

Fine tuning bacterial chemotaxis: analysis of *Rhodobacter sphaeroides* behaviour under aerobic and anaerobic conditions by mutation of the major chemotaxis operons and *cheY* genes

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***Rhodobacter sphaeroides* chemotaxis is significantly more complex than that of enteric bacteria. *Rhodobacter sphaeroides* has multiple copies of chemotaxis genes (two *cheA*, one *cheB*, two *cheR*, three *cheW*, five *cheY* but no *cheZ*), controlling a single 'stop-start' flagellum. The growth environment controls the level of expression of different groups of genes. Tethered cell analysis of mutants suggests that CheY₄ and CheY₅ are the motor-binding response regulators. The histidine protein kinase CheA₂ mediates an attractant ('normal') response via CheY₄, while CheA₁ and CheY₅ appear to mediate a repellent ('inverted') response. CheY₃ facilitates signal termination, possibly acting as a phosphate sink, although CheY₁ and CheY₂ can substitute. The normal and inverted responses may be initiated by separate sets of chemoreceptors with their relative strength dependent on growth conditions. *Rhodobacter sphaeroides* may use antagonistic responses through two chemosensory pathways, expressed at different levels in different environments, to maintain their position in a currently optimum environment. Complex chemotaxis systems are increasingly being identified and the strategy adopted by *R.sphaeroides* may be common in the bacterial kingdom.**

Keywords: chemotaxis/*cheY*/flagellar motility/*Rhodobacter (sphaeroides)*/signal transduction

Introduction

In order to survive in the dynamic and sometimes harsh environments they inhabit, most bacterial species are able to detect and move towards favourable and away from unfavourable conditions, a process known as taxis. To perform taxis, cells have an organelle for motility and a means to sense the environment and modulate that motility. The motility organelle in most bacteria is a rotating semi-rigid helical filament, the flagellum (reviewed by Macnab, 1996). Bacteria can carry out different types of taxis, e.g. aerotaxis, phototaxis and chemotaxis.

Bacterial chemotaxis has been studied extensively in *Escherichia coli*. In *E.coli*, chemotaxis relies on control-

ling the frequency at which the direction of flagellar rotation is switched. Counterclockwise flagellar rotation results in swimming, while clockwise rotation results in a tumble that changes the swimming direction of the cell. Unstimulated cells exhibit a random pattern of tumbling. A signal transduction pathway controls the frequency of tumbling. Cells tumble less frequently when swimming in a favourable direction, biasing the random swimming pattern so that there is a net movement towards attractant. In *E.coli*, membrane-spanning chemoreceptors, methyl-accepting chemotaxis proteins (MCPs), sense the concentration of chemoattractants (for a recent review see Mowbray and Sandgren, 1998). MCPs sense a decrease in attractant in the periplasm and transmit an activating signal to the MCP-associated cytoplasmic proteins CheW and CheA. CheW is thought to be a linker protein between the MCPs and the histidine protein kinase, CheA. Upon activation, CheA autophosphorylates at a conserved histidine (H48), using ATP as the phosphodonator (Hess *et al.*, 1988a). The phosphate group is then transferred to the response regulator CheY at a conserved aspartate (D57; Sanders *et al.*, 1989; Bourret *et al.*, 1990). The phosphorylated form of CheY (CheY-P) binds to the FliM component of the motor switch complex and stimulates clockwise rotation, resulting in tumbling (Welch *et al.*, 1993). The concentration of CheY-P determines whether cells run or tumble. CheY-P levels are reduced slowly by the autophosphatase activity of CheY-P, but dephosphorylation is greatly enhanced by CheZ, allowing rapid signal termination and thus gradient sensing (Hess *et al.*, 1988b). The importance of CheZ in signal termination is illustrated by the fact that *cheZ* null mutants are locked in tumbling and are chemotaxis deficient (Parkinson, 1978). Deletion of *cheW*, *cheA* or *cheY* in *E.coli* results in cells that are locked in smooth swimming and are also chemotaxis deficient (Parkinson, 1978).

Rhodobacter sphaeroides is a purple non-sulfur, photosynthetic bacterium belonging to the α -subgroup of proteobacteria. It is versatile in that it can grow under aerobic conditions, anaerobically in the light using photosynthesis or anaerobically in the dark using alternative electron acceptors. It is motile and chemotactic under all of these conditions. It has a single flagellum that rotates unidirectionally and stops periodically to allow the cell to reorient (Armitage and Macnab, 1987; Armitage *et al.*, 1999). A stop is equivalent to an *E.coli* tumble. A remarkable property of *R.sphaeroides* is that it has multiple homologues of the *E.coli* chemotaxis proteins. There are up to 12 chemoreceptors (MCP-like proteins), some of which are located in the cytoplasm (Ward *et al.*, 1995b; Harrison *et al.*, 1999; Wadhams *et al.*, 2000; G.Wadhams, A.C.Martin and J.P.Armitage, unpublished). At the time this study was initiated we had also identified two CheA, one CheB, two CheR, three CheW and four

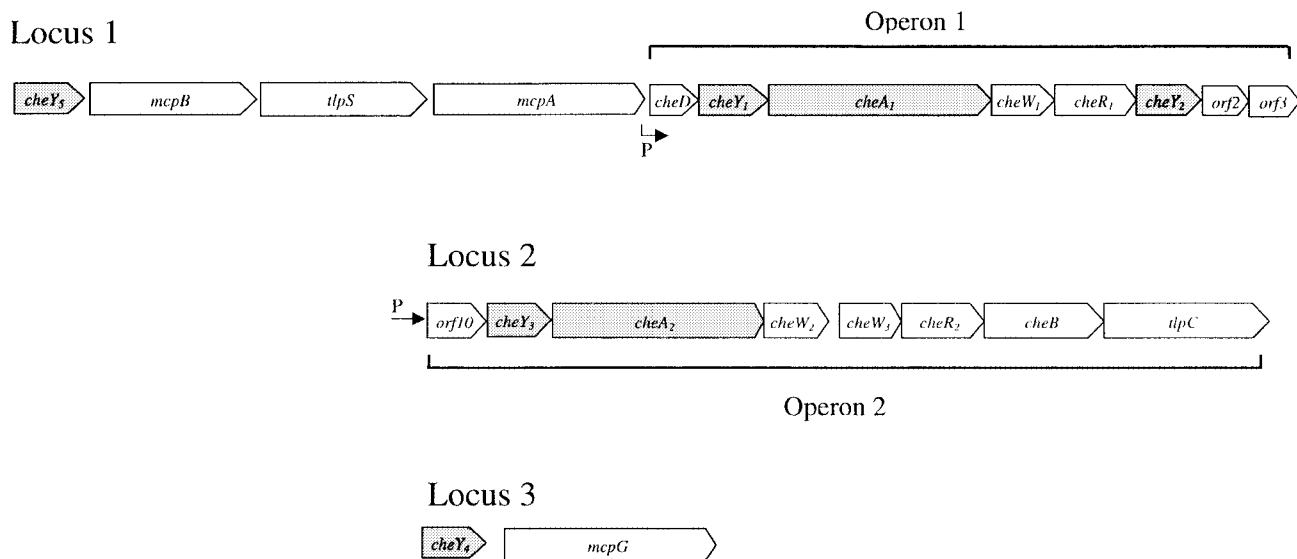


Fig. 1. Organization of the chemotaxis genes of *R.sphaeroides*. The locations of regions with detectable promoter activity are indicated with a 'P'. The associated arrows indicate the direction of transcription. *tlp*, transducer-like protein.

CheY homologues (Ward *et al.*, 1995a,b; Hamblin *et al.*, 1997b; Shah *et al.*, 2000). Interestingly, no CheZ homologues have been identified. The *che* genes and some of the receptor proteins are situated at two major loci with the exception of *cheY4*, which is located independently of the major loci (Figure 1). Multiple chemotaxis gene homologues are increasingly being found in a wide range of other bacterial species (Armitage, 1999). Many of these other species lack the signal terminator CheZ but all have multiple copies of CheY. Work on *Sinorhizobium meliloti* (previously *Rhizobium meliloti*), which has two CheY homologues, suggests that one of the CheYs (CheY₂-P) is more efficient at causing a change in motor behaviour than the other (CheY₁-P), which operates as a phosphate sink (Sourjik and Schmitt, 1996, 1998). To function as a phosphate sink, one CheY must have a higher affinity for the phosphate of CheA with a lower rate of reverse phosphotransfer to CheA than the other motor-binding CheY, thus allowing signal termination in the absence of CheZ. The use of a second CheY as a phosphate sink for signal termination may be a common strategy employed by species lacking CheZ.

