A common feature among all sensory cells is the ability to process information, an event that requires the function of many components, even in relatively simple prokaryotic cells. In mice, Drosophila, paramecia, and bacteria, one approach toward understanding the function of molecules involved in sensing has been to use a genetic dissection, usually by isolating mutants in which one sensory component has been eliminated (1, 2). The advent of recombinant DNA techniques provides additional permutations for perturbation of complex systems. First, recombinant plasmids can be used to increase dosage of a particular component to assess the phenotypes of overproducing mutants. Second, after removal of many sensory components by deletion mutations, individual proteins can be added back to the system to study them in a simpler context.

Genetic studies of bacterial chemotaxis have identified a total of eight genes directly involved in intracellular signaling between receptors and flagella (2-5). While many features of the receptors have recently been elucidated (6-9), the molecular details of signaling remain obscure. Since all of the chemotaxis genes have now been cloned (10-12) and a broad selection of deletion mutants exist (13-15), recombinant DNA addition experiments are feasible for the analysis of signaling.

One gene central to the processing system of Escherichia coli and Salmonella typhimurium is cheY. Mutants with lesions in this gene lack all sensory responses to both attractants and repellents. Their flagella rotate exclusively in the counterclockwise mode without clockwise reversals, resulting in constant smooth swimming without tumbling episodes to reorient swimming direction (16, 17). Furthermore, some cheY mutants fail to complement strains with mutations in the cheR gene, and allele-specific second-site suppressors of cheY can be isolated in the flaA locus, which suggests some type of interaction between these components (3, 17). The cheY gene has been shown to encode a soluble 14,000-Da protein (10, 11) and has recently been sequenced (18).

To probe the role of the Y protein, the gene has been subcloned under the control of a tac promoter (19) to allow cheY expression independent of the normal promoter. This plasmid was introduced into wild-type bacteria and into strains that were stripped of many other genes in the chemotaxis system. The results suggest a direct role for the Y protein in controlling rotational direction of flagellar motors.

MATERIALS AND METHODS

Bacterial Strains. RP437 (F thi, thr, leu, his, met, eda, rpsL) was considered wild type for chemotaxis (20). Mutants derived from this strain used in this study were RP4315 (cheY201), RP4372 (Atap-tar52Δ1), tsi-518), and RP1091 [acheA-cheW-tar-tap-cheR-cheB-cheY-cheZ (Δ2209)] (13). These strains were kindly supplied by . . .

For isolation and propagation of plasmids, strain MM294 hsdR17 (rK-, mK-) endul, thi-21) with a deletion in recA was used.

Recombinant DNA Methodology. Restriction endonucleases, T4 DNA ligase, and DNA polymerase were obtained from and used as recommended by the supplier. Plasmids were purified by the alkaline NaDodSO4 procedure ofagarose gel electrophoresis. Purification of DNA fragments, transformation of bacteria, and plasmid constructions were accomplished by standard methods (23). Minicells were purified and labeled as described (24), except that 35S)methionine (50-100 μCi/ml; 1 Ci = 37 GBq) and Difco methionine assay medium were used and minicells were labeled at an optical density (620 nm) of 1.

Behavioral Assays. Semisolid plates (0.3% agar) were made from Vogel-Bonner citrate minimal medium (26) supplemented with appropriate nutrients (100 μg/ml/1% glycerol/10 mM D-lactate, pH 7/ampicillin (100 μg/ml) for plasmid-containing strains. Plates were inoculated, incubated at 30°C, and the change in the diameter of the swarm was measured. For tethering analysis (27), cells were grown at 37°C in minimal medium with 0.4% glucose to inhibit flagellar synthesis. Tethered cells were constantly washed with fresh growth medium. Rotational behavior was analyzed by computer program written by flagellin antiserum was a gift of Fresh transformants were used for all behavioral experiments.

Immunoprecipitations and Gels. The procedures used to immunoprecipitate the Y protein have been described (28).

Abbreviation: IPTG, isopropyl β-D-thiogalactoside.
Proteins were analyzed on one-dimensional gels of the Laemmli type (29) and on two-dimensional gels according to O'Farrell (30).

