhibited polar chemoreceptor array assembly. Moreover, the increased resistance phenotype acquired by swarming cells was abolished by the presence of these antimicrobials. The same occurred in cells treated with these antimicrobial agents in combination with others that had no effect on swarming motility. Our results reveal the potential of targeting swarming inhibition in the development of strategies to enhance the therapeutic effectiveness of antimicrobial agents.

1. Introduction

Swarming motility is the rapid and coordinated multicellular migration of bacteria across a moist surface mediated by flagella (Henrichsen, 1972). This motility is widely distributed throughout flagellated bacteria and associated with their colonization of host surfaces, the increased expression of virulence factors, and antibiotic resistance (Kim and Surette, 2003, 2004; Lai et al., 2009; Overhage et al., 2008). Specifically, bacterial colonies exhibit a greater resistance to multiple antibiotics when swarming (Butler et al., 2010; Kim et al., 2003; Kim and Surette, 2003; Lai et al., 2009). This adaptive antibiotic resistance has been described for temperate swarmers such as *Salmonella enterica*, *Escherichia coli*, and *Pseudomonas aeruginosa*. In these species, swarming-based resistance is non-genetically conferred, since it ceases when the cells are grown under non-swarming conditions (Butler et al., 2010; Lai et al., 2009; Overhage et al., 2008). The mechanism underlying this transient resistance is poorly understood but it may be a physiological attribute of swarming cells, related, for example, to an altered outer membrane composition (Kim and Surette, 2003) or a decrease in membrane permeability (Kim and Surette, 2004) acquired in response to bacterial growth on moist surfaces. Furthermore, Multidrug-resistance seems to be a function of the bacterial cell density coupled with the swarming velocity of the bacterial colony (Butler et al., 2010).

S. enterica cells adapt their surface motility in response to the presence of direct or indirect DNA-damaging agents, by sensing these compounds through the so-called SOS response (Irazoki et al., 2016b). Among the consequences of SOS system activation is an increase in RecA protein concentration within the cells. RecA is both the main bacterial recombinase and the DNA-damage sensor of the SOS system (Cox, 2008; Little et al., 1980). An increase in RecA during the SOS response leads to an impaired swarming ability, via the titration of the CheW protein (Irazoki et al., 2016a, 2016b).

The CheW protein, together with other components of the chemotaxis pathway, plays a key role in swarming ability (Burkart et al., 1998; Mariconda et al., 2006). As the chemoreceptor adaptor, CheW couples transmembrane methyl-accepting chemoreceptor protein trimers of dimers to the histidine kinase CheA (Boukhvalova et al., 2002; Sourjik and Wingreen, 2012). Stabilized by CheW and CheA hexagonal rings, these signaling complexes aggregate at the cell poles to form the large chemoreceptor arrays that are essential for the surface motility of temperate swarmers (Cardozo et al., 2010; Santos et al., 2014). An increase in intracellular RecA levels due to SOS response activation hijacks CheW, thus preventing stabilization of the chemoreceptor cluster at the cell poles and impairing swarming motility (Irazoki et al., 2016a, 2016b).

Increases in bacterial resistance to antimicrobials have compromised the clinical utility of major chemotherapeutic antimicrobial agents. Other factors compromising the efficacy of these drugs are the administration of sub-optimal doses and poor pharmacokinetics, due, for example, to inefficient tissue penetration. To explore the possible inhibitory effect of antimicrobial compounds on both swarming motility and the transient acquisition of multidrug resistance, we analyzed the swarming ability and increased antibiotic resistance phenotype of *S. enterica* in experiments conducted using sub-lethal concentrations of several antimicrobial compounds differing in their mechanisms of action. We also examined the chemoreceptor array assembly and flagellation of antibiotic-treated cells. Our results

demonstrate that some antimicrobial agents, alone or in combination with others not affecting cell motility, prompt not only swarming inhibition but also the abolishment of transient multidrug resistance.

2. Materials and Methods

2.1 Bacterial strains and growth conditions

Salmonella enterica sv. Typhimurium ATCC14028 wild-type and *ΔrecA* strains (Medina-Ruiz et al., 2010) and their *ΔcheR* mutant derivatives carrying plasmid pUA1127, harboring an *eYFP::cheR* fusion (Mayola et al., 2014), were used in this work.

Except when indicated, the cells were grown at 37°C on either Luria–Bertani (LB) plates containing 1.7% agar or on swarming plates (1% tryptone, 0.5% yeast extract, 0.5% NaCl, 0.5% D-(+)-glucose, and 0.5% agar). These conditions are referred to in the following as non-swarming and swarming conditions, respectively.

When necessary, the plate media were supplemented with a sub-lethal concentration of amikacin (4 mg/L), cefotaxime (1.6 mg/L), chloramphenicol (2 mg/L), colistin (2.5 mg/L), tetracycline (4 mg/L), kanamycin (5 mg/L), ciprofloxacin (0.065 mg/L), and/or trimethoprim (1 mg/L).

