

In Vivo and In Vitro Characterization of *Escherichia coli* Protein CheZ Gain- and Loss-of-Function Mutants

M. GERMANA SANNA[†] AND MELVIN I. SIMON^{*}

Division of Biology, California Institute of Technology, Pasadena, California 91125

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Bacterial chemotaxis results from the ability of flagellated bacteria to control the frequency of switching between smooth-swimming and tumbling episodes in response to changes in concentration of extracellular substances. High levels of phosphorylated CheY protein are the intracellular signal for inducing the tumbling mode of swimming. The CheZ protein has been shown to control the level of phosphorylated CheY by regulating its rate of dephosphorylation. To identify functional domains in the CheZ protein, we made mutants by random mutagenesis of the *cheZ* gene and constructed a series of deletions. The map position and the in vivo and in vitro activity of the resulting gain- or loss-of-function mutant proteins define separate functional domains of the CheZ protein.

Peritrichously flagellated bacteria are able to move towards high concentrations of attractant substances by alternating the rotational direction of their flagella (for reviews, see references 1, 6, 16, and 22 to 24). Counterclockwise rotation results in unidirectional swimming (smooth swimming), whereas changes in the swimming direction (tumbling) are achieved by intermittent clockwise rotation of the flagella. Cells swimming toward increasing concentrations of attractant tend to tumble less frequently, thereby biasing their direction of movement towards regions of higher attractant concentration (3, 21). The signal transduction that ultimately controls the frequency of switches in flagellar rotation is mediated by several transmembrane receptors and by a series of cytoplasmic proteins. In *Escherichia coli* and *Salmonella typhimurium*, the external message is transmitted through transmembrane receptors to a cytoplasmic autophosphorylating histidine kinase, CheA, which transfers its phosphoryl group to the response regulator CheY (5, 9, 12, 26). CheY in its phosphorylated form (phospho-CheY) binds to the flagellar switch (2, 17, 25) and causes clockwise rotation of the flagella, resulting in a tumbling event (7, 15). Thus, high intracellular concentrations of phospho-CheY correlate with the tumbling motion of the cell. Smooth swimming is restored by dephosphorylation of CheY, which may be due to both the spontaneous hydrolysis of the phosphate group and the activity of the CheZ protein, which strongly accelerates the dephosphorylation of phospho-CheY (9, 11). It has also been established that phospho-CheY binds to the CheZ protein and that, following dephosphorylation, CheY is released from CheZ (4, 18).

CheZ is a ~24-kDa protein characterized in *E. coli* and *S. typhimurium* and does not seem to have significant homology with other bacterial proteins. Recently, a CheZ homolog has been described in *Pseudomonas aeruginosa* (13). It is able to complement an *E. coli cheZ* deletion strain. Proteins with analogous functions have been identified in other organisms. For example, Spo0E in *Bacillus subtilis* accelerates the dephosphor-

ylation of the response regulator Spo0A (14); however, this phosphatase is not homologous to CheZ.

The molecular mechanism through which CheZ acts on CheY has not been determined. It is not clear whether CheZ acts as a phosphatase, catalytically hydrolyzing the phosphate ester, or as an allosteric effector, changing the conformation of the CheY protein and therefore accelerating CheY autodephosphorylation (19). Thus, while CheZ plays a pivotal role in certain bacterial chemotaxis systems, much less is known about its structure and function than is known about the other proteins that are required for chemotaxis.

In this work, we characterized the in vivo and in vitro functions of a variety of CheZ mutants. We isolated several gain- or loss-of-function CheZ mutants whose mutations defined distinct domains of the CheZ protein.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial cultures were routinely grown in Luria broth (LB) at 37°C or in T broth (tryptone broth) at 30°C for the observation of the swimming behavior. Ampicillin, kanamycin, or tetracycline was added when needed to final concentrations of 100, 25, and 10 µg/ml, respectively.

