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Specific Inactivator of Flagellar Reversal in *Salmonella typhimurium*

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Specific inhibition of flagellar rotation reversal was observed after exposure of chemotactic *Salmonella typhimurium* to citrate autoclaved at neutral pH. The presence of a rotation reversal inactivator was established in autoclaved citrate-containing media and nutrient broth. Since modulation of flagellar rotation by attractants and repellents is the basis of chemotactic behavior, a specific inhibitor of rotation reversal, which is essential for tumble generation, provides a useful probe into the molecular mechanism of bacterial chemotaxis. The inactivator inhibits clockwise rotation without affecting counterclockwise rotation, speed of rotation, or the capacity of the cells to grow and divide. Inactivation of clockwise rotation is gradual and irreversible, differing from the transient inhibition of clockwise rotation by attractants, which is characterized by an immediate suppression followed by a return to normal rotation patterns. The rotation reversal inactivator is stable to acidification, rotary evaporation, lyophilization, and rehydration.

Bacteria migrate toward higher or lower concentrations of attractants and repellents by reorienting their swimming direction in gradients of these substances (see reviews by Koshland [4], Adler [1], and Berg [2]). Peritrichously flagellated bacteria such as *Escherichia coli* and *Salmonella typhimurium* reorient by a rapid erratic motion called "tumbling," which interrupts their unidirectional "smooth swimming." Since suppression and enhancement of tumbling by temporal gradients of attractants and repellents is the basis of chemotactic migration, a central question is the identity of the tumble generator and its chemical regulation.

Chemotactic responses can now be studied in bacteria with a single flagellar motor exposed to temporal changes in attractant and repellent concentrations. That bacteria swim by rotating their flagella was proved by Silverman and Simon (8), who attached individual flagella of *E. coli* to a glass surface coated with anti-flagellar antibody. Both clockwise (CW) and counterclockwise (CCW) rotation of the body with respect to the immobilized flagellum was observed. Subsequent studies (5) using the tethering technique showed the correlation of tumbling with reversal of flagellar rotation. In both *E. coli* (3, 5) and *S. typhimurium* (J. L. Spudich, Ph.D. thesis, University of California, Berkeley, 1976), a positive stimulus (an increase in attractant or decrease in repellent concentration) causes a transient period of sustained CCW rotation. After this response, the flagellar motor returns to its prestimulus state of an alternating CW/CCW rotation pattern. Similarly, a transient period of sustained CW rotation results from a negative stimulus (a decrease in attractant or increase in repellent concentration). This modulation of individual flagellar rotation direction results in control of tumbling frequency by the environmental stimuli.

In tethering studies we were surprised to find that under some conditions CW rotation (the repellent-stimulated direction of rotation) progressively disappeared in our tethered cells. Fields of up to 100 bacteria persisted in their continuous vigorous rotation, but only in one direction: counterclockwise. Since modulation of rotation is the basis of chemotactic behavior, this conversion of cells from CW/CCW reversing behavior to CCW-only behavior reflects a very fundamental change in the chemotactic machinery of the cells. Accordingly we sought the cause of this phenomenon and found specific inactivators of rotation reversal in autoclaved citrate-containing media and in autoclaved nutrient broth. Experiments demonstrating the presence of these inactivators and several of their properties are described in this report.

In this report CCW rotation is defined as counterclockwise rotation of the bacterial body when observed along the direction from the body

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to the flagellum. With this convention, CCW rotation is the attractant-stimulated direction, and CW rotation is the repellent-stimulated direction.

MATERIALS AND METHODS

Chemicals. Vogel-Bonner (VB) salts including citric acid·H_2O, $MgSO_4 \cdot 7H_2O$, K_2HPO_4, and $NaNH_4HPO_4 \cdot H_2O$ were obtained as Mallinckrodt analytical grade reagents. Glycerol was from Fisher, and *p*-hydroxybenzoic acid and chloramphenicol were from Sigma. L-Histidine was obtained from Mann Research; nutrient broth was from Difco.

