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RpoS and quorum sensing control expression and polar localization of *Vibrio cholerae* chemotaxis cluster III proteins *in vitro* and *in vivo*

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Summary

The diarrheal pathogen *Vibrio cholerae* contains 3 gene clusters that encode chemotaxis-related proteins, but only cluster II appears to be required for chemotaxis. Here, we present the first characterization of *V. cholerae's* "cluster III" chemotaxis system. We found that cluster III proteins assemble into foci at bacterial poles, like those formed by cluster II proteins, but the two systems assemble independently and do not colocalize. Cluster III proteins are expressed *in vitro* during stationary phase and in conjunction with growth arrest linked to carbon starvation. This expression, as well as expression *in vivo* in suckling rabbits, is dependent upon RpoS. *V. cholerae's* CAI-1 quorum sensing (QS) system is also required for cluster III expression in stationary phase and modulates its expression *in vivo*, but is not required for cluster III expression in response to carbon starvation. Surprisingly, even though the CAI-1 and AI-2 QS systems are thought to feed into the same signaling pathway, the AI-2 system inhibited cluster III gene expression, revealing that the outputs of the two QS systems are not always the same. The distinctions between genetic determinants of cluster III expression *in vitro* and *in vivo* highlight the distinctive nature of the *in vivo* environment.

Keywords

Vibrio cholerae; chemotaxis; RpoS; quorum sensing

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Introduction

One of the principal ways bacteria sense and respond to changing environmental conditions is by coupling chemotactic systems to motility, enabling them to bias their movement away from unfavorable chemical stimuli and towards favorable chemical compounds (Wadhams and Armitage, 2004; Sourjik and Armitage, 2010). Chemotactic apparatuses, which have been most fully characterized in *Escherichia coli*, are large, highly organized, multi-protein complexes that are generally associated with the bacterial inner membrane (Maddock and Shapiro, 1993; Sourjik and Berg, 2000). Typically, extracellular chemoeffectors are detected by the periplasmic ligand binding domains of transmembrane methyl-accepting chemotaxis proteins (MCPs). In response, MCPs modulate the activity of a phosphorelay pathway whose principal components are the cytoplasmic proteins CheW, CheA, and CheY. In *E. coli* (and many other organisms), phosphorylated CheY controls the flagellar switch complex that determines the direction of flagellar rotation, and thereby controls whether bacteria maintain or change their path of movement (Wadhams and Armitage, 2004; Sourjik and Armitage, 2010).

Notably, *E. coli* encodes only four MCPs and single copies of each of the other chemotaxis signaling proteins. In contrast, many other bacterial species encode far more MCPs and multiple homologues of chemotaxis proteins, which raises the possibility that they can sense a broader range of stimuli than *E. coli*, and/or respond to them in diverse ways (Szurmant and Ordal, 2004; Butler and Camilli, 2005; Hamer *et al.*, 2010). Indeed, studies in *M. xanthus*, which is non-flagellated but nonetheless encodes eight chemosensory operons, have revealed a variety of outputs for chemotactic signaling pathways, including flagellum-independent chemototaxis, motility, and developmental processes (Zusman *et al.*, 2007). Similarly, in the bacterium *Rhodospirillum centenum*, homologs of chemotaxis proteins have been found to mediate flagella biosynthesis and cyst development as well as classical chemotaxis (Berleman and Bauer, 2005). In other organisms, chemotaxis proteins have been found to regulate phototaxis, synthesis of Type IV pili, and several developmental processes (Zusman *et al.*, 2007).

Vibrio cholerae, the causative agent of the diarrheal disease cholera, is a highly motile, Gram-negative rod with a single polar flagellum. Its genome encodes 68 putative chemotaxis proteins, including multiple copies of the *che* genes and 45 MCP-like proteins (Heidelberg *et al.*, 2000). The *mcp* genes are scattered throughout the 2 chromosomes of the pathogen's genome, while the majority of the *che* genes are found in three clusters (I, II, and III) (Heidelberg *et al.*, 2000; Boin *et al.*, 2004). To date, only cluster II genes have been found to be required for chemotaxis in laboratory media (Gosink *et al.*, 2002; Hyakutake *et al.*, 2005). Additionally, only cluster II genes have been found to influence bacterial behavior in the suckling mouse model of infection, where they modulate the extent and localization of colonization (Butler and Camilli, 2004; Ringgaard *et al.*, 2011; Millet *et al.*, 2014). Both cluster I and cluster III genes have no known effect under these conditions (Millet *et al.*, 2014). In a recent study, cluster I chemotaxis proteins were observed to localize to polar and lateral membrane regions under microaerobic conditions (standing incubation). Localization was lost upon aeration (shaking incubation), suggesting that cluster I might be involved in sensing during oxygen deprivation (Hiremath *et al.*, 2014).

However, so far no function has been observed for cluster I proteins, and it is unknown whether they contribute to chemotaxis (under specific, yet to be identified growth conditions) or instead mediate a process other than motility. Neither localization nor function has yet been reported for cluster III proteins.

In *V. cholerae*, unlike in *E. coli*, localization of the principal chemotactic signaling arrays is not a stochastic process (Ringgaard *et al.*, 2011; Ringgaard *et al.*, 2014). Instead, two cluster II-encoded proteins, ParC and ParP, are required for reliable localization of cluster II proteins. In new born cells, cluster II chemotaxis proteins are targeted exclusively to the old (flagellated) cell pole, whereas in pre-divisional cells, ParC and ParP recruit cluster II proteins to the new pole as well, readying this site to become an old pole after cell division (Ringgaard *et al.*, 2011; Ringgaard *et al.*, 2014). Mislocalization of cluster II signaling complexes results in reduced chemotactic capacity and altered swimming behavior (Ringgaard *et al.*, 2011; Ringgaard *et al.*, 2014). The influence of ParC and ParP on localization of cluster I and cluster III chemotaxis proteins in *V. cholerae* has not been reported.

We initiated the current study with the goal of defining the subcellular localization and function of the products of *V. cholerae*'s cluster III chemotaxis-related genes. This cluster encodes all the usual components of chemotactic operons: two MCPs (one of which is predicted to be membrane associated and one that is predicted to be cytosolic), two *cheW*s, and one *cheA, cheY, cheR, cheB* and a putative *cheD* gene (Fig. 1A). Our efforts to link cluster III genes to chemotaxis under a variety of conditions were unsuccessful. However, we discovered that under typical laboratory culture conditions, expression of cluster III gene products is limited to stationary phase. Control of gene expression as bacteria transition into stationary phase is a complex process that is modulated by several factors, chief of which is the global stress regulator RpoS, an alternative sigma factor whose levels and activity are induced when nutrients are depleted and growth slows (reviewed in (Battesti *et al.*, 2011; Mika and Hengge, 2014)). RpoS can also be rapidly induced in growing cells in response to a variety of different stress conditions (reviewed in (Battesti *et al.*, 2011; Mika and Hengge, 2014)). Quorum sensing pathways, which respond to extracellular accumulation of autoinducers at high cell densities, also influence gene expression as cells enter into stationary phase (reviewed in (Ng and Bassler, 2009; Rutherford and Bassler, 2012)). *V. cholerae* releases two well characterized autoinducers, AI-2 and CAI-1, which are produced by the synthases LuxS and CqsA, respectively. Each autoinducer has its own receptor; however, AI-2 and CAI-I are thought to influence stationary phase gene expression via a common signaling pathway, in which the response regulator LuxO and the transcription regulator HapR play pivotal roles (reviewed in (Ng and Bassler, 2009; Rutherford and Bassler, 2012)).

We have described environmental as well as genetic factors that govern expression of *V. cholerae* cluster III proteins, as well as explored their subcellular distribution. As noted above, expression of cluster III proteins (unlike cluster II proteins, which are constitutively expressed) is induced by entry into stationary phase, and can also be induced by carbon starvation. Expression is positively regulated by RpoS (both in response to growth phase and carbon starvation) and CqsA (growth phase only), but unexpectedly appears to be repressed

by LuxS, countering the idea that these QS pathways have a single output. As with cluster II proteins, cluster III proteins form polar foci; however, they are not restricted to the old pole, consistent with their lack of a role in flagellum-mediated motility. Notably, we observed expression and polar localization of cluster III proteins during *V. cholerae* infection of infant rabbits. Localization, and most likely expression, of cluster III proteins *in vivo* required RpoS but was in part independent of CqsA. The distinct requirements for expression/localization of cluster III proteins *in vivo* and *in vitro* highlight the unusual physiologic state of *V. cholerae* during infection.

Results

Subcellular localization of chemotaxis cluster III proteins is growth phase dependent

We previously found that chemotaxis proteins encoded in *V. cholerae* chemotaxis cluster II localize to the cell pole in a cell cycle dependent manner (Ringgaard *et al.*, 2011; Ringgaard *et al.*, 2014). To investigate if proteins encoded in chemotaxis cluster III display a similar localization pattern, we compared the localization of ectopically expressed YFP-CheA3 and CFP-CheW3, translational fusions of two cluster III-encoded proteins and fluorescent proteins, with that of YFP-CheW1, a fluorescently tagged cluster II-encoded protein. In contrast to YFP-CheW1, which localized to the cell poles in both exponentially growing and stationary phase cells, we observed only a diffuse cytoplasmic signal from CFP-CheW3 and YFP-CheA3 in exponential phase cells (Fig. 1B). However, strikingly, in stationary phase cells, both cluster III-encoded proteins localized to the cell poles (Fig. 1B, right panels). These observations suggest that the polar localization of cluster III proteins depends on a factor that is only produced (or active) in stationary phase.