Plasmid pMGS2 (pBAC [20] carrying the *cheZ* wild-type gene and the IPTG [isopropyl-β-D-thiogalactopyranoside]-inducible promoter from plasmid pQE12 [Qiagen]) was obtained by introducing via PCR the *Hind*III and *Bam*HI sites at the 5' and 3' ends of the *cheZ* sequence, respectively. PCR products and the pBAC vector were then double digested with *Hind*III and *Bam*HI restriction enzymes, and ligation was performed by standard procedures. Construction of pMGS1 (*cheZ*/pQE12 wild-type recombinant vector) has already been described (19). The strains used in this work were the following: KO642 *recA* (Δ *cheZ*) (8), MGS55 (KO642 *recA*/pMGS1/pREP4) (19), MGS7 (KO642 *recA*/pREP4) (this study), MGS12 (KO642 *recA*/pQE12/pREP4) (this study), MGS64 (KO642 *recA*/pMGS2/pREP4) (this study), the library of mutants (KO642 *recA*/pMGS1*/pREP4) (this study), and the *cheZ*-deleted strains (KO642 *recA*/pMGS1 Δ /pREP4) (this study). Mutant proteins are named with the residue number followed by the single-letter abbreviations for the wild-type amino acid and the mutant amino acid, in that order (e.g., CheZ117FS is the CheZ mutant with an F-to-S mutation at residue 117).

Construction of the *cheZ* mutant library and in vivo assays. The *cheZ* library of mutants was obtained by PCR-mediated mutagenesis of the *cheZ* gene as described previously (18). The mutated PCR products were cloned into the vector pQE12, producing recombinant plasmid pMGS1*, and transformed into a Δ *cheZ* background (KO642 *recA* containing the pREP4 repressor plasmid which allows modulation of the expression of pQE12 in strain MGS7). Plasmid DNA was prepared from selected strains, and the corresponding mutated *cheZ* genes were sequenced with an Applied Biosystems model 373A sequencer. The promoter of each clone of pQE12 was also sequenced to ensure that any unwanted mutations were not introduced during the cloning process.

Miniswarm, swarm, and swimming assays were performed as described previously (18, 19). Briefly, for the swarming assays, colonies were stabbed on motility

* Corresponding author. Mailing address: Division of Biology 147-75, California Institute of Technology, Pasadena, CA 91125. Phone: (818) 395-3944. Fax: (818) 796-7066.

[†] Present address: Department of Immunology, The Scripps Research Institute, La Jolla, CA 92037.

plates (1% tryptone, 0.5% NaCl, 0.3% agar) and incubated overnight at 25°C. The diameter of the swarming colonies was measured after 17 h.

Purification of CheZ and in vitro assays. CheY, wild-type CheZ, and CheZ mutant proteins were purified as described previously (10, 19). Intracellular levels of CheZ were measured for each mutant before and after induction of fresh cultures with 1 mM IPTG for 3 h. Samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and gels were stained with Coomassie blue.

In vitro dephosphorylation assays of phospho-CheY were performed as described by Sanna et al. (19).

Construction of $\Delta cheZ$ mutant strains. Deletions of the *cheZ* gene were obtained by PCR with different sets of priming oligonucleotides. PCR products and the pQE12 vector were double digested with *EcoRI* and *BamHI* restriction enzymes and ligated by standard procedures. The truncated *cheZ* genes as well as the vector promoter were sequenced (Applied Biosystems model 373A sequencer) to eliminate the possibility that unwanted mutations had been introduced by PCR. Recombinant plasmids were transformed into strain KO642 *recA* ($\Delta cheZ$).

RESULTS

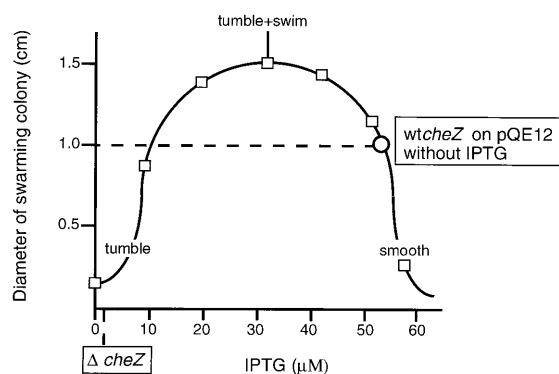
Strategy for the isolation of gain- and loss-of-function CheZ mutants. Mutations in the *cheZ* gene that cause smooth-swimming behavior are expected to enhance the activity of the CheZ protein, while mutations that cause tumbling swimming behavior should result from loss of CheZ function. Random mutagenesis of *cheZ* by PCR (18) was used to generate a library of *cheZ* mutants which was screened by use of a mini-swarm assay (18) to isolate strains with altered swarming ability.

