Two-component signal transduction is widely used by bacteria to sense environmental conditions (reviewed in Ref. 1); an extensively studied example of this is bacterial chemotaxis (reviewed in Ref. 2). Two-component systems comprise histidine protein kinases and response regulators. Histidine protein kinases are homodimeric proteins in which each subunit transphosphorylates the other subunit on a histidine residue using ATP as the phosphodonor. The rate of this autophosphorylation reaction is controlled by sensory stimuli. Phosphorylation-induced conformational change and their subsequent interactions with the flagellar motor. Caution should therefore be used when projecting from *E. coli* CheY onto novel response regulators.

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an unlinked locus (18). cheOp₂ and cheOp₃ have been shown to be essential for chemotaxis (17, 19), whereas deletion of cheOp₁ has only minor effects upon chemotaxis under laboratory conditions (20). With the exception of the CheYs, the cellular localization of the R. sphaeroides chemotaxis proteins has been determined; the sensory transducing proteins encoded by cheOp₂ form a complex with the transmembrane chemoreceptors at the cell poles, whereas the proteins encoded by cheOp₃ localize with putative cytoplasmic chemoreceptors to a cytoplasmic cluster (21). The polar chemotaxis cluster contains CheA₂, which has been shown in vitro to phosphorylate all six of the R. sphaeroides CheYs and both CheBs (22). In contrast, the chemotaxis cluster contains the atypical kinase formed from CheA₃ and CheA₄ (subsequently referred to as CheA₃A₄), which can only phosphotransfer to CheY₁, CheY₆, and CheB₂. Phosphosignaling from both chemotaxis clusters has been shown to be essential for chemotaxis (23).

In vitro assays have shown that all six R. sphaeroides CheYs are capable of binding to the FliM component of the flagellar motor, and that this binding is enhanced by CheY phosphorylation (24). If all six CheYs can bind to FliM, then an intriguing question is as follows: why does R. sphaeroides have six CheYs? The consequence of each of the CheYs binding to FliM is not known; the ability of a CheY to bind FliM in vitro does not necessarily imply that this interaction happens in vivo or that it mediates direction changing. In this study, we determined which of the six CheYs are important for controlling flagellar motor rotation. We identified the cellular localization of these important CheYs using a combination of CFP²/YFP tagging and immunogold electron microscopy. These CheYs were then targeted by site-directed mutagenesis with the aim of either preventing phosphorylation or stabilizing the activated conformation of the CheYs. One phosphorylation site mutant, CheY₁(D56N), caused a stopped phenotype (cells were flagellate but nonmotile). This mutant was shown to be phosphorylated at an alternative site, and in vivo data suggest that phosphorylation at this alternative site mediates the stopped phenotype.

EXPERIMENTAL PROCEDURES

Plasmids and Strains—The plasmids and strains used in this study are shown in Table 1 and supplemental Tables I and II. E. coli strains were grown in LB medium at 37 °C. R. sphaeroides strains were grown in succinate medium at 30 °C either aerobically with shaking without illumination or photoheterotrophically in an anaerobic cabinet (Don Whitley Scientific) with illumination at 50 μmol m⁻² s⁻¹. Where required, antibiotics were used at concentrations of 100 μg ml⁻¹ for ampicillin and 25 μg ml⁻¹ for kanamycin, nalidixic acid, and tetracycline.

Molecular Genetic Techniques—All standard genetic techniques were performed as described (25). Pfu polymerase (Promega) was used for all PCRs. All primers were synthesized by Sigma-Genosys. The plasmid midí-prep kit (Qiagen) was used to prepare sequencing quality DNA, which was sequenced by the DNA sequencing service (Department of Biochemistry, Oxford University). DNA sequence was analyzed using Clone Manager version 7 (Scientific and Educational Software).

Site-directed Mutagenesis of cheY Genes in the R. sphaeroides Genome—Overlap extension PCR was used to generate the constructs for mutating the cheY genes; these constructs contained the mutated cheY genes plus 500 bp of flanking genomic sequence on each side. The most frequently used R. sphaeroides codon was used for each of the desired amino acid substitutions. These constructs were cloned into the allelic exchange suicide vector pK18mob sacB to generate the mutation plasmids (supplemental Table II). The mutation plasmids were checked by sequencing and then used to introduce the point mutations into the genome of R. sphaeroides by allelic exchange as described previously (Table 1) (19, 26). Western blotting was used to confirm that the point mutations had no effect on protein expression levels.

Behavioral Analysis—Behavior was measured under both aerobic and photoheterotrophic growth conditions (Table 1 and supplemental Table I); cheY mutations that affected chemotaxis had similar effects under both sets of growth conditions. The swarm plate responses, the behavior of tethered cells in a flow chamber to the removal of propionate, and the photoresponses of R. sphaeroides strains were characterized as described previously (17). Nine data sets were obtained for swarm plate and photoresponse analysis. Three data sets that together contained at least 10 cells were obtained for each tethered cell analysis. Strains were described as chemotactic if swarm rings were visible on swarm plates. Swarm plates were also used to assess motility by reference to known nonmotile, nonchemotactic, and chemotactic strains; nonmotile cells form smaller colonies on swarm plates than motile but nonchemotactic cells which in turn form smaller colonies than chemotactic cells. Motility and free-swimming stopping frequency was assessed in photoheterotrophic cells at an OD₇₀₀ nm = 0.6 by microscopic examination of free-swimming cells.

Fluorescence Microscopy—Differential interference contrast and fluorescence images of CFP/YFP fusion expressing R. sphaeroides strains were acquired as described previously (21). At least five fields of view each containing at least 50 cells from independent cultures were analyzed for each strain.

