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Sensing wetness: a new role for the bacterial flagellum

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We have uncovered a new role for the bacterial flagellum in sensing external wetness. An investigation into why mutants in the chemotaxis signaling pathway of *Salmonella typhimurium* exhibit fewer and shorter flagella than wild-type when propagated on a surface, first showed that the mutants downregulate only a small set of genes on swarm media—class 3 or 'late' motility genes, and genes associated with the pathogenicity island SPI-1 TTSS (type three secretion system). Based on observations that swarm colonies of the mutants appear less hydrated, we tested a model in which the flagellum itself is a sensor: suboptimal external hydration interferes with secretion of flagellin subunits, inhibiting filament growth and blocking normal export of the class 3 transcription inhibitor FlgM. We provide strong experimental support for the model. In addition, the data show that the flagellar and SPI-1 TTSS are coupled via regulatory proteins. These studies implicate the flagellum, a bacterial organ for motility, in sensing the external environment to modulate not only its own biogenesis but other physiological functions as well.
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Introduction

Bacteria have evolved elaborate sensory pathways to monitor and respond to their environment, to direct motility, and to program development. A major mechanism of signal transduction is the 'two-component' system, where phosphorylation serves as a means of information transfer (Hoch and Silhavy, 1995). Most two-component systems regulate gene expression. However, the two-component signaling system employed for chemotaxis in *Escherichia coli/Salmonella typhimurium* (A newly published nomenclature suggests using the name *Salmonella enterica* subsp. *enterica* serovar *typhimurium* to describe this species; Tindall *et al*, 2005.) modulates flagellar rotational bias to direct motility (Stock and Surette, 1996). The present study was undertaken to determine whether this system has a hitherto unknown function in gene regulation as well, since mutants in the chemotaxis pathway of these organisms are developmentally impaired for swarming motility. The study has unexpectedly revealed a role for the flagellum in sensing external wetness to regulate its own biogenesis. A concomitant regulation of virulence gene expression was observed in addition.

Swarming is a flagella-driven form of motility used by a large number of bacteria to navigate across solid surfaces as a group (see Harshey, 2003). Swarmer cells have a distinct morphology, being generally longer and more flagellated than vegetative cells. Bacteria elaborate surfactants and wetting agents, which constitute the 'slime' surrounding a swarming colony, providing an aqueous milieu vital for movement over surfaces. The chemotaxis sensory system plays a critical role in the swarming motility of *Serratia marcescens* (O'Rear *et al*, 1992), *S. typhimurium* and *E. coli* (Harshey and Matsuyama, 1994), although chemotaxis is not required for outward migration of the bacteria (Burkart *et al*, 1998). Mutants in the chemotaxis pathway neither swarm nor display the hyperflagellated morphology associated with wild-type swarmer cells (Harshey and Matsuyama, 1994; Burkart *et al*, 1998; Toguchi *et al*, 2000).

Flagellar biogenesis in *S. typhimurium* involves a combination of transcriptional, translational and post-translational regulatory mechanisms (see Chilcott and Hughes, 2000). More than 50 genes are transcribed in operons of three temporal classes—early, middle and late. The early or class 1 genes are included in the master flagellar operon *flhDC*, whose proteins direct transcription of class 2 promoters of the middle and some late genes. The middle operons encode structural and assembly proteins required for the biosynthesis of the base of the flagellar structure called hook-basal-body (HBB). Two competing regulatory proteins, FlgM and FliA (σ^{28}), regulate flagellar morphogenesis at this stage. The FlgM protein binds directly to σ^{28} to prevent class 3 transcription until the HBB is completed, after which FlgM is secreted from the cell and class 3 transcription occurs. Thus, the external filament is not made until the HBB platform is complete. Late genes also include the motor proteins and the chemotaxis components. Early in flagellar assembly, after the inner membrane MS-ring and the cytoplasmic C-ring are assembled, a flagellar-specific type III secretion system (TTSS) docks at the base of this structure and directs the export of all extracytoplasmic flagellar subunits comprising the membrane-spanning rod, the external hook as well as the filament. A disordered N-terminal segment is of primary importance for export through the TTSS (Namba, 2001), which employs specific chaperones for substrate delivery. Additionally, there is evidence that special signals at the 5′ end of mRNA couple translation to secretion (Karlinsey *et al*, 2000). The flagellar TTSS shares sequence and structural

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similarities with secretion systems dedicated to the export of virulence proteins into host cells (Blocker *et al*, 2003; Macnab, 2003).

To test whether there is a genetic program specific to swarming, we recently determined global gene expression profiles of *S. typhimurium* over an 8-h time course of swarming, and compared the microarray data with a similar time course of growth in liquid media as well as on harder agar where the bacteria do not swarm (Wang *et al*, 2004). Most flagellar genes were not upregulated, except genes for the flagellar filament which were elevated approximately two-fold. The study concluded that bacteria growing on the surface of an agar plate experience a significantly altered physiology compared to bacteria growing in broth (see also Kim and Surette, 2004). Swarming bacteria have likely exploited the physiological changes affected by growth on surfaces to effect movement. The surface may serve as a concentrated sink for the accumulation of slime, the right consistency of which may induce the changes necessary to promote swarming.

To determine whether mutations in the chemosensory system alter specific gene regulation, we have carried out a microarray analysis of several *che* mutants of *S. typhimurium* on swarm agar. Surprisingly, their expression profiles showed differences in regulation of only a small set of genes when compared to wild-type. These included all flagellar class 3 genes, putative motility genes identified earlier as showing a class 3 expression pattern (Wang *et al*, 2004), and genes associated with the TTSS encoded in the pathogenicity island SPI-1. Besides confirming earlier findings that filament synthesis is only inhibited on swarm agar and not in broth in the *che* mutants, we have found that lawns made by these mutants on swarm agar have an abnormal phenotype and are less hydrated compared to wild-type. These data led us to a model where the flagellar filament senses external surface conditions that are nonconducive to subunit assembly in the mutant colonies, leading to a feedback inhibition of flagellin export. Thereupon, secretion of FlgM, an inhibitor of class 3 transcription, is also affected. We provide strong experimental support for the model. In addition, the data show that the flagellar and SPI-1 TTSS are coupled via regulatory proteins.

