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Phosphorylation-Independent Bacterial Chemoresponses Correlate with Changes in the Cytoplasmic Level of Fumarate

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Bacterial chemotaxis is based on modulation of the probability to switch the direction of flagellar rotation. Responses to many stimuli are transduced by a two-component system via reversible phosphorylation of CheY, a small cytoplasmic protein that directly interacts with the switch complex at the flagellar motor. We found that the chemorepellents indole and benzoate induce motor switching in *Escherichia coli* cells with a disabled phosphorylation cascade. This phosphorylation-independent chemoresponse is explained by reversible inhibition of fumarase by indole or benzoate which leads to an increased level of cellular fumarate, a compound involved in motor switching for bacteria and archaea. Genetic deletion of fumarase increased the intracellular concentration of fumarate and enhanced the switching frequency of the flagellar motors irrespective of the presence or absence of the phosphorylation cascade. These correlations provide evidence for fumarate-dependent metabolic signal transduction in bacterial chemosensing.

Motile procaryotes actively search for optimal living conditions. This phenomenon, called taxis, is based on the ability to sense environmental stimuli like chemicals, oxygen, and light and to respond by modulating the probability of switching the rotational sense of the motility organelle, the flagellar motor. The interplay of excitation and adaptation allows the cell to detect time-dependent changes in the strengths of different stimulus types; these changes are integrated to give a graded motor response (for reviews, see references 4, 12, and 22). Components of the bacterial sensory machinery have been identified and characterized by genetic, biochemical, and cell physiological methods. Bacterial chemotaxis provides a fascinating example of how computational information processing is performed by a network of proteins (8).

Interestingly, homologous molecular modules are found to process information in evolutionarily divergent bacteria and archaea. Stimulus input is transduced by a family of methyl-accepting transmembrane proteins that are specific for different stimulus types at the input site but feed into the same signalling pathway via a highly homologous output domain (for reviews, see references 16 and 33). This biochemical mechanism of sensory integration works with stimuli as different as light and chemical compounds, depending on whether the transducers are complexed to a photoreceptor or a ligand-binding protein (19, 20). Transducer activation feeds into a two-component system (28) that relies on CheW-mediated regulation of CheA histidine kinase activity in both bacteria and archaea (6, 30). CheA phosphorylates CheY, a small cytoplasmic protein that plays an important role in controlling the direction of flagellar movement (17, 30).

For the archaeon *Halobacterium salinarium*, we have identified fumarate as a factor required for switching the direction of rotation of the flagellar motor (24) and found that its cytoplasmic concentration is controlled by the photoreceptors (23, 26). In eubacteria, fumarate also has switch factor activity, as demonstrated in an in vitro system. Flagellar motors of envelopes prepared from *Escherichia coli* or *Salmonella typhimurium* cells which are totally devoid of cytoplasm but loaded with CheY protein do not switch their sense of rotation unless fumarate is added (1). Some chemical analogs of fumarate are also active, but to a lesser extent (3). Since the envelopes lack all cytoplasmic components of the chemotaxis signal transduction pathway, fumarate is likely to interact directly with the CheY protein and/or the switch complex at the base of the flagellar motor.

Bacteria sense two types of chemostimuli that act antagonistically: attractants and repellents. Attractants suppress motor switching and increase the counterclockwise (CCW) bias of the flagellar motor when applied to the cell, whereas repellents increase the clockwise (CW) bias. Many attractants are detected by binding to the external domains of methyl-accepting chemotaxis proteins (MCPs), transmembrane receptors whose cytoplasmic signalling domains regulate the activity of the CheA histidine kinase (6). Changes in the concentration of attractant have been shown to modulate the phosphorylation level of the CheY protein. Phosphorylated CheY causes CW rotation of the motor, which has a default CCW rotational sense.

Repellents often are hydrophobic substances that may enter the cell by diffusion. The mechanism by which repellents influence bacterial behavior is poorly understood. In contrast to stimulation by attractants, no change in CheY phosphorylation level in vitro has been described after stimulation with repellents. Since only some repellents change the methylation level of the MCPs (32), it is not clear whether a signal-processing mechanism different from the attractant-dependent pathway is involved.