Immunogold Electron Microscopy—Aerobic cells at an OD₇₀₀ nm = 0.6 (wild-type cells are motile at this optical density) were prepared for electron microscopy as described (27). Gold particles were scored according to their cellular position and their proximity to other particles as described (28). A total of 80 cell sections was analyzed for each strain.

Protein Purification—His-tagged CheA and CheY proteins were overexpressed and purified as described previously (22, 23, 29). Mutant CheY proteins were overexpressed and purified in the same way as the wild-type versions of the proteins. Protein purity and protein concentrations were measured as described (22). Purified proteins were stored at −20 °C; activity remained constant for at least 1 year.

Phosphotransfer from the CheAs to the CheYs—Phosphotransfer assays were performed at 20 °C in TGMNKD buffer (50 mM Tris-HCl, 10% (v/v) glycerol, 5 mM MgCl₂, 150 mM NaCl, 50 mM KCl, 1 mM dithiothreitol, pH 8.0). CheA₂ reaction mixtures contained 5 μM CheA₂, whereas CheA₃A₄ reaction mixtures...
**CheYs of *R. sphaeroides***

contained 2 μM CheA₂ and 10 μM CheA₄. The reactions mixtures were incubated at 20 °C for 1 h prior to addition of 0.5 mM [γ-32P]ATP (specific activity 14.8 GBq mmol⁻¹; Amersham Biosciences). The ATP-dependent phosphorylation of CheA₂ and CheA₄ was allowed to proceed for 30 min, and then the phosphotransfer reactions were initiated by the addition of 10 μM response regulator. Reaction aliquots of 10 μl were taken at the specified time points and quenched immediately in 5 μl of 3× SDS-PAGE loading dye (7.5% (w/v) SDS, 90 mM EDTA, 37.5 mM Tris-HCl, 37.5% glycerol, 3% (v/v) β-mercaptoethanol, pH 6.8). Quenched samples were analyzed using SDS-PAGE and PhosphorImaging as described previously (22).

*Mass Spectroscopy*—CheY₆(D56N)-P was prepared according to the above phosphotransfer method using CheA₁A₀ as the kinase (nonradioactive ATP was used). Following electrophoresis, the gels were stained with Cooomassie Blue, and the CheY₆(D56N)-P bands were excised and washed with water for 30 min. Samples were digested with trypsin, reductively alkylated on cysteine residues, and prepared for mass spectroscopy according to standard methods (30). Samples were analyzed using a nano-liquid chromatography-MS/MS system consisting of a reversed phase high pressure liquid chromatography (Ultimate Plus™, Dionex/LC Packings, The Netherlands) coupled to an ion trap tandem mass spectrometer (HCTplus™, Bruker Daltonics, Bremen, Germany) as described (31). Individual MS/MS data were searched against the Swiss-Prot database (version 11.12.2005) using Mascot™ software (Matrixscience, London, UK). Identification of the phosphorylated residue Ser-83 of the CheY₆(D56N)-P protein was based on the neutral loss (−97.98Da) observed in the tryptic peptide (M + 3H)⁺ 608.05 Da, and a loss of −18 Da mass at this particular residue because of a conversion of serine to dehydroalanine at this position. Interpretation of the data was performed in accordance with published guidelines (32).

**RESULTS**

*CheY₃ and Either of CheY₅ or CheY₆ Are Essential for *R. sphaeroides* Chemotaxis*—In-frame deletions of the cheY genes were generated to determine the minimal subset(s) of cheYs that could support chemotaxis. Deletion of all six *R. sphaeroides* cheY genes (strain JPA1337) abolished chemotaxis but not motility; the cells swarm but failed to respond to chemosensory and photosensory stimuli. Chemotaxis was observed in strains lacking any one of cheY₁, cheY₂, cheY₃, cheY₄, cheY₅, and cheY₆ (Table 1). Previously we have shown that cheY₆ is essential for chemotaxis (17); however, in this study we show that cheY₆ alone cannot support chemotaxis because the strain deleted for the other cheYs (JPA1025 (ΔcheY₁, ΔcheY₂, ΔcheY₃, ΔcheY₄, and ΔcheY₅)) was nonchemotactic. Because cheY₆ alone is not sufficient for chemotaxis, a series of five strains were created with four cheYs deleted but retaining cheY₆ plus one other cheY. Of these five strains, only JPA433 (ΔcheY₁, ΔcheY₂, ΔcheY₃, and ΔcheY₅) and JPA434 (ΔcheY₁, ΔcheY₂, ΔcheY₃, and ΔcheY₄) were chemotactic (Table 1), indicating that cheY₆ plus either of cheY₃ and cheY₄ can support chemotaxis. CheY₃ and CheY₄ appear to be functionally redundant for one another. A strain deleted for both cheY₃ and cheY₄ (JPA425) was nonchemotactic, suggesting that CheY₃, CheY₄, and CheY₆ are unable to partner CheY₅ in supporting chemotaxis. Thus, chemotaxis roles can only be assigned to three of the six *R. sphaeroides* cheYs: CheY₁, CheY₃, and CheY₄, these cheYs were therefore analyzed further.