Results

Gene expression profiles of che mutants on swarm agar

In an earlier experimental setup profiling global expression patterns of wild-type *S. typhimurium*, motility was first observed at 2.5 h and peaked at 4 h after inoculation of cells on swarm plates (Wang *et al*, 2004). Therefore in the current microarray experiments, a *cheY* mutant was monitored during a 4 h time course. A total of 29 motility-related genes were downregulated in the mutant, maximally between 3 and 4 h (Table I). Of these, 21 were known class 3 flagellar genes, and eight were putative class 3 motility genes (Wang *et al*, 2004).

Three other *che* mutants *cheA*, *cheB* and *cheZ* were also tested at the 3 h time point, when all cells are actively moving in the wild-type (Wang *et al*, 2004). These same motility genes were downregulated in all four mutants. Genome wide, nine other genes, all belonging to the pathogenicity island SPI-1 TTSS, or secreted through the TTSS, were downregulated in the mutants (Table II).

Table I Transcriptional ratio of class 3 motility genes

	0.0 h	1.0 h	1.5 h	2.0 h	2.5 h	3.0 h	4.0 h
Class III							
aer	1.0	**1.6**	1.4	1.3	**1.8**	**2.6**	**2.5**
cheA	1.0	**1.6**	1.4	1.4	1.4	**2.1**	**2.6**
cheB	1.2	**2.4**	**2.1**	**2.1**	**1.9**	**2.6**	**3.4**
cheR	1.0	**1.5**	1.4	1.3	1.2	**2.1**	**2.5**
cheW	1.1	**1.5**	1.3	**1.5**	**1.5**	**1.8**	**2.6**
cheY	—	—	—	—	—	—	—
cheZ	1.1	**2.5**	**2.2**	**2.3**	**2.3**	**2.6**	**3.8**
flgK	0.9	**1.5**	1.4	1.1	1.2	**1.7**	**2.1**
flgL	0.9	1.4	1.3	1.0	1.1	**1.6**	**2.2**
flgM	1.0	**1.5**	1.2	0.9	1.2	**1.6**	**2.3**
flgN	1.1	1.3	1.2	0.9	1.2	**1.7**	**2.3**
fliC	0.9	**2.0**	1.4	1.1	1.1	**1.6**	**2.6**
fliD	0.9	**1.5**	1.2	0.8	1.1	**2.0**	**2.7**
fliS	1.0	1.3	1.1	0.9	1.2	1.4	**2.5**
fliT	1.0	1.3	1.1	1.0	1.4	**1.5**	**2.4**
fljA	0.9	**1.8**	1.3	1.1	1.4	**2.5**	**3.1**
fljB	0.8	**2.2**	**1.6**	1.2	1.0	1.3	**2.5**
motA	0.9	**1.7**	1.4	1.3	1.4	**2.6**	**2.9**
motB	1.0	1.4	1.2	1.1	1.3	**2.3**	**2.8**
tcp	0.8	**1.8**	1.3	1.2	1.4	**2.2**	**2.8**
trg	0.9	**1.5**	1.3	1.4	**1.7**	**2.7**	**2.4**
tsr	1.0	**1.7**	1.4	1.3	1.4	**2.3**	**2.2**
Putative class III							
STM1300	1.0	1.3	1.2	1.3	**1.6**	**2.1**	**2.2**
STM1301	0.8	1.1	1.3	1.3	1.4	**1.8**	**1.6**
STM2314	0.9	**2.3**	**1.5**	1.2	1.4	**2.3**	**2.5**
STM3152	0.9	**1.9**	**1.5**	1.2	**1.5**	**2.0**	**2.2**
STM3216	0.8	**2.2**	1.4	1.0	1.4	**2.2**	**2.6**
ycgR	1.0	**1.5**	1.3	1.2	1.3	**1.8**	**2.2**
yghW	0.7	**1.8**	1.2	1.2	**1.6**	**2.1**	**2.3**
yhjH	0.9	**1.9**	1.3	1.4	**1.6**	**2.3**	**2.7**

These values were obtained by microarray analysis for wild-type versus *cheY* over a 4 h time course of swarming. Differences greater than 1.5-fold are indicated in bold. Signal intensities similar to background are indicated by a dash.

Microarray profiles of *che* mutants in broth showed a large experimental variation and hence could not be interpreted. Growth curves of the mutants were comparable to wild-type (Supplementary Figure 1).

Time course of flagellar filament synthesis in che mutants

We have reported previously that *che* mutants appear less flagellated than wild-type on swarm plates (Harshey and Matsuyama, 1994; Toguchi *et al*, 2000). Here, we have systematically compared filament synthesis during growth in broth and on swarm agar, in wild-type bacteria versus *che* mutants, by flagellar staining as well as by Western blots. Data for a representative smooth (*cheY*) and tumbly (*cheZ*) mutant are shown (Figures 1 and 2). Class 3 flagellar gene expression has been shown to increase between 1.5 and 3.5 h during exponential growth (Wang *et al*, 2004). Consistent with this, flagellation on wild-type cells also increased during this period under both broth and plate conditions (Figure 1; see WT (B) and WT (P)). While a similar pattern was observed for the *che* mutants in broth (Figure 1; compare *cheY* (B) and *cheZ* (B) to WT (B)), fewer and shorter flagella were observed on these mutants on swarm agar (Figure 1; compare *cheY* (B) to *cheY* (P), and *cheZ* (B) to *cheZ* (P)). Flagella on the *cheZ* mutant were shorter than on *cheY*. The flagellar phenotype of a *cheB* mutant was similar to *cheZ*,