Why are there multiple copies of *che* genes in many bacterial species? Previous studies on *R.sphaeroides* showed that deletion of *che* operon 1 results in only minor effects on chemotaxis whereas deletion of *cheA2* from *che* operon 2 results in an inverted behavioural response compared with wild type (Hamblin *et al.*, 1997b), suggesting that the two chemotaxis operons may have distinct roles. *Rhodobacter sphaeroides* CheA₂, CheW₁, CheW₃, CheB and CheR₂ can restore swarming to *E.coli* strains deficient in the corresponding proteins, whereas CheA₁ and CheW₂ cannot (Hamblin *et al.*, 1997a; Shah *et al.*, 2000; H.Jones, D.S.H.Shah, S.L.Porter and J.P.Armitage, unpublished observations). Thus, although some of the Che proteins of *R.sphaeroides* are compatible with the *E.coli* pathway, others are not. None of the *R.sphaeroides* CheYs can restore swarming to a CheY-deficient *E.coli* strain, suggesting that the *R.sphaeroides*

CheYs cannot bind to the *E.coli* motor. Surprisingly however, all the *R.sphaeroides* CheYs enhance swarming of a CheZ-deficient strain, indicating that they can substitute for CheZ and act as signal terminators (Shah *et al.*, 2000). Thus, studying the role of the CheY proteins in *R.sphaeroides* is of particular interest.

The metabolic versatility, the range of chemotactic responses and the possession of multiple chemotaxis genes make *R.sphaeroides* an interesting model system. Until now, no details about the molecular interactions during chemotaxis of *R.sphaeroides* were available. In this study the roles of the two major operons and the *cheY* genes of *R.sphaeroides* were investigated. We also present data on the relative expression levels of the two operons under aerobic and anaerobic conditions, and describe the discovery of a fifth *cheY* homologue. A model of the possible interactions within this complex chemotaxis system is presented.

Results and discussion

Discovery of a fifth *cheY* gene

During the course of this study a fifth *cheY* homologue was identified when the region upstream from *cheD* in chemotaxis locus 1 was sequenced (Figure 1). The *cheY5* open reading frame (ORF) is predicted to encode a protein of 122 amino acids with 50, 26, 69 and 74% amino acid identity with CheY₁, CheY₂, CheY₃ and CheY₄, respectively. It has 32% identity with *E.coli* CheY. The extended sequence of the first chemotaxis locus has been deposited in the DDBJ/EMBL/GenBank database under accession No. X80205.

Expression levels of operons

To interpret the behaviours shown by the mutant strains we needed to determine the relative expression levels of *che* operon 1 and *che* operon 2. The putative promoter regions of the two operons were cloned upstream from a promoterless *lacZ* reporter gene as described in

Table I. Mean \pm SD β -galactosidase activity (arbitrary units) from *lacZ* fused to the promoter regions of *che* operon 1 and *che* operon 2 in pUI523A under different growth conditions in wild-type strain WS8N

Plasmid	Aerobic	Photosynthetic
None	7.6 \pm 0.4	8.3 \pm 0.8
pUI523A	8.1 \pm 0.8	6.7 \pm 0.2
Operon 1	25.1 \pm 4.3	11.2 \pm 0.9
Operon 1 reverse	7.0 \pm 0.3	7.3 \pm 0.3
Operon 2	485.6 \pm 45.9	43.5 \pm 8.0
Operon 2 reverse	6.6 \pm 0.3	7.0 \pm 0.3

Materials and methods. The expression levels from these regions in the wild-type strain harbouring the test and control plasmids were assayed under anaerobic photosynthetic and aerobic conditions. The results are shown in Table I. *che* operon 1 and *che* operon 2 promoter activity was observed. *che* operon 2 expression was ~4- and 15-fold higher than that of *che* operon 1 under anaerobic and aerobic conditions, respectively. Therefore, under aerobic conditions the concentration of *che* operon 2 components would be very high relative to *che* operon 1 components. Although expression from plasmid fusions only provides a crude qualitative estimate of the genomic expression levels from these promoters, the data clearly indicate that the chemotaxis operons are under environmental control and that the cells can 'customize' their chemotaxis system according to the prevailing growth conditions. Indeed, previous studies have revealed a difference in the expression of MCP-like proteins under aerobic and anaerobic conditions (Harrison *et al.*, 1999). These data have a significant effect on the interpretation of the differences in behaviour of mutant strains seen under aerobic and anaerobic conditions.

Phenotypic analyses

Strains with all combinations of operon and *cheY*₁–*cheY*₄ mutations on the chromosome were constructed as described in Materials and methods. Note that the *cheY*₃ mutant is referred to as *cheY*₃* because it is an insertion mutant whilst all the others are in-frame deletions, and that Δ Op1 and Δ Op2 refer to strains that have been deleted for *che* operon 1 and *che* operon 2, respectively. Each strain was tethered in a flow cell and assayed for chemotactic responses to the addition and removal of 1 mM propionate, under aerobic and anaerobic conditions. Propionate was used because *R. sphaeroides* shows the largest chemotactic response to propionate on swarm plates incubated under both aerobic and anaerobic conditions (our unpublished observations). The cell behaviour was plotted as rotation rate with time (e.g. Figure 2). Each point on the plot is the average of 100 consecutive data points and represents the average rotation rate over 2 s. Thus, if a cell stops often over this time period the perceived rotation rate will be low and if it hardly ever stops the perceived rotation rate will be higher. Each experiment was carried out at least in duplicate with a total of at least 10 cells observed for each strain over several fields of view. The graphs shown represent the averaged tracks for 2–8 cells per strain, from a typical field of view in the microscope. The results

obtained are summarized in Table II and the phenotypes are discussed in greater detail below.

The wild-type response

Wild-type cells had a random pattern of runs and stops in the pre-stimulus state. Upon addition of propionate, the stopping frequency was markedly reduced, measured as an increase in rotation rate. Upon removal of propionate all the cells came to an abrupt stop and then adapted over a period of 1–2 min (Figure 2A). We refer to this type of response as the 'normal' response. Anaerobically and aerobically grown cells gave identical responses (Table II).

In *E. coli*, membrane-bound sensors activate CheA autophosphorylation via CheW. CheA-P then phosphorylates CheY, which binds to the flagellar motor. Since *R. sphaeroides* lacks a CheZ homologue, we assumed that the 'CheY as phosphate-sink' model for signal termination from *S. meliloti* may also apply to *R. sphaeroides*; this is supported by the data (see later). Therefore, in *R. sphaeroides* the addition of attractant should result in an inactivation of CheA causing a decrease in CheY-P levels, with a phosphate sink pool of CheYs reducing motor-binding CheY-P concentration and decreasing stopping frequency. Removal of attractant would result in activation of CheA and a large increase in CheY-P levels resulting in a prolonged stop, and then adaptation resulting from CheA-dependent changes in CheB-P concentration. The response seen here with propionate is identical to that shown when 1 mM acetate or 1 mM fructose is used as attractant (Packer *et al.*, 1996). Why does the above response require the numerous chemotaxis gene homologues identified in *R. sphaeroides*? Which of them contribute to this response?

Responses of mutant strains under anaerobic conditions

Operon-deleted strains

che operon 1 and *che* operon 2 have distinct roles. To determine the roles of the two major chemotaxis operons, we examined the behavioural phenotypes of operon deletion strains. Removal of *che* operon 1 resulted in only minor effects on the phenotype (Hamblin *et al.*, 1997b). Strain JPA117, which has *che* operon 1 deleted, gave normal wild-type responses to the addition and removal of propionate (Table II). *che* operon 1 is therefore dispensable for the normal response to a step change in attractant.

Deletion of *cheA*₂ resulted in 'inverted' responses to attractants under anaerobic conditions (Hamblin *et al.*, 1997b). Interestingly, deletion of the entire *che* operon 2 (JPA403) also showed an inverted response under anaerobic conditions (Figure 2B). In this case, the cells stopped on addition of 1 mM propionate and restarted when it was removed. There was no adaptation in any of the strains lacking *che* operon 2, presumably because of the lack of *cheB*. Thus, there appear to be two distinct and antagonistic chemotaxis signal transduction pathways. In the absence of *che* operon 2 proteins, addition of propionate appears to result in an increase in CheY-P levels, which may occur via CheA₁ activation and involve one or more of CheY₁, CheY₂, CheY₄ and CheY₅, but not CheY₃ (since JPA403 lacks CheY₃). In wild-type cells, the inverted

Table II. Phenotypes of the mutant strains in the tethered cell assay using 1 mM propionate