*Localization of CheY₃, CheY₅, and CheY₆*—Strains were produced in which the cheY genes were replaced with genes encoding the corresponding CFP/YFP fusions. The functionality of the fusions was tested using swarm plates and in *vitro* phosphotransfer to the purified fusion proteins from their cognate kinases (supplemental Tables I and II). N-terminal fusions to both CheY₃ and CheY₄ were fully functional in both wild-type backgrounds and in backgrounds retaining cheY₆, but deleted for the four remaining cheYs. However, strains with either N- or C-terminal fusions to CheY₆ were nonchemotactic. Western blotting using an antibody against CheY₆ showed that the CheY₆ fusions were expressed at much lower levels than wild type (undetactable in the case of the N-terminal fusion), which could explain why strains containing these fusions were nonchemotactic. Only the N-terminal fusions to the three CheYs were successfully expressed and purified from *E. coli*, and these behaved like the wild-type proteins in the *in vitro* phosphotransfer assays (Table 2).

Fluorescence microscopy showed that the majority of YFP-CheY₆ was delocalized throughout the cytoplasm; no polar clusters were observed, although some protein was localized to cytoplasmic clusters (Fig. 1). In contrast, CFP-CheY₄ mainly localized to discrete regions at both the cell poles and in the cytoplasmic cluster with some diffuse fluorescence throughout the cytoplasm. The fluorescence from CheY₃e-YFP and CheY₆(D56N)-YFP (a phosphorylation site mutant of CheY₆) was mainly localized to cytoplasmic clusters, although a substantial amount of diffuse fluorescence was seen throughout the cytoplasm; no polar clusters were visible. Because the CheY₃ and CheY₄ fusions were nonfunctional, the localization of CheY₃ and CheY₆(D56N) was also determined by immunogold electron microscopy (Fig. 1). This method suggested that CheY₃ and CheY₆(D56N) are distributed diffusely throughout the cytoplasm (72 and 75% of gold particles were cytoplasmic and unassociated, respectively).

*Site-directed Mutagenesis of CheY₃, CheY₅, and CheY₆*—To assess the contributions of CheY₃, CheY₅, and CheY₆ to the control of flagellar motor rotation, point mutations were made in the cheY genes in the *R. sphaeroides* genome (Table 1). These were in sites which in *E. coli* CheYs have been shown to either prevent phosphorylation or to cause activation. A variety of behavioral assays were performed on the mutants, and the *in vitro* phosphorylation of the CheYs was measured (Tables 1 and 2). For convenience, we have used the equivalent *E. coli* CheY residue numbers when referring collectively to mutations in the *R. sphaeroides* CheYs (Fig. 2). The phosphorylation site mutations D57A and D57N were made with the aim of preventing phosphorylation, whereas the D13K, X95V, and X106W mutations were made with the aim of activating the CheYs. Each mutated cheY was introduced into an otherwise wild-type background. However, because CheY₃ and CheY₄ are redundant...
TABLE 1
Summary of the phenotypic analyses of the *R. sphaeroides* cheY mutants