Table II Differentially regulated genes common to all *che* mutants

	cheA	*cheB*	*cheY*	*cheZ*	*flgM*	*cheYflgM*
Known motility genes						
aer	**1.8**	1.4	**2.6**	**1.8**	0.2	0.2
cheA	—	**1.6**	**2.1**	**1.7**	0.4	0.3
cheB	**1.7**	—	**2.6**	**1.5**	0.4	0.6
cheR	**1.6**	1.2	**2.1**	1.4	0.4	0.4
cheW	0.6	**1.7**	**1.8**	**1.6**	0.4	0.3
cheY	**1.8**	**1.5**	—	**1.5**	0.4	—
cheZ	**1.7**	1.3	**2.7**	—	0.4	1.0
flgK	**1.8**	**1.7**	**1.7**	**1.7**	0.3	0.3
flgL	**1.8**	**1.9**	**1.6**	**1.6**	0.4	0.4
flgM	**1.8**	**1.7**	**1.6**	1.3	—	—
flgN	**1.8**	**1.7**	**1.7**	1.4	0.2	0.3
fliC	**1.7**	**1.9**	**1.6**	**2.1**	0.3	0.3
fliD	**1.9**	**1.7**	**2.0**	**1.5**	0.3	0.3
fliS	**1.8**	**1.8**	1.4	1.4	0.4	0.4
fliT	**1.8**	**1.8**	**1.6**	1.4	0.4	0.4
fliZ	**1.7**	**1.8**	1.4	**1.7**	0.4	0.4
fljA	**2.2**	**1.9**	**2.5**	**1.7**	28.6	24.8
fljB	**1.8**	**1.7**	1.3	**1.6**	1.2	2.2
motA	**1.9**	**1.9**	**2.6**	**1.9**	0.4	0.2
motB	1.0	**1.8**	**2.3**	**1.5**	0.4	0.3
tcp	**2.2**	**2.0**	**2.2**	**1.8**	0.2	0.2
trg	**1.6**	1.3	**2.7**	**1.7**	0.2	0.2
tsr	**1.7**	**1.5**	**2.3**	**1.8**	0.3	0.3
Putative motility genes						
STM1300	**1.6**	**1.5**	**2.1**	1.4	0.4	0.3
STM1301	**1.6**	**1.5**	**1.8**	**1.5**	0.3	0.2
STM2314	**2.1**	**1.8**	**2.3**	**1.7**	0.2	0.2
STM3152	**1.7**	**1.5**	**2.0**	**1.8**	0.2	0.2
STM3216	**2.0**	**1.6**	**2.2**	**2.2**	0.2	0.1
ycgR	**1.6**	**1.5**	**1.8**	**1.5**	0.5	0.4
yghW	**1.9**	**1.7**	**2.1**	**1.9**	0.3	0.2
yhjH	**1.7**	**1.5**	**2.3**	**1.8**	0.3	0.3
Pathogenicity genes						
pipC	**2.8**	**2.6**	1.3	**2.2**	0.5	0.5
sicA	**2.5**	**2.3**	1.3	**1.8**	0.6	0.4
sipA	**2.6**	**2.3**	1.4	**2.5**	0.5	0.5
sipB	**3.0**	**2.8**	1.4	**2.8**	0.5	0.5
sipC	**2.8**	**2.5**	1.1	**1.9**	0.5	0.5
sipD	**2.9**	**2.7**	1.4	**3.1**	0.5	0.5
sopB	**2.7**	**2.6**	1.3	**2.4**	0.6	0.4
sopE2	**2.4**	**2.3**	1.3	**1.8**	0.5	0.4
sscA	**1.8**	**1.8**	1.1	**1.6**	0.9	0.6

Transcription ratio between wild-type versus *che* or *flgM* mutants at the 3 h time point on swarm plates. Other symbols as in Table I. The *flgM* mutation appears to affect expression of the *fljBA* operon.

while that of *cheA* was more similar to *cheY* (not shown); *cheA* and *cheY* mutants rotate their flagella counterclockwise (CCW), whereas *cheB* and *cheZ* mutants do so predominantly clockwise (CW).

We also determined filament protein FljB levels in whole cells using Western blots. These are shown from 1.5 to 4.5 h for wild-type and *che* mutants on swarm agar (Figure 2). In broth, flagellin levels were similar between wild-type and mutants (0 h). However, during the time course of growth on swarm plates, the mutants showed substantially lower flagellin levels compared to wild-type. Consistent with the staining data (Figure 1), flagellin levels in the *cheZ* mutant were lower than in *cheY*.

We conclude that *che* mutants have fewer and shorter flagellar filaments on surfaces as determined by three measures: number of filaments (Figure 1), filament protein levels (Figure 2) and flagellar transcription profiles (Tables I and II).

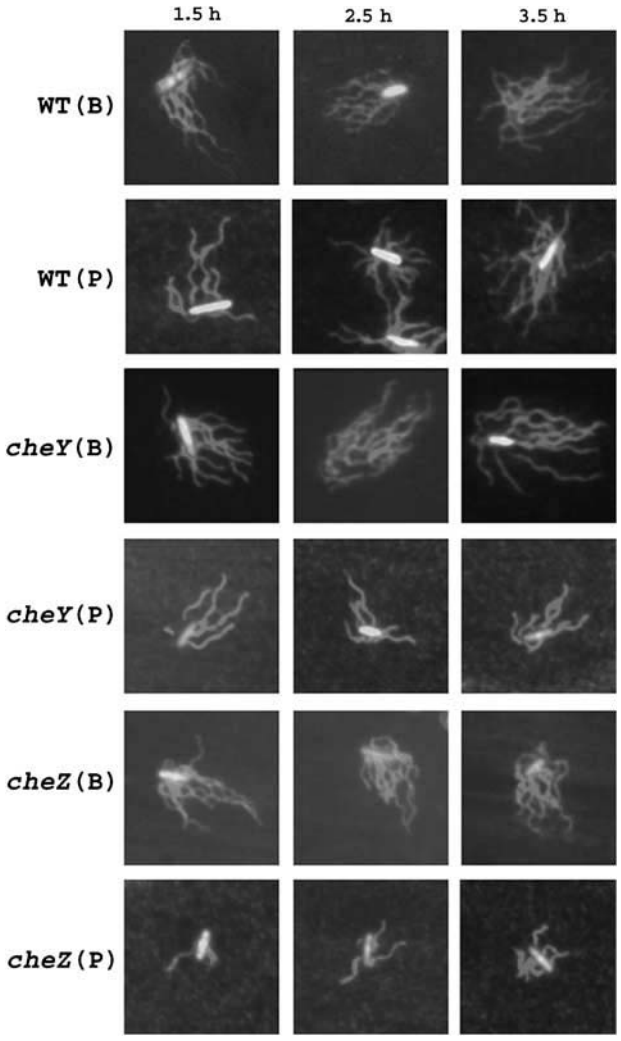

Figure 1 Flagellar filament visualization. Cells growing for indicated times in broth (B) or swarm plates (P) were stained with filament antibodies as described in Materials and methods. A color version of this figure is available at *The EMBO Journal* Online.