Strain	Mutations	Anaerobic	Aerobic
WS8N	none	normal	normal
JPA117	Δ Op1	normal	normal
JPA403	Δ Op2	inverted	weak inverted
JPA404	Δ Op1, Δ Op2	smooth	smooth
JPA109	Y_1	normal	normal
JPA114	Y_2	normal	normal
JPA410	Y_3^*	normal	stopped/adapts to addition
JPA421	Y_4	no response	stopped/adapts to addition
JPA115	Y_1, Y_2	normal	normal
JPA412	Y_1, Y_3^*	normal	stopped/adapts to addition
JPA413	Y_2, Y_3^*	inverted/normal (see text)	stopped/adapts to addition
JPA414	Y_1, Y_2, Y_3^*	stopped	stopped
JPA422	Y_1, Y_4	normal (delayed recovery)	stopped/adapts to addition
JPA423	Y_2, Y_4	no response	stopped/adapts to addition
JPA425	Y_3^*, Y_4	inverted (delayed recovery)	weak normal
JPA424	Y_1, Y_2, Y_4	no response	stopped
JPA427	Y_1, Y_3^*, Y_4	no response	weak normal
JPA428	Y_2, Y_3^*, Y_4	weak inverted	weak normal
JPA429	Y_1, Y_2, Y_3^*, Y_4	inverted (delayed recovery)	weak normal
JPA415	Δ Op2, Y_1	inverted (delayed recovery)	no response
JPA416	Δ Op2, Y_2	inverted (delayed recovery)	no response
JPA417	Δ Op2, Y_1, Y_2	inverted (delayed recovery)	weak inverted
JPA411	Δ Op1, Y_3^*	stopped	stopped/adapts to addition
JPA418	Δ Op1, Y_4	normal	normal
JPA426	Δ Op1, Y_3^*, Y_4	normal	normal
JPA432	Δ Op2, Y_1, Y_2, Y_4	inverted (delayed recovery)	no response
JPA420	Δ Op1, Δ Op2, Y_4	smooth	smooth
JPA419	Δ Op2, Y_4	inverted (delayed recovery)	no response
JPA430	Δ Op2, Y_1, Y_4	inverted	weak inverted
JPA431	Δ Op2, Y_2, Y_4	inverted	no response

See the text and figures for details of the different phenotypes. In general, a normal response is a stop-on-removal of propionate whereas an inverted response is a stop-on-addition. Strains described as having no response are able to stop but fail to respond to the addition and removal of propionate. Smooth strains are those that stop very rarely or not at all and do not respond to propionate. Note that Y_1 refers to a mutation in *cheY₁*, Y_2 refers to a mutation in *cheY₂* and so on. Δ Op1, deletion of *che* operon 1; Δ Op2, deletion of *che* operon 2.

response to propionate is masked. What then is the role of the inverted response in chemotaxis? Photosynthetic wild-type tethered cells show inverted responses when switched from anaerobic to aerobic buffer (Gauden and Armitage, 1995). Oxygen is a strong repellent for photosynthetic cells. It is therefore possible that *che* operon 1 is involved in repellent responses. Indeed, in enteric bacteria repellent responses are characterized by increased tumbling on exposure to repellents (Tsang *et al.*, 1973).

Propionate is a strong attractant for *R.sphaeroides*, yet in the absence of *che* operon 2 it caused an apparent repellent response. Thus, propionate can elicit both an attractant (normal) response and a repellent (inverted) response, but under gradient conditions the attractant response must normally override any repellent signal. The expression data suggest that *che* operon 2 components are present at higher levels than *che* operon 1, under both aerobic and anaerobic conditions. This may explain how the *che* operon 2 pathway masks the *che* operon 1 pathway. The purpose of having an attractant and a repellent response to the same compound is unclear. However, when high concentrations (>5 mM) of attractant were used in the tethered cell assay, wild-type cells showed an inverted response (H.Packer, C.Wood and J.P.Armitage, unpublished observations). Therefore, it may be that the complex chemotaxis system of *R.sphaeroides* is designed to enable the cells to move towards concentrations that are optimal for growth, i.e. to nutrient concentrations that are neither too high nor too low, but allow maximal growth rates.

Strain JPA404, which is deleted for both *che* operon 1 and *che* operon 2, had a 'smooth' phenotype, i.e. the cells stopped very rarely and did not respond to the addition and removal of propionate (Figure 2D). This suggests that *che* operons 1 and 2 encode the essential components for the chemotactic response.

Role of CheYs in the inverted response in the absence of che operon 2. Under our experimental conditions, strains with *che* operon 2 deleted showed inverted responses. In the absence of *che* operon 2 and therefore CheB, cells could still respond but not adapt. This allowed the rates of signal termination in different strains to be determined and, therefore, the roles of CheY₁, CheY₂, CheY₄ and CheY₅ in the inverted response. *cheY₁*, *cheY₂* and *cheY₄* were deleted in different combinations in a *che* operon 2-deficient background (strains JPA415, JPA416, JPA417, JPA419, JPA430, JPA431 and JPA432, Table II). When grown under anaerobic conditions, all these strains showed an inverted response. Examples are shown in Figure 3A and B. The JPA432 (Δ Op2, *cheY₁*, *cheY₂*, *cheY₄*) result suggests that CheA₁ and CheY₅ are sufficient for the inverted response. What role, if any, do the other CheYs play? Some indication of this is given by looking at the time taken to respond to removal of attractant (Figure 3C). In the presence of CheY₁, CheY₂ and CheY₄ (JPA403) the response time was 1–1.5 min. When either *cheY₁* or *cheY₂* was deleted (JPA415 and JPA416, respectively), this increased to 2–2.5 min. When both *cheY₁* and *cheY₂* were

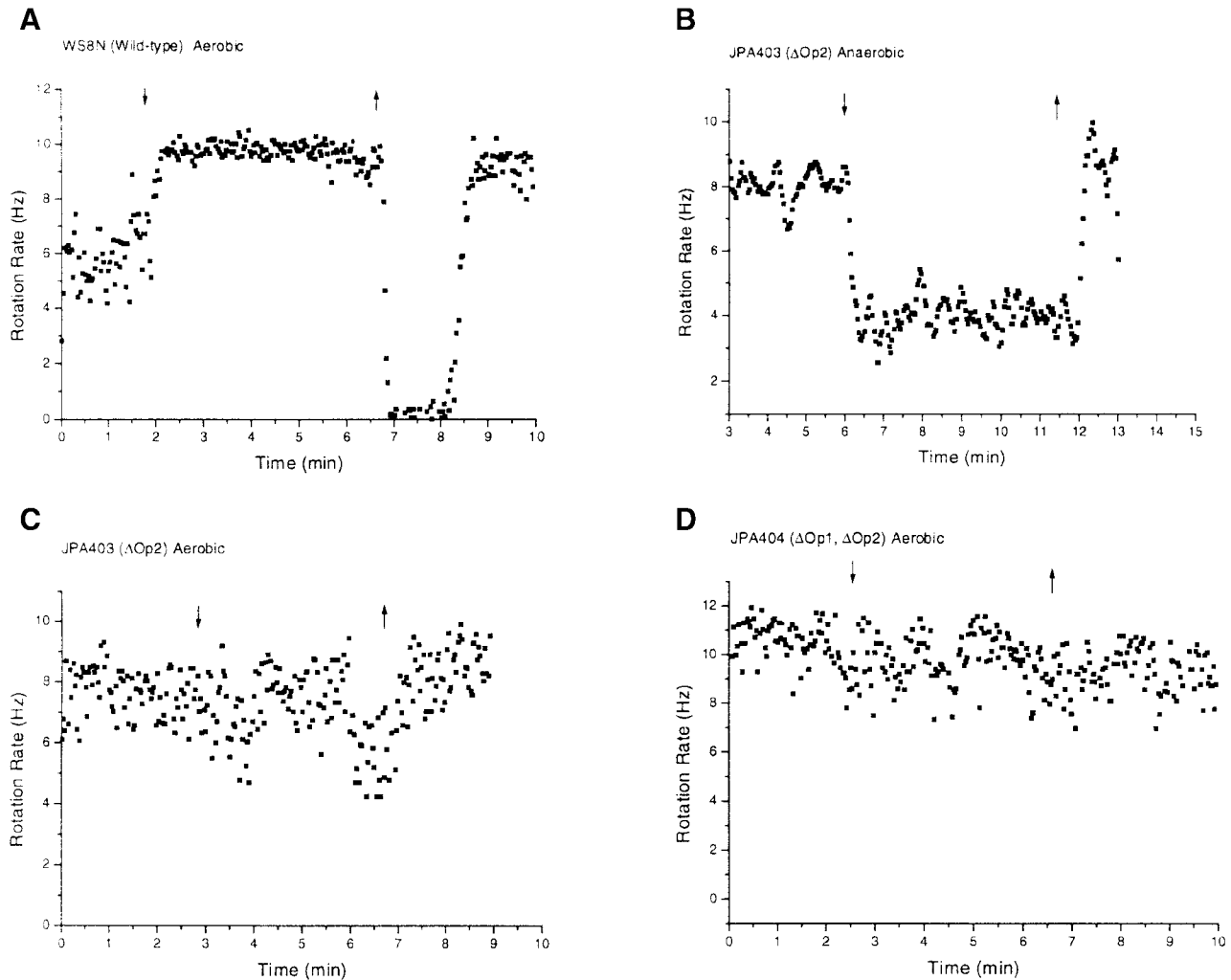


Fig. 2. Behaviour of tethered cells. The strains and conditions are indicated above each graph. Mutated operons/genes are indicated in parentheses. Δ Op1, deletion of *che* operon 1; Δ Op2, deletion of *che* operon 2. Down arrow, addition of 1 mM propionate; up arrow, removal of propionate. Other details in text.

deleted (JPA417), the recovery time was increased to 2.5–3 min. This suggests that under these conditions the level of motor-binding CheY remained high, presumably because the phosphate sinks were missing and signal termination had to rely on the inherent rate of CheY autodephosphorylation. This indicates that both CheY₁ and CheY₂ can act as phosphate sinks. If CheY₄ was also acting as a phosphate sink then deleting *cheY₄* in the *cheY₁* and/or *cheY₂* mutants should lead to a further increase in recovery time. In fact, there was a reduction in recovery time to 1.5–2 min. Thus, in the absence of CheY₄ the concentration of motor-binding CheY-P was reduced more quickly, indicating that CheY₄-P was part of a motor-binding CheY-P pool along with CheY₅-P. CheY₅ was probably the major contributor to this pool since deletion of CheY₄ had no discernible effect on the response (e.g. JPA419, Table II). It is possible that CheA₁ is more efficient at phosphorylating CheY₅ than CheY₄ or that the concentration of CheY₅ is greater in the cell. Future biochemical and expression studies should resolve these possibilities.