| Mutant           | Strain name | Ref.      | Motile | Normal free swimming stopping frequency | Chemotactic |
|------------------|-------------|-----------|--------|-----------------------------------------|-------------|
| Wild type        | WSN         | 57        | Yes    | Yes                                     | Yes         |
| ΔcheY            | JPA109      | 43        | Yes    | Yes                                     | Yes         |
| ΔcheY3           | JPA104      | 43        | Yes    | Yes                                     | Yes         |
| ΔcheY4           | JPA421      | 43        | Yes    | Yes                                     | Yes         |
| ΔcheY5           | JPA437      | 58        | Yes    | Yes                                     | Yes         |
| ΔcheY6           | JPA1336     | 17        | Yes    | No                                      | No          |
| ΔcheY7           | JPA1025     | This study| Yes    | Yes                                     | No          |
| ΔcheY8           | JPA436      | This study| Yes    | No                                      | No          |
| ΔcheY9           | JPA435      | This study| Yes    | No                                      | No          |
| ΔcheY10          | JPA434      | This study| Yes    | Yes                                     | Yes         |
| ΔcheY11          | JPA429      | 43        | Yes    | Yes                                     | No          |
| ΔcheY12          | JPA425      | 43        | Yes    | Yes                                     | No          |
| cheY(D10K)       | JPA1003     | This study| Yes    | Yes                                     | Yes         |
| cheY(D53A)       | JPA1024     | This study| Yes    | Yes                                     | Yes         |
| cheY(D53N)       | JPA1218     | This study| Yes    | Yes                                     | Reduced     |
| cheY(K91I)       | JPA1387     | This study| Yes    | Yes                                     | Yes         |
| cheY(K91V)       | JPA1389     | This study| Yes    | Yes                                     | Yes         |
| cheY(W102Y)      | JPA1395     | This study| Yes    | Yes                                     | Yes         |
| ΔcheY1, ΔcheY2, ΔcheY3, ΔcheY4, ΔcheY5, ΔcheY6, ΔcheY7, ΔcheY8, ΔcheY9, ΔcheY10, ΔcheY11, ΔcheY12 | JPA4103 | This study | Yes | No |
| ΔcheY(D10K)      | JPA1033     | This study| Yes    | No                                      | No          |
| ΔcheY(D53A)      | JPA1016     | This study| Yes    | No                                      | No          |
| ΔcheY(D53N)      | JPA1219     | This study| Yes    | No                                      | No          |
| ΔcheY(K91I)      | JPA1388     | This study| Yes    | Yes                                     | Yes         |
| ΔcheY(K91V)      | JPA1390     | This study| Yes    | Yes                                     | Yes         |
| ΔcheY(W102Y)     | JPA1396     | This study| Yes    | Yes                                     | Yes         |
| cheY(D10K)       | JPA1004     | This study| Yes    | Yes                                     | Yes         |
| cheY(D53A)       | JPA1024     | This study| Yes    | Reduced                                 | No          |
| cheY(D53N)       | JPA1220     | This study| Yes    | Reduced                                 | No          |
| cheY(K91I)       | JPA1074     | This study| Yes    | Yes                                     | Yes         |
| cheY(K91V)       | JPA1084     | This study| Yes    | Yes                                     | Yes         |
| cheY(W102Y)      | JPA1064     | This study| Yes    | Yes                                     | Yes         |
| ΔcheY1, ΔcheY2, ΔcheY3, ΔcheY4, ΔcheY5, ΔcheY6, ΔcheY7, ΔcheY8, ΔcheY9, ΔcheY10, ΔcheY11, ΔcheY12 | JPA1034 | This study | Yes | No |
| ΔcheY(D10K)      | JPA1034     | This study| Yes    | No                                      | No          |
| ΔcheY(D53A)      | JPA1020     | This study| Yes    | Yes                                     | Yes         |
| ΔcheY(D53N)      | JPA1221     | This study| Yes    | Yes                                     | Yes         |
| ΔcheY(K91I)      | JPA1114     | This study| Yes    | Yes                                     | Yes         |
| ΔcheY(K91V)      | JPA1124     | This study| Yes    | Yes                                     | Yes         |
| ΔcheY(W102Y)     | JPA1154     | This study| Yes    | Yes                                     | Yes         |
| cheY(D10K)       | JPA1006     | This study| Yes    | Yes                                     | Yes         |
| cheY(D56A)       | JPA1216     | This study| Yes    | Yes                                     | No          |
| cheY(D56N)       | JPA1213     | This study| Yes    | Reduced                                 | No          |
| cheY(V104W)      | JPA1007     | This study| Yes    | Yes                                     | Yes         |
| cheY(V104Y)      | JPA1008     | This study| Yes    | Yes                                     | Yes         |
| cheY(D10K, V106W) | JPA1013     | This study| Yes    | Yes                                     | No          |
| ΔcheY1, ΔcheY2, ΔcheY3, ΔcheY4, ΔcheY5, ΔcheY6, ΔcheY7, ΔcheY8, ΔcheY9, ΔcheY10, ΔcheY11, ΔcheY12 | JPA1212 | This study | No | No |
| cheA(D51Q)       | JPA1210     | 23        | Yes    | Yes                                     | No          |
| cheA(D51Q, cheY(D56N) | JPA1246 | This study | Yes | No |
| cheA(G220K)      | JPA1211     | 23        | Yes    | Yes                                     | No          |
| cheA(G220K, cheY(D56N) | JPA1260 | This study | Yes | No |
| cheY(D56N, S83D) | JPA1255     | This study| Yes    | Yes                                     | No          |
| cheY(D56N, S83T) | JPA1257     | Reduced   | Yes    | No                                      | No          |
| cheY(D56N, S83A) | JPA1259     | This study| Yes    | Yes                                     | No          |
| cheY(S83D)       | JPA1254     | This study| Yes    | Yes                                     | No          |
| cheY(S83T)       | JPA1256     | This study| Yes    | Yes                                     | No          |
| cheY(S83A)       | JPA1258     | This study| Yes    | Yes                                     | Yes         |
| yfp-cheY         | JPA729      | This study| Yes    | Yes                                     | Yes         |
| ΔcheY1, ΔcheY2, ΔcheY3, ΔcheY4, ΔcheY5, ΔcheY6, ΔcheY7, ΔcheY8, ΔcheY9, ΔcheY10, ΔcheY11, ΔcheY12 | JPA1629 | This study | Yes | Yes |
| ΔcheY(D10K)      | JPA1629     | This study| Yes    | Yes                                     | Yes         |
| ΔcheY(D53A)      | JPA1630     | This study| Yes    | Yes                                     | Yes         |
| ΔcheY(D53N)      | JPA1631     | This study| Yes    | Yes                                     | Yes         |
| ΔcheY(K91I)      | JPA1632     | This study| Yes    | Yes                                     | Yes         |
| ΔcheY(K91V)      | JPA1624     | This study| Yes    | Yes                                     | Yes         |
| ΔcheY(W102Y)     | JPA1621     | This study| Yes    | Yes                                     | No          |

CheYs of *R. sphaeroides*

Dant for one another, it is probable that the phenotype of a null point mutation in either one of these *cheYs* would be masked by the other *cheY*. Therefore, the *cheY1* and *cheY4* mutations were also introduced into strains deleted for *cheY1*, *cheY2*, *cheY3*, and *cheY4* and *cheY1*, *cheY2*, *cheY3*, and *cheY4*, respectively.

**Mutation of Asp-13 and Xaa-106—Asp-13** is strictly conserved in all CheYs and has been shown to be involved in Mg$^{2+}$ binding and catalysis. Mutation of this residue to lysine in *E. coli* prevents phosphorylation by CheA, yet increases the CW motor bias (33, 34). Conversion of both Asp-13 to lysine and Tyr-106 to tryptophan further increases the CW motor bias. These mutations strongly favor the activated conformation of *E. coli* CheY, increasing the FliM binding affinity to that of CheY-P (10). The equivalent of the Asp-13 residue is present in...
**CheYs of *R. sphaeroides***