Bacterial strains and growth conditions. Bacterial strains used in this study were *S. typhimurium* LT2 (wild type), SL4041 (a tumbly mutant derived from *S. typhimurium* LT2), and SL3625, a leaky *flaAIII* mutant derived from LT2 and previously described as LT2 *hisC527*(Am) *xyl-416 fla-401*(Am) (10). The latter strains were obtained from B. A. D. Stocker (Department of Medical Microbiology, Stanford University, Stanford, Calif.). SL3625 cultures were grown with shaking at 30°C to middle or late logarithmic phase (2×10^8 to 8×10^8 cells per ml) in either nutrient broth or VB citrate (VBC) minimal medium (11) made 1% glycerol, 200 μM *p*-hydroxybenzoic acid, and 0.05 mg/ml L-histidine. VBC contains 10 mM citrate (pH 6.95). Absence of the *p*-hydroxybenzoic acid in VBC results in a totally immotile culture. In the presence of *p*-hydroxybenzoic acid and under these conditions, electron micrographs show one to three flagella per SL3625 bacterium and full flagellation of LT2 and SL4041. When peritrichously flagellated bacteria (e.g., *E. coli* and *S. typhimurium*) are used for tethering, a high percentage of rotating bacteria is not observed unless the average number of flagella per cell in the population is reduced from the usual value of about eight to a value close to one. For tethering LT2 and SL4041, the numbers of flagella per cell were reduced by using the temperature shift conditions exactly as described by Quadling and Stocker (7).

Tethering procedures. Chambers for tethering were made by attaching a lucite block with an oblong hole in the center (as described for temporal gradient observation cells [6]) with epoxy to a slide surface. Each lucite block contained inlet and outlet channels to which tubing could be attached, and the upper surface was open. The outlet tubing led to a peristaltic pump, and the inlet tubing led to a flask containing the wash solution (for delivering temporal gradient stimuli, the inlet tubing was connected to a stopcock that allowed connection with either of two flasks; in this way, when the pump was on, the incoming solution could be changed by simply turning the stopcock).

Bacteria were tethered by incubating about 0.1 ml of bacterial suspension for 3 to 5 min on a cover slip containing flagellar antibody, prepared as described by Spudich (Ph.D. thesis). This cover slip with attached bacteria was inverted (letting the drop of bacterial suspension hang from the cover slip) and placed on the open chamber. The chamber and inlet tubing were previously filled with liquid to allow a liquid seal to form immediately when the cover slip was placed (time = 0). The volume of the chamber was 0.09 ml. A microscope at ×500 magnification was focused on the cover slip. The bacteria were observed along the direction from the flagellum to the body, and the observed rotation was corrected to accord with the convention discussed in the Introduction. This procedure gives about 40 to 100 vigorously rotating cells per microscopic field. The behavior of the bacteria was recorded on a video tape recorder attached to the microscope so that the results could be replayed for examination. In all experiments, unless otherwise noted, the cells were continuously washed at a rate of 0.70 ml/min. Deviation from this flow rate and the acquisition and processing of data from tethered cells are described in the appropriate figure legends and table notes. Measurements involving histidine starvation were performed with the smaller chamber and flow rates described in Fig. 2. All instruments and solutions were equilibrated to 30°C before the cells were tethered.

RESULTS

Inactivation of rotation reversal in tethered bacteria with autoclaved nutrient broth. When SL3625 bacteria were tethered and observed as described in Materials and Methods, initially 75 to 95% of the rotating bacteria showed alternate CW and CCW rotation, with most switching between the two modes occurring between 2 and 10 s. The remaining 5 to 25% of the rotating bacteria showed no CW rotation. Under certain conditions (details below) the number of these CCW-only bacteria increased in the observation cell so that by typically 3 h after tethering none of the cells showed CW rotation. CW responses were not elicited from these bacteria by subjecting them to the relatively strong repellent stimuli 0 → 50 mM acetate or 10 → 0 mM citrate.