Here we present evidence for a repellent signal transduction pathway in *E. coli* cells, disabled in phosphorylation-dependent chemotaxis, that works by causing changes in the cellular concentration of fumarate.

MATERIALS AND METHODS

Growth of bacterial cultures and strains used. Bacterial cultures were grown in tryptone broth by inoculation with 1% of an overnight culture in tryptone broth and were shaken at 250 rpm at 37°C until an optical density at 590 nm of 0.5 was reached. *E. coli* strains are listed in Table 1. Strains with mutations in citric acid cycle enzymes were grown either in tryptone broth with 0.4% glycerol as a supplementary carbon source or in H1 minimal medium supplemented with 0.4% glycerol (18) as the sole carbon source.

Extraction of fumarase and estimation of fumarase activity. Cells were har-

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TABLE 1. Bacterial strains used in this study

Strain	Relevant genotype	Mutated TCA enzymes[a]	Reference or source
RP437	Wild type for chemotaxis	None	29
CP362	Δ*tsr* Δ*tar* Δ*tap* Δ*trg*	None	27
EW13	Δ(*cheA–cheZ*)2209 <pJH120>	None	12a[b]
EW13ΔF_{ac}	Δ(*cheA–cheZ*)2209 <pJH120> Δ*fumA* Δ*fumC*	FumA, FumC	This study
RP437ΔF_{ac}	Wild type for chemotaxis; Δ*fumA* Δ*fumC*	FumA, FumC	This study
RP437 CheYD13KD57A	Wild type for chemotaxis; *cheYD13KD57A*	None	7

[a] FumA, fumarase A; FumC, fumarase C; TCA, tricarboxylic acid cycle.
[b] EW13 was obtained by transformation of RP1091 (29) with pJH120, which carries the CheY gene under the control of the arabinose promoter (10).

vested by centrifugation (1,650 × *g*, 20 min, 4°C), washed twice in one volume of 100 mM KP_i buffer (pH 7.5), and resuspended in the same buffer to a final density of 2.5×10^9/ml. Cells were disrupted by ultrasonication, and the debris was centrifuged for 30 min at 28,000 × *g* at 4°C. Fumarase activity was assayed in the supernatant.

Fumarase activity was determined photometrically at 240 nm by its ability to convert L-malate to fumarate. For kinetic measurements, fumarase A (the enzyme that contributes 80% of the total fumarase activity in wild-type cells) was overexpressed and partly purified according to the method of Flint et al. (13) to obtain a sufficient amount of the enzyme. Fumarase A was overexpressed in the deletion strain RP437Δ*fumAC* (see below) which was transformed with pGS57 (35), resulting in a 30-fold overexpression compared with the wild-type level. Cells were harvested by centrifugation, washed twice in a solution containing 50 mM Tris-HCl, 10 mM $MgCl_2$, and 5 mM β-mercaptoethanol (pH 8.0), and resuspended in the same buffer, which now contained 0.2 mg of lysozyme per ml. Cells were disrupted by sonication, and the debris was removed by centrifugation (28,000 × *g*, 20 min, 4°C). Fumarase was adsorbed to DEAE-cellulose and eluted from the column by a linear KCl gradient (0 to 500 mM). The fumarase-containing fraction had a considerably lower background level of UV absorbance than the total cell extract, which greatly facilitated the activity measurements. Fumarase was stabilized by the addition of 5 mM β-mercaptoethanol, 0.5 mM ferrous ammonium sulfate, and 2 mM ascorbate and was maintained anaerobically on ice.

Estimation of cytoplasmic fumarate. Cells were harvested by centrifugation, washed twice with motility buffer (10 mM KP_i [pH 7.3], 0.1 mM EDTA), and resuspended in the same buffer to a final optical density at 590 nm of 10. Forty milliliters of the bacterial suspension was shaken in a 100-ml Erlenmeyer flask at 260 rpm at room temperature for 45 min. Control measurements revealed that the cell suspension remained saturated with oxygen under this condition.