2.2 MIC and sub-lethal concentration determination

The minimal inhibitory concentrations (MICs) of the antibiotics used in this work for *S. enterica* ATCC14028 *ΔcheR* pUA1127 containing the *eYFP::cheR* fusion were determined by the standard microdilution method using tryptone broth (TB), as described previously (Wiegand et al., 2008). Strain growth ability was tested in microtiter plate wells containing two fold-serial dilutions of the antibiotic in TB (Table S1, Supplementary material). The sub-lethal concentration was defined as the antimicrobial concentration inhibiting growth by 20–30% compared to non-treated cells. Bacterial growth reduction was determined by measuring the optical density of bacterial cultures at 600 nm (Figure S1, Supplementary material) as described previously (Kohanski et al., 2010). In all cases, the established sub-lethal concentration reduced *S. enterica* viability by ~30% after 2 h of treatment.

2.3 Swarming motility assays

The swarming phenotype of wild-type *S. enterica* or its Δ*cheR* derivative was tested in the presence of the above-listed antimicrobial agents. In all cases, the observed swarming phenotype of the two strains was exactly the same. When needed, the *S.enterica* Δ*recA* strain, which is unable to swarm (Medina-Ruiz et al., 2010), was also used as a non-motile control. Swarming assays were carried out as described previously (Gómez-Gómez et al., 2007; Irazoki et al., 2016b; Mayola et al., 2014). Briefly, a single colony picked using a sterile toothpick from bacterial strains grown on LB plates was inoculated in the center of a freshly prepared swarming plate containing medium supplemented with the antimicrobial compound(s) of interest. The plates were incubated overnight at 37°C for 14 h and then imaged using a ChemiDocTM XRS+ system (Bio-Rad).

2.4 Chemoreceptor clustering assay

Chemoreceptor clustering assays were performed using *S. enterica ΔcheR* carrying plasmid pUA1127 containing the *eYFP::cheR* fusion under the control of an IPTG-inducible promoter (Ptac) (Mayola et al., 2014). The fusion protein served as a reporter of polar cluster localization (Cardozo et al., 2010; Kentner et al., 2006; Santos et al., 2014). The clustering experiments were carried out as described previously (Mayola et al., 2014). Briefly, the bacterial cells were grown on swarming plates supplemented with the sub-lethal concentration of the corresponding antimicrobial agent. After 14 h of incubation at 37ºC, the cultures were sampled as described previously (Kim and Surette, 2004), using ice-cold tethering buffer (10 mM potassium-phosphate pH 7, 67 mM NaCl, 10 mM Na-lactate, 0.1 mM EDTA, and 0.001 mM L-methionine). The cells in the samples were harvested by 15 min of low-speed (5000 g) centrifugation, washed once, resuspended in 20–100 μL of tethering buffer, and applied onto thin 1% agarose pads.

Fluorescence microscopy was performed using a Zeiss AxioImager M2 microscope (Carl Zeiss Microscopy) equipped with a Zeiss AxioCam MRm monochrome camera (Carl Zeiss Microscopy) and a filter set for enhanced yellow fluorescent protein (eYFP; excitation BP500/25; beam splitter FT 515; emission BP535/30). Cell fields were photographed and the number of clusters then quantified using ImageJ software (National Institutes of Health). At least 500 cells were visually inspected. Each experiment was performed at least in triplicate using independent cultures, resulting in the examination of a minimum of 1500 cells from each growth condition.

2.5 ELISA for RecA quantification

Samples for RecA quantification were obtained by recovering the cells directly from the colony edge of the corresponding swarming plates, following the same procedure and conditions as described above. In this case, the cells were resuspended in sonication buffer (PBS 1×, cOmplete mini EDTA-free tablets, pH 7.3) and whole-cell lysates were obtained by sonication (two 30-s pulses of 20% amplitude, Digital sonifierR 450, Branson). The supernatants were recovered, centrifuged (12000 g for 10 min), and the total protein concentration of each sample was then quantified using the Bradford method (protein reagent DyeR, BioRad). A standard curve was generated using bovine serum albumin (range: 1.5–200 μg/mL).

Pre-treated 96-well microtiter plates (Nunc-Immunoplate F96 Maxisorp, Nunc) were coated with serial dilutions of the whole-cell lysates. The RecA concentration in the polar cluster assays was determined by ELISA, as previously described (Irazoki et al., 2016b). A standard quantification curve was obtained using purified RecA protein. A rabbit anti-RecA antibody (ab63797, Abcam) served as the primary antibody, and a goat anti-rabbit-IgG horseradish-peroxidase-conjugated antibody (IgG, IgM, IgA, polyclonal antibody, YO Proteins) as the secondary antibody. The BD OptEIA TMB substrate reagent kit (BD Biosciences) was used as the developing solution. The plate reactions were read at 650 nm using a multi-plate reader (Sunrise, Tecan).

2.6 Disc-diffusion sensitivity test

To evaluate the antibiotic susceptibilities of the cells, LB non-swarming or swarming plates supplemented when needed with the corresponding antibiotic were surface-inoculated using a sterile swab with either *S. enterica* Δ*cheR* pUA1127 or *S. enterica* Δ*cheR* Δ*recA* pUA1127 freshly grown on LB plates. Antimicrobial susceptibility test discs (amikacin, 30 µg; trimethoprim, 25 µg; and tetracycline, 30 µg; Pronadisa) were placed in the middle of the inoculated plates. After a 14 h incubation at 37°C, the size of the bacterial growth inhibition zone was determined based on photographic images of the plates (ChemiDoc XRS + system, Bio-Rad). Each of these experiments was performed at least in triplicate. The images shown in the figures are representative of the entire image set.