Figure 1 illustrates this phenomenon and the principal assay we used to follow the loss of CW rotation. The bacteria were recorded on video tape for 1-min intervals at various times after tethering. The recordings were then examined to determine the proportion of rotating bacteria that were CCW-only during each 1-min interval. In Fig. 1 are shown plots of the fraction of bacteria that were CCW-only for cells grown and washed in nutrient broth compared to those grown in defined medium and washed with buffer. These two particular conditions are shown here to illustrate that the loss of CW rotation is clearly not attributable simply to mechanical effects of tethering or washing the bacteria, since in that case the loss should occur in the buffer-washed cells.

From the data in Fig. 1, it is not clear whether the increase in CCW-only bacteria for the nutrient broth cells is due to selective loss of CW/CCW alternating cells from the population or to the conversion of CW/CCW alternating bacteria

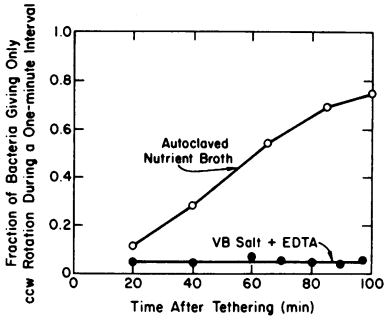

FIG. 1. *Loss of CW rotation in tethered cells. SL3625 bacteria were grown and tethered as described in the text, except (i) the cells were grown and the experiment was performed at room temperature; (ii) wash conditions were as described for the "continuous wash" case of Fig. 2. At various times after tethering, bacteria grown in autoclaved nutrient broth and washed with autoclaved nutrient broth* (○) *and bacteria grown in autoclaved VBC minimal medium + 1% glycerol + 50 μg/ml L-histidine + 200 μM p-hydroxybenzoic acid and washed with VB salts + 10^{-5} M EDTA* (●) *were examined for a period of 1 min to ascertain the fraction of the bacteria that rotate continuously throughout the interval and show no CW rotation in the interval. The 1-min periods were videotaped, and the recording was repetitively played back for analysis. The average number of bacteria analyzed for each point in these experiments was 24.*

to the CCW-only state; i.e., do individual bacteria show a loss of the CW rotation mode with time? To answer this question, data were collected from one microscopic field of bacteria grown and washed in nutrient broth for four 1-min periods at various times after tethering. The mean CW (CW) and mean CCW (CCW) interval lengths in each period were measured for individual bacteria. The results (data are in Spudich's Ph.D. thesis) were as follows. (i) Individual bacteria showed a gradual and progressive loss of the CW rotation mode with time. (ii) The inactivation of rotation reversal differed from that induced by attractants, since the latter resulted in immediate suppression of CW rotation followed by a gradual return to normal reversal frequencies. (iii) For an individual bacterium an increase in the average length of CCW runs (CCW) was accompanied by a decrease in the average length of CW runs (CW). Analogous measurements performed on the buffer-washed cells (Fig. 1) showed no systematic decrease in CW nor increase in CCW over time.

To test whether this phenomenon is a peculiarity of the mutant SL3625, the number of flagella per bacterium was reduced by the temperature shift method of Quadling and Stocker (7) to allow tethering of wild type (LT2) and a tumbly mutant, SL4041. Each of these strains was tethered and washed with fresh nutrient broth for 50 min, and their rotation was observed. Both strains developed predominantly CCW-only behavior, and therefore the phenomenon is not restricted to the SL3625 strain.