Cells were lysed by injecting five aliquots of 1 ml into 5 ml of boiling water while stirring. The cells were boiled for a further 5 min. The suspension was cooled to room temperature and, after the addition of 1,500 U of lysozyme per ml, was incubated at room temperature for 20 min to ensure complete lysis. Lysed cells and debris were centrifuged for 30 min at 28,000 × *g* at 4°C. No residual fumarate could be detected in the pellet after homogenization by ultrasound. The supernatant was lyophilized and redissolved in 1 ml of 10 mM KP_i buffer, pH 8.0. After the addition of 1 ml of 4 M NaCl, 50 μl of concentrated HCl, and 2 ml of ice-cold diethyl ether, fumaric acid was extracted by vigorous vortexing at 4°C for 5 min. The ether phase was quantitatively removed and evaporated under vacuum. The residual was redissolved in 0.2 ml of 0.1 M 3-amino-1-propanol buffer, pH 9.9, and assayed for fumarate as described by Montrone et al. (26). The procedure was calibrated by adding fumarate as an internal standard to the boiling water used for lysing the cells. When fumarate from indole-treated cells was to be analyzed, indole had to be removed from the sample because it inhibits the pig heart fumarase used in the assay. This was achieved by ether extraction as described above prior to the addition of HCl. The ether phase containing indole was discarded and, after the addition of 50 μl of concentrated HCl to the remaining aqueous phase, fumarate was extracted with 2 ml of diethyl ether.

Behavioral measurements. Cells were resuspended in motility buffer and tethered as described previously (31). Chemotactic stimulation took place in a flow chamber (5) at 23°C on a thermostated stage. The behavior of the cells was videotaped and played back for visual analysis. Alternatively, spinning cells were digitized on-line with a video frame grabber (Motion Analysis Corp., Santa Rosa, Calif.) at a frequency of 60 frames per s. Sequences of 10 s were recorded and evaluated with respect to rotational sense and switching by using motion analysis algorithms (24a) yielding a time resolution of 50 ms. The system was calibrated by the analysis of cells locked in the CCW mode.

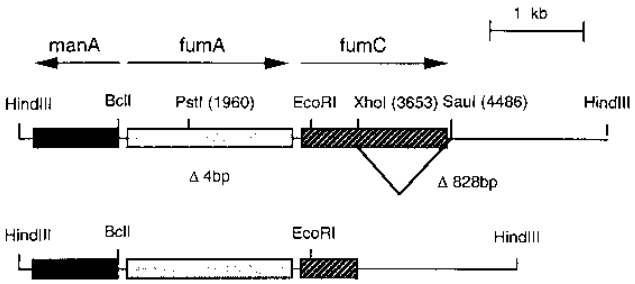

FIG. 1. Genetic maps of the fumarase operon before (top) and after (bottom) introduction of specific deletions.

Deletion of fumarases A and C. Deletion of the genes for fumarase was performed according to the method of Hamilton et al. (15). Plasmid pGS54 (25) was digested with *Xho*I and *Sau*I and the restriction sites were blunt-ended and religated with T4 ligase, which gave a deletion of 828 bp in *fumC* (Fig. 1). The mutated fragment was excised by *Hin*dIII digestion and cloned into pMAK700 deletion vector, which has a temperature-sensitive origin of replication (15). The *fumA* gene was then cut with *Pst*I at its unique restriction site, and the fragments were polished by the exonuclease activity of T4 DNA polymerase and religated as above, resulting in a 4-bp deletion (pMAK700Δ*fumAC*).

Strains RP437, RP1091, and CP362 (Table 1) were transformed with pMAK700Δ*fumAC* by the TSS method (9). Transformants were selected by growth on Luria-Bertani plates containing chloramphenicol (20 μg/ml) at 30°C. The plates were shifted to 44°C to select for cells that integrated the plasmid into the chromosome by homologous recombination, as the plasmid is unstable at this temperature. Subsequent growth of these cointegrates at 30°C selects for cells that have undergone a second recombination event resulting in the loss of the additional, temperature-sensitive origin of replication (whose presence is lethal at 30°C), eventually accompanied by a replacement of the adjacent genes. Candidate clones were screened for the loss of ability to grow on H1 minimal medium (18) supplemented with 20 mM fumarate (14). The specific deletions were then verified by chromosomal sequencing and enzymatic assay of fumarase as described above.