Altered colony morphologies of che mutants

Besides flagellation, the *che* mutants also show altered colony morphologies (Figure 3A). As described earlier (Wang *et al*, 2004), between 2 and 2.5 h and just prior to the onset of motility, lawns of wild-type cells display a 'swiss cheese' morphology consisting of low and high regions. This morphology was conspicuously absent in all *che* mutants (Figure 3A; *cheY* and *cheZ* are shown). Visually, the mutant lawns did not look as hydrated as wild-type. We attempted to quantitate this observation by dropping 1 μl Drummond glass capillaries on the swarm surface at 3 h, and measuring the height of the fluid rising in the capillary. Lawns of *che* mutants had less fluid as measured by this assay (Figure 3B). Rehydration of the mutant lawns also rescued swarming motility (see below). We suggest therefore that poor hydration is likely responsible for the aberrant colony morphologies of the *che* mutants as well as their flagellar phenotype, as proposed below.

A model for surface sensing

The striking result from the microarray experiments in Tables I and II is the downregulation of only the class 3 motility

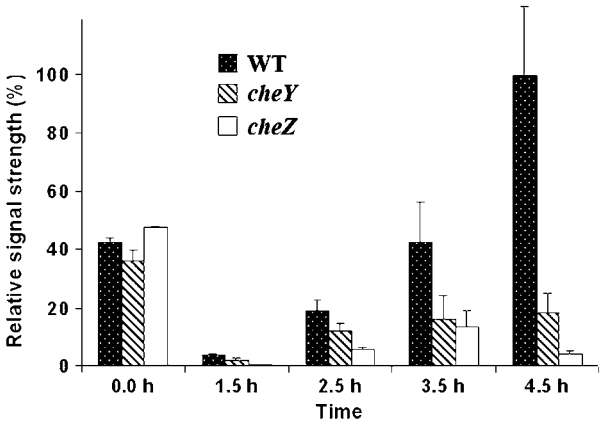

Figure 2 Relative flagellin amounts in strains grown on swarm plates. Cells from exponentially growing broth cultures were used as inoculum for plates (0 h). Samples were normalized to an OD_{600} of 0.6 prior to electrophoresis, and Western blots developed using anti-FljB antibodies as described in Materials and methods. Signal intensities are plotted as a function of total OD_{600}, and relative to wild-type at 4.5 h.

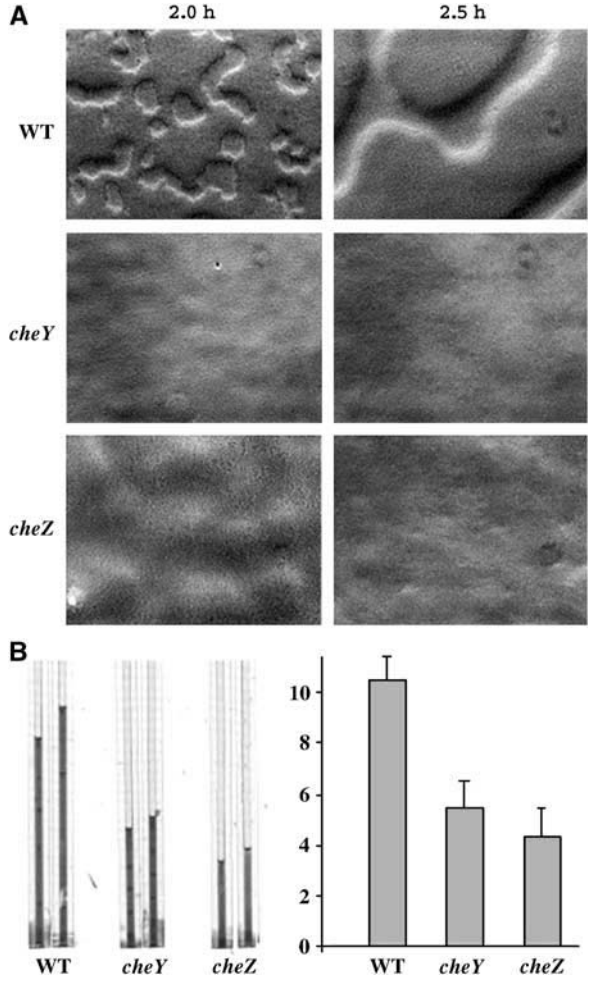

Figure 3 Lawn morphologies of wild-type and *che* mutants propagated on swarm agar. (**A**) At indicated times, plates were photographed with a DP-12 digital camera attached to an Olympus BH-2 microscope. Magnification, ×25. (**B**) Left: comparison of fluid retention in 3 h-lawns of indicated strains by the capillary-drop method, as described in Materials and methods. Two capillaries are shown for each strain. Height of capillary in mm is indicated on the *y*-axis (center). Right: data from triplicate plates measured on the same day; error bars indicate standard variation.

genes. On the basis of these data as well as those in Figures 1–3, we propose the following model (Figure 4). Downregulation of flagellar genes on swarm agar is due to a feedback mechanism in which the flagellar filament itself is a sensor for unfavorable external conditions, for example, poor hydration. The flagellum could specifically control class 3 transcription by controlling the delivery of its normal cargo, the class 3 inhibitor FlgM, to the outside. Initially, interference of filament subunit polymerization at the growing end