RESULTS

Construction of a Plasmid with Adjustable cheY Expression. The appropriate position of the cheY gene was mapped by making deletions of a plasmid containing cheY and the other genes in the meche (for methylation and chemotaxis) operon (31), and then assaying for protein products in minicells (Fig. 1). The plasmid pWK53 contains a deletion to the right of the HindIII site and fails to make the cheB, cheY, and cheZ gene products. This places the HindIII site within the cheB gene and, assuming the genes are contiguous, places the cheY gene between the HindIII and Pvu II sites. This fragment was purified and inserted behind the tac promoter in the plasmid pKK204 (Fig. 2). The tac promoter [a trp-lac hybrid (32)] includes the binding site for the lac repressor, so transcription can be controlled by an inducer. Since the tac promoter is on a multicopy plasmid, a higher than normal amount of repressor must be supplied to control expression. This was accomplished by adding the repressor gene, lacI, encoded by pMC7 (25) to the plasmid. Since this tac-cheY construct makes its own supply of repressor, it can be regulated in a variety of strains.

To ensure that the effects of the plasmid were due to cheY, a plasmid was constructed that was identical to the first plasmid except for a frameshift mutation that was introduced into the cheY gene. The sticky ends of the unique Sal I site were filled in, and the plasmid was religated, resulting in an 8-base pair insertion that altered the reading frame. This plasmid was used as a control in all behavioral experiments to ensure that effects of the plasmid outside the cheY gene were not responsible for the behavioral effects.

The tac-cheY plasmid (pCK63) directed the expression of Y protein that was indistinguishable from wild-type Y protein on two-dimensional gels. When cells containing this plasmid were grown in the presence of 1 mM isopropyl β-D-thiogalactoside (IPTG) and analyzed on NaDodSO4/polyacrylamide gels stained with Coomassie blue, a prominent band corresponding to 14,000 Da was observed. The control plasmid (pCK65) with a frame shift mutation in cheY did not produce any detectable Y product.

cheY Overproduction Affects Chemotactic Behavior. As a general indication of the effects of altered Y protein dosage on behavior, the wild-type strain containing the overproducing plasmid was seeded into semisolid agar, and its ability to swarm away from the initial inoculum was compared to the same strain harboring the control plasmid or the plasmid pBR322. Bacteria that are chemotactically competent detect gradients in the agar that are created as nutrients are consumed and waste products accumulate. It was observed that, even in the absence of IPTG, the tac-cheY construct inhibited the rate of swarming (Table 1). Cells containing this plasmid spread through the agar at approximately half the rate observed for the controls. Thus, read-through expression of

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Proteins Encoded</th>
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<tbody>
<tr>
<td>pWK51</td>
<td>Tar, Tap, R, B, Y, Z</td>
</tr>
<tr>
<td>pWK56</td>
<td>Tar</td>
</tr>
<tr>
<td>pWK54</td>
<td>Tar fragment (56 kDa)</td>
</tr>
<tr>
<td>pWK53</td>
<td>Tar, Tap, R, 24 kDa</td>
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Fig. 2. Construction of a plasmid with adjustable cheY expression. The 0.85-kilobase HindIII/Pvu II fragment was purified and inserted behind the double tac promoter elements in pKK204. The resulting plasmid, pCK62, was fused to the plasmid pMC7, which encodes the lac operator (lacI) (25), by cutting at the BamH I sites in both plasmids and ligating them together. As a control, pCK62 was cleaved at the single Sal I site, and the sticky ends were made flush by incubating with dNTPs and the polymerase. The resulting plasmid was named pCK64 and was fused to pMC7 as described above.
Table 1. Swarming behavior of wild type (RP437) containing cheY plasmids

<table>
<thead>
<tr>
<th>Swarm-plate composition</th>
<th>Swarm rate, mm/hr</th>
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<tbody>
<tr>
<td></td>
<td>pBR322</td>
</tr>
<tr>
<td>VBC minimal medium</td>
<td>1.5</td>
</tr>
<tr>
<td>VBC minimal medium</td>
<td>4.4</td>
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RP437 was transformed with the plasmids indicated and the resulting colonies were inoculated onto 0.3% minimal agar supplemented with nutrients or supplemented with nutrients and aspartate and serine (100 μM each). In the case of RP437 pCK65 (tac-cheY<sup>-</sup>), five individual colonies were examined, and the mean ± SEM is reported. In the other cases, the inoculum was mixed, consisting of ~10 colonies scraped from the transformation plate.

The Y protein in the absence of inducer inhibits chemotaxis ability. Addition of IPTG resulted in a filamentous, mostly immotile phenotype, presumably because of extremely high levels of overproduction. For this reason, bacteria were grown without IPTG to analyze the effects of moderate overproduction.