We also explored whether the polar localization of CFP-CheW3 was dependent on additional chemotaxis proteins. First, to test whether CheW3 localization required additional cluster III gene products, we imaged ectopically expressed CFP-CheW3 in SR29, a strain lacking chemotaxis cluster III. Polar CFP-CheW3 foci were not observed in stationary phase cells in this background (Fig 1C), suggesting that interactions between cluster III proteins are required for generating detectable CheW3 foci. Since MCPs are required for array formation in other organisms, it is likely to be the absence of the cluster III MCPs that leads to diffuse CFP-CheW3 localization in the cluster III mutant background. In contrast, when we imaged CFP-CheW3 in a strain lacking chemotaxis clusters I and II (SR36), we found that CFP-CheW3 localized to the poles (Fig. 1D). Thus, polar localization of cluster III proteins is independent of the other two chemotaxis clusters; there is no apparent crosstalk between cluster III localization and the ParC/ParP-mediated polar localization of cluster II proteins (Ringgaard *et al.*, 2011; Ringgaard *et al.*, 2014).

Consistent with the idea that cluster II and cluster III proteins form independent complexes, simultaneous visualization of cluster II and III proteins (in strain SR15) revealed that YFP-CheW1 and CFP-CheW3 did not always localize to the same pole (Fig. 1E, white arrowheads). Furthermore, even when both proteins were found at the same pole (Fig. 1E, yellow arrowheads), foci of YFP-CheW1 and CFP-CheW3 did not always perfectly co-localize. Thus, although cluster III proteins, like those of cluster II, appear to form

macromolecular signaling complexes at cell poles, there is no evidence for crosstalk between these complexes or any shared function.

Cluster III proteins are only expressed in stationary phase

To further explore the growth phase dependent localization of CheW3, we used a strain (SR9) that contains CFP-CheW3 expressed from its native chromosomal site under the control of its native promoter. When overnight cultures (late stationary phase) of SR9 were diluted into fresh LB medium, ~65% of stationary phase cells (at time 0) had a polar CFP-CheW3 focus (Fig. 2A, B). Afterwards, the percentage of cells with a CFP-CheW3 focus decreased as the cells started growing, until no cells were observed to possess CFP-CheW3 foci at the onset of exponential growth. As cultures approached their maximal density (OD600 ~ 3.5), polar CFP-CheW3 foci reappeared, and within one generation reached the starting level of ~65% of cells with polar CFP-CheW3 foci (Fig. 2B). A similar growth phase-dependent localization pattern was observed when cells were grown in M9 minimal medium supplemented with thiamine and glycerol. However, in minimal medium nearly 100% of cells possessed CFP-CheW3 foci in stationary phase (Fig. 2C and Fig. S1).

We also monitored CFP-CheW3 expression in different growth phases by western blotting with antisera to CFP (Fig. 2D). There was an excellent correlation between detection of CFP-CheW3 foci and of CFP-CheW3 by western. No CFP-CheW3 was detected in exponentially growing cells, and the protein became detectable when the culture reached stationary phase (OD600 ~ 3.6). We used expression of FlaA, the major *V. cholerae* flagellin, to control for the total protein in the samples from the different time points (Fig. 2D). Although there was somewhat less FlaA apparent at the early time points, the differences cannot account for the marked increase in CFP-CheW3 detected during stationary phase. Since cluster III genes are thought to be expressed as an operon, and since formation of CFP-CheW3 foci is dependent upon the presence of cluster III, this result suggests that additional proteins of cluster III are also induced as cultures become saturated. RNAseq analyses provided additional evidence that numerous cluster III genes, including *cheW3*, are more highly expressed in stationary phase than in exponential phase growth (Fig. 2E). Markedly more transcripts (5 – 35 fold more) for eight cluster III genes were detected in stationary phase RNA. In contrast, we observed only a minor increase in cluster II gene expression upon entry into stationary phase (Fig. 2E). Thus, the increase in CheW3 upon entry into stationary phase is at least in part due to changes in transcriptional regulation that is specific to cluster III chemotaxis genes.

RpoS and quorum sensing regulate expression of chemotaxis cluster III genes in stationary phase cells

Since the stationary phase sigma factor RpoS and quorum sensing pathways are key modulators of gene expression in stationary phase, CFP-CheW3 reporter strains lacking RpoS or auto inducer synthases CqsA (CAI-1 synthesis) or LuxS (AI-2 synthesis) were constructed to address whether these factors influence cluster III gene expression. In marked contrast to wild type stationary phase LB cultures, which had polar CFP-CheW3 foci in ~65% of cells, the *rpoS* and the *cqsA* mutants (strains SR71 and SR48, respectively) completely lacked CFP-CheW3 foci (Fig. 3A, B). However, a higher percentage of Δ*luxS*

cells (strain SR49) than wild type cells contained polar CFP-CheW3 foci (~90%), and foci were brighter than in the wild type background (Fig. S2). Thus, RpoS and CqsA are required for formation of CFP-CheW3 foci in stationary phase cells, while LuxS appears to inhibit focus formation. The Δ*cqsA*Δ*luxS* double mutant (strain SR47) completely lacked CFP-CheW3 foci, indicating that the CqsA-mediated phenotype is dominant over the LuxS phenotype regarding formation of CFP-CheW3 foci in stationary phase cultures.

We constructed a translational fusion between full-length CheW3 and *Renilla reniformis* luciferase (Rluc) expressed from the native *cheW3* locus to address if changes in expression of CheW3 (monitored with luminescence) could account for the differences in the frequency of CheW3 foci observed above. In these experiments, expression of *rluc-cheW3* in the Δ*rpoS*, Δ*cqsA*, Δ*luxS*, and Δ*cqsA*Δ*luxS* backgrounds was compared to that in the wild type, which was set at 1 (Fig. 3C). There was very little expression of Rluc-CheW3 observed in Δ*rpoS*, Δ*cqsA*, and Δ*cqsA*Δ*luxS* strains, suggesting that the absence of CFP-CheW3 foci in these background is due to lack of CheW3 expression. In contrast, there was a greater than tenfold increase in expression of Rluc-CheW3 in the Δ*luxS* mutant, suggesting that the increased frequency of focus formation and fluorescence intensity in this background are due to elevated levels of CFP-CheW3 and other cluster III proteins. Both the reduced reporter activity in the Δ*cqsA* and Δ*cqsA*Δ*luxS* strains and the elevated reporter activity in the Δ*luxS* strain could be restored to wild type levels by co-culture with wild type cells, presumably due to provision of autoinducers *in trans* (Fig. 3D). Collectively, these observations suggest that, in stationary phase cells, RpoS and CqsA (via CAI-1) promote expression of CheW3 (and likely other chemotaxis cluster III proteins), whereas LuxS (via AI-2) inhibits its expression. The opposite effects of LuxS and CqsA on expression of cluster III genes in stationary phase was unexpected, since their autoinducer products are both thought to feed into the same regulatory pathway, ultimately leading to dephosphorylation of LuxO and increased expression of HapR at high cell density (Ng and Bassler, 2009; Rutherford and Bassler, 2012).

To further decipher this regulatory process, we investigated cluster III expression and focus formation in a HapR-deficient strain. In a Δ*hapR* mutant (strain SR12), we did occasionally observe polar localization of CFP-CheW3 and cells appeared to have a more intense cytosolic CFP signal (Fig. 3A); however, the percentage of cells with foci was much reduced compared to wild type (Fig. 3B). Furthermore, expression of Rluc-CheW3 was much lower in the Δ*hapR* mutant than in the wild type, although significantly higher than that of the Δ*cqsA* and the Δ*cqsA*Δ*luxS* mutants (Fig. 3C), which is consistent with the microscopy data of CFP-CheW3 in the Δ*hapR* mutant. This finding, coupled with our earlier observations, suggests that cluster III genes are downstream of HapR in the *V. cholerae* quorum sensing pathway, and that the CqsA-synthesized CAI-1 is the dominant activator of HapR expression in these cultures. A more prominent role for CqsA than LuxS has also been observed in analyses of quorum-regulated biofilm formation (Zhu and Mekalanos, 2003). However, it is not clear why the LuxS-deficient strain shows enhanced expression of cluster III genes and production of cluster III foci. We note that cluster III foci were still detected in ~10% of stationary phase HapR-deficient cells (Fig. 3B), and that there is some HapR-independent expression of cluster III genes (Fig. 3C). Thus, it is possible that LuxS/

AI-2 exert a negative regulatory effect on a HapR-independent pathway, which enables elevated cluster III expression in the *luxS* mutant.