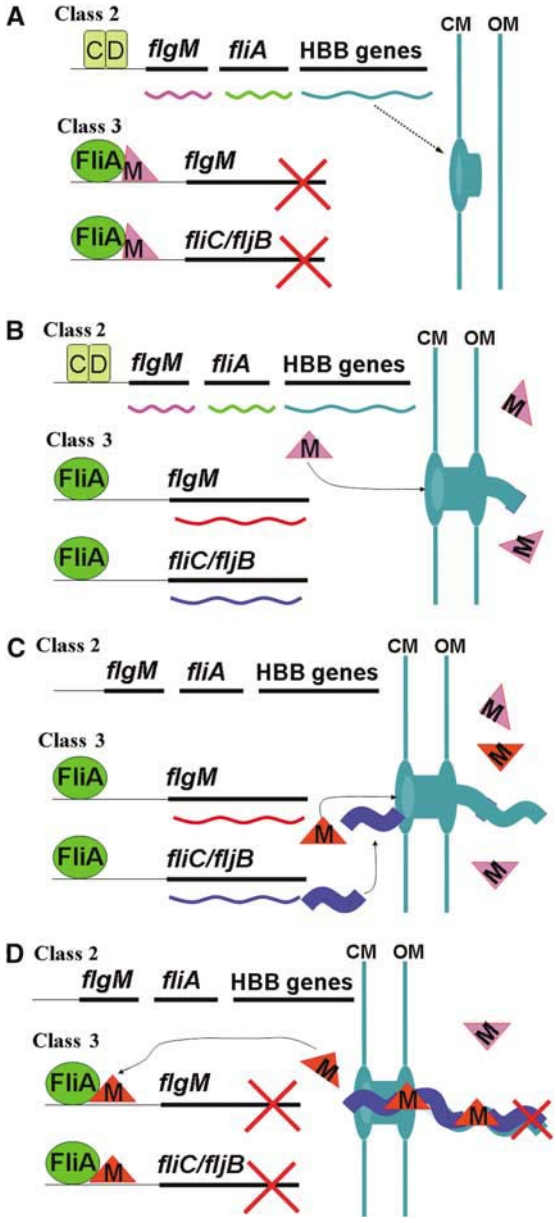

Figure 4 The flagellum as an environment sensor, and FlgM as a dual developmental checkpoint. (**A**) The FlhCD regulator activates class 2 transcription (only relevant genes shown). Class 2 FlgM inhibits FliA while HBB construction is in progress (Chilcott and Hughes, 2000). (**B**) Completion of HBB allows FlgM export, lifting FliA inhibition and allowing class 3 expression (e.g. filament genes *fljB/fliC* and class 3 *flgM*). (**C**) Class 3 FlgM is primarily secreted during filament biogenesis (Karlinsey *et al*, 2000). (**D**) An external block to filament growth interferes with FlgM secretion and re-establishes repression of FliA. CM, cytoplasmic membrane; OM, outer membrane; X, block to transcription (A, D) or filament growth (D); wavy lines, transcripts. Nascent flagellin subunits in purple.

would lead to a back-up of nascent subunits in the hollow channel, signaling the flagellar TTSS to inhibit flagellin translation (Karlinsey *et al*, 2000). FlgM levels inside the cell would consequently start to rise, turning down class 3 transcription by virtue of the anti-FliA (σ^{28}) activity of FlgM.

Testing the model

A prediction of this model is that FlgM levels should be lower outside/higher inside cells in *che* mutants, when propagated on swarm agar but not in broth. However, because FlgM is both a class 2 and class 3 gene (80% of FlgM comes from the class 3 promoter; Chilcott and Hughes, 2000), elevation of internal FlgM levels could be transient since increases in intracellular FlgM would be expected to turn down its own expression. We determined FlgM levels in the spent medium as well as in whole cells during growth in broth versus on swarm agar. In broth, extracellular (EC) and intracellular (IC) FlgM levels were similar between wild-type and representative *che* mutants (Figure 5A, left). There was an observable difference, however, in the extracellular levels of FlgM in wild-type versus *che* on swarm plates (Figure 5A, right); as conjectured, a change in intracellular levels was not apparent. The difference in extracellular FlgM between wild-type and *cheY* was then followed in a time course experiment (Figure 5B). The difference was first apparent at 2.5 h, and continued to widen until the last time point measured at 5 h.

Assuming that hydration is the main defect in the *che* mutant lawns, a second way to test the model might be to restore hydration. After several trials, we settled on a method in which the lawn was exposed to a fine mist of water at various times (0.5–3 h) after inoculation of the *cheY* mutant on swarm agar (see Materials and methods). Swarming motility was restored within 60 min after spraying, and optimal rescue was obtained when lawns were sprayed 2 h postinoculation. This time point was therefore used to follow extracellular FlgM levels 0.5–2 h after spraying (Figure 5C).

Figure 5 (**A**) FlgM levels of wild-type and *che* mutants in broth versus on swarm plates 3 h postinoculation. EC, extracellular; IC, intracellular. *fliM* affects formation of the initial C-ring, and serves as a negative control for extracellular FlgM (Macnab, 2003). (**B**) EC FlgM in WT versus *cheY* during a 5 h time course. (**C**) EC FlgM in a *cheY* mutant at indicated times after hydration (+) of a lawn grown for 2 h prior. (−) is a nonhydrated control at the 1.5 h time point equivalent.

They were found to be substantially restored. Additionally, the mutant regained a wild-type flagellar phenotype concomitant with the onset of motility (Figure 6; compare with CheY (P) and WT (P) in Figure 1).

Mutation of flgM simultaneously restores class 3 and virulence gene expression

A definitive test of the model presented above would be restoration of class 3 gene expression in the *che* mutants upon mutation of *flgM*. A microarray analysis was therefore performed on a *cheY flgM* double mutant (Table II, last two columns). Class 3 expression was seen to be restored to higher than wild-type levels in the double mutant, similar to levels in the *flgM* mutant alone. Additionally, virulence gene expression was also restored. We note that although the 3 h time point is optimal for motility gene expression, it is not so for SPI-1 genes that are turned on very early (Wang *et al*, 2004). Indeed, data from earlier times in the *che* mutants (1 and 1.5 h in *cheY*, and 2 h in *cheA*, *cheB* and *cheZ*) indicate that all the SPI-1 genes are downregulated in the mutants, and that they are all restored in *cheY flgM* (not shown). These data are consistent with an earlier study, which found that in the absence of FliA, SPI-1 genes were downregulated approximately 50% in *S. typhimurium* and more severely affected in *S. typhi* (Eichelberg and Galan, 2000).