2.7 Fluorescence flagellar labeling

The flagella were labeled as described previously (Turner et al., 2010) but with modifications. Cells from the corresponding swarming plates were collected in 0.5 mL of tethering buffer (described above), washed three times by centrifugation (1500 × g for 10 min) at 15ºC, and resuspended first in 1 mL and then in 0.5 mL of PBS. To label the flagella, 100 µL of PBS, 25 µL of 1 M $NaHCO_3$, and 0.5 µL of Cy3b dye (0.1 mg/µL) were added to the cell suspension. After a 1-h incubation at room temperature in the dark with gyrorotation, the labeled cells were washed three times with 1 mL of PBS before they were resuspended in 0.2–0.5 mL of tethering buffer.

To visualize the cells, they were immobilized and fixed on the same focal plane using thin 1% agarose pads in tethering buffer. Fluorescence microscopy was performed using a Zeiss AxioImager M2 microscope (Carl Zeiss Microscopy) equipped with a Zeiss AxioCam MRm monochrome camera (Carl Zeiss Microscopy) and a filter set for Cy3b protein (excitation BP 546/12; beam splitter FT 560; emission BP 575-640). All fluorescence images were obtained at a 1000× magnification under identical conditions. Each experiment was performed in triplicate using independent cultures. The images presented are representative of the entire image set. ImageJ software (National Institutes of Health) was used to prepare images for publication. In all cases, at least 100 cells were visually inspected. Each experiment was performed at least in triplicate using independent cultures, resulting in the examination of a minimum of 300 cells from each growth condition.

2.8 Statistical analysis

Data from the chemotaxis clustering assays and measurements of the RecA concentration were analyzed using a one-way analysis of the variance (ANOVA) with Prism (GraphPad). In all cases, the analyses were followed by Bonferroni multiple comparison post-hoc tests. A p-value < 0.01 was considered to indicate statistical significance. The error bars in each of the figures indicate the standard deviation.

3 Results

3.1 Swarming ability in the presence of sub-lethal concentrations of antimicrobial agents

The effect on swarming of sub-lethal concentrations of the following antimicrobial agents differing in their mechanisms of action was analyzed: (i) inhibitors of translation (chloramphenicol, tetracycline, and the aminoglycosides kanamycin and amikacin); (ii) an inhibitor of cell-wall synthesis (the cephalosporin cefotaxime); (iii) an inhibitor of DNA replication (the fluoroquinolone ciprofloxacin); (iv) a disruptor of the outer cell membrane (colistin); and (v) an inhibitor of thymidine synthesis (trimethoprim). Swarming plates containing sub-lethal concentrations of the corresponding antimicrobial agent were inoculated with *S. enterica* wild-type strain in the middle of the plate. In all cases, it was determined that the antimicrobial concentration used produces about 30% reduction in cell viability (see Materials and Methods section). In addition, the swarming ability of the wild-type strain and a *ΔrecA* mutant derivative, which is not able to swarm (Medina-Ruiz et al., 2010), were tested in the absence of any antimicrobial agent, as swarming and non-swarming controls, respectively (Figure 1A).

Our results indicated that the presence of kanamycin, amikacin, colistin, and tetracycline did not affect the swarming ability of *S. enterica* (Figure 1B); rather, the phenotype of the respective bacterial colonies was the same as that of wild-type non-treated cells (Figure 1A). By contrast, the addition of sub-lethal concentrations of cefotaxime, ciprofloxacin, trimethoprim, and chloramphenicol completely abolished swarming motility (Figure 1B). In these cases, the sub-lethal concentration of antimicrobial treatment gave rise to the same non-swarming phenotype as observed for non-treated *ΔrecA* cells (Figure 1A).

3.2 Identification of the antibiotic mediated swarming abolition mechanism

To identify the mechanism responsible for impaired swarming, the dynamics of chemosensory array assembly and the flagellation of swarming cells during antibiotic treatment were evaluated, since both are essential for the surface motility of *S. enterica* (Cardozo et al., 2010; Partridge and Harshey, 2013; Santos et al., 2014).

Chemosensory arrays were observed using a *S. enterica* strain expressing the *eYFP::cheR* fusion under the control of an IPTG-inducible promoter (Irazoki et al., 2016b; Mayola et al., 2014). This strain exhibits the same swarming phenotype as the wild-type strain. In concordance with their motility behavior, cells treated with the antimicrobials that allowed swarming (kanamycin, amikacin, tetracycline, and colistin) did not exhibit altered polar chemosensory array assembly (Figure 2B) compared to non-treated cells (Figure 2A). Consistent with this finding, there was no alteration in the RecA concentration in cells treated with the compounds that did not impair surface motility (Figure 2B).

However, among the agents that blocked swarming, treatment with cefotaxime, ciprofloxacin, and trimethoprim but not chloramphenicol induced a decrease in polar cluster assembly (Figure 2B). Furthermore, the induction of the SOS response mediated by cefotaxime, ciprofloxacin, and trimethoprim gave rise to an increase in the intracellular concentration of RecA (Figure 2B). The percentage of cells with polar clusters at the end of the corresponding treatment was similar to that observed in the *ΔrecA* strain (Figure 2A).

Previous reports showed that either the increase in the RecA concentration following SOS response induction by mitomycin-C treatment or the absence of this protein within *ΔrecA* mutant cells prevents polar chemosensory array formation due to CheW-RecA titration (Irazoki et al., 2016a; Mayola et al., 2014).