Role of CheYs in the normal response in the absence of che operon 1. The strain deleted for *che* operon 1 retains only

cheY₃, *cheY₄* and *cheY₅*. These three *cheY* genes together with the other *che* operon 2 components are sufficient for the normal response. To determine their roles we examined *che* operon 1-deleted strains carrying combinations of *cheY₃* and *cheY₄* mutations (JPA411, -418 and -426, Table II). Both JPA418 (Δ Op1, *cheY₄*) and JPA426 (Δ Op1, *cheY₃*^{*}, *cheY₄*) stop upon removal of propionate (Figure 4A and B). The JPA426 result demonstrates that CheY₅ and the *che* operon 2 components (excluding CheY₃) were sufficient for a response. We can conclude that CheA₂ phosphorylated CheY₅. However, the responses of both JPA418 and JPA426 were different to those of the wild type (Figure 2A). The stops after removal of propionate were much shorter than the wild type, and had a delay before the response (~60 s for JPA426 compared with <10 s for the wild type). Thus, in the absence of CheY₃ and CheY₄, the accumulation of enough CheY-P to stop the motor took longer. This, together with the much reduced stop length, suggests that CheY₃ and CheY₄ are part of the motor-binding pool of CheY. These observations also suggest that CheA₂ is less efficient than CheA₁ at phosphorylating CheY₅ since the stop-on-addition of the *che* operon 2, *cheY₁*, *cheY₂*, *cheY₄* mutant (JPA432, Figure 3B) was almost immediate. In strain

JPA418, which contained CheY₃ and CheY₅, the delay between removal and stopping was increased to ~90 s indicating that CheY₅-P levels took even longer to accumulate in the presence of CheY₃, demonstrating that CheY₃ was acting as an efficient phosphate sink. This was confirmed by the behaviour of JPA411 (Δ Op1, *cheY₃**). The cells were stopped in the pre-stimulus state but on addition of propionate they began to rotate, stopping again on removal (Figure 4E). We refer to this as a ‘stopped’ phenotype. We conclude that in the absence of CheY₁, CheY₂ and CheY₃, the cell lacked all the components that could act as phosphate sinks and, therefore, a basal level of CheA₂ activation in unstimulated cells could generate enough CheY₄-P and CheY₅-P to give the motor a very high stopping frequency. When CheY₅ alone was present, the cells did not have a stopped phenotype (Figure 4B), suggesting that CheY₄-P is necessary for the stopped phenotype of JPA411 (Figure 4E). This observation is consistent with the idea that CheA₂ phosphorylates CheY₄ more efficiently than it does CheY₅ (indeed strain JPA421, in which CheY₄ alone was deleted, had a non-responsive phenotype, see later). Therefore, CheA₁-CheY₅ and CheA₂-CheY₄ probably form functional kinase-effector couples.

Operon intact strains

From the phenotypes of *che* operon deletion strains it seems that the inverted response is mediated by CheA₁-CheY₅, with CheY₁ and CheY₂ serving as signal terminators. The normal response appears to be mediated by CheA₂-CheY₄ with CheY₃ acting as a signal terminator. This is the default response in wild-type cells and dominates over the inverted response under the conditions used. In considering operon-deleted strains we have simplified the interpretation of the data by eliminating ‘cross-talk’ between operon components.

Single or combinations of mutations of *cheY* genes in strains that have all the other components of the operons present were investigated. The phenotypes were not as clearly defined as those of the operon-deleted strains. Within any population of cells there were differences in the behaviour of individual cells, e.g. for strain JPA422 (*cheY₁*, *cheY₄*), out of 15 cells analysed 13 had a ‘normal’ response and two showed no response. In each case, we assigned the phenotype as the behaviour shown by the majority (>75%) of cells in the population. Differences in behaviour of individual cells observed in *E.coli* have been attributed to subtle differences in the levels of expression of the components of the chemotaxis transduction pathway between individual cells (Levin *et al.*, 1998). The greater number of genes in *R.sphaeroides* is likely to multiply such effects.

CheY₃ is the major phosphate sink but *CheY₁* and *CheY₂* can also act as phosphate sinks. When *cheY₁* and/or *cheY₂* were deleted (JPA109, JPA114, JPA115, Table II), the cells had responses to propionate that were almost identical to the wild type (graphs not shown). This is not surprising in the light of the result that JPA117 (Δ Op1) also has a normal response.

When *cheY₃* alone was mutated (JPA410), the cells showed a normal response (Table II; Figure 4C) although JPA411 (Δ Op1, *cheY₃**) had a stopped phenotype

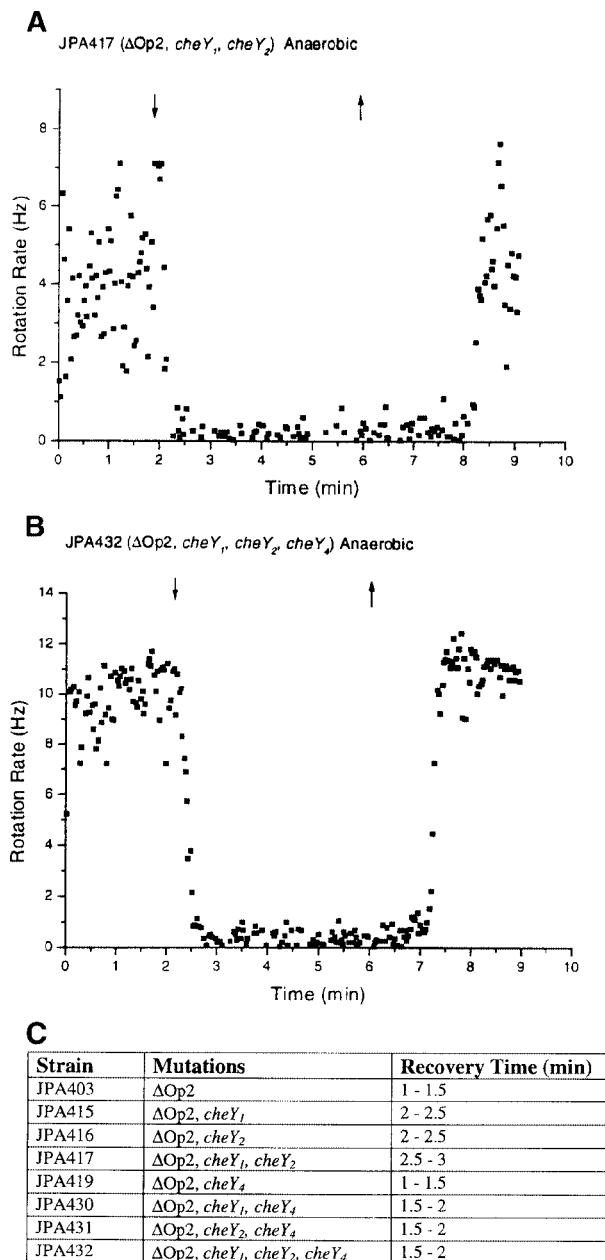


Fig. 3. (A and B) Behaviour of tethered cells. (C) Table showing recovery times after removal of propionate shown by strains with inverted responses. Note that there is no delay in response to the addition of propionate. Other details in text and legend to Figure 2.