The swarming phenotype of the *cheY flgM* mutant is similar to that of *cheY* (Figure 7). We note that in these assays, the swimming and swarming zones of the *flgM* mutant alone lag behind wild-type, likely due to slower growth rates of the *flgM* mutant, which have also been reported earlier (Kutsukake and Iino, 1994). The *cheY flgM* mutant, while more flagellated than *cheY*, had less flagella compared to either *flgM* alone or wild-type (Figure 8; compare also to *cheY* (P) in Figure 1). Moreover, like the *cheY*

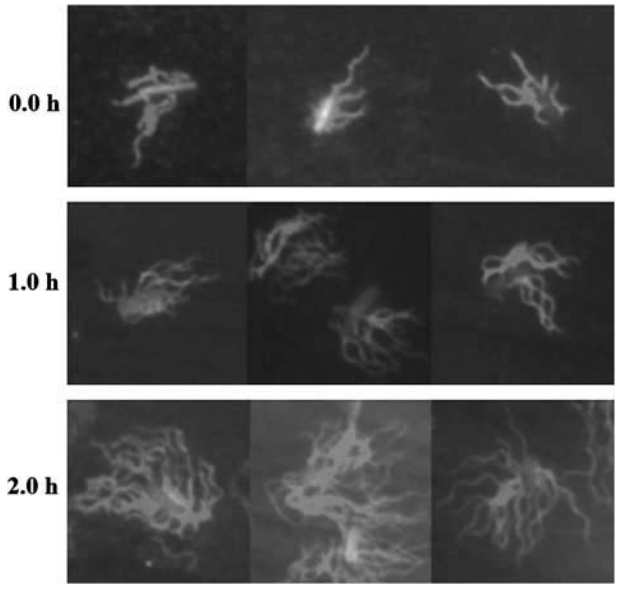

Figure 6 Flagellar staining following hydration and restoration of swarming motility to a *cheY* lawn. The mutant was propagated on swarm plates for 2 h (0 h time point) before spraying with a fine mist of water as described in Materials and methods. Cells stained for flagella 1 and 2 h after spraying are shown. A color version of this figure is available at *The EMBO Journal* Online.

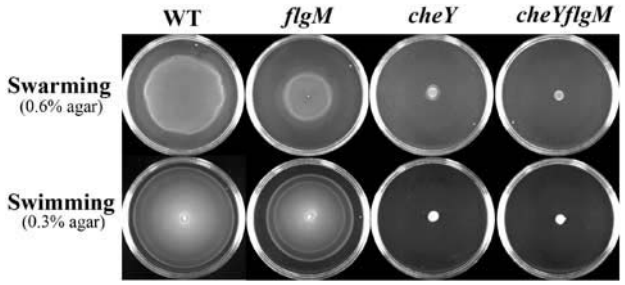

Figure 7 Swarming and swimming morphology of wild-type, *flgM*, *cheY* and *cheY flgM* strains.

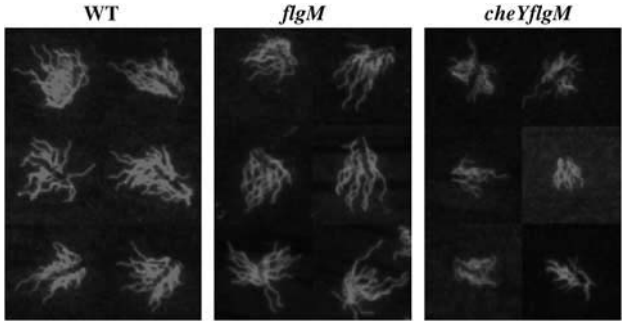

Figure 8 Flagellar staining of wild-type, *flgM* and *cheY flgM* strains propagated on swarm plates for 3 h. A color version of this figure is available at *The EMBO Journal* Online.

mutant, the flagella were shorter on the double mutant. The swarming defect and shorter flagella exhibited by the *cheY flgM* mutant suggests that the inhibitory external environment encountered in the *che* mutants is dominant. Thus, the flagellum is the primary sensor of unfavorable external conditions.

Discussion

The work reported here implicates a new sensory role for an organ of motility in controlling not only its own biogenesis, but also the expression of other bacterial functions; in this case, bacterial virulence. The original impetus for this study was to understand why mutants in the chemotaxis signaling pathway of a number of organisms (*S. marcescens*, *S. typhimurium*, *E. coli*) do not swarm, even though chemotaxis is not required for their surface motility (Burkart *et al*, 1998; Harshey, 2003). These mutants are developmentally impaired, that is, they have fewer and shorter flagella than wild-type (Figures 1 and 2). This is surprising because the chemotaxis system is not known to control gene expression in these bacteria. Also puzzling is that this phenotype is manifested only on swarm media and not in broth (Toguchi *et al*, 2000). We have solved much of the puzzle in the present study starting with our initial observation that only class 3 motility genes are downregulated in the mutants (Tables I and II). Aberrant morphologies of *che* mutant colonies likely resulting from poor hydration (Figure 3) led us to a model in which the flagellar filament senses unfavorable external conditions (Figure 4): interference with filament subunit secretion/polymerization sends a feedback signal to the flagellar TTSS to inhibit further flagellin translation, leading in turn to inhibition of FlgM secretion and thus, inhibition of class 3 transcription. Strong support for this model was provided by the following observations: extracellular FlgM levels in *che* mutants were lower than in wild-type (Figure 5A and B); restoration of hydration in mutant lawns concomitantly restored external FlgM levels, a normal flagellar phenotype and swarming motility (Figures 5C and 6); mutation of *flgM* restored class 3 gene expression in the *cheY* mutant (Table II). Dominance of the *cheY* phenotype in the *cheY flgM* double mutant (Figures 7 and 8) was in keeping with the proposal that the initial defect is an inhibition of filament growth, which subsequently interferes with FlgM secretion. We have found in addition, that at the time of near-maximal downregulation of motility genes, nine virulence genes whose products are secreted through the SPI-1 TTSS were downregulated. The SPI-1 genes are normally maximally expressed early during the time course of swarming (Wang *et al*, 2004); when examined at these earlier time points, all of the SPI-1 genes were affected in the *che* mutants (data not shown). Mutation of *flgM* simultaneously restored expression of the virulence genes in the *cheY* mutant (Table II, last two columns). We discuss these results in greater detail below.