**TABLE 2**

| CheY mutant                  | In vitro phosphorylation* | Supports chemotaxis in vivo |
|------------------------------|---------------------------|-----------------------------|
| CheY3                        | Phosphorylated by CheA3-P | Yes                         |
| CheY6                        | Phosphorylated by CheA3-P | No                          |
| CheY3(D10K)                  | No                         | No                          |
| CheY6(D53A)                  | No                         | No                          |
| CheY3(D53N)                  | Yes*                       | Yes                         |
| CheY6(S83T)                  | Yes*                       | No                          |
| CheY6(S83D)                  | Yes*                       | Yes                         |
| CheY6(D56N)                  | Yes*                       | Yes                         |
| CheY6(V102Y)                 | Yes*                       | Yes                         |
| CheY6(V104Y)                 | Yes*                       | Yes                         |
| CheY6(D10K, V106W)           | No                         | No                          |
| CheY6(D56N, S83D)            | No                         | No                          |
| CheY6(D56N, S83T)            | No                         | No                          |
| CheY6(D56N, S83A)            | Yes*                       | Yes                         |
| YFP-CheY3                    | Yes*                       | Yes                         |
| YFP-CheY6                    | Yes*                       | Yes                         |
| YFP-CheY6(D56N)              | Yes*                       | Yes                         |
| CheY3(W102Y)                 | Yes*                       | Yes                         |
| CheY6(W102Y)                 | Yes*                       | Yes                         |
| CheY3(K91I)                  | Yes*                       | Yes                         |
| CheY6(K91I)                  | Yes*                       | Yes                         |
| CheY3(W102Y)                 | Yes*                       | Yes                         |
| CheY6(W102Y)                 | Yes*                       | Yes                         |
| CheY3(V91)                   | Yes*                       | Yes                         |
| CheY6(V91)                   | Yes*                       | Yes                         |
| CheY3(Y104)                  | Yes*                       | Yes                         |
| CheY6(Y104)                  | Yes*                       | Yes                         |
| CheY3(Y91)                   | Yes*                       | Yes                         |
| CheY6(Y91)                   | Yes*                       | Yes                         |
| CheY3(Y91)                   | Yes*                       | Yes                         |
| CheY6(Y91)                   | Yes*                       | Yes                         |
| CheY3(Y91)                   | Yes*                       | Yes                         |
| CheY6(Y91)                   | Yes*                       | Yes                         |
| CheY3(Y91)                   | Yes*                       | Yes                         |
| CheY6(Y91)                   | Yes*                       | Yes                         |
| CheY3(Y91)                   | Yes*                       | Yes                         |
| CheY6(Y91)                   | Yes*                       | Yes                         |
| CheY3(Y91)                   | Yes*                       | Yes                         |
| CheY6(Y91)                   | Yes*                       | Yes                         |
| CheY3(Y91)                   | Yes*                       | Yes                         |
| CheY6(Y91)                   | Yes*                       | Yes                         |
| CheY3(Y91)                   | Yes*                       | Yes                         |
| CheY6(Y91)                   | Yes*                       | Yes                         |
| CheY3(Y91)                   | Yes*                       | Yes                         |
| CheY6(Y91)                   | Yes*                       | Yes                         |
| CheY3(Y91)                   | Yes*                       | Yes                         |
| CheY6(Y91)                   | Yes*                       | Yes                         |
| CheY3(Y91)                   | Yes*                       | Yes                         |
| CheY6(Y91)                   | Yes*                       | Yes                         |
| CheY3(Y91)                   | Yes*                       | Yes                         |
| CheY6(Y91)                   | Yes*                       | Yes                         |
| CheY3(Y91)                   | Yes*                       | Yes                         |
| CheY6(Y91)                   | Yes*                       | Yes                         |
| CheY3(Y91)                   | Yes*                       | Yes                         |
| CheY6(Y91)                   | Yes*                       | Yes                         |
| CheY3(Y91)                   | Yes*                       | Yes                         |
| CheY6(Y91)                   | Yes*                       | Yes                         |
| CheY3(Y91)                   | Yes*                       | Yes                         |
| CheY6(Y91)                   | Yes*                       | Yes                         |
| CheY3(Y91)                   | Yes*                       | Yes                         |
| CheY6(Y91)                   | Yes*                       | Yes                         |
| CheY3(Y91)                   | Yes*                       | Yes                         |
| CheY6(Y91)                   | Yes*                       | Yes                         |
| CheY3(Y91)                   | Yes*                       | Yes                         |
| CheY6(Y91)                   | Yes*                       | Yes                         |
| CheY3(Y91)                   | Yes*                       | Yes                         |
| CheY6(Y91)                   | Yes*                       | Yes                         |
| CheY3(Y91)                   | Yes*                       | Yes                         |
| CheY6(Y91)                   | Yes*                       | Yes                         |

*Yes indicates that extent of phosphorylation was the same as for the corresponding wild-type CheY. Yes* indicates that phosphorylation was observed but at a different level to the corresponding wild-type CheY.

**FIGURE 1.** Subcellular localization of the *R. sphaeroides* CheY proteins. Fluorescently tagged CheY3(A), CheY6(B), CheY4(C), and CheY6(D56N)(D) are shown. Immunogold electron micrographs of *R. sphaeroides* cells using an anti-CheY3 antibody are shown. E, wild-type cell (WS8N); F, cheY3(D56N) mutant (JPA1213).  

all *R. sphaeroides* CheYs (Fig. 2). However, CheY3 and CheY4 already have tryptophan instead of tyrosine at their equivalent of the 106† position, and CheY6 has valine at this position. Strains containing the cheY3(D10K) and cheY4(D10K) alleles in otherwise wild-type backgrounds were chemotactic; however, these alleles were unable to support chemotaxis in strains deleted for all of the other cheYs except cheY6. The cheY6(D10K) and cheY6(D10K, V104W) mutants were nonchemotactic. All of the strains expressing D13K† mutants of the *R. sphaeroides* cheYs exhibited wild-type stopping frequency (Table 1). In summary, strains containing the cheY3(D10K), cheY3(D10K, V104W), or cheY6(D10K, V104W) alleles were phenocopies of their corresponding cheY deletion mutants (Table 1). This suggests that D13K† mutations make the CheY3, CheY4, and CheY6 proteins nonfunctional. This contrasts with *E. coli*, where the CheY(D13K) mutant causes a very different phenotype (CW rotation) to the cheY deletion mutant (counter-clockwise rotation).