RESULTS

Indole inhibits fumarase and enhances the cellular fumarate concentration. Exponentially growing *E. coli* cells were harvested and lysed, and the fumarase activity in the lysate was estimated. Fumarase was assayed photometrically by its ability to convert L-malate to fumarate, and a K_m of 2 mM for malate was determined. The addition of indole inhibited the enzyme by shifting its V_{max} value to lower levels. Half-maximal inhibition was found at a concentration of 1 mM indole (Fig. 2A). The inhibition was fully reversible when indole was removed from the fumarase preparation (data not shown). Benzoate, another repellent for *E. coli*, inhibited fumarase slightly more effectively than indole did (Fig. 2B).

There are only a few metabolic reactions that turn over fumarate. The main reaction path is the oxidation of succinate by succinate dehydrogenase and the hydration of fumarate to malate by fumarase in the course of the citric acid cycle. Inhibition of fumarase is therefore expected to increase the cytoplasmic concentration of fumarate.

Indole at a concentration of 2 mM was added to a cell suspension, and the metabolism was subsequently quenched by rapid injection of the suspension into an excess of boiling water. The fumarate concentration in the lysate was assayed enzymatically. Upon the addition of indole to strain RP437 cells, which display the wild-type chemotaxis response, the cellular concentration of fumarate of about 6,000 molecules per cell increased by a factor of three compared with the concentration in the control (Fig. 3). The increase was also observed in CP362, a strain from which all four MCPs had been deleted (Fig. 3), and it therefore cannot be mediated by

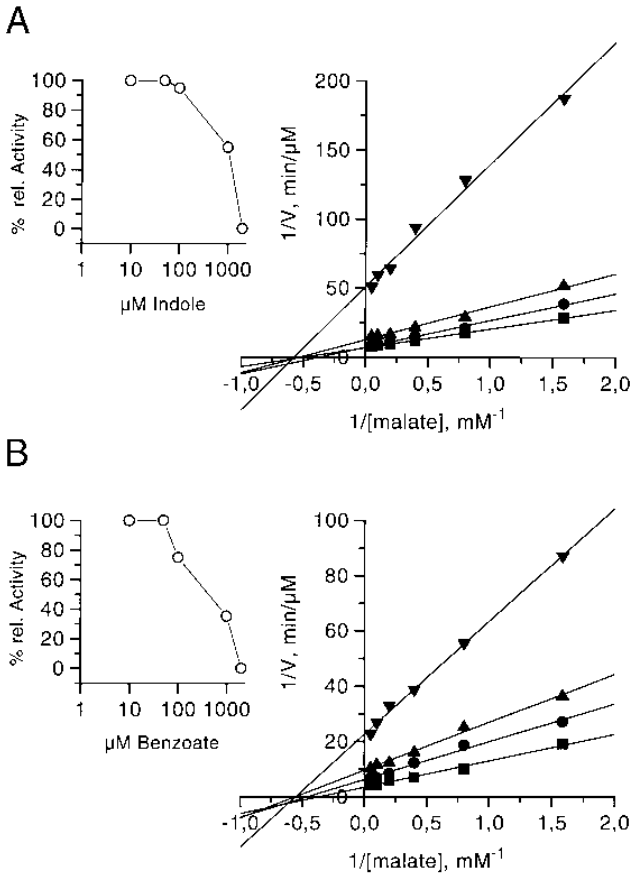

FIG. 2. Lineweaver-Burk plots of fumarase A and its inhibition by indole and benzoate. Fumarase A was overexpressed and partly purified as described in Materials and Methods. The enzymatic activity was measured and plotted against the concentration of its substrate, L-malate. (A) Inhibition by 0.5 mM (●), 1 mM (▲), and 1.5 mM (▼) indole compared with the control (no indole addition) (■). (B) Inhibition by 0.5 mM (●), 0.75 mM (▲), and 1 mM (▼) benzoate compared to the control (no benzoate addition [■]. The graphs at the left of each panel show the inhibition of total fumarase activity mediated by fumarases A and C, as determined with whole-cell lysates of RP437 (i.e., without overexpression of fumarase A), by indole and benzoate, respectively, taken at substrate saturation (20 mM malate).