Flagellum as a sensor and FlgM as a dual checkpoint in flagellar biogenesis

Bacterial motility on a surface is critically dependent on a hydrated external milieu (see Harshey, 2003). Since enormous cellular resources are invested into building motility organelles such as flagella or pili, a regulatory mechanism to gauge external water content would seem judicious. Our studies implicate the motility organelle itself in providing this sensing function, primarily by controlling the secretion/polymerization of filament subunits, which occurs outside the cell. They also suggest a new role for the transcriptional regulator FlgM in controlling flagellar biogenesis from the outside.

The proposed surface-sensing model predicts that FlgM levels would be lower outside/higher inside cells in *che* mutants, only when propagated on swarm agar and not in broth. The observed levels of external FlgM were consistent with the model, but a consequent elevation of intracellular FlgM was not seen (Figure 5). However, this was not surprising, and was even expected, because *flgM* itself is also a class 3 gene. The interaction between FlgM and FliA (σ^{28}) is very tight, with a dissociation constant (K_d) on the order of 2×10^{-10} M (Chilcott and Hughes, 2000). Even a small rise in FlgM levels is likely sufficient to shut down class 3 transcription due to its high affinity to σ^{28} and/or its ability to destabilize the σ^{28}-RNA polymerase holoenzyme complex (Chilcott and Hughes, 2000).

FlgM is transcribed from both class 2 and class 3 promoters. Class 2 FlgM is primarily retained inside the cell, whereas class 3 FlgM (which constitutes 80% of the total FlgM) is primarily destined for secretion (Karlinsey *et al*, 2000). What is the significance of this design? Why is 80% of the cellular FlgM directed to the outside? While the role of class 2 FlgM in developmental regulation of gene expression is well established, the role of class 3 FlgM is not known. As we saw, FlgM is continuously secreted into the medium during the time course of swarming (Figure 5B). One

suggested role for FlgM is regulation of filament length; thus, the longer the filament grows the harder it is to transport FlgM, the final length representing a balance between the two processes. However, such a mechanism can be specified by the filament subunits themselves, where filament length can be auto-regulated by the distance the subunits must travel. Another suggested role for FlgM is to regenerate flagella if they are accidentally sheared off; this idea has not as yet been put to the test. From the results presented here, we propose that the continued secretion of class 3 FlgM is designed to test the favorability of external environments for motility. This may be particularly relevant on surfaces where moisture content is so critical for movement. Filament synthesis is energetically the most costly step in flagellar biogenesis, and it would seem prudent to regulate expression only at the class 3 level when conditions are not optimal, ensuring a fast response when they are. Thus, while class 2 FlgM serves as an internal checkpoint, we propose that class 3 FlgM is an external checkpoint for flagellar morphogenesis. We envision that surface conditions that facilitate secretion of FlgM could signal cells to increase flagellar filament numbers by regulating the class 3 step. Similar secreted regulatory proteins could govern other aspects of swarmer cell differentiation such as inhibition of cell division (Harshey, 2003).

The sensory role for the flagellum proposed here is distinct from its proposed role as a mechanosensor in *Vibrio parahaemolyticus*, where all conditions that slow down polar flagellar rotation lead to swarmer cell differentiation (McCarter *et al*, 1988; Kawagishi *et al*, 1996). How a slowed motor may signal for gene expression in this organism is not known. If swarmer cell induction is due to an increased secretion of FlgM or FlgM-like regulators in this bacterium, it might be useful to consider whether interference with flagellar rotation somehow promotes their secretion.

Why are che mutants less hydrated?

We have provided a plausible mechanism for the poor flagellation of the *che* mutants by identifying a block in secretion of the flagellar class 3 negative regulator FlgM in these mutants. We have also identified poor hydration as the likely primary cause of the block to filament growth; simultaneous rescue of external FlgM levels, flagellation, and swarming in the *che* mutants by external hydration is consistent with this observation. However, the data do not address why *che* mutants are less hydrated, or why all of the mutants show a similar defect. Based on the results of an ongoing genetic study, we favor a model in which the *che* mutants are mechanically impaired for flagellar rotation on the surface. We imagine that flagellar filaments normally stick to the surface of swarm agar, and the ability to switch motor direction is important for them to un-stick. Flagellar rotation may be critical in the initial stages for stirring-up the surface moisture or whipping off lipopolysaccharide (LPS) from the surface of neighboring cells in order to generate the right amount of wetting required for movement (LPS has been implicated in a surfactant/wetting function; Toguchi *et al*, 2000). According to this two-step model (first un-stick, then stir), Mot (paralyzed motor) or Fla (absence of flagella) mutants should display hydration defects similar to the *che* mutants. In preliminary experiments with *motA*, *motB* and *fliM* (Fla^-) mutants, this was indeed observed to be the case (our unpublished data). Flagellar staining and microarray data from the *motA* and *motB* mutants appear to be consistent with the observed hydration defects, but inconsistent with reported filament protein levels of a *motB* mutant, which were found to be comparable to wild-type on swarm media (Toguchi *et al*, 2000). We are currently re-examining these data.

Communication between flagellar and SPI-1 secretion systems

S. typhimurium has one TTSS to build flagella, and two such systems to build needle structures and direct translocation of virulence proteins into host cells (Blocker *et al*, 2003; Macnab, 2003). There are sequence and structural similarities between these systems, yet each is highly specific for its substrates. Virulence TTSS are found only in Gram-negative bacteria and there are several reports of regulatory connections between flagellar and virulence gene expression (Goodier and Ahmer, 2001; Lostroh and Lee, 2001; Ellermeier and Slauch, 2003).