Carbon starvation induces expression and localization of chemotaxis cluster III proteins

Our observation that a higher percentage of cells possess CheW3 foci in minimal medium (~100%, Fig. 2C, S1) than in LB (~65%) (Fig. 2AB) suggested that starvation could also be a factor modulating expression of chemotaxis cluster III genes. The Rluc-CheW3 luciferase reporter strain was used to monitor CheW3 expression when cells were depleted for carbon sources (Fig. 4A). In these experiments, overnight cultures of a wild type strain harboring the *rluc-cheW3* reporter (SR76) were used to inoculate fresh MSR6 minimal medium supplemented with 0.01% to 0.4% (w/v) D-glucose. Cells were grown in microtiter plates and the OD600 and total luminescence of each well was measured as a function of time. A concentration of 0.4% glucose was sufficient for cells to grow into saturated stationary phase cultures, while at lower concentrations bacterial growth arrested at lower culture densities, presumably due to exhaustion of D-glucose (Fig. 4A). There was a clear correlation between the time when growth ceased and markedly increased expression of Rluc-CheW3 (Fig. 4A), such that elevated CheW3 expression was detected first in cultures with 0.01% glucose and last in cultures that contained 0.4% glucose (Fig 4A). Thus, growth arrest linked to carbon starvation is a potent inducer of CheW3 expression, even when cells are not grown to high density.

To further explore the relationship between CheW3 expression/focus formation and carbon metabolism/cell growth, we set up a carbon catabolite repression assay (Madigan, MT, Martinko, JM, Clark, 2000). Cells were grown in a very minimal medium (MSR6) supplemented with 0.04% w/v D-glucose and 0.4% w/v succinate. In these conditions, the cells initially only metabolize D-glucose; when it is used up, they arrest growth temporarily, then resume growth by metabolizing succinate. An overnight culture (late stationary phase) was diluted into fresh MSR6 medium, and growth and focus formation was monitored as in Fig. 2C (Fig. 4B-C). As observed in M9 minimal medium, 100% of stationary phase cells had a polar CFP-CheW3 focus in MSR6 medium. The percentage of cells with a CFP-CheW3 focus decreased as the cells progressed through lag phase, so that by the onset of exponential growth no cells with CFP-CheW3 foci were observed. The subsequent growth arrest (at OD~0.3), indicative of glucose depletion, was rapidly followed by an increase in the percentage of cells with CFP-CheW3 foci; within 20 minutes of arrest, 100% of cells had polar CFP-CheW3 foci (Fig. 4B-C). When cell growth resumed, presumably as cells started metabolizing succinate (at ~400 minutes), there was a concomitant decrease in the percentage of cells with CFP-CheW3 foci. Finally, as the culture entered stationary phase (after 500 minutes), the percentage of cells with CFP-CheW3 foci increased again to 100% (Fig. 4B-C). Together, these observations illustrate the remarkable correlation between growth arrest (caused either by carbon starvation or by culture saturation) and induction of expression and polar localization of *V. cholerae* chemotaxis cluster III proteins.

We used the Rluc-CheW3 based luminescence assay to test whether RpoS or CqsA are required for the expression of cluster III genes that follows growth arrest induced by carbon starvation (Fig. 4D). When wild type, Δ*cqsA*, and Δ*rpoS* strains were grown in MSR6

minimal medium supplemented with 0.04% w/v D-glucose (Fig 4D left, arrow), expression of CheW3 increased rapidly after growth ceased in the wild type and the Δ*cqsA* backgrounds (Fig. 4D, arrow). However, no luminescence was detected in the Δ*rpoS* background, indicating that RpoS, but not CqsA, is absolutely required for expression of cluster III proteins following growth arrest induced by carbon starvation. Thus, the requirements for expression and localization of cluster III proteins differ between stationary phase LB, where both RpoS and CqsA are necessary, and growth arrest due to carbon starvation, for which only RpoS is necessary (Fig. 3).

Since carbon starvation induces cluster III expression, we analyzed if a cluster III mutant would be out-competed by the wild type in competition experiments when cells were starved for carbon over longer periods. Wild type (*lacZ*+) and cluster III mutant (*lacZ*−) were co-inoculated 1:1 in MSR6 minimal supplemented with 0.04% w/v L-glucose (which induces growth arrest due to carbon limitation during exponential growth), cells were incubated over night, and the ratio between wild type and mutant was determined by plating on X-gal indicator plates. Additionally, the culture was back-diluted into three different growth conditions: MSR6 minimal supplemented with 0.04% w/v L-glucose, MSR6 minimal supplemented with 0.04% w/v L-glucose, and LB rich medium and incubated over night. This cycle continued for seven days and each day the ratio between wild-type and mutant was determined. We observed no effect of the absence of cluster III (Fig. S3) when cells were starved for carbon. Furthermore, when we performed chemotactic assays with stationary phase cells (expressing cluster III), we observed no chemotactic defect of cluster III mutants when compared to wild type. As expected, a cluster II mutant was outcompeted by wild type in chemotactic assays (Fig. S4).

Both RpoS and CqsA contribute to expression/polar localization of CheW3 during *V. cholerae* intestinal colonization

Infant rabbits infected with *V. cholerae* develop severe cholera-like diarrhea (Ritchie *et al.*, 2010), and we found it is possible to image the intracellular localization of fluorescently tagged *V. cholerae* proteins in cells collected from cecal fluid, in which they accumulate to densities of $>10^9$ cfu/ml. Rabbits were inoculated with the YFP-CheW1 and CFP-CheW3-producing strain SR15, which colonizes and causes disease equivalent to its wild type parental strain C6706 (Fig. S5, (Ritchie *et al.*, 2010)). Notably, nearly 90% of intestinal bacteria contained polar foci of YFP-CheW1 (a cluster II protein), and ~35% of cells had detectable polar foci of the cluster III protein CFP-CheW3 (Fig. 5A, 5B). These observations demonstrate that cluster III genes are expressed during infection, corroborating previous RNA-Seq and microarray based analyses (Nielsen *et al.*, 2006; Mandlik *et al.*, 2011), and show for the first time that it is possible to monitor the subcellular localization of bacterial proteins during infection. We also observed polar localization of cluster III proteins in homogenized tissue from the distal small intestine of infected rabbits (Fig. S6), although quantification of foci in these samples was not possible due the presence of autofluorescent tissue residues in these samples. However, when SR15 cells were taken from exponentially growing LB cultures and inoculated into filtered (cell-free) cecal fluid, CFP-CheW3 foci were not detected after 2hrs of growth at 37° (Fig. 5C). This observation suggests that *V. cholerae* survival, growth and passage through the small bowel may be required to induce

cluster III genes, rather than simply the presence of an inducing signal within cecal fluid. It is also noteworthy that cells continued to grow and divide during the 2hr observation period, suggesting that cecal fluid is not a nutrient-limited condition, and thus that *in vivo* expression of cluster III genes, unlike *in vitro* expression, may not be association with cessation of growth.

We used CFP-CheW3 foci as a marker to explore the *in vivo* requirements for expression of cluster III proteins. In particular, we tested whether RpoS and CqsA, which are required for stationary phase expression of cluster III genes *in vitro* (Fig. 3), are also required for *in vivo* expression/localization of CFP-CheW3. Mutants carrying a chromosomal *cfp-cheW3* reporter and lacking *rpoS* or *cqsA* (SR71 and SR48, respectively) colonized the rabbit intestine and induced cecal fluid accumulation equivalent to wild type *V. cholerae* (strain SR9) (Fig. 6A). However, we did not detect any CFP-CheW3 foci in RpoS-deficient cells taken from cecal fluid of infected rabbits (Fig. 6B,C). These observations indicate that *rpoS* is essential for CheW3 expression/focus formation *in vivo*, and confirm that expression of cluster III genes is not important for *V. cholerae* pathogenicity, as previously reported for infection of infant mice, where deletion of cluster III has no influence on colonization (Millet *et al.*, 2014). In contrast to *rpoS*, *cqsA* was not required for CFP-CheW3 expression/localization in cells from cecal fluid. However, the percentage of cells with foci was reduced compared to that of wild type cells (Fig. 6B, C), suggesting that CqsA also contributes to expression of cluster III *in vivo*. The different genetic requirements for CheW3 expression in the gut compared to *in vitro* cultures suggest that there may be multiple stimuli/pathways that can lead to cluster III induction.

Discussion

Many bacterial species encode several independent chemotaxis operons, suggesting that their chemotaxis pathways are much more complex than that of the model organism *E. coli*. However, the regulation and purpose of multiple chemotaxis systems has only been studied in a few bacterial species. We show here that the *in vitro* expression of *V. cholerae* chemotaxis cluster III proteins coincides with cessation of growth, either due to carbon starvation or to culture saturation, and is dependent in both circumstances upon RpoS. The CAI-1 and AI-2 quorum sensing pathways were also found to regulate cluster III gene expression, although in opposite direction, which counters prevailing dogma. Visualization of fluorescently tagged proteins revealed that cluster III genes are expressed in a subset of *V. cholerae in vivo*, potentially independently of growth cessation. As for cluster II chemotaxis proteins, foci of cluster III chemotaxis proteins were observed at cell poles, both *in vitro* and *in vivo*; however, the two systems appears to localize independently. Our data highlight the diversity of stimuli and regulatory processes that govern expression of these chemosensory proteins.