To visualize the flagella, the cells were directly labeled with Cy3b fluorescent dye, as previously described (Turner et al., 2010). As expected, treatment with the antimicrobial compounds that allowed swarming had no effect on cell flagellation, which was the same as in non-treated cells (data not shown). However, despite the inhibitory effect of cefotaxime, ciprofloxacin, and trimethoprim on swarming, cells treated with these compounds had the same flagellar phenotype as non-treated bacteria (data not shown). Only in cells treated with a sub-lethal concentration of chloramphenicol was there a clear reduction in the number of flagella (Figure 3), which explained the abolishment of swarming by this antibiotic.

3.3 Drug-resistance phenotype of swarming-impaired cells

S. enterica swarming cells exhibit elevated resistance to a variety of antibiotics, including those that target the cell envelope, protein translation, DNA replication, and transcription (Butler et al., 2010; Kim et al., 2003; Kim and Surette, 2003). To determine whether the antibiotics that impaired surface motility also inhibited the drug increased resistant phenotype, the antibiotic susceptibility of swarming cells to trimethoprim, amikacin, and tetracycline was tested in the presence or absence of sub-lethal concentrations of either non-swarming affecting or swarming impairing compounds (Figure 4A). These three antimicrobial agents (trimethoprim, amikacin and tetracycline) were selected based on the high level of resistance to them exhibited by cells grown on swarming plates (Figure 4C) as well as their different modes of action (Brogden et al., 1982; Chopra et al., 1992; Davis, 1987).

Disc-diffusion sensitivity tests revealed that the increased-resistance phenotype was preserved in the presence of a sub-lethal concentration of kanamycin, which did not affect swarming behavior (Figure 4A). In this case, kanamycin-treated cells had an increased resistance to trimethoprim, amikacin, and tetracycline when growing on swarming plates, as occurred in the absence of treatment (Figure 4B). The same was observed when, instead of kanamycin, sub-lethal concentrations of either amikacin, colistin, or tetracycline were used (data not shown). These results confirmed that compounds unable to inhibit swarming also did not affect the increased swarming-mediated drug resistance phenotype.

By contrast, this increased antibiotic resistance was dramatically abolished in *S. enterica* wild-type strain treated with a sub-lethal concentration of cefotaxime when growing on swarming plates (Figure 4B). Likewise, the *recA* defective mutant, which is unable to swarm (Medina-Ruiz et al., 2010), also did not increase its resistance sensitivity to trimethoprim, amikacin, and tetracycline when growing under swarming conditions (Figures 4A). Furthermore, the increased drug-resistance phenotype was also inhibited by other antimicrobials that hindered swarming as ciprofloxacin, trimethoprim, and chloramphenicol (data not shown).

Together, these data indicated that the restoration of antimicrobial sensitivity is directly associated with the absence of swarming and not specifically with the inhibition of chemosensory array assembly (induced by cefotaxime, ciprofloxacin, or trimethoprim) or the reduction in the number of flagella (chloramphenicol).

3.4 Synergistic effect of combined antibiotic treatment

Combination antibiotic therapy takes advantage of possible synergistic effects between antibiotics (Tamma et al., 2012; Tängdén, 2014). For this reason, and considering the above results, we asked whether compounds that impaired swarming and hindered the increased drug-resistance phenotype maintained their effects when provided together with antimicrobials that did not alter motility. Thus, the cells were treated with sub-lethal concentrations of cefotaxime in combination with sub-lethal treatments of either kanamycin or colistin. Swarming ability, chemosensory cluster assembly, and the increased resistance phenotype were then examined.

As shown in Figures 5 and 6, combined treatment with sub-lethal concentration of the antimicrobial agents yielded the same results as obtained with cefotaxime alone (Figures 1, 2, and 4). Thus, in all cases, cells treated with cefotaxime and colistin or cefotaxime and kanamycin were unable to swarm (Figure 5A). In both cases, the number of cells with assembled polar chemosensory arrays was reduced (Figure 5B), and the swarming-mediated increased resistance phenotype abolished (Figure 6). The same results were obtained when, instead of kanamycin or colistin, other non-swarming-impairing compounds, such as tetracycline or amikacin, were used in the combined treatment or when cefotaxime was replaced by other swarming-impairing compounds, such as trimethoprim or ciprofloxacin (data not shown).

Taken together these observations showed that the presence of an antibiotic with no effect on swarming (kanamycin, amikacin, colistin, or tetracycline) did not alter the effects prompted by a swarming-impairing agent (cefotaxime, ciprofloxacin, or trimethoprim).

4. Discussion

The results reported herein demonstrate that sub-lethal concentrations of chloramphenicol, cefotaxime, ciprofloxacin, and trimethoprim abolish the swarming ability of *S. enterica* (Figure 1B). They also provide evidence for two different mechanisms associated with the loss of surface motility. The first is the effect of cefotaxime, ciprofloxacin, and trimethoprim, which caused a defect in chemosensory array assembly (Figure 2B). As reported in a previous study (Laureti et al., 2013), sub-lethal concentrations of these drugs induce the SOS response, in turn prompting an increase in the RecA concentration (Figure 2B). It has been described that an increase of RecA within the cell impairs polar chemoreceptor array assembly by the RecA-mediated titration of CheW (Irazoki et al., 2016a, 2016b).