(Figure 4E). The implication of these data is that when *che* operon 1 was present CheY₁ and CheY₂ compensated for the absence of CheY₃ and operated as a phosphate sink for the CheA₂-dependent pathway. This conclusion is supported by strain JPA414 (*cheY₁*, *cheY₂*, *cheY₃**), which had a stopped phenotype (Table II) while strain JPA412 (*cheY₁*, *cheY₃**) had a relatively normal phenotype (Table II). Strain JPA413 (*cheY₂*, *cheY₃**) showed an increased stopping frequency upon addition of propionate, similar to the inverted response, but adapted and showed a stop upon removal of propionate, similar to the normal response (Figure 5A). The CheA₁-CheY₅ pathway probably mediated the stop-on-addition, and the CheA₂-CheY₄ pathway mediated the stop-on-removal. Strains JPA410 (*cheY₃**) and JPA412 (*cheY₁*, *cheY₃**), in which CheY₂

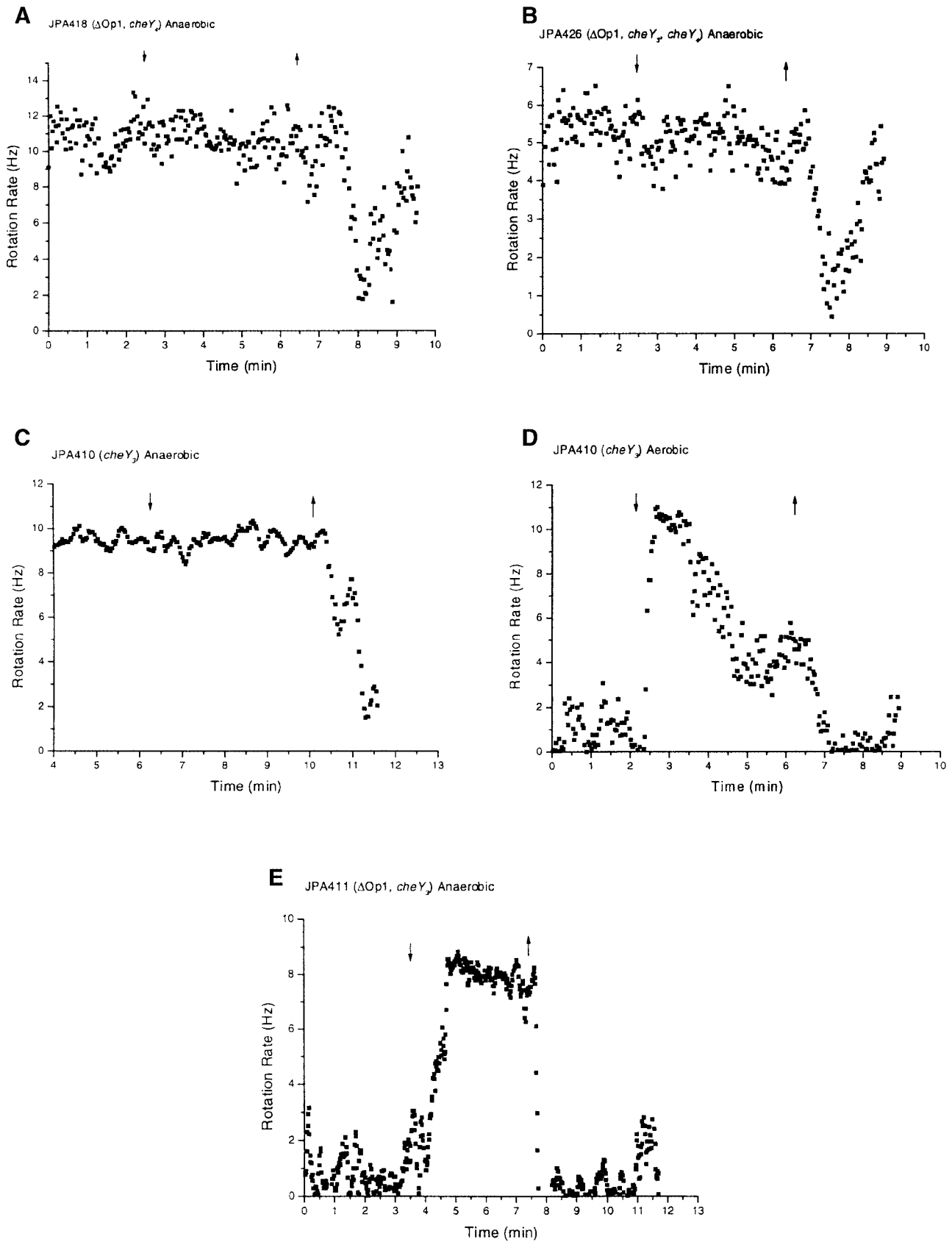


Fig. 4. Behaviour of tethered cells. See legend to Figure 2.

was present, did not show a stop-on-addition of propionate indicating that CheY₂ could prevent stop-on-addition seen in strain JPA413. Strain JPA428 (*cheY₂, cheY₃^{*}, cheY₄*), in

which only CheY₁ and CheY₅ were present, had a weak inverted response (Table II). These results indicate that in the absence of CheY₃, CheY₂ was the major phosphate

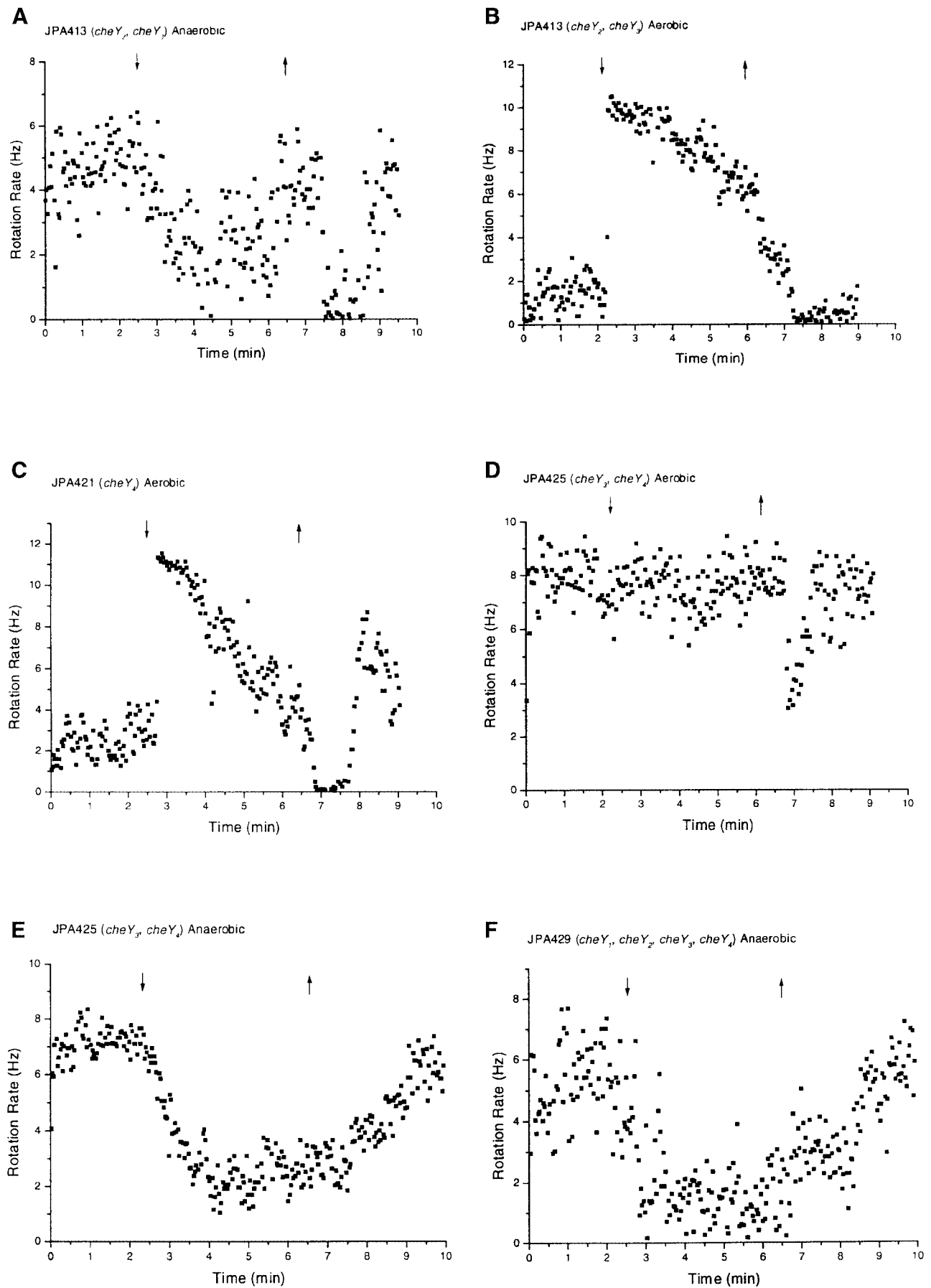


Fig. 5. Behaviour of tethered cells. See legend to Figure 2.

sink for the CheA₁–CheY₅ pathway and CheY₁ had only a supplementary role. Nevertheless, the data from the *che* operon 2-deleted strains suggest that this role of CheY₁ is important because its deletion caused longer recovery times.

More evidence that CheY₁ and CheY₂ have different roles as phosphate sinks is provided by an examination of strains JPA421 (*cheY₄*), JPA422 (*cheY₁*, *cheY₄*) and JPA423 (*cheY₂*, *cheY₄*). In all these strains CheY₃ was present, but the motor-binding pool of CheY was depleted by the deletion of CheY₄. JPA421 (*cheY₄*) had a non-responsive phenotype with some cells showing a brief slight increase in stopping frequency on addition, but the majority of cells not responding (Table II). This result is consistent with the idea that CheY₅ is the only motor-binding CheY in this strain, and that the three phosphate sinks did not allow the accumulation of enough CheY₅-P to cause the flagellum to stop in the majority of cells. However, strain JPA422 (*cheY₁*, *cheY₄*) had a normal response (Table II); therefore, the loss of CheY₁ allowed CheY₅-P levels to increase to active levels on the activation of CheA₂ by the removal of attractant, even though CheY₃ was present. This indicates that CheY₁ is a more efficient sink for the CheA₂-mediated responses than CheY₂ since JPA423 (*cheY₂*, *cheY₄*), which contained CheY₁, showed no responses (Table II). In the absence of CheY₄, CheY₁ appeared to act as a strong phosphate sink for the CheA₂–CheY₅-mediated stop-on-removal, even when CheY₃ was present. These data indicate a hierarchy of CheA₂-P affinities. CheY₁ had a higher affinity than CheY₂, but not as high as CheY₄ (and CheY₃). Therefore, under anaerobic conditions CheY₁ was an important supplement to CheY₃ in the pool of CheYs able to act as phosphate sinks.