The measurements above in which loss of the CW mode was observed were made with nutrient broth-grown cells washed with nutrient broth after tethering. To rule out that some substance specific only to this complex medium (nutrient broth) causes the effect, SL3625 bacteria were grown in the defined VBC minimal medium plus glycerol and washed with this medium (see Materials and Methods). The fact that this defined medium also induces loss of the CW mode (see "continuous wash" curve in Fig. 2) enabled us to establish the presence of a rotation reversal inactivator (RRI) and to identify its source in the defined medium components (see below).

To test whether this phenomenon is growth related, tethered bacteria were washed with complete medium + 100 μg of chloramphenicol per ml. Such nongrowing cells did show the phenomenon of loss of the CW mode when washed with chloramphenicol in either nutrient broth or VBC + glycerol (Table 1) and rotated vigorously for at least 4 h. Since data analysis is simpler when no newly produced bacteria are attaching to the cover slip during the experiment, the measurements reported below were all made with chloramphenicol in the wash medium.

Autoclaved citrate-containing medium also contains the RRI. The RRI activity of fresh VBC medium was produced by autoclaving the medium (Table 1). Bacteria continuously washed with VBC + glycerol that was not autoclaved showed no tendency to lose their rotation reversal, whereas autoclaved VBC + glycerol inactivated the reversal. Autoclaving 1% glycerol or water alone did not produce RRI; hence the RRI-forming compound(s) is in VBC. VB salts (i.e., VBC without citrate) when autoclaved produced little or no RRI. Inorganic phosphate and citrate were shown to be sufficient components of VBC + glycerol to generate RRI by autoclaving (Table 1), and autoclaved citrate alone produced RRI activity.

Properties of RRI from autoclaved citrate and its action. Bacteria induced into a CCW-only state by RRI in the absence of chloramphenicol continued to grow and divide, indicating that RRI is not a general inhibitor of cellular metabolism. Furthermore, bacteria induced into a CCW-only state showed essentially no change in their rotation speed, indicating that

TABLE 1. *Autoclaving citrate produces RRI*[a]

Autoclaved component in minimal medium	Mean % increase of CCW-only bacteria (± SD)	No. of bacteria analyzed
VB salts + citrate + glycerol	72 ± 9	1,150
None	8 ± 2	266
Glycerol	−6	60
Water	3	83
VB salts	24 ± 10	260
Citrate + K_2HPO_4	79 ± 10	301
Citrate	80 ± 9	160

[a] For each assay SL3625 bacteria were tethered and continuously washed as described in the text. Autoclaved components of our minimal medium were autoclaved in bakelite-capped glass bottles at their concentrations in minimal medium at pH 7.0 for 40 min in an American CyclomatiControl laboratory autoclave. After cooling, nonautoclaved components were added so that the wash solution in all cases was VB salts + citrate + glycerol + histidine + *p*-hydroxybenzoic acid at the concentrations reported in Fig. 1 and chloramphenicol at 100 μg/ml. Percentage increases (± standard deviation [SD]) were calculated based on measurements at 20 and 140 min after tethering; a 20-min value of 22% CCW-only bacteria was obtained as the average of 17 assays (washing with used medium or buffer) in which no increase in CCW-only cells between 20 and 140 min was observed. The number of CCW-only cells at a given time was determined as described in Fig. 1.

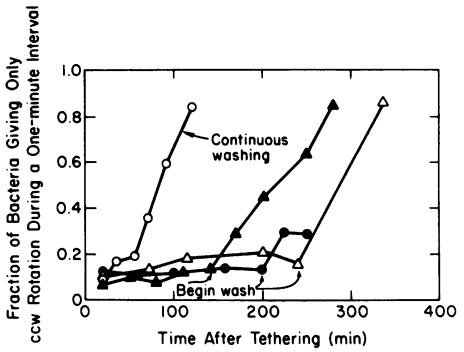

FIG. 2. *Washing induces loss of CW rotation. SL3625 bacteria were grown and tethered as described in Fig. 1 and washed at 12.7 chamber volumes per min for 5 min with the minimal medium described in the text plus 100 μg of chloramphenicol per ml. For the curve designated "continuous washing," the initial 5-min wash was immediately followed by a continuous wash at 1.6 chamber volumes per min (1 chamber volume = 0.07 ml). For the other curves no washing was performed after the initial 5 min until the times designated by arrows, at which time a continuous wash at 1.6 chamber volumes per min was commenced. Data were collected as described in the legend to Fig. 1.*

the motility apparatus was not generally inhibited.