Our work provides the first demonstration that cluster III chemotaxis proteins, like those of clusters I and II, assemble into cellular foci, as well as the first visualization of chemotactic signaling arrays during infection. Cluster III foci form at bacterial poles; however, they do not precisely colocalize with cluster II foci and in some cells are instead at the opposite pole. Their distinction from the flagellum-associated cluster II foci suggests that this putative

chemosensory apparatus may have an output other than flagellar motility. Further supporting this idea, despite intensive efforts using cells grown under cluster III-inducing conditions, we have been unable to detect any chemotaxis-related phenotype associated with the presence or absence of cluster III genes. Diverse outputs from chemosensory pathways have been identified in other organisms that contain multiple chemotaxis gene clusters (Berleman and Bauer, 2005; Zusman *et al.*, 2007); however, additional work is needed to define the targets of *V. cholerae's* cluster III proteins.

Expression of cluster III genes is governed by a variety of regulatory processes. Previous microarray-based analyses of mRNA levels have demonstrated that expression of cluster III genes increases at stationary phase *in vitro* and during growth within ligated intestinal loops from adult rabbits, and that both HapR and RpoS contribute to this increase (Nielsen *et al.*, 2006). However, earlier analyses have not reported whether gene expression correlated with the presence of chemotactic proteins and protein complexes or was detectable during “uninduced” conditions, nor explored the influence of quorum sensing. Our analyses suggest that the dominant regulatory factor may be the alternative sigma factor RpoS, as it is required for expression under all conditions tested. However, the specific carbon-starvation signal that triggers the RpoS-dependent expression of cluster III remains to be elucidated. It is, however, likely that cluster III is part of the RpoS-mediated general stress response, which leads to general stress resistance of cells upon nutrient deprivation, stress or entry into stationary phase (Battesti *et al.*, 2011). During exponential growth RpoS translation is inhibited and RpoS protein is rapidly degraded, resulting in a very low pool of RpoS. The RpoS response is induced by a rapid increase in levels of RpoS and is regulated at both the transcriptional and translational levels, and through degradation, and regulation of RpoS activity.

Expression of RpoS is regulated by a variety of signals, including (at the transcriptional level) HapR (Joelsson *et al.*, 2007). Since the autoinducer synthase CqsA (whose product induces HapR expression) is also required for most or all expression of the cluster III gene CheW3 in stationary phase cultures, it is possible that the key role of CqsA (with respect to cluster III) is induction of RpoS. However, CqsA is not required for cluster III gene expression in response to carbon starvation, and it contributes to but is not essential for cluster III gene expression *in vivo*. The fact that CqsA modulates cluster III expression *in vivo* suggests that intraintestinal *V. cholerae* cell densities are sufficient to activate quorum sensing pathways, at least in a subset of cells, and that carbon starvation cannot be the only stimulus for *in vivo* induction of cluster III genes. Furthermore, since cecal fluid can support continued *V. cholerae* replication, it seems likely that stimuli not identified here (i.e., neither carbon starvation nor quorum sensing) also contribute to RpoS induction *in vivo*.

Given the role we have described for CqsA, as well as previously reported overlapping roles for CqsA and *V. cholerae's* second autoinducer synthase, LuxS (Ng and Bassler, 2009; Rutherford and Bassler, 2012), our observation that LuxS is a negative regulator of cluster III expression is unexpected. The products of both CqsA and LuxS are thought to signal through the same LuxO- and HapR-mediated pathway, although previous analyses indicate that CqsA provides a more powerful signal (Miller *et al.*, 2002; Joelsson *et al.*, 2007). However, our data suggests that LuxS also influences an additional regulatory pathway that

likely counteracts the HapR-dependent stimulus. One possibility is that LuxS influences post-transcriptional regulation of RpoS, which is known to be governed by a variety of sRNAs. The dominant phenotype of the *cqsA* mutation in the *cqsA luxS* double mutant might then indicate that such regulation is only effective after activation of RpoS transcription (e.g, via HapR). The fact that LuxS is thought to be an agent of interspecies, rather than intraspecies, quorum sensing (Federle and Bassler, 2003), also suggests that the presence of species other than *V. cholerae* will suppress expression of cluster III chemotaxis genes. Future studies exploring the process by which LuxS negatively regulates cluster III expression should provide a more complete understanding of the complexity of *V. cholerae* quorum sensing. Such studies might also enable identification of additional stimuli associated with expression of cluster III or provide clues as to the cellular role of cluster III signaling complexes.

Experimental Procedures

Growth conditions and media

V. cholerae, and *E. coli* were grown in LB media or on LB agar plates at 37°C containing antibiotics in the following concentrations: streptomycin 200 μg/ml; kanamycin 50 μg/ml; ampicillin 100 μg/ml; carbenicillin 50 μg/ml; chloramphenicol 20 μg/ml for *E. coli* and 5 μg/ml for *V. cholerae*. For experiments in minimal medium, *V. cholerae* was grown two different media; MSR6 minimal medium: (50 mM KH2PO4, 50 mM Na2HPO4, 7.5 mM (NH4)2SO4, 2 mM MgSO4, 0.1 mM CaCl2, 25 μM FeSO4) supplemented with varying concentrations of D-glucose and succinate as indicated in the figures and figure legends; M9 minimal medium supplemented with 0.2% glycerol, and 1 μg/mL thiamine.

Strains and plasmids

The strains and plasmids used in this study are listed in Table 1. Primers used are listed in Table 2. *E. coli* strains DH5αλ*pir* and SM10λ*pir* were used for cloning. *E. coli* strain SM10λ*pir* was used to transfer DNA into *V. cholerae* by conjugation (Miller and Mekalanos, 1988). Throughout this study all *V. cholerae* strains used were derived from the El Tor clinical isolate C6706. Construction of *V. cholerae* deletion or insertion mutants was performed with standard allele exchange techniques using derivatives of plasmid pCVD442 (Donnenberg and Kaper, 1991). Strain SR9 was created by insertion of *cfp-cheW3* on the chromosome replacing the *cheW3* at its native locus using plasmid pSR1011 in strain C6706. Strain SR15 was created by insertion of *yfp-cheW1* and *cfp-cheW3* on the chromosome and replacing the *cheW1* and *cheW3* at their native locus using plasmids pSR1010 and pSR1011 respectively in strain C6706. Strain SR29 was created by deleting the entire cluster III operon using plasmid pSR1158 in strain C6706. SR36 was created by consecutive deletion of chemotaxis clusters I and II in strain SR9 using plasmids pSR1157 and pSR1020 respectively. Strains SR47, SR48, and SR49 was created by insertion of *cfp-cheW3* on the chromosome replacing the cheW3 at its native locus using plasmid pSR1011 in strains MM883, MM893, and KSK1059 respectively. Strain SR71 was created by deletion of *rpoS* in strain C6706 using plasmid pCVD442-Δ*rpoS*. Strain SR71 was created by deletion of *hapR* in strain C6706 using plasmid pSR1217. Strains SR76, SR80, SR81, SR82, and SR83 were created by insertion of *rluc8-cheW3* on the chromosome replacing the

cheW3 at its native locus using plasmid pSR1213 in strains C6706, MM883, MM893, KSK1059, and SR71 respectively. Plasmid pSR1228 was constructed by PCR amplification of the *vca1094* (*cheW3*) gene using primers CheW3-III-1-cw / CheW3-III-2-ccw and chromosomal DNA from *V. cholerae* as template. The PCR product was digested with BsrGI and HincII and ligated into the equivalent sites of plasmid pMF391 resulting in plasmid pSR1228. Plasmid pSR1224 was constructed by PCR amplification of the *vca1095* (*cheA3*) gene using primers CheA3-III-cw/CheA3-III-ccw and chromosomal DNA from *V. cholerae* as template. The PCR product was digested with BsrGI and XbaI and ligated into the equivalent sites of plasmid pMF390 resulting in plasmid pSR1224. Plasmid pSR1011 was constructed by PCR amplification of the up- and down-stream regions of *vca1094* (*cheW3*) using primers CheW3-a/CheW3-bb and CheW3-cc/CheW3-d respectively and *V. cholerae* chromosomal DNA as template. In a third PCR reaction *cfp-vca1094* was amplified using primers CheW3-b/CheW3-c and plasmid pSR1228 as template. A fourth PCR was then performed using primers CheW3-a/CheW3-d and products from all three of the former PCR reactions as template. The resulting PCR product was digested with XbaI and inserted into the equivalent site in pCVD442 resulting in plasmid pSR1011.

Fluorescence and time-lapse microscopy

Fluorescence microscopy was carried out essentially as described in references (Ringgaard *et al.*, 2009; Ringgaard *et al.*, 2011); cells were mounted on 1% agarose pads in 20% LB on microscope slides. Microscopy was performed using a Zeiss Axioplan 2 microscope equipped with a 100x a-plan lens and Hamamatsu cooled CCD camera. Microscopy of CFP-CheW3 in strain SR29 was performed using a Nikon eclipse Ti inverted microscope equipped with a 100x lens and an Andor Zyla sCMOS cooled camera. Microscopy images were analyzed using ImageJ imaging software and the percentage of cells with polar foci was enumerated by hand using the cell counter plug-in for ImageJ.