The nature of the chemotaxis defect caused by excess Y protein was analyzed by tethering unflagellated cells on coverslips coated with anti-Flagellin antibody and recording their rotational behavior (Fig. 3). The overproducing strain spends an inordinate percentage of time in the clockwise rotational mode. Whereas wild-type cells without any plasmid or with the control tac-cheY plasmid were mostly counterclockwise (70%), cells with excess Y protein rotated in the clockwise direction 90% of the time. If IPTG was added to tethered cells, all counterclockwise rotation was abolished within 10 min. The counterclockwise cheY mutant RP4315 was also made almost entirely clockwise by the tac-cheY plasmid, indicating that an excess of one gene dosage was produced in the absence of inducer. The increased clockwise rotational bias caused by Y overproduction appeared as increased tumbling behavior in free-swimming bacteria. These bacteria did not spread through semisolid agar at wild-type rates presumably because of improper run lengths (32, 33).

The next step was to ask whether other chemotaxis gene products were required to produce the clockwise bias brought about by overproduction of Y protein. The tac-cheY plasmid was introduced into several mutant strains and the behavior of individual cells was assayed by tethering. The results are summarized in Figure 3. In both cases examined, a dramatic effect on motor bias was observed. A strain lacking most of the major receptors (RP4372), which is 97% counterclockwise in the absence of the tac-cheY plasmid, was made to rotate mostly clockwise by Y overproduction. Furthermore, even when most other chemotaxis-specific gene products were deleted (strain RP1091), Y protein produced predominantly clockwise motors.

It was possible that the Y protein could be released from the receptor and influence motor direction in the absence of the other chemotaxis gene products. To test this, serine was added to the cheA<sup>-Z</sup> deletion mutant containing the tac-cheY plasmid. This strain contains an intact tsr gene, which has been shown to encode the serine receptor (6, 34). However, no interruption of clockwise rotation was observed. The clockwise rotation caused by overproduction of the cheY was reversed by serine only if the other chemotaxis gene products were present (RP4315 cheY and pCK63). Thus, other genes in addition to cheY are required for communication between the receptor and the flagella.

**DISCUSSION**

The experimental approach and initial results obtained can be summarized as shown in Figure 4. By fusing a titratable promoter to a chemotaxis-signaling gene with unknown function (cheY), we constructed a plasmid from which the expression of this gene could be maintained at different levels. This construct was then introduced into wild-type and mutant strains to assess the effects of altered cheY expression on behavior. A similar approach has been successfully used to elucidate some of the properties of the λ repressor and cro protein (35-37).

The behavioral consequences of Y protein overproduction establish a phenotypic activity for this protein. In wild-type cells, increased Y protein leads to an increase in clockwise flagellar rotation. In strains lacking the Y protein, incessant counterclockwise rotation is observed (3, 16). In fact, while other counterclockwise processing mutants can reverse direction upon repellent addition, cheY mutants cannot (13). These facts suggest that the Y protein generates clockwise rotation. They are also consistent with cell envelope studies (40).

The clockwise influence of the Y protein does not require the presence of the major receptors. This argues against models in which these receptors release a ligand or synthesize a molecule that is essential for the Y protein to influence the flagella. Nor does the clockwise activity require any components of the methylation system (38, 39), cheA, cheW, or cheZ, because the deletion mutant RP1091 lacking all these genes was found clockwise by the tac-cheY plasmid. At least some of these other genes are required, however, to transmit responses from receptors to motors, because the deletion mutant could not respond to serine.

A reasonable model, consistent with these findings and supported by previous genetic studies (17), is that the Y protein binds to a detector in the flagellar motor, probably consisting of the flaA (also called cheC) and the flaB (also called cheV) gene products. Binding imparts a clockwise bias to the motor. While no other processing gene products are...
Fig. 4. Experimental design and summary of results. The circled letters represent chemotaxis gene products. The only receptors shown are those for aspartate and serine (6, 34). The cheC/flaA and cheV/flaB gene products are pictured as part of the flagellar machinery (3, 4). Overproduction of Y protein is indicated by repetition of Y in the cytoplasm. The wild-type cell shows random swimming and the deletion mutant is counterclockwise (CCW). Both become clockwise (CW) when cheY is overproduced.

...quired for this interaction, they may enhance or lessen the influence of the Y protein to alter the counterclockwise/clockwise ratio as needed for sensory responses. Alternatively, Y could produce some small molecule that reacts with the motor, but that alternative is less probable in view of the allele-specific suppression of cheY mutations.

The finding that Y protein can make smooth-swimming (counterclockwise) cells tumble (clockwise) clarifies its role in information processing. It also adds an important experimental approach. Since the "stripped down" system shows counterclockwise rotation without cheY protein but shows clockwise rotation with cheY protein only, other components can be added back separately to these two systems to test their roles in altering the rotational sense of the motor.