Flagellar reversal inactivation by RRI is apparently irreversible: CCW-only bacteria continuously washed for 100 min with a medium not containing RRI but containing chloramphenicol did not recover to an alternating CW/CCW state.

Two types of measurements show that RRI is either consumed or inactivated by the bacteria. First, loss of the CW mode absolutely requires continuously confronting the bacteria with fresh RRI. The results of Fig. 2 demonstrate this requirement for continuous washing. In each of the measurements in Fig. 2, beginning at the time of tethering, the bacteria were washed continuously for 5 min at a relatively high rate (which completely replaced the medium in which the bacteria were grown) with fresh medium containing RRI. After 5 min for the "continuous washing" curve, the washing was changed to a continuous relatively slow rate of flow which confronted the bacteria with constantly fresh RRI but did not affect their motion. If washing was stopped after the initial 5 min, little or no increase in CCW-only bacteria was observed. When washing was recommenced at a later time in the experiment, CCW-only behavior began to appear in the bacteria (Fig. 2). This requirement for washing shows that the bacteria either consume or inactivate RRI. This also explains why wild-type cultures grown in flasks in autoclaved citrate minimal media or nutrient broth do not become smooth swimmers. A second way to demonstrate this is by washing bacteria continuously with used medium, from which bacteria have been removed by centrifugation. Bacteria washed with used medium (in which bacteria were grown overnight to stationary phase, "used medium, early stationary phase,") showed no loss of rotation reversal (Table 2). Thus RRI is either consumed or inactivated by the bacteria. Boiling or steaming of used medium (Table 2) either (i) produced RRI de novo or (ii) destroyed a RRI inactivator secreted by the bacteria. Favoring alternative (i) is the fact that late-stationary-phase medium (in which bacteria were incubated for 2 days after reaching stationary phase) did not show regeneration of RRI activity upon boiling (Table 2). Such a late medium might be expected to be depleted of citrate. However, favoring alternative (ii) is the fact that boiling nonautoclaved VBC (data not shown) or citrate + K_2HPO_4 (Table 2) did not generate RRI. In view of this, if alternative (i) is correct, then some compo-

TABLE 2. *Used medium does not contain RRI*[a]

Wash solution	Mean % increase of CCW-only bacteria	No. of bacteria analyzed
Used medium, early stationary phase	4 ± 5[b]	912
Used medium, early stationary phase; boiled	83	109
Used medium, early stationary phase; steamed	78	120
Used medium, late stationary phase; boiled	0	60
Citrate + K_2HPO_4; boiled	14	57

[a] For each assay SL3625 bacteria were tethered and continuously washed with the wash solution indicated, reconstituted with fresh components at their concentrations in fresh medium. Percentage increases were determined as described in Table 1. "Used medium, early stationary phase" was obtained by growing SL3625 bacteria to an optical density at 650 nm of 0.6 to 0.9 in the minimal medium described in the text, pelleting the bacteria by centrifugation at ambient temperatures, and collecting the supernatant. "Used medium, late stationary phase" was obtained the same way except the bacteria remained in the medium for 2 days (at 30°C with shaking) after reaching the early stationary phase condition. Boiling was for 5 min in open flasks. Steaming was for 2 min in a closed glass container.

[b] Standard deviation.