To obtain a detailed profile for CFP-CheW3 localization during growth into stationary phase, we diluted 100 μL of an overnight culture (late stationary phase) into 25 mL fresh LB medium and followed growth by measuring optical density. Furthermore, each sample was analyzed by fluorescence microscopy and the percentage of cells with CFP-CheW3 cluster was counted. For microscopy at low optical densities 5 mL culture was harvested and resuspended in 5-50 μL PBS (depending on cell density) and immediately analyzed by fluorescence microscopy.

For the carbon catabolite repression assay, cells were grown in MSR6 medium supplemented with 0.04% w/v D-glucose and 0.4% w/v succinate. Due to catabolite repression, cells will initially only metabolize D-glucose, however, when D-glucose is used up they will adjust and start metabolizing succinate. 100 μL of an overnight culture from MSR6 medium was diluted into 25 mL fresh MSR6 medium. Growth was followed growth by measuring optical density. Furthermore, each sample was analyzed by fluorescence microscopy and the percentage of cells with a CFP-CheW3 cluster was counted.

Infection experiments of infant rabbits by *V. cholerae*

Infant rabbit infections were carried out as previously described (Ritchie *et al.*, 2010) with minor variation. Briefly, overnight cultures of *V. cholerae* were diluted 1:100 in LB and shaken at 37°C for 3 hours. The three-hour cultures were harvested by centrifugation and resuspeneded in 2.5% Sodium Bicarbonate (pH 9) at a final concentration of ~2×10^9 CFU/mL. Rabbits were treated with Cimetidine (50mg/kg, intraperitoneal injection) 3 hours prior to orogastric inoculation with 10^9 CFU of *V. cholerae*. Rabbits were monitored and sacrificed immediately following the onset of disease symptoms: diarrhea and staining of the ventral surface. Onset ranged from 14-20 hours. The rabbits that didn't develop symptoms were sacrificed by 24 hours. Following removal of the entire gastrointestinal tract at necropsy, cecal fluid was extracted using needle and syringe, and tissue homogenates were spread on LB agar plates with 200 μg/mL Streptomycin for enumeration of bacterial colonization.

Renilla luciferace assays

For single read stationary phase experiments 5mL LB medium was inoculated with a bacterial colony of the relevant strain. Cultures were incubated at 37°C over night (approximately 16 hours) with shaking. Next day 200 μL cells were sampled and added coelenterazine to a final concentration of 7.5 μM. The 200 μL cells were then added to a well in a 96 well microtiter plate. Samples were then incubated at ambient temperature for 30 minutes to allow for the luciferase generated luminescence signal to stabilize (Hatzios *et al.*, 2012). The total luminescence was then measured for each well with an integration time of 2 seconds using a SpectraMax L Luminescence Microplate Reader from Molecular Devices.

For time course and combined OD600/luminescence experiments, 10 μL cells were added to 1 mL LB with a final concentration of coelenterazine of 7.5 μM. Three times 200 μL cells for each strain were then added to a well in a 96 well microtiter plate. Again, samples were then incubated at ambient temperature for 30 minutes to allow for the luciferase generated luminescence signal to stabilize. Using a Promega GloMax®Multi Detection System with fluorescence and luminescence module the OD600 and the total luminescence measured over time for each well with an integration time of 2 seconds for the luminescence readings.

Western blotting to determine presence of CFP-CheW3

V. cholerae strain SR9 was inoculated in 5 mL LB and incubated overnight at 37°C shaking. 100 μL was then transferred to 50 mL of fresh LB medium at 37°C and shaking. OD600 was measured and samples taken for western blotting as a function of time. The amount of culture harvested and analyzed was normalized for OD600. Samples were then analyzed by SDS-PAGE and western blotting was performed using JL-8 anti-GFP antibodies, which also recognizes CFP. As a loading control samples were also analyzed by western blotting using anti bodies against the flagellar protein FlaA.

RNAseq experiment

RNA-seq was performed basically as described in (Livny *et al.*, 2014). RNA samples were generated from mid-exponential phase (O.D. 600 = ~0.4 – 0.6) and stationary phase (overnight) cultures of C6706 grown in LB. Libraries for each condition were prepared in duplicate from biological replica cultures.

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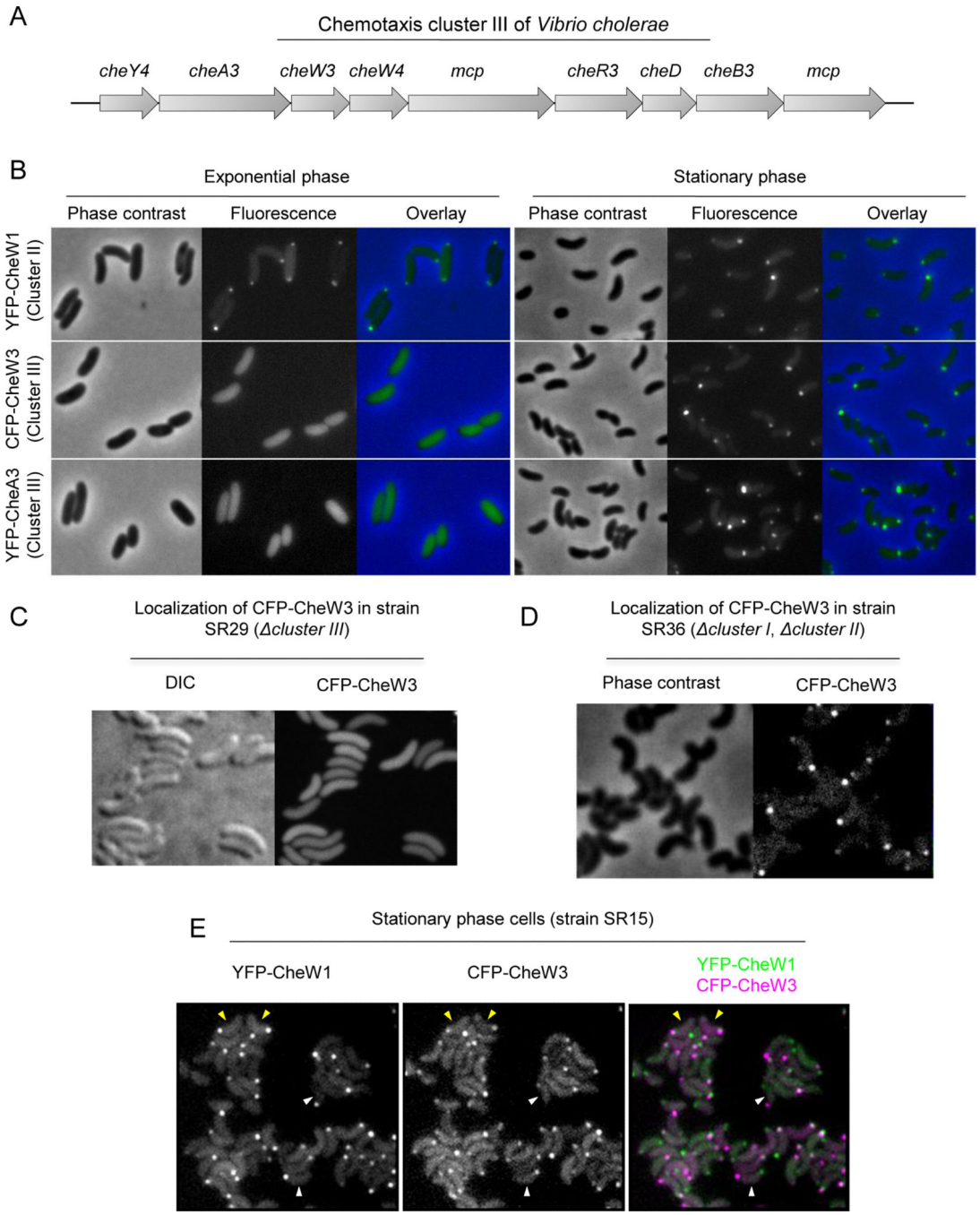

Figure 1. Growth phase dependent polar localization of chemotaxis cluster III proteins

(A) Schematic of chemotaxis cluster III. (B) Localization of ectopically expressed YFP-CheW1, CFP-CheW3, and YFP-CheA3 in cells from exponential (left) and stationary (right) phase cultures of wild type *V. cholerae*. (C, D) Localization of CFP-CheW3 in cluster III-deficient strain SR29 (C) and in SR36, a strain deleted for chemotaxis clusters I and II (D). Cells were grown to stationary phase. (E) Localization of YFP-CheW1 and CFP-CheW3 expressed from their respective native loci (in strain SR15) in stationary phase cells. Yellow arrowheads indicate cells with YFP-CheW1 and CFP-CheW3 localized to the same pole.

White arrowheads indicate cells where YFP-CheW1 and CFP-CheW3 localize to opposite poles.