The role of the residue at position 106† was probed by mutating it to either phenylalanine (for CheY3, CheY4, and CheY6) or to tryptophan (for CheY6 only). None of these mutations had any effect on chemotaxis, stopping frequency, or *in vitro* phosphorylation (Tables 1 and 2). This suggests that unlike *E. coli* CheY, the *R. sphaeroides* CheYs are tolerant of substitutions at position 106†.

**Mutation of Residue 95—**Another mutant that has been shown to activate *E. coli* CheY is the I95V mutation. This mutant is phosphorylatable and shows an increased affinity for FlhM (35). CheY3 and CheY4 have a lysine residue at this position (Fig. 2). Interestingly, an *E. coli* CheY(I95K) mutant behaves like the ΔcheY mutant and is unable to stimulate CW rotation of the flagellum (36, 37). Mutation of the Lys-95† residue in CheY3 and CheY4 to either valine or isoleucine had no detectable effects upon CheY in *in vitro* phosphorylation, stopping frequency, or the ability of these CheYs to support chemotaxis (Table 1). These data suggest that the Lys-95† residue is not important for the function of CheY3 or CheY4.

**Mutation of the Phosphorylatable Aspartate—**The D57A† and D57N† mutations remove the phosphorylatable aspartate residue from CheY. These mutations have been widely used to prevent response regulator phosphorylation, allowing the *in vivo* properties of unphosphorylated response regulators to be studied (38). The D57A† mutants of CheY3, CheY4, and CheY6 were all nonphosphorylatable *in vitro* and were *in vivo* phenocopies of their corresponding cheY deletion mutants (Table 1). This indicates that phosphorylation of these CheYs is required for chemotaxis.

Surprisingly, the D57N† mutants behaved differently to the D57A† and the cheY deletion mutants. Like the cheY3(D53A) and cheY3(D53A) mutations, the cheY3(D53N) and cheY3(D53N) mutations were not capable of supporting chemotaxis in strains retaining cheY6 but lacking the remaining cheYs. However, unlike the D57A† mutations, the cheY3(D53N) and cheY3(D53N) mutations partially inhibited chemotaxis in otherwise wild-type backgrounds on swarm plates but behaved like the wild-type strain in the tethered cell and phototaxis assays (Table 1).

Unlike the cheY6 deletion and the cheY6(D56A) mutant, the cheY6(D56N) mutant had a stopped phenotype, i.e. it did not swim in any of the behavioral assays. Electron microscopy revealed that this mutant was flagellate suggesting that the flagellar motor was stopped in this strain (data not shown). Unlike the *E. coli* flagellar motor, the *R. sphaeroides* flagellar
motor is a stop-start motor; it does not reverse, i.e. clockwise rotation in E. coli is replaced with a stop in R. sphaeroides. It is likely that the stopped phenotype of the cheY6(D56N) strain is analogous to the E. coli tumbly phenotype which is caused by exclusive clockwise rotation of the flagella. A strain containing cheY6(D56N) but lacking the remaining cheYs is also stopped, whereas a strain containing wild-type cheY6 but lacking the remaining CheYs is motile, indicating that CheY6(D56N) is capable of causing the stopped phenotype in the absence of the other CheYs.

Although the D57A† mutants of CheY3, CheY4, and CheY6 were nonphosphorylatable, the D57N mutants were phosphorylatable by their cognate kinases (Table 2 and Fig. 3). This difference in phosphorylation between the D57A† and D57N† mutants might account for the different behavior of the D57A† and D57N† mutants. The phosphorylation of the D57N† mutants occurred on a much slower time scale than for the wild-type CheYs, suggesting that the mutant CheYs were being phosphorylated at a different site to the wild-type CheYs (Fig. 3). Even though the phosphotransfer reactions were slow for the D57N† mutants, the final phosphorylation levels of CheY4(D53N) and CheY6(D56N) were higher than those of the corresponding wild-type proteins, suggesting that the D57N† mutants dephosphorylate more slowly than the wild-type proteins. The E. coli phosphorylation site mutant, CheY(D57N) is phosphorylated at an alternative site, Ser-56, by CheA (39); CheY3 and CheY4 both have a threonine at this position, which is likely to be the alternative phosphorylation site for these proteins. However, CheY6 has a nonphosphorylatable leucine residue at this position, and so the phosphorylation site of CheY6(D56N) must be elsewhere on the protein.

Phosphorylated CheY6(D56N) Stops the Flagellar Motor—The cheY6(D56N) mutant was the only stopped (flagellate, but nonmotile) mutant found in this study. Unlike wild-type cells, this mutant did not form an expanding colony in the swarm plate assays. However, after prolonged incubation of the swarm plates (4 days) flares could be seen emerging from the colony. Microscopic examination of the cells in these flares revealed that they had regained motility. Cells recovered from the flares were used to inoculate new swarm plates, where they formed...
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CheA4 (G220K) do not participate in phosphorylation reactions in vitro nor do they support chemotaxis in vivo (23). Both of the product strains lacked the stopped phenotype, being motile but nonchemotactic, which is consistent with the hypothesis that CheY6(D56N) needs to be phosphorylated by CheA3A4 to stop the motor.

