

# The Wetting Agent Required for Swarming in *Salmonella enterica* Serovar Typhimurium Is Not a Surfactant<sup>∇</sup>

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**We compared the abilities of media from agar plates surrounding swarming and nonswarming cells of *Salmonella enterica* serovar Typhimurium to wet a nonpolar surface by measuring the contact angles of small drops. The swarming cells were wild type for chemotaxis, and the nonswarming cells were nonchemotactic mutants with motor biases that were counterclockwise (*cheY*) or clockwise (*cheZ*). The latter strains have been shown to be defective for swarming because the agar remains dry (Q. Wang, A. Suzuki, S. Mariconda, S. Porwollik, and R. M. Harshey, *EMBO J.* 24:2034–2042, 2005). We found no differences in the abilities of the media surrounding these cells, either wild type or mutant, to wet a low-energy surface (freshly prepared polydimethylsiloxane); although, their contact angles were smaller than that of the medium harvested from the underlying agar. So the agent that promotes wetness produced by wild-type cells is not a surfactant; it is an osmotic agent.**

Bacterial swarming is a specialized form of motility that develops when cells that can swim are grown in a rich medium on a moist surface. The earliest work on swarming was done with *Proteus*, whose cells build large terraced colonies by switching periodically between a swarming migratory phase and a vegetative nonmigratory phase (19). During swarming, the cells become multinucleate, elongate, synthesize large numbers of flagella, excrete agents that promote wetness, and advance across the surface in coordinated packs. Upon reverting to the vegetative phase, they produce shorter, nonswarming cells by division. This cycle of migration and consolidation also occurs with other swarming bacteria (12); however, *Escherichia coli* and *Salmonella*, which were found to swarm by Harshey and Matsuyama (11), produce large, unterraced colonies. This apparently simpler migration, as well as the advantage of working with organisms for which so much of genetics and biochemistry are known (18), suggested to Harshey that “questions that are of central importance in understanding the swarming phenomenon. . . should soon be within reach” (10). For recent reviews on swarming, see Fraser et al. (8), Sharma and Anand (20), Harshey (9), Daniels et al. (4), and Kaiser (13).

Transposon mutagenesis of *Salmonella* turned up a number of swarm-negative mutants that were defective in lipopolysaccharide (LPS) synthesis, and many of these could be rescued by addition of surfactin (see below) or by growth on Eiken agar, which is more wettable than Difco agar; however, a smooth-swimming *Salmonella* strain (*cheA*) could not be rescued by addition of either LPS or surfactin (23). Secreted surfactants have been identified for a variety of organisms (5), including

serrawettins (lipopeptides) for *Serratia* (17, 25), surfactin (another lipopeptide) for *Bacillus subtilis* (14), and rhamnolipids (glycolipids) for *Pseudomonas aeruginosa* (2, 6), but such compounds have not been characterized for *E. coli* or *Salmonella*. However, a flagellar gene required for swarming (*flhE*) and encoding a protein thought to affect the wettability of the outer cell surface has been found in *Salmonella* (21).

Harshey and colleagues found that cells of most chemotactic mutants swarm poorly because the cell lawn remains dry (24). This leads to a defect in secretion of the filament protein FliC and the transcription inhibitor FlgM, yielding cells with fewer and shorter flagella. These defects can be cured simply by exposing the swarm plate to a fine mist of water. Wild-type behavior also can be restored by selecting for mutations in *fliM* that increase the frequency of motor reversals—FliM is a protein at the base of the motor required for assembly, rotation, and direction control—leading to the suggestion that these reversals promote wetness by causing the cells to shed LPS (16).

We thought it would be useful to know whether we are dealing with wetness or wettability. Wetness refers to the amount of fluid that a swarm contains, while wettability refers to the ability of that fluid to wet a nonpolar surface. Presumably, the wetness depends upon the hydrophilicity of the LPS shed by the cells and the osmolarity of the resulting fluid, leading to movement of water from the underlying agar, while the wettability depends upon the concentration of surface-active agents in this fluid. It is possible that both functions are supported by the same molecules. For a brief introduction to wetting, see Courbin and Stone (3); for a more extensive review, see Zisman (26).