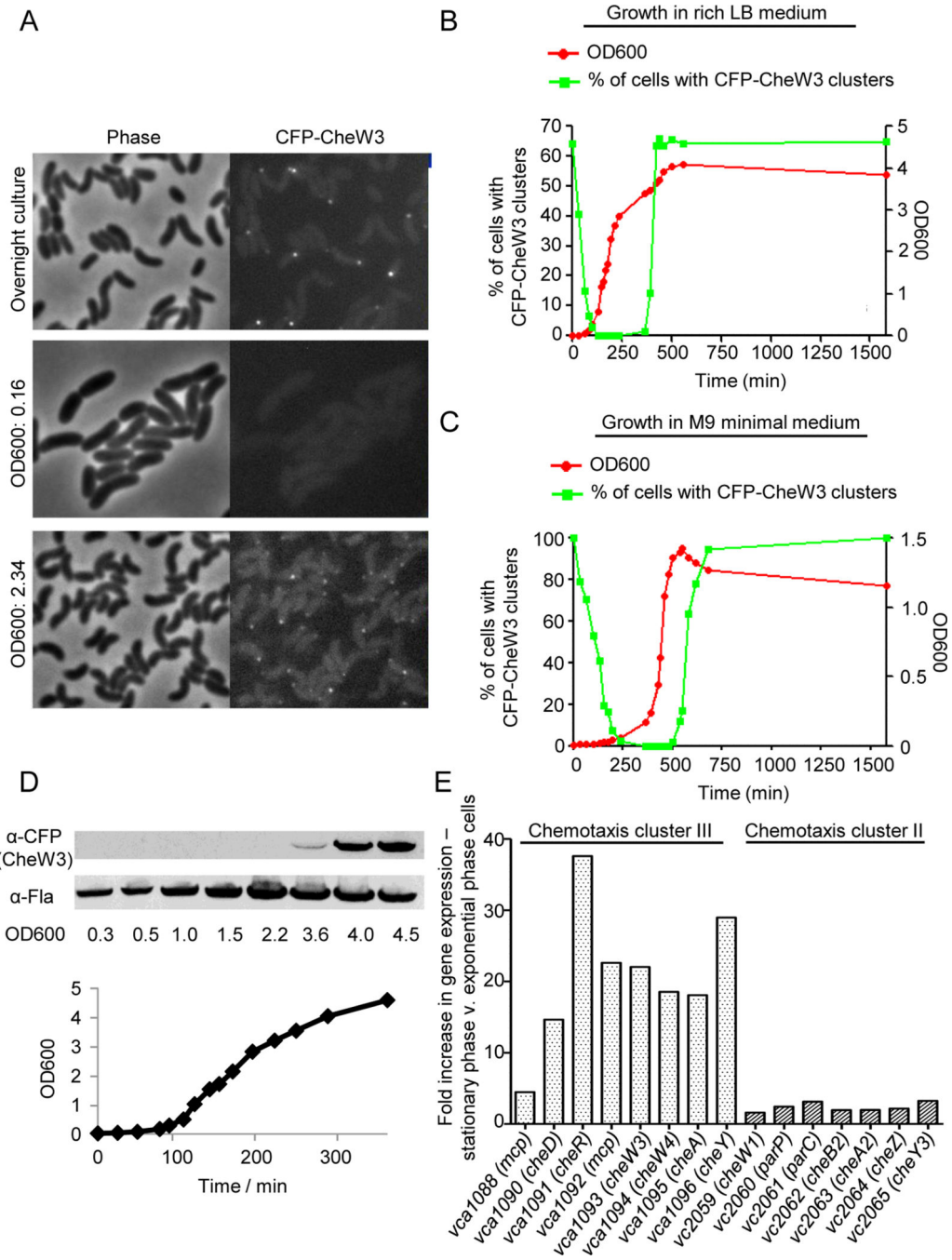

Figure 2. Correlation between stationary phase specific polar localization and expression of chemotaxis cluster III proteins

(A) Localization of CFP-CheW3 expressed from its native locus and promoter (in strain SR9) at different optical densities during growth in LB. (B) Growth curve (filled green squares) of strain SR9 in LB medium and the corresponding percentage of cells with CFP-CheW3 foci (filled red circles). (C) Growth curve (filled green squares) of strain SR9 in minimal M9 medium and the corresponding percentage of cells with CFP-CheW3 foci (filled red circle). See Figure S1 for micrographs. (D) Western blot against CFP-CheW3 and FlaA using anti-CFP and anti-FlaA specific antibodies on SR9 cultures from the indicated

optical densities. Samples were normalized and loaded with equal optical density. The corresponding growth curve is shown. (E) RNAseq experiment of the transcription levels of cluster II and III chemotaxis genes in stationary and exponentially growing cells. The Bar-graph shows the fold change in gene expression between stationary phase cells versus exponentially growing cells.

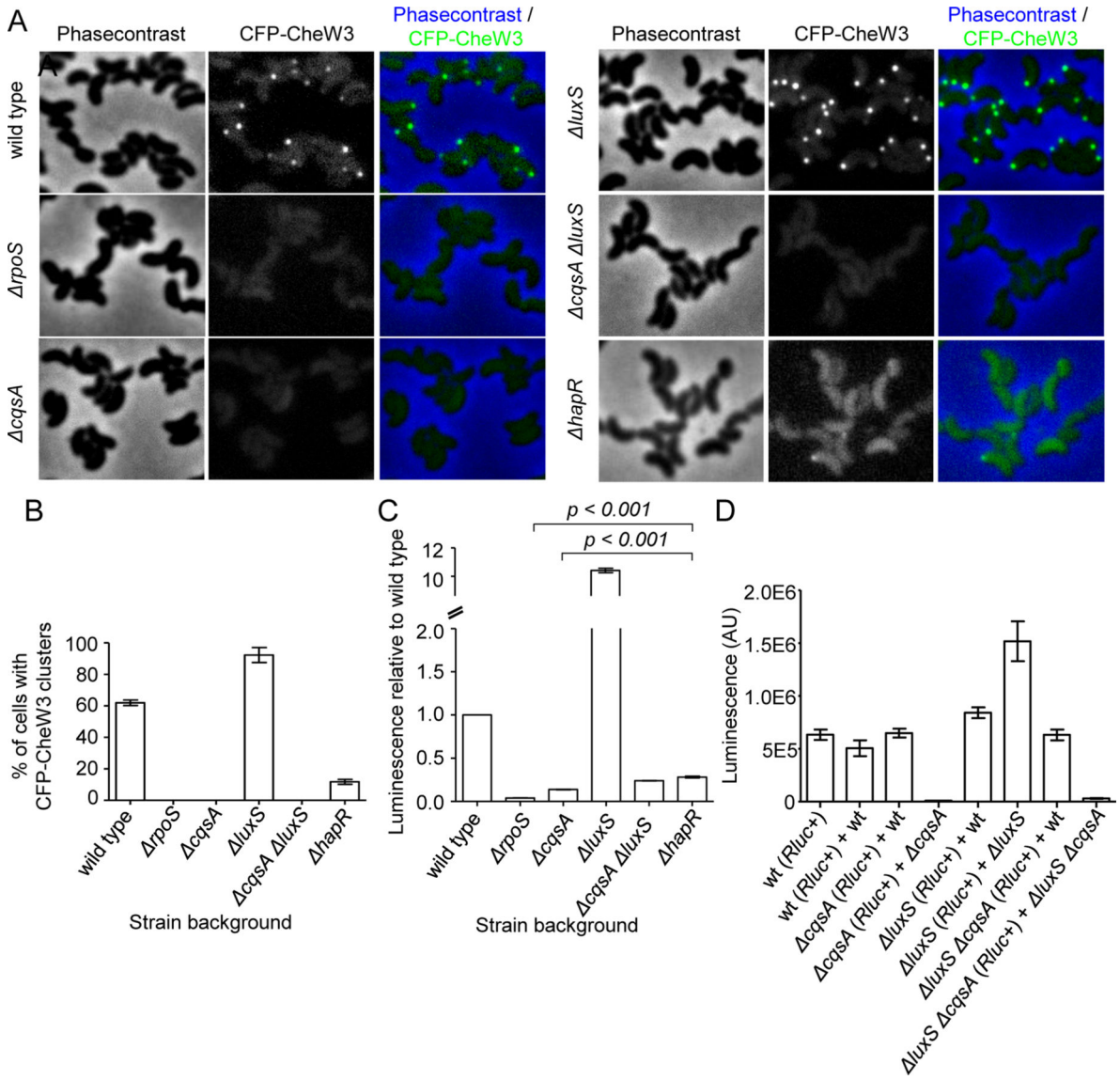

Figure 3. RpoS and quorum sensing signaling modulate expression of CheW3

(A) Visualization of CFP-CheW3 expressed from its native locus in late stationary phase wild type (strain SR9) cells and in various mutant derivatives: Δ*rpoS* (strain SR71), Δ*cqsA* (strain SR48), Δ*luxS* (strain SR49), Δ*cqsA*Δ*luxS* (strain SR47), Δ*hapR* (strain SR12). (B) Bar-graph showing the percentage of cells with CFP-CheW3 foci in strain SR9 and in various mutant derivatives: Δ*rpoS* (strain SR71), Δ*cqsA* (strain SR48), Δ*luxS* (strain SR49), Δ*cqsA*Δ*luxS* (strain SR47), Δ*hapR* (strain SR12). (C) Total luminescence of the mutant strains Δ*rpoS* (strain SR84), Δ*cqsA* (strain SR81), Δ*luxS* (strain SR82), Δ*cqsA*Δ*luxS* (strain SR80), Δ*hapR* (strain SR89) relative to wild type *V. cholerae* strain SR76 (Rluc8-CheW3). All cultures were grown in LB medium. (D) Total luminescence of stationary phase cells from co-cultures of cells. Wild type (SR76), Δ*cqsA* (SR81), Δ*luxS* (SR82), and Δ*cqsAluxS* (SR80) *rluc-cheW3*$^{+}$ strains were grown in co-cultures with *rluc-cheW3*$^{-}$ wild type (C6706), Δ*cqsA* (SR48), Δ*luxS* (SR49), and Δ*cqsAluxS* (SR47) in order to test if production

and release of AI-1 and AI-2 from wild type could complement expression (luminescence) of cluster III in mutants.