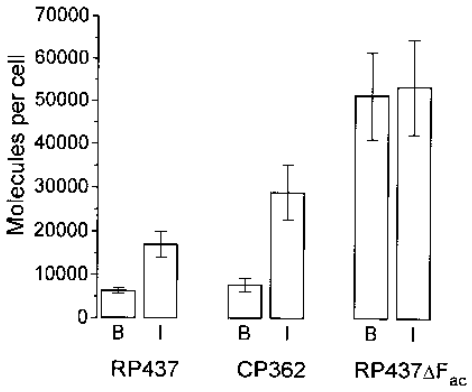

FIG. 3. Effect of indole on the cellular concentration of fumarate. Cells were stimulated by the addition of 1/10 volume of a 20 mM solution of indole (I) in motility buffer while being shaken vigorously. Cells were quenched 10 s after the addition, and the fumarate level was determined as described in Materials and Methods. In control experiments, buffer (B) was added in place of the indole solution. Error bars indicate the standard errors of the means of nine determinations from three independent experiments.

the MCP-dependent chemosensory system present in RP437 cells.

Under aerobic conditions, fumarases A and C are expressed while fumarase B is not. In order to block fumarase activity genetically, the genes for fumarases A and C were deleted by the method of Hamilton et al. (15) (see also Fig. 1) and the phenotype of the deletion strain was verified by a determination of fumarase activity. Deletion in RP437 cells (strain RP437ΔF_{ac}) increased the cytoplasmic steady state to about 50,000 molecules of fumarate per cell and abolished changes of fumarate level upon addition of indole (Fig. 3). This result is expected if the indole-mediated rise in fumarate concentration is caused by the inhibition of fumarase activity.

Indole and benzoate induce motor switching in cells defective in the MCP-dependent phosphorylation cascade. Wild-type *E. coli* cells which are stimulated with indole respond by switching the rotational sense of the flagellar motors to cause tumbling. The response to indole has been attributed to the methyl-accepting chemotaxis protein Tsr (32). Given that indole and benzoate increase the cellular level of fumarate and that fumarate is required for motor switching in cell envelopes (1), an indole-mediated increase in fumarate concentration might cause tumbling even in cells that are defective in the MCP-dependent chemotaxis machinery, provided that the steady-state concentration of fumarate is nonsaturating with respect to switching. Tethered cells of strain CP362, from which all four MCPs had been deleted, were stimulated with indole, and the behavior of the cells was analyzed. Indole induced motor switching, and a half-maximal response was obtained at a concentration of about 1 mM (Fig. 4). This value corresponds to the concentration which gives 50% inhibition of fumarase in vitro. RP437 cells which display a wild-type MCP-mediated chemotaxis response are more sensitive to indole and responded maximally at a concentration of 0.5 mM (data not shown). Since the response to indole in CP362 occurs in spite of the deletion of all four MCPs, it cannot be mediated by activation of the two-component system. This conclusion is supported by the additional finding that EW13 cells that express CheY (encoded by pJH120), but from which all other *che*

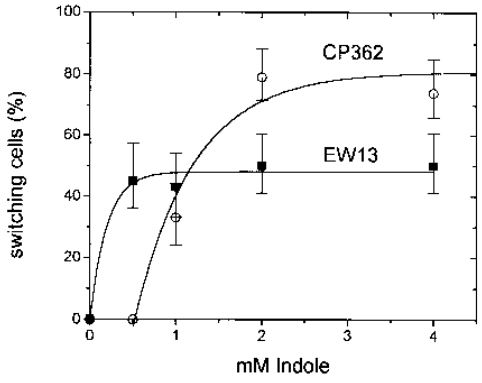

FIG. 4. Indole-induced motor switching in tethered cells from which different parts of the chemotaxis machinery were deleted. Stimulation was in a flow chamber and proceeded by step-like increases in indole concentration, as indicated on the abscissa. Cells were videotaped, and 2-min intervals were evaluated by visual inspection. The total number of continuously rotating cells was considered to be 100%, and the number of switching cells was counted. In the absence of the chemostimulus no switching event was observed. Each datum point represents the average of at least 30 cells. Error bars indicate the standard deviations of the means.