The second mechanism is that promoted by chloramphenicol, which reduces the flagellation of *Salmonella* (Figure 3) without affecting polar chemoreceptor assembly (Figure 2B). In a previous report, chloramphenicol and tetracycline caused the defective motility of some *S.*

enterica multidrug resistant clinical isolates, via a reduction in flagellar number (Brunelle et al., 2014). However, under the conditions of our experiments using *S. enterica* ATCC14028 swarming cells, this effect was observed in bacteria grown on plates containing a sub-lethal concentration of chloramphenicol but not of tetracycline, which, unlike chloramphenicol, did not abolish swarming motility (Figure 1B). Further work is needed to elucidate the molecular mechanisms associated with the flagellar decrease induced by chloramphenicol treatment.

The transient multidrug resistance phenotype exhibited by swarming cells is well-established (Butler et al., 2010; Kim et al., 2003; Kim and Surette, 2003). Our data are the first to demonstrate that the presence of sub-lethal concentration of an antimicrobial that blocks swarming (such as cefotaxime) also abolishes the increased swarming-mediated drug resistance to amikacin, tetracycline, and trimethoprim of *S. enterica* cells growing on swarming plates (Figure 4A). These antimicrobial agents present different modes of action: amikacin binds to the 30S ribosomal sub-unit, blocking mRNA translation (Davis BD 1987); tetracycline also inhibits bacterial protein synthesis, but by preventing the association of aminoacyl-tRNA with the bacterial ribosome (Chopra I Hawkey P Hinton M, 1992); trimethoprim exerts antimicrobial activity by blocking the production of tetrahydrofolate, the active form of folic acid (Gleckman et al.). These differences indicate that the inhibition of increased drug resistance is not associated with the abolition of a specific antimicrobial resistance mechanism but with the multidrug resistance phenotype of swarming cells.

It should also be noted that, the results reported herein pointed out that *S. enterica ΔrecA*, which is unable to swarm (Medina-Ruiz et al., 2010), displays the same antibiotic susceptibility to trimethoprim, amikacin ,and tetracycline when grown under swarming or non-swarming conditions (Figure 4B). This finding confirms that it is the ability of the bacterial colony to swarm, and not the cellular physiological changes associated with swarming growing conditions or the presence of sub-lethal concentration of swarming-impairing compounds, that accounts for the increased multidrug resistance of swarming cells.

The recent emergence of antibiotic resistance has greatly limited the therapeutic options available for treating bacterial pathogens, especially those that are multidrug-resistant. In this context, the combination therapy can offer a strategy for the treatment of severe bacterial infections if the synergistic effect of two or more antimicrobial agents in combination is greater than the sum of their individual activities (Doern, 2014). It is therefore of note that in our study the abolition of swarming-mediated multidrug resistance due to the presence of a swarming-impairing compound (such as cefotaxime) was maintained even when the latter drug was used together with antimicrobials that do not affect surface motility (such as kanamycin or colistin) (Figure 6).

The relationship between swarming and bacterial virulence involves, in addition to the multidrug resistant phenotype, host surface colonization and the increased expression of virulence factors (Lai et al., 2009; Medina-Ruiz et al., 2010; Overhage et al., 2008). In fact, mutants unable to swarm are usually attenuated and often display a reduced invasiveness that can be crucial during the first steps of bacterial infection (Burall et al., 2004; Butler and Camilli, 2004; Dons et al., 2004; Medina-Ruiz et al., 2010; Stecher et al., 2004; Terry et al., 2005). Accordingly, treatment with swarming-impairing antimicrobials, alone or in

combination with others, may abolish not only the ability to swarm but also the transient multidrug resistance associated with this motility.

However, swarming-impairing antibiotics may also cause unwanted effects. For instance, although an increase in the RecA concentration reduces the invasiveness of *S. enterica* (Medina-Ruiz et al., 2010), activation of the SOS response by some antimicrobials increases mutagenesis and may lead to the acquisition of antibiotic resistance (Cirz and Romesberg, 2007; Petrosino et al., 2009). In the case of chloramphenicol, the induced decrease in flagellation was shown to be associated with an increase in the virulence of *S. enterica* (Brunelle et al., 2014). Therefore, the identification of compounds able to abolish swarming but not exhibiting non-desirable effects, such as the induction of SOS-mediated mutagenesis or an enhancement of virulence, would likely improve the treatment of bacterial infections.

Taken together, our study showed that compounds able to inhibit swarming motility also abolish the transient multidrug-resistant phenotype. Thus, approaches that promote the inhibition of surface motility may result in novel strategies to increase the effectiveness of antibiotic treatments targeting swarming-associated bacterial host colonization.

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Figure legends

Figure 1. (A) Swarming ability of *S. enterica* wild-type and a *ΔrecA* mutant derivative grown on swarming-plates **(B)** Effect of sub-lethal concentrations (indicated in parentheses) of several antimicrobial agents on the swarming ability of the *S. enterica* wild-type strain. Representative images of a swarming bacterial colony are shown.