In studying wetness, Wang et al. (24) prepared lawns of wild-type and mutant cells by inoculating the entire surfaces of agar plates. To find out whether the dryness of lawns of chemotactic mutants might be due to a paucity of surface-active agents, we followed the procedures of Wang et al. and pre-

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pared lawns of the same *Salmonella* strains, specifically the wild-type strain and *cheY* and *cheZ* mutants. CheY is the response regulator that when phosphorylated binds to FlhM, and CheZ is its phosphatase; when in liquid media, *cheY* cells rotate their flagella exclusively counterclockwise and swim smoothly, while *cheZ* cells rotate their flagella predominantly clockwise and tumble. We harvested the fluid surrounding the cells of these three strains and measured the wetting angles of small drops on a clean, low-energy surface (freshly prepared polydimethylsiloxane [PDMS]) with a surface energy of 19 dynes/cm (7). For similar experiments with swarms of *Serratia* on polystyrene (surface energy, 33 dynes/cm), see references 17 and 15. We also harvested fluid from the underlying agar. The fluid surrounding the cells yielded contact angles smaller than those found for the growth medium or for the fluid extracted from the agar and thus contained surface-active agents, but the results for each strain were identical. Therefore, the differences in wetness observed by Wang et al. (24) are not due to differences in wettability.

**Measurements of interfacial tensions of bulk material.** Wild-type *Salmonella enterica* serovar Typhimurium strain 14028 and strains carrying *cheY* and *cheZ* deletions derived from this strain, a gift from Rasika Harshey (24), were grown to saturation overnight in LB broth (Difco Bacto tryptone, 1%; yeast extract, 0.5%; and NaCl, 0.5%) at 37°C and then diluted 1:100 and grown again to an optical density at 610 nm of ~0.6. The interfacial tensions of these cultures were measured with a commercial tensiometer (Fisher Scientific tensiometer, model 20), which senses the force required to pull a platinum-iridium ring out of the liquid. The readings for different cultures were indistinguishable (~55 dynes/cm) and not very different from that for LB alone (~58 dynes/cm). Solutions of the detergent Triton X-100 (TX1566; EM Science) gave tensiometer measurements that decreased smoothly from ~71 to 33 dynes/cm at concentrations increasing from 0 to 0.013%, the critical micelle concentration (data not shown).

**Measurements of contact angles.** Since the tensiometer requires ~25 ml of fluid, we chose to study the wetting of a low-energy surface by small drops (5  $\mu$ l), a scale more suitable for assays of fluid from bacterial swarms. A Parafilm surface gave erratic results, so we prepared fresh films of PDMS. We mixed Dow Corning Sylgard-184 with its curing agent in the ratio 10:1 (wt/wt), removed air bubbles under soft vacuum, spin coated 22-mm-diameter coverslips at 5,000 rpm for 1 min (using a Headway Research spinner, model PWM32-PS-R790), and cured the films at 100°C in an oven for 45 to 60 min. The cured coverslips were stored in a covered dish for at least 1 h before use or overnight under vacuum.

Swarm agar (0.6% Difco agar in LB broth, stored in sterile aliquots of 100 ml/bottle) was melted in a microwave oven at low power and mixed with glucose (final concentration, 0.5%), pipetted (10 ml) into petri plates (8.5-cm diameter), spread by gentle swirling, and allowed to cool uncovered in a large Plexiglas box (to minimize contamination by dust) for ~15 min. Then, following Wang et al. (24), 10 ml of the culture with an optical density at 610 nm of ~0.6 was poured over the plate, allowed to sit for ~40 s, and poured off, with the excess at the side of the plate removed with a Kimwipe. The plates were returned to the Plexiglas box and allowed to dry for ~10 min (at which time they were free of noticeable puddles) and then