gene products have been deleted (Δ*cheA* to -*Z*), still respond to indole (Fig. 4). We conclude that an indole-induced rise in fumarate concentration might directly cause the observed switching response of the flagellar motor.

To test this hypothesis, a change in steady-state phosphorylation of CheY by metabolic sources in response to enhanced fumarate concentration had to be excluded. This is possible by using the CheY protein carrying the double mutation 13D→K (aspartic acid to lysine at position 13) 57D→A, which, although it cannot be phosphorylated at 57D, retains some phosphorylation-independent switch-promoting activity through its second site mutation (7). Consequently, cells expressing this mutated CheY from a single-copy gene in a background that is wild type with respect to the other components of the chemotaxis machinery spin their flagellar motor predominantly CCW and do not respond to specific stimulation of the MCPs. The identity of this strain was verified by genomic sequencing, and its nonchemotactic phenotype was verified by swarm plates (data not shown) and, in the flow chamber, by the removal of attractant or by the lowering of the pH of the incubation buffer (Fig. 5A), both of which are strong repellent stimuli. Stimulation with indole induced switching when applied in the same concentration range found to be effective for CP362 (Fig. 5B). The cells did not adapt to indole during an observation time of 30 s. If, however, indole was removed by washing out, CCW rotation without switching was resumed. The strain was responsive to the fumarase inhibitor benzoate as well (Fig. 5C).

These experiments demonstrate that an increase in the cellular concentration of fumarate caused by external stimuli (indole and benzoate) is correlated with the switching of the flagellar motor. This holds true for strains from which different components of the MCP-dependent phosphorylation cascade have been deleted and which therefore are nonresponsive through the classical pathway.

As expected, the frequency of spontaneous switching was enhanced drastically when the fumarate level was permanently raised by genetically deleting fumarases A and C in EW13 cells that are deficient in CheA to -Z but that express CheY encoded by a plasmid (Fig. 6).