These data suggest that CheY₁ and CheY₂ acted as phosphate sinks, with CheY₂ being a more efficient signal terminator for the CheA₁-mediated response, while CheY₁ was better for signal termination in the CheA₂-mediated response. However, these roles for CheY₁ and CheY₂ were only revealed in the absence of CheY₃ and, in some circumstances, CheY₄.

Inverted responses in the presence of CheA₂. All of the *che* operon 2-deficient strains had inverted responses under anaerobic conditions, whereas in the wild-type strain the inverted response was masked, possibly because *che* operon 2 was expressed at higher levels than *che* operon 1. It is possible that CheA₁ and CheA₂ compete for binding to chemoreceptors, with CheA₂ occupying more sites because of its higher concentration. Alternatively, the high levels of CheY₃, encoded on *che* operon 2, might prevent phosphorylation of CheY₅ by removing phosphate from CheA₁, thereby preventing the stop-on-addition. The phenotypes of the mutant strains allow us to distinguish between these possibilities.

If the dominance of the normal response were due to competition between CheA₁ and CheA₂ for binding sites on chemoreceptors, we would not expect to observe inverted responses in the presence of CheA₂ because of its higher expression under all conditions tested. However, strains JPA413 (*cheY₂*, *cheY₃**), JPA425 (*cheY₃**, *cheY₄*), JPA427 (*cheY₁*, *cheY₃**, *cheY₄*), JPA428 (*cheY₂*, *cheY₃**, *cheY₄*) and JPA429 (*cheY₁*, *cheY₂*, *cheY₃**, *cheY₄*), which

all contain *cheA₂*, showed the stop-on-addition typical of inverted responses (Table II and e.g. Figure 5A, E and F), implying that CheA₁ and CheA₂ do not compete for binding to chemoreceptors. Indeed, the loss of inverted responses under aerobic conditions (see below) suggests that there may be specific receptors for the inverted and normal responses.

A common feature of all these strains is that they lack *cheY₃*, highlighting its importance as a phosphate sink. However, in each case deletion of *cheY₂* and/or *cheY₄* was necessary to obtain the inverted phenotype. Thus, the dominance of *che* operon 2-mediated responses in the wild type appears to be due mainly to CheY₃, with CheY₂ and CheY₄ playing supplementary roles.

Strain JPA413 contains CheY₄ and showed both a sustained stop-on-removal and a stop-on-addition (Figure 5A), demonstrating that the CheA₁–CheY₅ and CheA₂–CheY₄ pathways can act independently in the same cell. Strains JPA425 and JPA429 both showed a more sustained stop-on-addition with a delayed recovery (Figure 5E and F), probably due to CheA₂ phosphorylation upon removal of propionate. Thus CheA₂, in the absence of CheY₃ and CheY₄, probably phosphorylated CheY₅ causing a delay in recovery. The importance of CheA₁ in the inverted response is highlighted by the fact that JPA426 (Δ Op1, *cheY₃*, *cheY₄*) did not show inverted responses (Figure 4B; compare with JPA425, Figure 5E).

Responses of mutant strains under aerobic conditions

The responses of strains grown and tethered in aerobic conditions to the addition and removal of propionate were tested. Many of the strains (including wild type) showed similar responses under both aerobic and anaerobic conditions (see Table II). However, there were some notable and interesting differences as described below.

Loss of inverted responses under aerobic conditions. Inverted responses were weak or lost altogether under aerobic conditions (Table II; e.g. compare Figure 2B and C). This result is most surprising for the *che* operon 2-deleted strains, JPA403, JPA415, JPA416, JPA417, JPA419, JPA430, JPA431 and JPA432, where there is no CheA₂ or CheY₃ to overcome the *che* operon 1-mediated inverted response (Table II). Therefore, under aerobic conditions CheA₁ was probably unable to generate enough CheY-P to stop the motor on addition of propionate. However, expression of *che* operon 1 was higher under aerobic than anaerobic conditions. The reduction in CheA₁-mediated phosphorylation could be the result of a reduction in chemoreceptors to activate CheA₁, or of a severe reduction in CheY₅ expression under aerobic conditions. However, the data for strain JPA426 (Δ Op1, *cheY₃**, *cheY₄*), which gave a CheA₂–CheY₅-mediated stop-on-removal under aerobic conditions (Table II; graph not shown), suggest that CheY₅ expression under aerobic conditions was sufficient to allow a response. The reduced level of CheA₁ activation by chemoreceptors under aerobic conditions suggests that there may be CheA₁-specific chemoreceptors whose expression is much lower under aerobic conditions, thus abolishing inverted responses.

CheY₃ is the major phosphate sink under aerobic conditions. Under aerobic conditions the behaviour of JPA410 (*cheY₃**), JPA412 (*cheY₁*, *cheY₃**) and JPA413 (*cheY₂*, *cheY₃**) was markedly different to that under anaerobic conditions. All three strains had a stopped phenotype under aerobic conditions (Table II; e.g. compare Figure 4C with D and 5A with B), indicating that *CheY₃* was essential for aerobic signal termination and that *CheY₁* and *CheY₂* are unable to complement the function. The relative level of *che* operon 2 components was much higher under aerobic conditions; therefore, *CheA₂* was probably able to phosphorylate *CheY₄* (and to a lesser extent *CheY₅*) at a rate which could not be compensated by the phosphate sink kinetics of the much lower levels of *CheY₁* and *CheY₂*. Since *CheY₃* is located in *che* operon 2, its expression was similar to that of *CheA₂*; therefore, under normal circumstances there must always be enough *CheY₃* to cope with *CheA₂*-mediated phosphorylation of motor-binding *CheY*s. Thus, *CheY₃* is the major phosphate sink for the *CheA₂*–*CheY₄* pathway and cannot be replaced by *CheY₁* and *CheY₂* under aerobic conditions.

CheY₃ is, however, a motor-binding *CheY* in the absence of *CheY₄*. Deletion of *cheY₄* in operon intact strains gave unexpected phenotypes under aerobic conditions. Strain JPA421 (*cheY₄*) had a stopped phenotype under aerobic conditions (Figure 5C). Strains JPA422 (*cheY₁*, *cheY₄*), JPA423 (*cheY₂*, *cheY₄*) and JPA424 (*cheY₁*, *cheY₂*, *cheY₄*) also had stopped phenotypes (Table II). Since the only known motor-binding *CheY* present in these strains is *CheY₅*, we could assume that *CheY₅*-P is responsible for the high stopping bias. However, strain JPA425 (*cheY₃**, *cheY₄*) did not have a stopped phenotype (Table II; Figure 5D). Therefore, the stopped phenotype of JPA421, JPA422 and JPA423 must be due to *CheY₃*-P binding to the motor in the absence of *CheY₄*. These data imply that *CheY₄* can operate as a phosphate sink and *CheY₃* as a motor-binding *CheY*, but all the other evidence suggested a motor-binding role for *CheY₄* and a phosphate sink role for *CheY₃*. This apparent conflict can be resolved by considering the model of Sourjik and Schmitt (1998). According to their model, a phosphate sink *CheY* must have a lower affinity for the motor than a motor-binding *CheY*-P. In the case of *R.sphaeroides* we assume that *CheY₃*-P has a lower affinity for the motor than *CheY₄*-P; therefore, the amount of *CheY₃*-P required to stop the motor would be much higher than the amount of *CheY₄*-P required. In the absence of *CheY₄*, *CheY₃*-P can build up to higher levels and stop the motor, as observed. *Sinorhizobium meliloti* *CheY₁*, which is the phosphate sink, can also cause the motor to stop in the absence of *CheY₂*, the motor-binding *CheY* (Sourjik and Schmitt, 1996).

A model for signal transduction in *R.sphaeroides* chemotaxis

Rhodobacter sphaeroides has two distinct chemotactic responses: a normal response exhibited by wild-type cells when challenged with a step change in the concentration of chemoattractants, and an inverted response exhibited by wild-type cells to high concentrations of certain chemoattractants (Packer and Armitage, 1996, 2000). The inverted response is also shown by anaerobic strains deficient in *che*

operon 2 components, when challenged with effector concentrations that would elicit a normal response in wild-type cells.