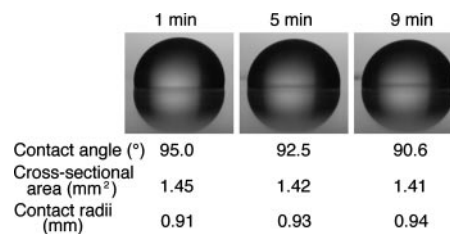


FIG. 1. Three images of a drop in the *cheY* sequence of Fig. 2A, together with results for different drop parameters. The lower half of each image is a reflection from the PDMS surface. The indentations at the left and right edges of the first image at the level of the PDMS surface indicate contact angles of  $>90^\circ$ . Eventually, these indentations disappear, as the contact angles fall below  $90^\circ$  (not shown).

covered and incubated at 37°C. Vigorous motion among the wild-type cells, typical of swarming, was evident after ~2 h, but the mutant cells remained nonmotile. At 3 h, the cells were scraped off the plates with the long edge of a microscope coverslip (22 by 60 mm, no. 1) and transferred to an Eppendorf tube, and the cell bodies were removed by centrifugation at  $\sim 8,000 \times g$  for 20 min, yielding a supernatant fraction of 100 to 150  $\mu$ l. A volume of agar similar to that of the cells was harvested from a region from which cells had been removed and was processed in the same way. To be certain that *cheY* and *cheZ* cells were defective when grown under these conditions, plates were inoculated at the center; the wild-type cells spread vigorously, but the mutant cells did not.

Drops of the supernatant fractions were placed on a PDMS-coated coverslip on a black Delrin platform and covered with a rectangular glass-walled box (with a 25- by 27-mm opening at the bottom, 18 mm high) made from a black Delrin frame and pieces of no. 1 coverslips attached with silicone rubber cement (GE RTV-118). A 10- by 20-mm piece of moist filter paper was inserted at each end of this box to raise the humidity. The drops were backlit with a diffuse light source and photographed from the front with a Nikon Coolpix E4500 digital camera equipped with a time lapse attachment set to release the shutter every 2 min. One drop was placed when the camera was started (time zero), and a second was placed at 1 min, so the data displayed a systematic variation between even and odd data points. We used an ImageJ plugin, DropSnake (22), to find the contact angles. This plugin utilizes both the image of the drop and its reflection to compute left and right contact angles, cross-sectional area, and left and right contact radii; here, the means of the left and right values are reported.

Three images of drops from a *cheY* experiment are shown in Fig. 1, taken at 1, 5, and 9 min. Note that the contact angles gradually decreased with time. The cross-sectional areas also decreased with time but more slowly, while the contact radii increased. We believe that the changes that occurred over time were due to gravity-driven spreading.

Contact angles for supernatant fractions of cells from swarm plates and for the underlying agar are shown in Fig. 2A. The fluid obtained from the agar displayed contact angles essentially identical to those found for unused agar or for pure water (see below). The fluid obtained from the different cell fractions displayed contact angles that were smaller, indicating the presence of surface-active agents. However, no differences were observed between fluid obtained from wild-type cells and that