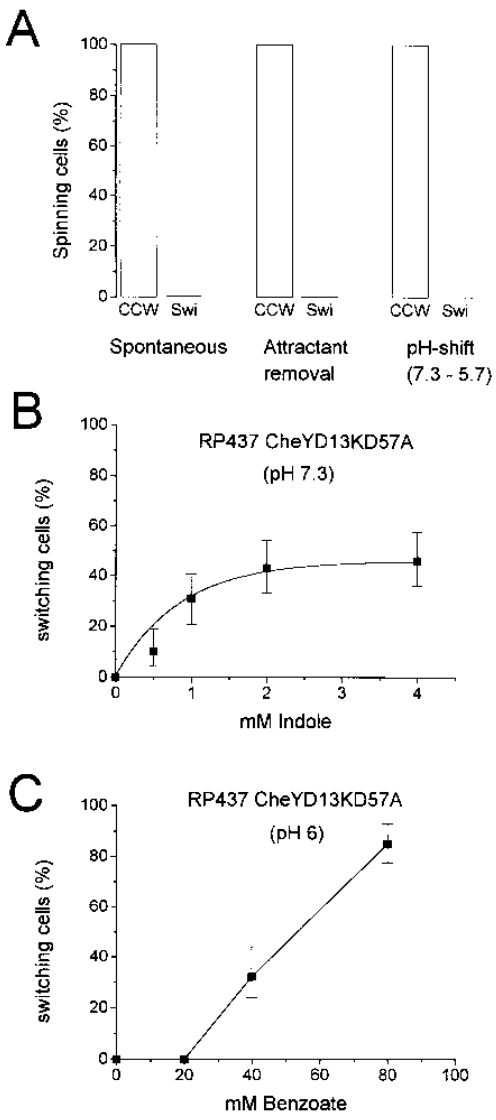

FIG. 5. Response of RP437 cells expressing nonphosphorylatable CheY D13K D57A to indole and benzoate. Cells tethered in a flow chamber were exposed to a step down in an attractant mix (100 μM *N*-methylaspartate and 100 mM α-aminobutyrate) and a step down in the pH of the flow buffer (7.3 to 5.7) (A) or a step up in indole (B) or benzoate (C) concentration. Cells were videotaped, and 2-min intervals were evaluated as described in the legend to Fig. 4. The total number of continuously rotating cells was considered to be 100%. The time between two successive stimulations was 10 min. Each datum point represents the average of at least 30 cells. A concentration of 80 mM benzoate at pH 6 corresponds to 1.25 mM benzoic acid, which can enter the cell. Swi, switching. Error bars indicate the standard errors of the means.

DISCUSSION

We have shown that the repellent indole, which acts through an MCP, is, in addition, active by a pathway different from the classical two-component system cascade (MCP→CheW→CheA→CheY). This surprising finding is based on the following experimental evidence: (i) a mutant defective in all four MCPs (CP362) is responsive to indole, and (ii) response to indole occurs in cells from which all other *che* genes (Δ*che*A to -Z) have been deleted but which express CheY.

We believe that the cytoplasmic fumarate concentration is a factor that determines the probability of motor switching at a constant CheY phosphate level in vivo. This is concluded from two findings, which will be discussed in detail below. (i) Fumarate is directly involved in motor switching in cytoplasm-free cell envelopes. (ii) Flagellar motors of cells with enhanced cellular fumarate levels have an enhanced probability of switching. This in vivo effect is not caused by an increase in the phosphorylation of CheY.

Fumarate is directly involved in motor switching in cytoplasm-free cell envelopes. Cytoplasm-free cell envelopes prepared by osmotic lysis retain their ability to spin the flagellar motor when energized with lactate. Unless CheY is added to the lysis buffer, the flagellar motors rotate exclusively CCW. Switching, i.e., the transition from CCW to CW rotation and vice versa, only occurs when CheY and fumarate are included in the lysis buffer (1). The specificity of fumarate as a switch factor has recently been investigated (3). Besides fumarate, maleate, malate, and succinate are active, though to a lesser extent. Aspartate and lactate were inactive. There are several arguments why fumarate acts per se in envelopes and not by changing the phosphorylation level of CheY. (i) Cells only spin if they are energized; i.e., if there are minimal cytoplasmic remnants in the envelopes, ATP should be formed by ATP synthase much more efficiently than by the metabolism of fumarate, and as a consequence the metabolic phosphorylation rate of CheY should not change. More important, photore-