Figure 2. Evolution of the polar chemoreceptor cluster assembly (expressed as a percentage of the entire population) of the *S. enterica* wild-type strain grown in the absence **(A)** or presence **(B)** of sub-lethal concentrations of the indicated antibiotics. Cluster assembly was measured at several time points after the addition of each antibiotic (white dots, continuous line). The antibiotic concentration is indicated in parentheses. The RecA concentration in each sample, quantified by ELISA, is also shown (black dots, discontinuous line). *S. enterica ΔrecA* cells cultured in the absence of antibiotic were included as a non-swarming strain control. In all cases, the results are the mean of at least three independent imaging, swarming, or ELISA experiments. Error bars indicate the standard deviation. *$p < 0.01$ compared to the initial sample.

Figure 3. Representative fluorescence microscopy images of Cy3b-labeled wild-type *S. enterica* grown on swarming plates in the presence or absence of chloramphenicol (2 mg/L).

Figure 4. (A) Multidrug resistance phenotype of vegetative and swarming *S. enterica* wild-type cells growing in the presence of sub-lethal concentrations of antibiotics that either inhibit (cefotaxime) or have no effect on swarming (kanamycin). Disc-diffusion sensitivity tests were used to determine the sensitivity to trimethoprim (upper disc), amikacin (left disc), and tetracycline (right disc) of *S. enterica* wild-type cells grown on plates containing 1.7% (non-swarming plates) or 0.5% (swarming plates) agar supplemented with the corresponding antibiotic. **(B)** As controls, the same disc-diffusion sensitivity tests were performed using *S.enterica* wild-type and *ΔrecA* mutant derivative grown on plates lacking supplemented antimicrobial agents.

Figure 5. (A) Swarming ability of wild-type *S. enterica* treated with sub-lethal concentrations of kanamycin (5 mg/L) or colistin (2.5 mg/L) in combination with a sub-lethal concentration of cefotaxime (1.6 mg/L). Representative images of a swarming bacterial colony are shown for each treatment. As a control, the swarming ability of untreated cells is included. **(B)** Evolution of polar chemosensory cluster assembly (expressed as a percentage of the entire population) in *S. enterica* cultured in the presence of sub-lethal concentrations of both kanamycin and cefotaxime or colistin and cefotaxime. Error bars indicate the standard deviation. *$p < 0.01$ compared to the initial sample.

Figure 6. Disc-diffusion sensitivity tests comparing the trimethoprim (upper disc), amikacin (left disc), and tetracycline (right disc) resistance of *S. enterica cells* grown on plates containing 1.7% (non-swarming plates) or 0.5% (swarming plates) agar supplemented with a sub-lethal concentration of both kanamycin and cefotaxime **(A)** or both colistin and cefotaxime **(B).** Representative images of each condition are shown

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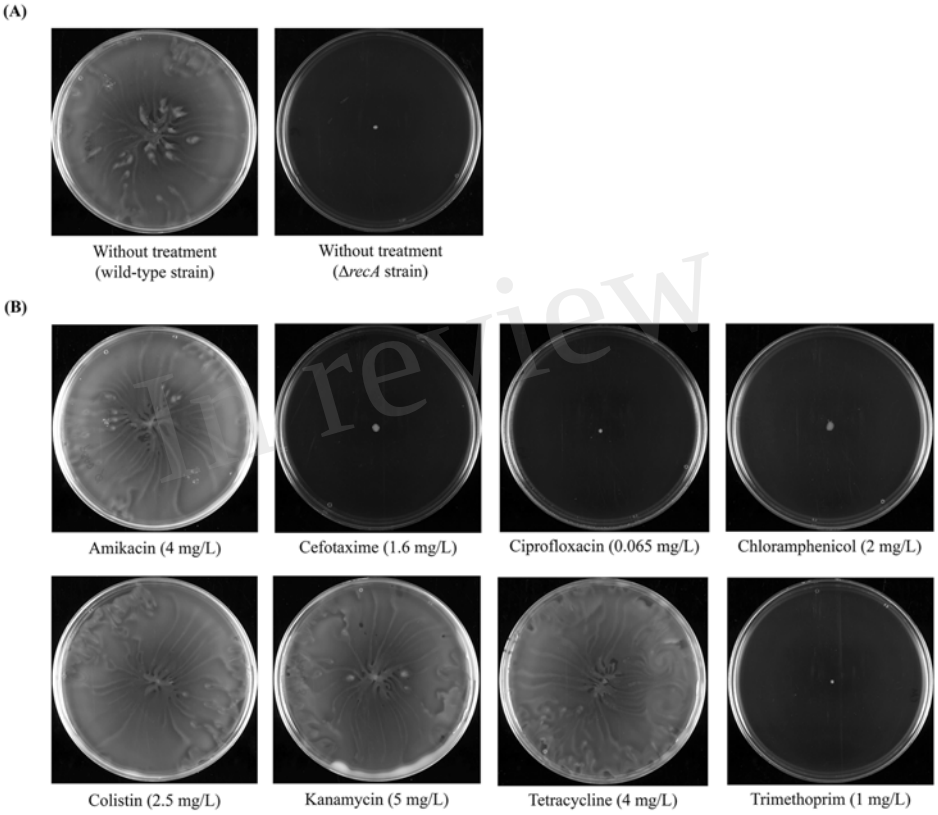