**CheY6(D56N) Is Phosphorylated at Ser-83**—The *E. coli* phosphorylation site mutant, CheY(D56N), is phosphorylated at Ser-56 by CheA; however, *R. sphaeroides* CheY6 has a nonphosphorylatable leucine residue at this position. The phosphorylation site was determined by mass spectroscopy. Phosphorylated CheY6(D56N) was prepared as described under “Experimental Procedures.” Following digestion with trypsin, the resulting peptides were analyzed using MS/MS spectrometry. The peptide ICML(S*)SV-AVSGSPHAAR was found to be phosphorylated at the serine residue highlighted with an asterisk, which corresponds to residue Ser-83 in CheY6(D56N) (Fig. 4).

**In Vitro Phosphorylation of Ser-83 Mutants of CheY6**—The predicted phosphorylation site of CheY6—Ser-83. This residue was mutated in both wild-type CheY6 and in CheY6(D56N). Three different substitutions were made as follows: for S83D, aspartate substitutions have been successfully used in other proteins to mimic phosphoserine (40, 41); for S83T, this is a conservative substitution, which should be phosphorylatable; and for S83A, this mutant should be nonphosphorylatable. Phosphotransfer from CheA3-P to the CheY6 mutants was measured under multiple turnover conditions in the presence of CheA3 and excess ATP, allowing CheA3 phosphorylation to continue throughout the course of the reactions. The progress of these phosphotransfer reactions after 30 min is shown in Fig. 5; phosphotransfer occurred in reactions where a decrease in CheA3-P levels was accompanied by an increase in CheY6-P levels.

Phosphotransfer occurred to a similar extent for the CheY6(D56N) and CheY6(D56N,S83D) mutants (Fig. 5). However, no phosphotransfer was observed to the CheY6(D56N,S83T) mutant, and only a small amount of phosphotransfer was seen to the CheY6(D56N,S83A) mutant (there was ~200-fold less CheY6(D56N,S83A)-P than CheY6(D56N)-P). In *E. coli*, the asparagine 57 residue of the CheY(D57N) mutant has been shown to spontaneously deamidate to aspartate on a slow time scale (42). If the CheY6(D56N,S83A) mutant underwent a similar deamidation reaction, then the product of this deamidation would be CheY6(S83A), which could be phosphorylated at Asp-56 by CheA3-P. Deamidation and subsequent phosphorylation of a
small fraction of the CheY6(D56N,S83A) mutant could account for the residual phosphotransfer seen for this mutant. Presumably, the CheY6(D56N,S83D) mutant does not deamidate, and consequently no phosphorylation was observed. These results are again consistent with Ser-83 being the phosphorylation site of CheY6(D56N).

Phosphotransfer from CheA3-P to wild-type CheY6 and subsequent dephosphorylation of CheY6-P was complete within 30 s, i.e. after 30 s no CheA3-P or CheY6-P was detectable (Fig. 5). Phosphotransfer to the CheY6(S83D) mutant and its subsequent dephosphorylation was much slower than wild type. Both the CheY6(S83D) and CheY6(S83A) mutants were detectable after 30 min. Phosphotransfer to CheY6(S83T) and CheY6(S83A) was much faster than for CheY6(S83D) but still slower than wild type because detectable levels of CheA3-P remained after 30 min. These results show that Ser-83 is an important residue in CheY6 where mutations affect both the rate of phosphotransfer from CheA3-P and the rate of CheY6-P dephosphorylation.

In Vivo Behavior of the CheY6 Ser-83 Mutants—The wild-type cheY6 gene in the R. sphaeroides genome was replaced with the genes encoding the D56N and/or Ser-83 mutants analyzed above. The chemotactic behavior of the resulting strains was analyzed. Unlike the stopped cheY6(D56N) mutant, the strains containing the nonphosphorylatable CheY mutants CheY6(D56N,S83D) and CheY6(D56N,S83A) were motile but nonchemotactic, i.e. they behaved like the cheY6 deletion. Interestingly, cells containing the phosphorylatable mutant, CheY6(D56N,S83T) showed severely reduced motility where the cells were predominantly stopped with occasional very brief periods of movement. The CheY6(D56N,S83T) mutant therefore has a slightly less severe motility defect than the CheY6(D56N) mutant, which could indicate that the precise geometric configuration of the alternative phosphorylation site is critical for causing the stopped phenotype. These data are consistent with the hypothesis that phosphorylation of Ser-83 is required for CheY6(D56N) to cause the stopped phenotype.

Ser-83 was mutated in an otherwise wild-type CheY6 to assess the role of this residue in wild-type CheY6. The CheY6(S83T) and cheY6(S83A) mutants both supported wild-type levels of chemotaxis, whereas the cheY6(S83D) mutant phenocopied the cheY6 deletion, i.e. was motile but nonchemotactic. The lack of chemotaxis of the strain expressing the CheY6(S83D) protein can be explained by the reduced rates of phosphotransfer and dephosphorylation of this mutant protein. However, the normal behavior of the CheY6(S83A) mutant indicates that phosphorylation of Ser-83 is not essential for wild-type chemotaxis.

DISCUSSION

Prior to the sequencing of its genome, it was believed that R. sphaeroides had only four CheYs (CheY1, CheY2, CheY3, and CheY4) (18). A genetic study of the roles of these CheYs was performed; signaling events that could not be explained by these CheYs were attributed, by the process of elimination, to the newly discovered CheY protein CheY6 (43). However, there was yet another undiscovered CheY protein, CheY7 (17). In this study we demonstrate that CheY6 plus either of CheY3 and CheY4 are essential for chemotaxis in R. sphaeroides. No chemotaxis roles could be found for the cheO1-encoded CheY1, CheY2, or CheY4.