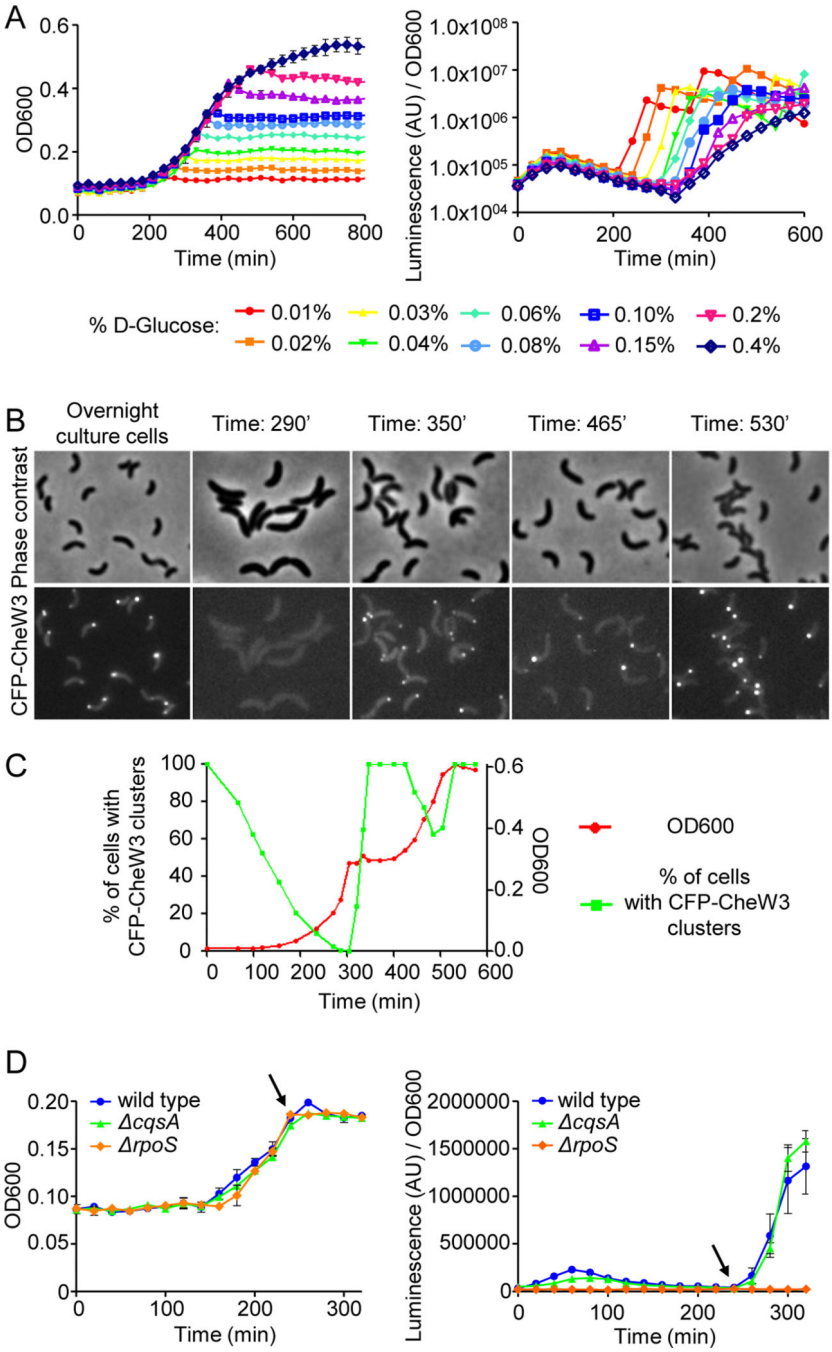

Figure 4. Growth arrest following carbon starvation induces expression/localization of CheW3 in an RpoS dependent and CqsA independent fashion

(A) Optical density (OD600) and total luminescence were measured over time for wild type *V. cholerae* strain SR76 (Rluc8-CheW3). Cells were grown in MSR6 minimal medium with the indicated concentrations of D-glucose. (B) Detection of CFP-CheW3 foci at different time points during the carbon catabolite experiment shown in (C). (C) Growth-curve (filled green squares) of strain SR9 (*cfp-cheW3*) in MSR6 minimal medium supplemented with 0.04% w/v D-glucose and 0.4% w/v succinate and the corresponding percentage of cells with CFP-CheW3 foci (filled red circle) at the indicated optical densities. (D) Kinetics of

CheW3 expression, detected using Rluc8-CheW3 luminescence, as a function of growth in wild type *V. cholerae* (strain SR76), Δ*rpoS* (strain SR84) and Δ*cqsA* (strain SR81) backgrounds.

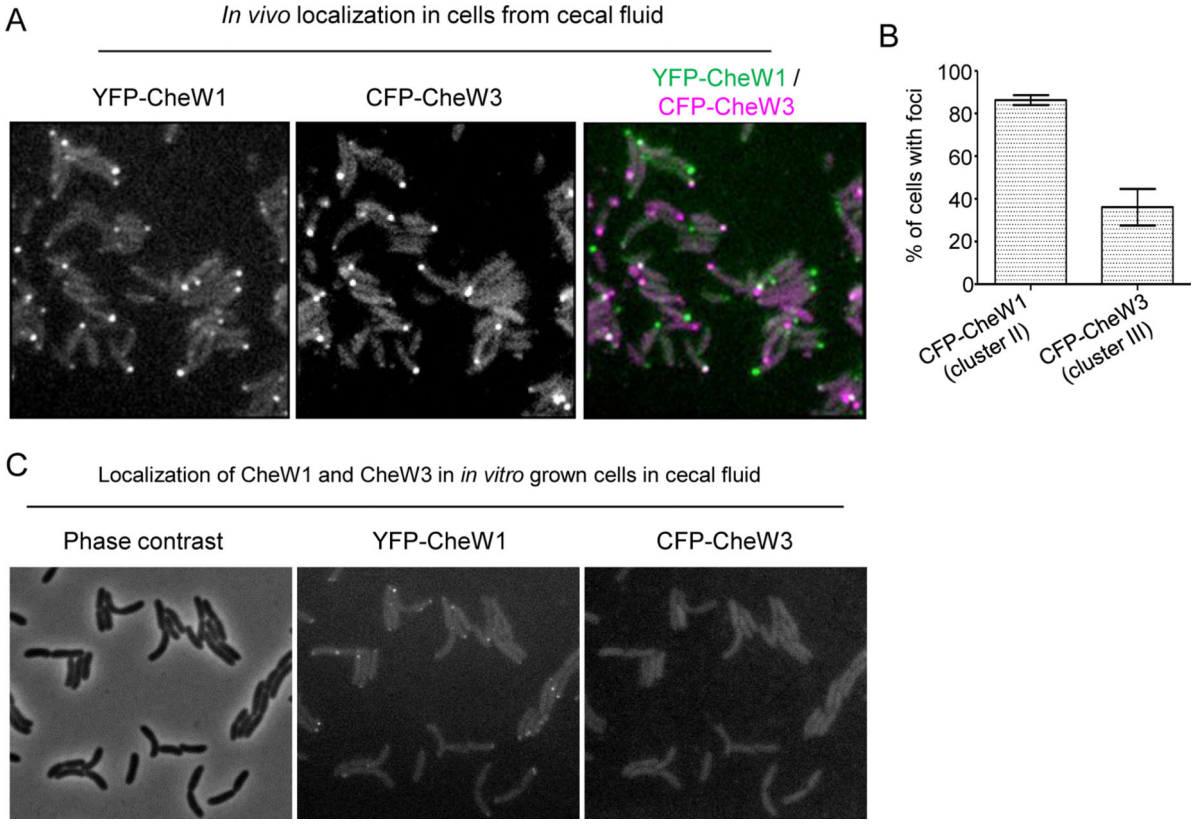

Figure 5. Intracellular localization of YFP-CheW1 and CFP-CheW3 in cells from cecal fluid during *V. cholerae* colonization of infant rabbits

(A) Fluorescent micrographs and (B) percentage of cells from cecal fluid with YFP-CheW1 and CFP-CheW3 clusters from cecal fluid infant rabbits infected with *V. cholerae* strain SR15 (YFP-CheW1, CFP-CheW3). (C) Localization of YFP-CheW1 and CFP-CheW3 in SR15 cells initially grown to exponential phase in LB and then transferred to filtered cecal fluid an imaged after 2hr incubation at 37° with shaking.