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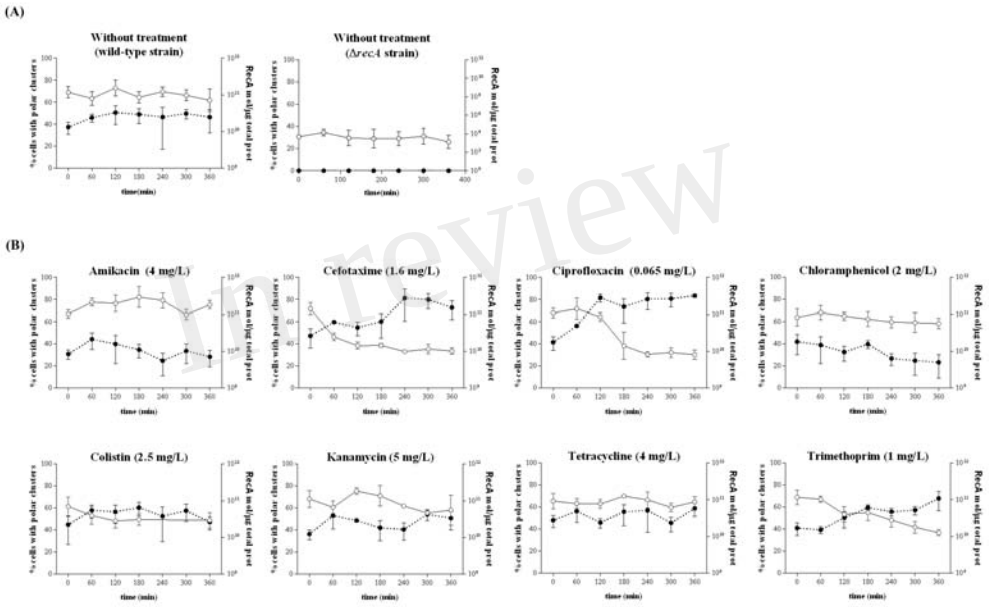

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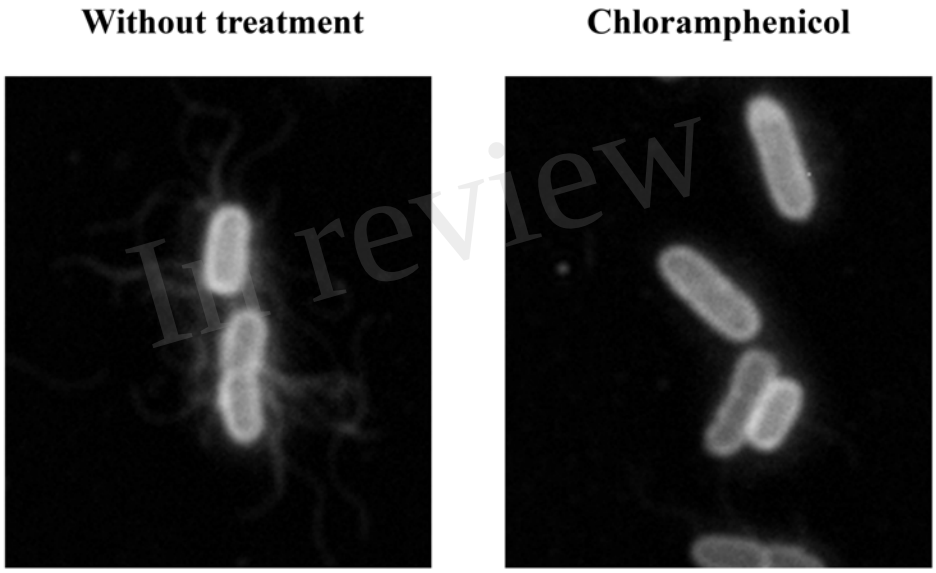

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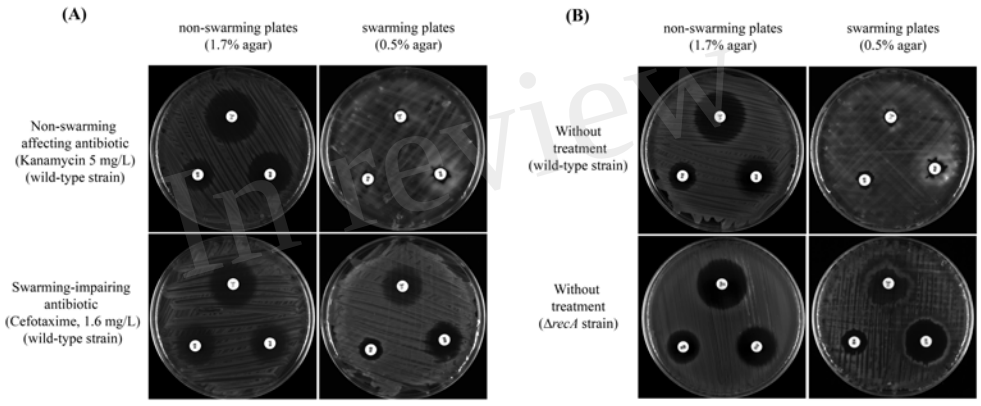