TABLE 3. *RRI stability and lack of formation at low pH*[a]

Wash solution	Mean % increase of CCW-only bacteria	No. of bacteria analyzed
Citrate + K_2HPO_4, autoclaved at pH 1.4	0 ± 1[b]	140
Citrate + K_2HPO_4, autoclaved at pH 7.0 and incubated for 20 min at 30°C, pH 1.4	64	93
Citrate + K_2HPO_4, autoclaved at pH 7.0, rotary evaporated, lyophilized, and rehydrated	72	76
Citrate + K_2HPO_4, at pH 7.0 (nonautoclaved), rotary evaporated, lyophilized, and rehydrated	9	56

[a] For each assay SL3625 bacteria were tethered and continuously washed, as described in the text, with complete minimal medium containing the components indicated in the table plus 100 μg of chloramphenicol per ml. Percentage increases were measured as described in Table 1.

[b] Standard deviation.

nent(s) of used medium must facilitate the reaction producing RRI de novo.

In Table 1 RRI is shown to be produced by autoclaving citrate and K_2HPO_4 at pH 7.0. When citrate and K_2HPO_4 were autoclaved at pH 1.4, no RRI was formed (Table 3). Since RRI was not destroyed by incubation at pH 1.4 (Table 3), the reaction leading to RRI formation must be inhibited at low pH. At pH 7.0 citric acid is trianionic, whereas at pH 1.4 it is fully protonated. Therefore, the reaction leading to RRI requires either one, two, or three of the carboxyl groups to be ionized. The data in Table 3 also demonstrate that RRI is stable to rotary evaporation, lyophilization, and rehydration.

When histidine was omitted from the wash solution containing RRI, tethered SL3625 cells showed little or no loss of CW rotation (percent increase of CCW-only bacteria = 5%, determined as described in Table 1). SL3625 is a histidine auxotroph. To determine whether this protection against RRI inactivation by histidine starvation is related to the auxotrophic requirement, a spontaneous *his*$^+$ revertant was selected from SL3625. The revertant, grown in the absence of histidine and washed with autoclaved VBC-glycerol without histidine, lost CW rotation (percent increase of CCW-only bacteria = 73%, determined as described in Table 1). Therefore the presence of histidine in the wash solution is not essential for RRI activity, but histidine starvation protects a histidine auxotroph against rotation reversal inactivation. RRI inactivation and the protection of SL3625 by histidine starvation were both observed in the presence and absence of chloramphenicol. Thus histidine starvation protects a *his* auxotroph even though chloramphenicol does not.

DISCUSSION

The results presented above show that autoclaving citrate at neutral pH produces a substance (or substances) which inactivates chemotaxis of *S. typhimurium* by specifically inhibiting flagellar reversal. The presence of RRI was established in autoclaved citrate-containing media, and several of its properties were determined by studies of rotating *S. typhimurium* tethered by their flagella. An inhibitor of rotation reversal was also found in autoclaved nutrient broth.

RRI inhibits CW rotation without affecting CCW rotation, speed of rotation, or the capacity of the cells to grow and divide. This inhibition appears to be irreversible, since removal of RRI did not restore CW rotation. This phenomenon differs from the transient inhibition of CW rotation induced by attractants, which is charac-

terized by an immediate suppression followed by a return to normal rotation patterns.

RRI was effective on all the strains tested: the *S. typhimurium* wild type (LT2), a tumbly mutant derived from LT2 (SL4041), and a leaky *fla* mutant derived from LT2 (SL3625). That RRI is either consumed or inactivated by the bacteria was shown by the observations that bacteria must be exposed to an excess of autoclaved medium to demonstrate an effect and that excess bacteria could deplete the medium of RRI.

Single-cell tethering is becoming an increasingly more important procedure in studies of chemotaxis. The presence of RRI in commonly used media makes an appreciation of RRI action important to potential uses of the tethering technique.

Since modulation of flagellar rotation direction by attractants and repellents is the basis of chemotactic behavior in bacteria, a specific inhibitor of rotation reversal with the properties of RRI would provide a useful probe into the molecular mechanism of bacterial chemotaxis.

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