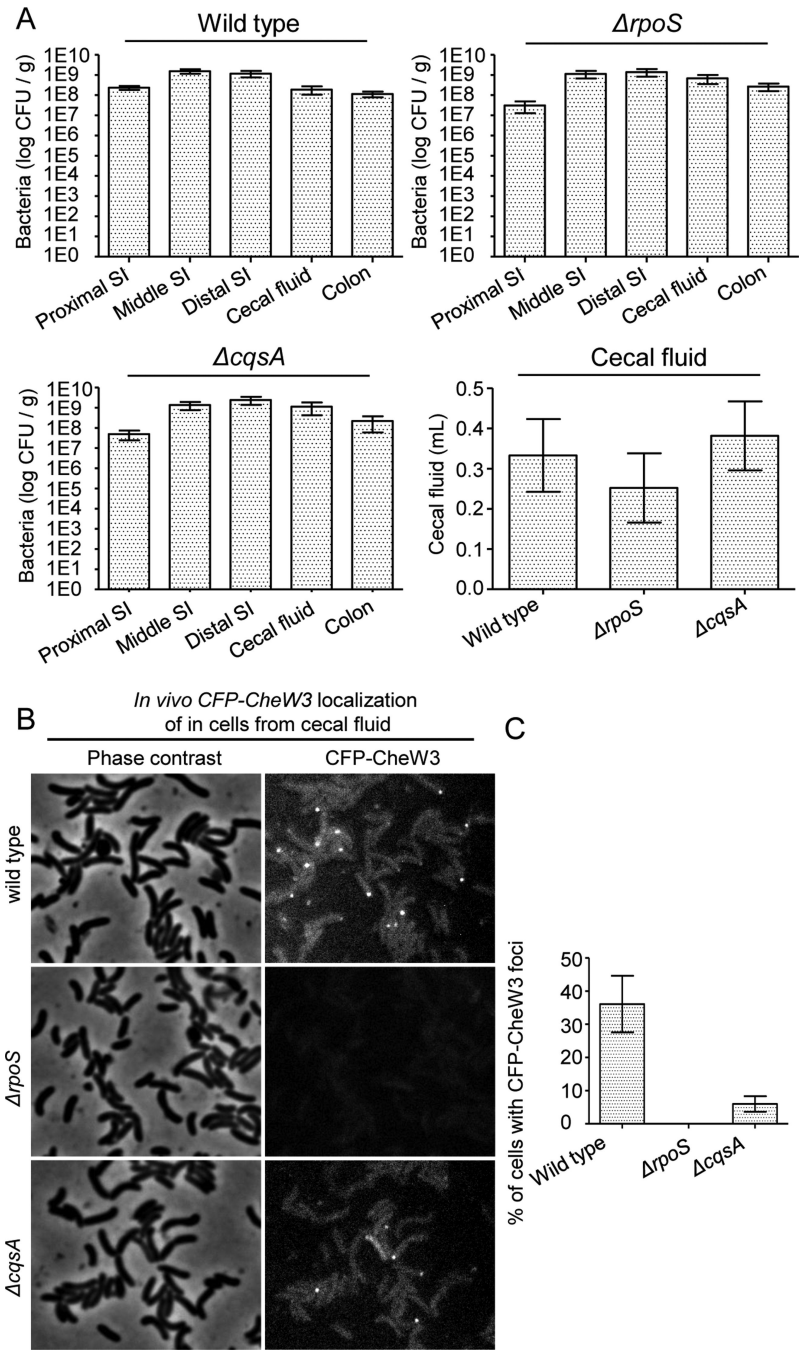

Figure 6. RpoS is required for expression/localization of CheW3 during *V. cholerae* colonization of the infant rabbit intestine

(A) Number of colony forming units (CFUs) per gram from homogenates of the indicated sections of the small intestine (SI), colon or the cecal fluid. Cecal fluid accumulation in rabbits infected with wild type (SR9), SR71 (Δ*rpoS*, *cfp-cheW3*), or SR48 (Δ*cqsA*, *cfp-cheW3*) *V. cholerae*. (B) Fluorescent micrographs and (C) percentages of cells from cecal fluid with CFP-CheW3 foci from infant rabbits infected with SR9, SR76, and SR48.

Table 1

Strain and plasmid list.

Strain name	Genotype	Reference
Vibrio cholerae C6706 (wild type)	Clinical isolate	
SR6	C6706 *lacZ- ΔcheW1::yfp-cheW1*	(Ringgaard *et al.*, 2011)
SR9	C6706 *lacZ- ΔcheW3::cfp-cheW3*	This work
SR12	C6706 *lacZ- ΔhapR ΔcheW3::cfp-cheW3*	
SR15	C6706 *lacZ- ΔcheW1::yfp-cheW1 ΔcheW3::cfp-cheW3*	This work
SR28 (Δ*cluster II*)	C6706 *lacZ- ΔcheY3 ΔcheZ ΔcheA2 ΔcheB2 ΔparC ΔparP ΔcheW1*	(Hatzios *et al.*, 2012)
SR29 (Δ*cluster III*)	C6706 *Δvca1088 Δvca1090 Δvca1091 Δvca1092 Δvca1093 Δvca1094 Δvca1095 Δvca1096*	This work
SR36 (Δ*clusters I and II*)	C6706 *lacZ- ΔcheW3::cfp-cheW3 Δvc1394 Δvc1396 Δvc1397 Δvc1398 Δvc1399 Δvc1400 Δvc1401 Δvc1402 Δvc1403 Δvc1404 Δvc1405 Δvc1406 Δvc2059 Δvc2060 Δvc2061 Δvc2062 Δvc2063 Δvc2064 Δvc2065*	This work
SR47	C6706 *ΔcqsA ΔluxS ΔcheW3::cfp-cheW3*	This work
SR48	C6706 *ΔcqsA ΔcheW3::cfp-cheW3*	This work
SR49	C6706 *ΔluxS ΔcheW3::cfp-cheW3*	This work
SR71	C6706 *lacZ- ΔrpoS ΔcheW3::cfp-cheW3*	This work
SR76	C6706 *lacZ- ΔcheW3::rluc8-cheW3*	This work
SR80	C6706 *ΔcqsA ΔluxS ΔcheW3::rluc8-cheW3*	This work
SR81	C6706 *ΔcqsA ΔcheW3::rluc8-cheW3*	This work
SR82	C6706 *ΔluxS ΔcheW3::rluc8-cheW3*	This work
SR83	C6706 *lacZ- ΔcheW3::cfp-cheW3 ΔrpoS*	This work
SR84	C6706 *lacZ- ΔcheW3::rluc8-cheW3 ΔrpoS*	This work
SR89	C6706 *lacZ- ΔcheW3::rluc8-cheW3 ΔhapR*	This work
MM883	C6706 *ΔcqsA ΔluxS*	(Miller *et al.*, 2002)
MM893	C6706 *ΔcqsA*	(Miller *et al.*, 2002)
KSK1059	C6706 *ΔluxS*	(Miller *et al.*, 2002)
Escherichia coli DH5αλpir		
Escherichia coli SM10λpir		

Plasmid name	Relevant genotype / description	Reference
pCVD442		(Donnenberg and Kaper, 1991)
pCVD442-ArpoS	Plasmid for deletion of *rpoS*	
pSR1010	Plasmid for insertion of *yfp-cheW1* on the chromosome	(Ringgaard *et al.*, 2011)
pSR1011	Plasmid for insertion of *cfp-cheW3* on the chromosome	This work
pSR1020	Plasmid for deletion of chemotaxis cluster II	(Hatzios *et al.*, 2012)
pSR1033	*P*BAD::*yfp-cheW1*	(Ringgaard *et al.*, 2011)
pSR1157	Plasmid for deletion of chemotaxis cluster I	(Hatzios *et al.*, 2012)
pSR1157	Plasmid for deletion of chemotaxis cluster III	(Hatzios *et al.*, 2012)
pSR1213	Plasmid for insertion of *rluc8-cheW3* on the chromosome	This work
pSR1217	Plasmid for deletion of *hapR*	This work
pSR1228	*P*BAD::*cfp-vca1094* (*cheW3*)	This work
pSR1224	*P*BAD::*yfp-vca1095* (*cheA3*)	This work

Plasmid name	Relevant genotype / description	Reference
pMF390	*P*BAD::*yfp*	(Yamaichi *et al.*, 2007)
pMF391	*P*BAD::*cfp*	(Yamaichi *et al.*, 2007)

Table 2

Primers list.

Primer name	Primer sequence
CheW3-III-1-cw	ccccgagctctgtacaagatgaattcagcgaatttgaccacatc
CheW3-III-2-ccw	cccctctagatcatgcttgtccctcattaaggatt
CheA3-III-cw	ccccgagctctgtacaagatggctttagatatggaacaactgc
CheA3-III-ccw	cccctctagactatgccgctttgcctttggtc
CheW3-a	cccctctagaatattcgcatgctaccgatgagtt
CheW3-b	caaagcggcataggagcaggtatggtgagcaagggcgagga
CheW3-c	cgaaactgagaaattcacgctgcatcatgcttgtccctcattaaggat
CheW3-d	cccctctagacatcaaattcgtcgtcatgccttc
CheW3-bb	tcctcgcccttgctcaccatacctgctcctatgccgctttg
CheW3-cc	atccttaatgagggacaagcatgatgcagcgtgaatttctcagtttcg