All pathogenic *Salmonella* strains depend on the SPI-1 TTSS for entry into epithelial cells (Galan, 1999). This system translocates an array of effector proteins, which stimulate cellular responses such as production of proinflammatory cytokines, reorganization of the actin cytoskeleton and programmed cell death. Motility is thought to aid the entry process indirectly by facilitating initial contact between the bacterium and the host cell. In some strains of *S. typhi*, however, there is epidemiological evidence suggesting a direct correlation between motility, tissue culture cell invasion and virulence (Grossman *et al*, 1995). This is supported by a previous study showing that mutations in FliA significantly reduce expression of components of the SPI-1 TTSS in *S. typhi* (Eichelberg and Galan, 2000). Our data showing that SPI-1 genes respond to FlgM levels, in accordance with its known effect on FliA (Table II), are consistent with these findings. While these two studies implicate the FliA-FlgM system in regulation of virulence, the nature of this regulation is not immediately apparent since virulence gene promoters do not resemble class 3 promoters. There are studies linking *fliZ*, the second gene in the *fliA* operon, to regulation of *hilA*, a positive regulator of SPI-1 genes (Lucas *et al*, 2000; Iyoda *et al*, 2001). Whatever the mechanism, it is evident that the two systems are linked, the regulatory arrow pointing thus far from the motility to the virulence system and not vice versa (Lostroh and Lee, 2001). Under what conditions would it be useful for the virulence system to be tuned to the motility system? In our study, the bacteria were monitoring the surface for its capacity to support swarming motility, in particular for its hydration. This property may be important for needle structure biogenesis and/or may serve as a gauge for assessing the health of a cell during the decision to invade. It is clear, however, that external hydration is a critical component and a common signal for both systems.

Materials and methods

Bacterial strains, growth conditions and reagents

The wild-type *S. typhimurium* strain 14028 (Toguchi *et al*, 2000) was propagated as described (Wang *et al*, 2004). Individual *che* or *flgM* mutants were generated by one-step mutagenesis of strain 14028 (Datsenko and Wanner, 2000). Primers were designed to replace most of the gene with a kanamycin resistance (Kan^R) cassette flanked by the FLP recombinase sites *FRT*. For *cheA*, the

replaced region covers 1866 bp starting from nucleotide (nt) 82 (1 refers to A of the start codon), for *cheB* this covers 936 bp from nt 58, for *cheY*, 301 bp from nt 46, for *cheZ*, 542 bp from nt 50 and for *flgM*, 294 bp from nt 1. The Kan^R cassette was later deleted via recombination (Datsenko and Wanner, 2000). The *cheY flgM* double mutant was constructed by transducing *flgM*::Kan into the *cheY* strain with phage P22 (Toguchi *et al*, 2000), followed by excision of the Kan cassette.

Anti-FljB, *Salmonella* H serum (single factor 2) was from DIFCO. Texas-Red conjugated goat anti-rabbit serum and DAPI (4′,6-diamidino-2-phenylindole; for staining the nucleoid) were from Molecular Probes. FlgM polyclonal antiserum was a gift from Dr Kelly Hughes.

Hydration experiments

Swarming cells were prepared as described (Wang *et al*, 2004). Briefly, broth cells at exponential phase ($OD_{600} = 0.6$) were inoculated on plates by pouring, and subsequently drained. After 2 h at 37°C, plates were moved to the bench top and the agar surface exposed to a fine mist of water by spraying with a common wash bottle with the nozzle adjusted to generate the finest spray. Two plates at a time were subjected to 15 sprays from about 30 cm above. Approximately 170 μl of water was delivered to the surface, although no visible water droplets were seen. This whole process took 90 s. The plates were moved back to 37°C, and removed at various times for observing motility and sampling for extracellular FlgM.

Hydration of mutant lawns was measured by dropping 1 μl capillary tubes (Drummond Scientific Company, Broomall, PA,USA) on a 3 h-lawn, allowing them to stand upright for 3 s without disturbing the agar surface. The procedure was repeated 10 times to sample different areas of the lawn. Capillaries were finally photographed using a Gel-Doc system (Bio-Rad^R). Swarm plates made with Bacto agar gave more consistent results than those with Eiken agar, hence the former were used for these experiments.

Microarray experiments

Details of RNA preparation, hybridization and data analysis have been described (Wang *et al*, 2004). Wild-type and *che* or *flgM* mutants were compared on the same array using different colors. Genes showing differential expression of at least 1.5-fold or above were considered significant. Each slide had three repeats of the entire array. Data in Table I represent several samples from a time course, while those in Table II were acquired for a specific time point from two independently prepared samples. The microarray data were deposited in Gene Expression Omnibus (GEO) of The National Center for Biotechnology Information (NCBI). The accession numbers are: GSM23883–85, GSM23150–52, GSM23876–81, GSM23896–98, GSM23889–91, GSM23126–28, GSM43870–96, GSM23900–02, GSM24267–69, GSM24273–75.

Flagellar staining, Western blots and photography

Staining. Cells from broth were centrifuged (200 *g*) and washed gently in water. 5 μl of the cell suspension was transferred onto a precleaned slide (Esco) and spread evenly with the blunt end of a pipette tip. From plates, cells were transferred to 5 μl of water already applied to the slide, lightly touching with a pipette tip end. After air-drying, cells were fixed for 30 min with 100 μl of Solution 1 (see Harshey and Matsuyama, 1994), followed by 100 μl of BSA (1 mg/ml) for 30 min, 20 μl of 1st antibody (Anti-FljB, 100 × diluted) over-night, 20 μl of 2nd antibody (Texas-Red^R, 100 × diluted) for 2 h and 30 μl of DAPI (1000 × diluted) for 30 min. The slide was washed with phosphate buffered saline (PBS) (Sambrook *et al*, 1989) between each treatment. All reagents were diluted in PBS, and all incubations were at room temperature in a wet and dark chamber. After a final wash with water, the slide was viewed under Olympus BS60 with × 1000 magnification. The fluorescence image was captured by Quantix (Roper Scientific, Inc.) and associated software MetaMorph.

Western blots. Each plate was washed with 2.3 ml of ice-cold LB (Luria broth). For FljB blots, cells were diluted to an OD_{600} of 0.6, and 30 μl of cells used for SDS–PAGE. For FlgM blots, cells were pelleted at 12 000 *g* for 10 min, and the pellets and supernatants analyzed separately—pellets were resuspended in LB to OD_{600} of 1.0, and 25 μl used for SDS–PAGE. Supernatants were precipitated with TCA (Sambrook *et al*, 1989), and protein was estimated by the Protein Assay reagent (Bio-Rad). Samples were normalized to OD_{595} of 0.3, and 20 μl subjected to SDS–PAGE. Western blots were performed using standard protocols (Sambrook *et al*, 1989) and according to the manufacturer's instructions (Amersham Pharmacia Biotech). Developed blots were quantitated with QuantityOne software (Bio-Rad).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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