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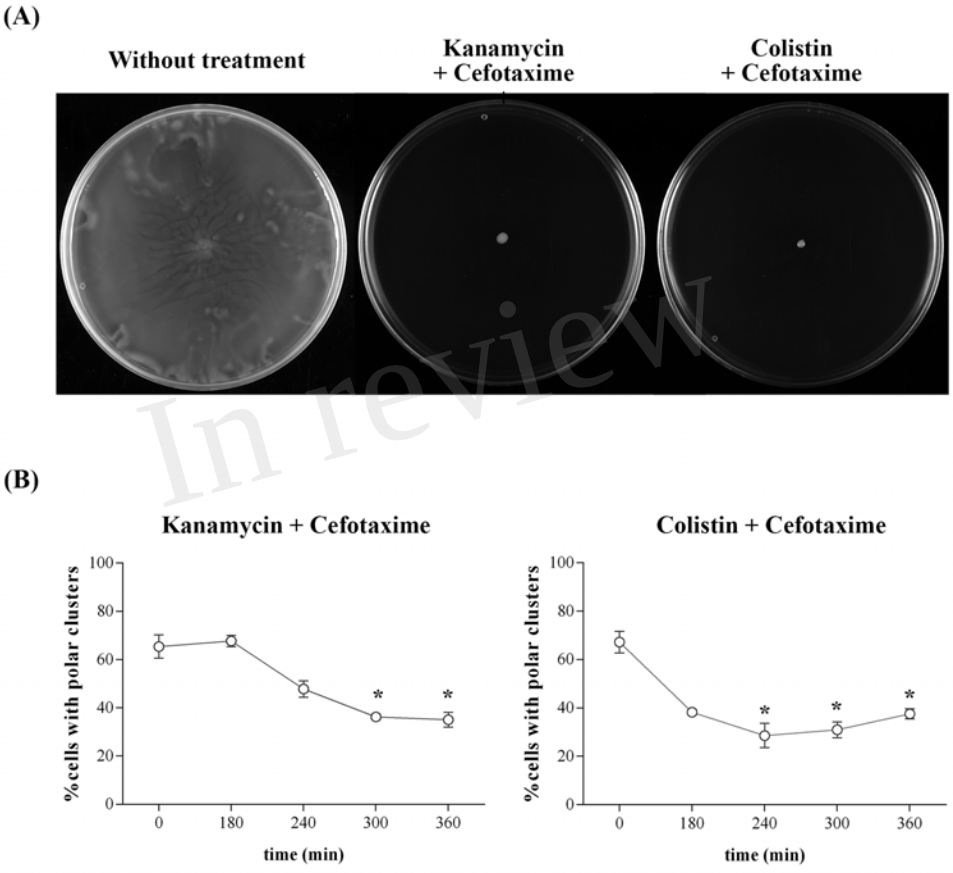

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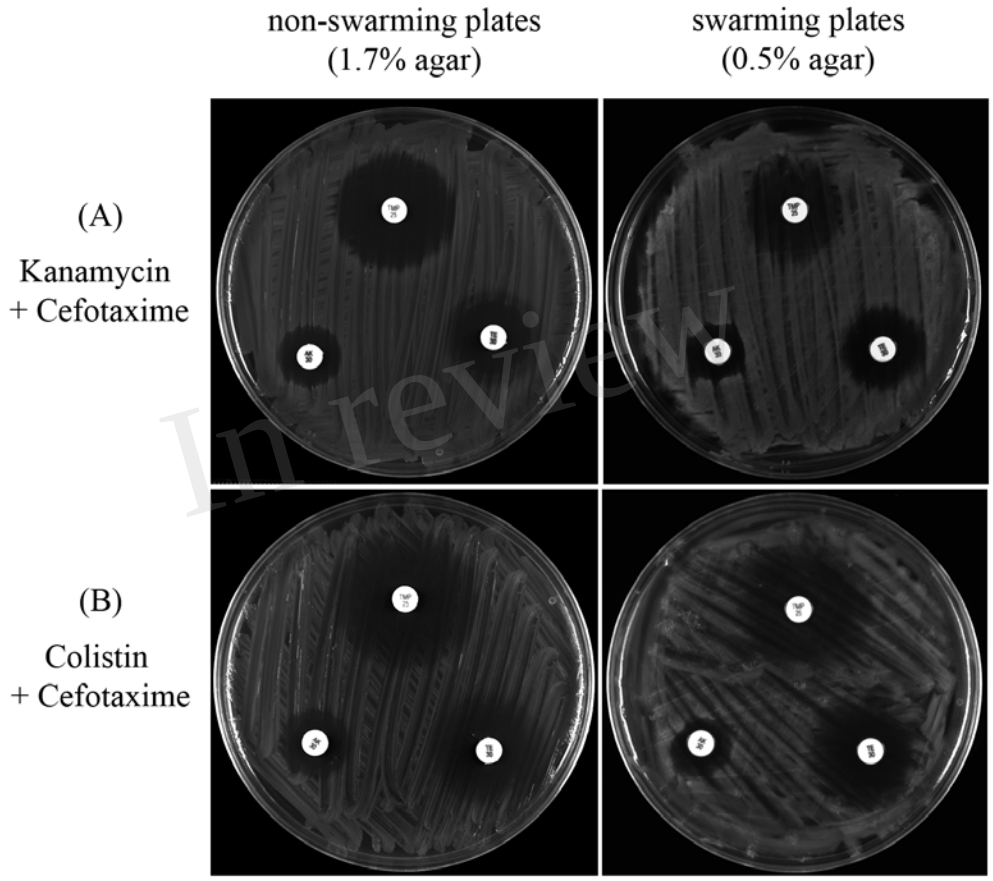

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