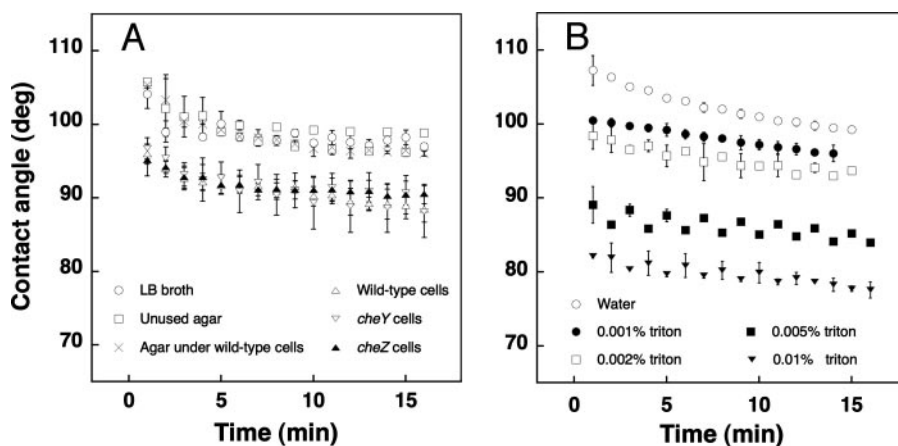


FIG. 2. (A) Contact angles for wild-type cells, *cheY* and *cheZ* mutants, LB broth, and agar removed either from the bottle or from a wild-type plate (or from a *cheZ* plate, which gave identical results [data not shown]). Averages are for 4 to 6 drops from each of three series of experiments. The error bars are standard deviations, with each drop weighted equally. (B) Contact angles for water and various solutions of the detergent Triton X-100. As expected, the contact angles decrease with the concentrations of the detergent. They also decrease with time. The interfacial tensions for water and these solutions, measured at 23.7° in triplicate with the Fisher tensiometer, were  $70.7 \pm 1.0$ ,  $53.9 \pm 1.6$ ,  $48.7 \pm 0.2$ ,  $41.7 \pm 0.4$ , and  $37.3 \pm 0.7$  dynes/cm, respectively.

from cells of *cheY* or *cheZ* mutants. Therefore, the differences in “wetting” observed by Wang et al. (24) for these strains are not due to differences in concentrations of surface-active agents. They must be due to differences in the concentrations of agents that draw water out of the underlying agar.

Results for water and a series of control solutions of the detergent Triton X-100 are shown in Fig. 2B. Contact angles measured for supernatant fractions of cells from swarm plates (Fig. 2A) fell between those observed for 0.002% and 0.005% Triton X-100 (Fig. 2B). And the wetting angles for water were similar to those measured previously for Sylgard-184 (7).

**Conclusions.** Lawns of wild-type cells are relatively wet, while lawns of *cheY* and *cheZ* cells are relatively dry (24), yet the fluid surrounding both kinds of cells wet PDMS to the same extent. They do so more than the medium from the agar on which the cells were grown; therefore, they contain surface-active agents derived from the cells. As noted above, the agent that makes the wild-type lawn wet must be something other than a surface-active agent: it is something that draws fluid out of the underlying agar, i.e., it is an osmotic agent. If surface-active agents are present in the fluid on top of the agar, why are they not found in the fluid extracted from the underlying agar (Fig. 2A)? These fluids are in intimate contact with one another, and agar is very porous, so any water-soluble substance in one should diffuse into the other. We believe that the surface-active agents secreted by the cells are adsorbed by the agar.

If the ideas of Harshey and colleagues (16), namely, that flagellar reversals promote wetness by causing the cells to shed LPS, are right, then this material is doing precisely that, i.e., promoting wetness. It is not acting as a surface-active agent (in common parlance, as a wetting agent); it is acting as an osmotic agent that draws water out of the underlying agar. Thus, our measurements support the hypothesis of Rauprich et al. (19), that swarming requires a fluid environment generated as bacteria extract water from the underlying agar gel, “achieved by the elaboration of the acidic capsular polysaccharide observed

to accompany swarming.” The role of surfactants, such as ser-rawettin or surfactin, not required for swarming in *Salmonella* might then be to promote the spreading of this polysaccharide (1).

Flagella are designed to work in an aqueous environment: swarming cells move in a thin layer of fluid over the surface of the agar. The fluid comes from the agar. If cells generate a medium of high osmolarity by secreting or shedding an osmotic agent, presumably LPS, water will move from the agar into this medium until the osmolarities match. In addition, if cells are to move freely over the agar, they must not adhere to the agar or to one another. If this is a problem, one can change the wettability of the cells, e.g., by adding FlhE, or the wettability of the agar, e.g., by using Eiken agar, or one can add surfactants, as practiced by *Serratia*, *Bacillus*, or *Pseudomonas*. For *Salmonella*, LPS appears to be an essential additive.

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