The normal response to an attractant results in reduced stopping when swimming up a gradient of attractant, enabling the cells to move into favourable microenvironments. The inverted response appears to be the equivalent of a 'repellent' response causing the cells to stop more frequently when swimming up a gradient of 'repellent', enabling cells to avoid unfavourable microenvironments. No 'true' repellents, i.e. chemicals that elicit only the inverted response, have been identified in *R.sphaeroides* (with the exception of oxygen in photosynthetic cells); therefore, a 'repellent' is defined as a high concentration of a compound that is normally an attractant at concentrations below a certain threshold. This threshold lies between 1 and 5 mM for propionate (H.L.Packer and J.P.Armitage, submitted).

The mutational analysis of behavioural responses presented here enables us to formulate a model for the molecular interactions that take place during these chemotactic responses. In the pre-stimulus state the cells have a random pattern of runs and stops mediated by a basal level of phosphorylation of motor-binding *CheY*s. Mutational analysis suggests that only *CheY₄*-P and *CheY₅*-P are able to bind to the motor under normal circumstances, and that *CheA₂* predominantly phosphorylates *CheY₄* while *CheA₁* phosphorylates *CheY₅*. *che* operon 2 components are present in greater amounts than *che* operon 1 components; therefore, most of the pre-stimulus stopping is probably caused by *CheA₂*–*CheY₄* phosphorylation. The other *CheY*s act as signal terminators, possibly using a phosphate sink mechanism. We predict that on addition of attractant, *CheA₂* activity is inhibited, the levels of *CheY₄*-P are rapidly reduced by the action of the phosphate sinks (*CheY₃*, *CheY₂* and *CheY₁*) and the motor stopping frequency is therefore reduced. At the same time, under anaerobic conditions, *CheA₁* shows low-level activity (as seen in *che* operon 2-deleted strains) but the action of the phosphate sinks prevents the accumulation of enough *CheY₅*-P to bind the motor. Therefore, the cells maintain a high swim bias. When the attractant is removed there is an increase in *CheA₂* activity and a consequent increase in *CheY₄*-P, causing a sustained motor stop. Under anaerobic conditions, high concentrations of propionate (or deletion of *che* operon 2) cause a stop-on-addition (inverted response), probably as a result of increased *CheA₁* activity and a consequent increase in *CheY₅*-P to levels high enough to overcome the activity of the phosphate sinks, causing a motor stop.

The observed behaviour of *R.sphaeroides* mutants strongly suggests that, in addition to the two chemotaxis operons, there are two pools of chemoreceptors: a *CheA₂*-specific pool that activates *CheA₂* upon a step-down in attractant levels and a *CheA₁*-specific pool that activates *CheA₁* upon a step-up in attractant concentration. *Rhodobacter sphaeroides* has both membrane-spanning and cytoplasmic chemoreceptors, whose expression levels are known to change under different growth conditions (Harrison *et al.*, 1999). Subsets of these may provide the *CheA₁*- and *CheA₂*-specific receptors. How can an attractant compound cause a repellent response at higher concentrations? This would be possible if the *CheA₁*-

specific chemoreceptors had a lower affinity for the compound or its binding protein than the CheA₂-specific chemoreceptor (or if one type of membrane-bound chemoreceptor senses the compound directly while the cytoplasmic chemoreceptor senses subsequent metabolites and, therefore, the metabolic or redox state of the cell). Thus, when a cell is swimming up a gradient, the CheA₂-specific chemoreceptor will bind the ligand first. The bound chemoreceptors will be inactive, resulting in a swim bias. When the cells move into attractant concentrations that can act as repellents (or the concentration of cytoplasmic metabolites increases above a certain threshold), the CheA₁-specific chemoreceptors are bound and cause an activation of CheA₁-CheY₅ phosphorylation, resulting in a high stop bias. In this way the cell could maintain itself in an ideal environment where nutrient concentrations are neither too low nor too high.

Conclusions

The data presented in this paper suggest a preliminary model for the molecular interactions involved in the *R.sphaeroides* chemotactic response allowing cells to maintain themselves in optimum microenvironments, which may be different under different growth conditions, by using antagonistic responses through two distinct chemosensory pathways expressed at different levels. Alternative models have been considered, but this is the simplest that fits all the current observations. It is interesting to note that in a computer simulation Bray and Lay (1994) showed that a pathway composed of a network of mutable receptors trained to respond maximally to a certain concentration of ligand 'evolved' two forms of receptor: a high affinity form with an excitatory output and a low affinity form with an inhibitory output. This fits well with the model developed here based on experimental data. Interestingly, a recent study on *Pseudomonas aeruginosa* phosphate sensing has revealed the presence of two chemotactic transducers, one of which is essential for responses to high concentrations of phosphate and the other is essential for responses to low phosphate concentrations (Wu *et al.*, 2000). The genome sequence of *P.aeruginosa* reveals that it also has multiple homologues of chemotaxis proteins (see <http://pseudomonas.bit.uq.edu.au>).

There are many specific predictions from the *R.sphaeroides* model; for example, the model predicts two types of receptors with either different ligand affinities or responses to cytoplasmic metabolites. The high affinity receptors are predicted to act via CheA₂ and thus CheY₄, and the low affinity or metabolite receptors are predicted to act via CheA₁ and CheY₅, the latter being at reduced levels under aerobic conditions. Having identified both membrane-spanning and cytoplasmic receptors, we are identifying their role in chemotaxis and their levels of expression under different conditions. This, together with protein-protein interaction assays using purified receptors and CheAs, should provide data to support or disprove the model. The data support the hypothesis that bacteria lacking CheZ, but with multiple CheYs, use CheYs for signal termination. We are carrying out phosphotransfer assays using purified proteins to test this.

Our model is built around known genes and it is possible that yet more chemotaxis genes may be discovered. However, the smooth and stopped phenotypes suggest that we have identified the majority of genes important for chemotaxis. Only the responses to 1 mM propionate using aerobically or photosynthetically grown cells were tested. However, this model is likely to be applicable to a wider range of compounds and growth conditions since wild-type cells show similar responses to many other compounds (Packer and Armitage, 1996). We have not considered the roles of the CheW proteins or the adaptation response in relation to this model. We are able to make only limited speculation about stimulus sensing and the very important role of sensory transducers in chemotaxis. Current work using genetic, biochemical and structural approaches to study all aspects of the chemotaxis system of *R.sphaeroides* should lead to a more detailed and complete model. The model presented in this paper will be subject to many refinements and modifications. Nevertheless, it is an important step in the elucidation of the mechanisms in complex chemotaxis systems that are increasingly being discovered in the bacterial kingdom.

Materials and methods

Bacterial strains and media

The *R.sphaeroides* strains used in this study are listed in Table II. *Escherichia coli* strain DH5 α (Woodcock *et al.*, 1989) was used for cloning procedures. *Escherichia coli* strains were grown in Luria-Bertani medium at 37°C with shaking. Bacto agar was added to 1.5% to solidify media. Ampicillin, kanamycin and streptomycin were added at 100, 50 and 25 μ g/ml, respectively, where necessary. *Rhodobacter sphaeroides* strains were grown in succinate (sux) medium (Sistrom, 1960) at 30°C aerobically with shaking, or anaerobically in the light in airtight bottles. Kanamycin, nalidixic acid and tetracycline were added at 50, 25 and 1 μ g/ml, respectively, where necessary.

Molecular genetic techniques

All standard cloning steps were carried out as described in Sambrook *et al.* (1989). *Escherichia coli* cells were transformed with plasmid DNA according to the protocol of Hanahan (1983). Restriction and modification enzymes were obtained from NEB or Boehringer Mannheim and used according to the manufacturer's instructions. Recombinant plasmids were introduced into *R.sphaeroides* by conjugation with the *E.coli* donor strain S17-1 as described (Moore and Kaplan, 1989). Plasmid DNA was extracted using the alkaline lysis method (Birnboim and Doly, 1979). Large-scale plasmid extractions for DNA sequencing were carried out using the Plasmid Midi-Kit from Qiagen. DNA sequencing was carried out by the Automated Sequencing Service (Department of Biochemistry, University of Oxford) using ThermoSequenaseTM (Amersham) dye terminators on an ABI377 sequencer. DNA sequence was assembled and analysed using the Clone Manager 5 program (Scientific and Educational Software) and the GCG package (Devereux *et al.*, 1984). PCR reactions were carried out using *Pfu* DNA polymerase (Lundberg *et al.*, 1991; Stratagene) according to the manufacturer's instructions with the following modifications: between 0.1 and 0.01 μ g of template DNA were used; 100 pmol of each primer were added per 50 μ l reaction; and deoxynucleotides were added to a final concentration of 0.25 mM each. Thermocycling was carried out in a Thermal Mini-Cycler (MJ Research). The cycling conditions were as follows: step 1, 98°C for 5 min; step 2.1, 98°C for 2 min; step 2.2, 55°C for 1.5 min; step 2.3, 72°C for 3 min (25 cycles); step 3, 72°C for 5 min. Before cloning, PCR products were electrophoresed and purified from the agarose gel using the GeneCleanIIITM kit (Bio101). All PCR primers were synthesized by Genosys Biotechnologies Inc. *Rhodobacter sphaeroides* genomic DNA was extracted using the method of Giuliano *et al.* (1988). Southern blotting of DNA onto nylon membranes was carried out as described (Sambrook *et al.*, 1989) with the following modifications: probes for Southern blotting were labelled using the DIG DNA labelling kit

Table III. Primer sequences

Name	Sequence
PUCFOR	GTTTTCCAGTCACGAC
PUCREV	CAGGAAACAGCTATGAC
ΔCHEY1PR9	CCGGCC <u>CCCGGG</u> AGCGGATGAAGCGAAGCTGGTTTCCG
ΔCHEY1PR10	CGCTCCCGGGGCCGGAAGAACGGTCAGCGGCATGATC
ΔCHEY2PR7	CCGGCC <u>CCCGGG</u> AGCGCAAGCTGTCTAGGCGCTCTATGA
ΔCHEY2PR8	CGCTCCCGGGGCCGATGTCATCGACCACCATGATCC
CHEY4DNF	GCGC <u>GAA</u> TTCCGGCCCCGCCGCCCGCTC
CHEY4DNR	GCGCGGATCCGGCCTTGCCCTCCGCCGATC
CHEY4DCF	GCGCGGATCCCTGACCTGGCTTTCGACCAG
MCPAR	GCGC <u>AAGCTT</u> CACGCGACGCGCGCTCGC

Restriction sites incorporated in the primers are underlined.