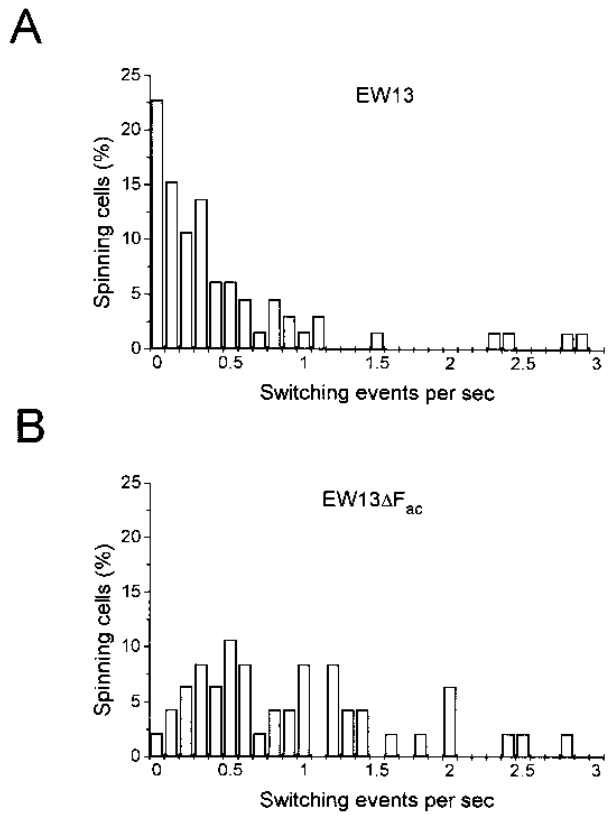

FIG. 6. Spontaneous switching of cells with a gutted background but expressing CheY with (A) and without (B) functional fumarases. CheY was expressed by plasmid pJH120 without additional induction by arabinose. Cells were grown in H1 minimal medium supplemented with 0.4% glycerol as the sole carbon source. For EW13 and EW13ΔF_{ac} cells, 66 and 53 cells, respectively, from five independent clones were evaluated by automatic computer-assisted motion analysis as described in Materials and Methods.

lease of caged ATP does not promote switching (2). (ii) It is known that the phosphorylation of CheY promotes CW rotation (28). Since in the absence of fumarate but in the presence of CheY there are envelopes that rotate exclusively CW and never switch (1, 3), a hypothetical increase in CheY phosphorylation by the metabolism of fumarate should retain CW rotation rather than cause these envelopes to switch. (iii) Maleate- and succinate-induced switching is observed in envelopes prepared from a mutant defective in fumarase and succinate dehydrogenase (3). Hence, these analogs cannot act by driving metabolic reactions in the envelopes.

Taken together, these findings confirm that fumarate is active in envelopes as a factor that promotes switching and that CheY alone is inactive in this respect. In addition, these experiments restrict the possible targets of fumarate to either CheY or the proteins of the switch complex.

Flagellar motors of cells with enhanced fumarate levels have an enhanced probability of switching. Indole raises the cellular concentration of fumarate by reversible inhibition of fumarase. This is the conclusion from four experimental observations: (i) addition of indole to a cell suspension increases the cytoplasmic concentration of fumarate; (ii) indole reversibly inhibits fumarase in vitro; (iii) genetic deletion of the fumarase genes increases the cytoplasmic fumarate level; and (iv) at the same time, genetic deletion of the fumarase genes abolishes the sensitivity of the fumarate level to indole.

Indole also induces motor switching in cells that contain fumarase but that are defective in MCP-dependent chemoresponses because of deletions in different *che* genes. Hence, induction of motor switching cannot be the result of CheA-mediated phosphorylation of CheY. Alternatively, CheY can be phosphorylated by metabolic sources, e.g., acetylphosphate (2, 11, 21, 34). Inhibition of fumarase reduces the turnover number of the citrate cycle and should not increase the concentration of acetylphosphate or any other phosphoryl donor in the cytoplasm. However, since the metabolic network is complex, predictions may not necessarily hold true and experimental evidence against fumarate-mediated CheY phosphorylation had to be obtained.

Essential to this is the experiment demonstrating that cells expressing mutated, nonphosphorylatable CheY (13D→K 57D→A) do not show MCP-dependent chemoresponses (7) but are responsive to indole and benzoate, both of which increase the cellular fumarate level (present study). Thus, these compounds cannot be active in the mutant through changes in the phosphorylation level of the CheY pool. This, together with the finding that both CheY and fumarate are required for motor switching in bacterial envelopes (see above), gives correlative evidence that indole and benzoate act through fumarate, which by itself coregulates switching. This conclusion, however, predicts that the steady-state concentration of fumarate in the cell is not saturating in determining the switching probability at the CheY phosphate level of an adapted cell. Indeed, when the fumarate level is enhanced by deleting the fumarase genes, a drastic increase in the switching frequency was obtained in the absence of the chemotactic machinery.

We have provided evidence that fumarate can regulate the switching probability at a constant CheY phosphate level and that metabolic changes in fumarate concentration under these conditions can cause chemoresponses. It is suggestive that cooperation of fumarate and CheY might have been an ancient mechanism of chemotaxis, long before a highly tunable MCP-controlled phosphorylation cascade evolved to control CheY. If this were true, an interaction between fumarate and targets of other two-component systems might be expected.

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