Why would an organism have six CheYs when two are sufficient? One possibility is that all six CheYs are used in the wild-type strain but the robustness of the R. sphaeroides chemotaxis system allows the system to cope even when some of those CheYs are removed. Alternatively, because the chemotaxis genes in cheO1 are expressed at very low levels under the conditions used in our assays (43), they may have little or no role in chemotaxis; however, it is possible that under alternative growth conditions, the CheYs encoded by cheO1 may be more highly expressed and may adopt a more significant role in chemotaxis. A further possibility is that cheO1 and the CheYs encoded within it are not involved in chemotaxis but instead control some other cellular process; there is a precedent for this in the α-subdivision of proteobacteria, where Rhodospirillum centenum uses chemotaxis-like systems to control both flagella biosynthesis and cyst development (44).

Why are a minimum of two CheYs required for R. sphaeroides chemotaxis when many bacteria, e.g. E. coli, Bacillus subtilis, and Vibrio cholerae, can manage with only one (45, 46)? Sinorhizobium meliloti and R. sphaeroides lack the CheZ, CheC, CheX, and FliY signal...
terminating CheY phosphatases that have been described in other bacteria (47–49). Like R. sphaeroides, S. meliloti also requires two CheYs for normal chemotaxis; S. meliloti CheY2-P can bind to the FliM component of the flagellar motor and brings about direction changing, whereas CheY1 cannot bind to the motor and acts as a signal terminating phosphate sink (50).

Could R. sphaeroides be using a similar system? All six R. sphaeroides CheYs can bind to FliM in vitro, and this binding is enhanced by CheY phosphorylation (24). If all six CheYs bind FliM in vivo, then this would suggest that any CheY acting as a phosphate sink would be capable of binding to FliM. However, the binding of a CheY to FliM may not result in a change in flagellar rotation. Some of the R. sphaeroides CheYs may be unable to stop flagellar rotation, but they might compete for binding to FliM with CheYs that can stop flagellar rotation. Structural studies on S. meliloti CheY2 also suggest that the phosphorylation-induced conformational change that occurs upon activation of E. coli CheY may not be universally applicable (52).

In vivo studies of the signaling role of the dephosphorylated forms of the CheYs using strains where the wild-type cheY gene had been replaced with a phosphorylation site mutant had unexpected results. The two mutations commonly used to prevent phosphorylation of response regulators, D57A† and D57N†, caused different phenotypes. The D57N† mutants could be phosphorylated at an alternative site by their cognate kinase, whereas the D57A† mutants could not be phosphorylated. This difference was most pronounced in the case of CheY6, where the cheY6(D56A) allele produced a motile but nonchemotactic strain, whereas the cheY6(D56N) allele produced a stopped strain, i.e. one that was flagellate but did not swim, suggesting that CheY6(D56N) could bind to and stop the flagellar motor. CheY6(D56N) can be phosphorylated at Ser-83 by the cognate kinase for CheY6, CheA3A4. Furthermore, the stopped phenotype of the cheY6(D56N) mutant requires functional CheA3 and CheA4, suggesting that CheY6(D56N) needs to be phosphorylated at Ser-83 to stop the flagellar motor. Therefore, although chemotaxis requires a minimum of two
CheYs, CheY₆ plus either of CheY₃ or CheY₄. CheY₆(D56N)-P alone is capable of stopping the flagellar motor.

What is the role of the alternative phosphorylation site (Ser-83) in wild-type CheY₆? There is no evidence suggesting that alternative site phosphorylation occurs in wild-type CheY₆. Even if it did, the slow phosphorylation kinetics are inconsistent with the ability of _R. sphaeroides_ to respond to a stimulus in less than 1 s (53). Phosphorylation of Ser-83 is not essential for wild-type chemotaxis, because the _cheY₆(S83A) _mutant supports wild-type levels of chemotaxis. Despite this, substitutions of Ser-83 altered both the rate of phosphotransfer from the kinase and the rate of response regulator dephosphorylation. This was most obvious for the _CheY₆(S83D) _mutant which failed to support chemotaxis. Ser-83 must therefore either be an important residue in the CheY₆ active site, or Ser-83 substitutions must affect its conformation.

A previous study on _E. coli_ CheY has shown that the CheY(D57N) mutant is partially activated by phosphorylation of Ser-56 (39). Phosphorylation of mutants where the primary phosphorylation site has been replaced with asparagine has also been detected for the response regulators _FixJ_ from _S. mellioti_ and NtrC from _E. coli_ (54, 55). No cases of response regulator phosphorylation where the primary phosphorylation site was mutated to alanine have been reported. A likely explanation for this has been proposed (39); the carbonyl group of the phosphorylatable aspartate is required to chelate the Mg²⁺ ion that is essential for catalysis (56); asparagine mutants retain this carboxyl group, whereas alanine mutants do not.

In many studies of response regulator action either the equivalent of the D57A or D57N mutation is made to facilitate the study of the signaling role of the dephosphorylated response regulator. Our results and those of other researchers suggest that caution should be used when interpreting the results of analogues of the D57N mutation. We have demonstrated that even if no phosphorylatable residue is present at position 56, it is still possible for the D57N mutant of the response regulator to be phosphorylated and to be active. Investigators should confirm, rather than assume, that D57N mutants of response regulators are not subject to alternative site phosphorylation. Other mutations that activated _E. coli_ CheY did not appear to activate the _R. sphaeroides_ CheYs, suggesting that not all CheYs undergo identical conformational changes upon phosphorylation. _E. coli_ CheY is often used as a model for response regulators. We have shown that even among CheYs, there is not necessarily conservation of critical residues and that activating/inactivating point mutations do not automatically translate from one response regulator to another.

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