(Boehringer Mannheim); all of the Southern blots were carried out using high stringency washes ($0.1 \times$ SSC/0.1% SDS at 65°C); and hybridized probe was detected using anti-DIG alkaline phosphate antibody conjugate (Boehringer Mannheim).

Determination of expression levels

Translational fusions of the putative promoter regions of *che* operon 1 and *che* operon 2 with a promoterless *lacZ* gene were constructed in the broad host range vector pUI523A (Tai *et al.*, 1988). The copy number of this tetracycline^R RSF1010 replicon is 4–6 in *R.sphaeroides* and does not vary with the growth condition. A 1.4 kb fragment covering the 3' end of *mcpA*, the putative promoter region and the 5' end of *cheD* was used to give the *che* operon 1 promoter fusion, such that *lacZ* would be translated as a fusion with *cheD*. A 1.5 kb fragment containing the 5' end of *cheY*₃ and upstream regions was used to give the *che* operon 2 fusion, such that *lacZ* would be translated as a fusion with *cheY*₃. The test plasmids and negative controls (promoter region inserted in the reverse orientation and no insert) were introduced into *R.sphaeroides* WS8N by conjugation. Cells were grown aerobically or photosynthetically and cultures sampled at an OD₇₀₀ of 0.1. β-galactosidase activity was determined using the Promega assay system according to the manufacturer's instructions.

Construction of mutant genes/operons

The deletion of *che* operon 1 has been described previously (Hamblin *et al.*, 1997b). Other mutations were constructed as detailed below.

che operon 2. Plasmid pJPA106 contains the 5' end of *che* operon 2 including *orf10*, *cheY*₃ and the 5' end of *cheA*₂, cloned into pUC18. pJPA132 contains the 3' end of *che* operon 2 including the 3' end of *tlpC* cloned into pUC18. The *Pst*I–*Bgl*II fragment from pJPA132 was cloned into *Pst*I- and *Bgl*II-cut pJPA106 to give pSLP2. Thus, regions upstream and downstream of *che* operon 2 were brought together and all of *che* operon 2, except for the 5' end of *orf10* and the 3' end of *tlpC*, was deleted. The *Eco*RI–*Hind*III fragment from pSLP2 was cloned into the suicide vector pK18*mobsacB* to give pKSLP3.

*cheY*₁. PCR was used to generate fragments on either side of *cheY1* using pCHB1.1 as template for both reactions. The sequences of the primers used are given in Table III. The upstream fragment was generated by PCR using primers PUCREV and ΔCHEY1PR10. This fragment had a *Bam*HI site (from the polylinker) at its 5' end and an *Xma*I site (incorporated from the primer) at the 3' end. The downstream fragment was generated using primers PUCFOR and ΔCHEY1PR9. This fragment had an *Xma*I site (incorporated from the primer) at its 5' end and an *Eco*RI site from the polylinker at the 3' end. The two PCR fragments were cloned together into pUC19 to give pAUL33, which resulted in a clone with a large deletion within *cheY*₁. The predicted peptide coded by the remaining *cheY*₁ sequence is MPLTVLFDEAKLVLSALRRVAVA. This lacks the phosphorylation site and most of the other regions important for function. The *Bam*HI–*Eco*RI fragment of pAUL33 was cloned into pK18*mobsacB* to give pAUL34.

*cheY*₂. This was deleted in a similar way to *cheY*₁. Again, the flanking regions were PCR-amplified using PUCREV, PUCFOR and two *cheY*₂-specific primers (ΔCHEY2PR8 and ΔCHEY2PR9) using pAUL38 as a template. The PCR fragments were cloned into pUC19 to give pAUL39. The predicted peptide coded by the remaining *cheY*₂ sequence is

MSTRGIQAVVVGAL, which lacks the residues important for function. The *Bam*HI–*Eco*RI fragment from pAUL39 was cloned into pK18*mobsacB* giving the plasmid pAUL40.

*cheY*₃. The *cheY*₃ gene was insertionally inactivated by cloning a *Mlu*I linker (5'-GACGCGTC-3') into the *Eco*RV site near the beginning of the *cheY*₃ ORF on pBM15 to give plasmid pDS2. This insertion introduced a stop codon at the site of insertion with a new *Mlu*I restriction site. The CheY₃ protein expressed from this construct was expected to be a truncated peptide of 26 N-terminal amino acids (named CheY₃*), which lacks the phosphorylation site and other functional domains. It was possible that the insertion of foreign DNA to create a stop codon in the middle of the *cheY*₃ ORF would have polar effects on downstream genes, i.e. *cheA*₂, *cheW*₂, etc. However, the phenotypes of *cheY*₃* strains suggest that this is not the case. The *Eco*RI fragment containing *cheY*₃* from pDS2 was cloned into pK18*mobsacB* to give plasmid pDS4.

*cheY*₄. The *cheY*₄ gene was deleted using PCR amplification from c301. The upstream flanking region was amplified using primers CHEY4DNF and CHEY4DNR to give a PCR fragment with an *Eco*RI site at the 5' end and a *Bam*HI site at the 3' end, both incorporated in the primer sequence. The downstream region was purified using primers CHEY4DCF and MCPAR, giving a PCR fragment with a primer incorporated *Bam*HI site at the 5' end and a *Hind*III site at the 3' end. Since the downstream region has an internal *Bam*HI site, a series of ligation steps had to be carried out to construct the deleted *cheY*₄ plasmid clone. The upstream PCR product was cloned into pUC19 first, and the *Bam*HI (internal)–*Hind*III fragment of the downstream region was cloned into this plasmid. Then the small *Bam*HI–*Bam*HI fragment of the downstream region was cloned into the *Bam*HI site of the previous construct. The isolate with the correct order and orientation of fragments was identified by restriction mapping and sequencing, and named pDS8. This construct had no remaining *cheY*₄ sequence. The *Eco*RI–*Hind*III fragment from pDS8 was cloned into pK18*mobsacB* to give pDS9.

The mutations were transferred into the chromosome by allelic exchange using the pK18*mobsacB* system as described previously (Schafer *et al.*, 1994; Hamblin *et al.*, 1997b).

Tethered cell analysis

Aerobic conditions. *sux* medium (100 ml in a 1 l flask) was inoculated with cells from a frozen stock and grown with vigorous shaking until the culture was at an OD₇₀₀ of between 0.4 and 0.6 when the cells were most motile. One millilitre of culture was harvested and resuspended in sterile, aerated (by vigorous shaking) tethering buffer (10 mM Na–HEPES pH 7.2 containing chloramphenicol at 50 μg/ml). After a 10 min incubation, the cells were tethered in a humidity chamber by incubation of 10 μl of cell suspension on a coverslip with 0.5 μl of anti-flagellar antibody for 10 min. The coverslip was loaded onto a flow chamber and tethering buffer flowed through for 5 min to remove free cells. The tethered cells were observed under phase contrast at 1000× magnification. Real time recordings were made on videotape. Buffers were flowed through the chamber at a rate of 0.09 ml/min. The cells were recorded in tethering buffer for 2 min after which 1 mM propionate (sodium salt) in tethering buffer was flowed through the chamber for 4 min. The propionate was removed by flowing tethering buffer through the chamber.

Anaerobic conditions. sux medium (100 ml in an airtight medical flat bottle) was inoculated with cells from a frozen stock. The bottle was incubated, with illumination at a light intensity of 50 $\mu\text{M}/\text{m}^2/\text{s}$, until the OD₇₀₀ reached 0.8–1.0. The tethering protocol was essentially the same as that under aerobic conditions. However, anaerobic (nitrogen-sparged) tethering buffer was used throughout. The propionate solution was also made using anaerobic tethering buffer.

The video recordings were analysed with the Hobson Bactracker (Hobson Tracking Systems) using the program Arot7. The rotation rate of the cells was measured by detecting the position of the cell every 50 ms for ~9 min. The data obtained were smoothed (100 points), averaged (for as many cells as available) and plotted.

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