The following strategy was adopted for identifying the strains demonstrating smooth-swimming and tumbling behavior on motility plates. At first, we determined the swarming pattern of strain MGS64 (see Material and Methods), which has a *cheZ* deletion and carries the wild-type *cheZ* gene on a single-copy pBAC plasmid (pMGS2) under an IPTG-inducible promoter. MGS64 was grown in the presence of increasing amounts of IPTG. The CheZ concentration was found to increase as a function of increasing levels of the inducer IPTG added in the range of 10 to 70 μ M. The swarming and swimming capacity, as a function of increasing IPTG and thus of increasing CheZ concentration, was quantified by measuring the diameter of the swarming colony at each single IPTG concentration and observing the swimming behavior of a corresponding strain in a liquid culture (Fig. 1A). Strain MGS64 shows wild-type chemotactic behavior when ~ 30 μ M IPTG is present in the medium. Decreasing or increasing the level of CheZ causes the bacteria to show tumbling or smooth-swimming behavior, respectively, presumably as a result of high or low levels of intracellular phospho-CheY. Extreme phenotypes, e.g., constant tumbling or smooth swimming, are manifest as nonswarming colonies on motility plates.

We compared the chemotactic behavior of MGS64 with that of MGS55, which has the same genetic background as MGS64 but carries the *cheZ* gene on the multicopy plasmid pQE12 (pMGS1) (see references 18 and 19). This comparison was necessary to determine the effect of multiple plasmid copies on the swimming behavior of MGS55 before screening the *cheZ* library (which was in pQE12) for smooth-swimming or tumbling mutants.

Clearly, MGS55 is somewhat defective in swarming. It has a bias toward smooth-swimming behavior even in the absence of IPTG, probably as a result of the presence of multiple copies of the *cheZ* gene (Fig. 1A). The diameter of a swarming MGS55 colony without induction is ~ 1.0 cm, and it becomes ~ 0.2 cm (nonswarming) when IPTG (as little as 5 μ M) is added to the plate. This correlates with enhancement of the bias toward smooth-swimming behavior observed in liquid cultures as well (Fig. 2A and B).

A



B

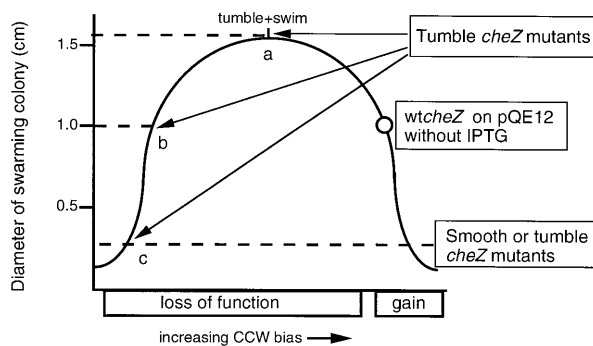


FIG. 1. Screening strategy for isolation of smooth-swimming and tumbling *cheZ* mutants. (A) The effect of an increasing level of CheZ in a $\Delta cheZ$ background was analyzed with the single-copy plasmid pBAC which carries the *cheZ* wild-type gene under an IPTG-inducible promoter (pMGS2, strain MGS64). The bell-like curve (solid line) results from plotting the diameter (in centimeters) (\square) of the MGS64 (wild-type *cheZ* on pBAC) swarming colony relative to the IPTG concentration present in the plate. Swarm diameters were measured after 17 h (see Materials and Methods). The maximum swarm capacity was obtained at 30 μ M IPTG induction in comparison with the $\Delta cheZ$ strain, which is unable to swarm. Concentrations of IPTG lower than 30 μ M caused a bias toward tumbling, whereas higher concentrations favored bacteria with smooth-swimming behavior (as observed in liquid culture). The dashed line represents the swarming behavior, without IPTG induction, of control strain MGS55, which carries the *cheZ* wild-type gene on multicopy plasmid pQE12 (pMGS1). Because of the multiple copies of the *cheZ* gene, MGS55 has a smooth swimming bias even without IPTG induction. (B) Tumbling *cheZ* mutant (loss-of-function) strains were isolated as colonies that swarm more than (a), equal to or somewhat less than (b), or markedly less than (c; i.e., nonswarming colonies) the control strain MGS55. Smooth-swimming *cheZ* mutant (gain-of-function) strains were isolated as nonswarming colonies. To distinguish between colonies that were extremely tumbling biased (b and c) or extremely smooth-swimming biased, it was necessary to analyze the swarming pattern formation of cells in the presence of IPTG as well as the swimming behavior of cells in liquid cultures. The arrow on the x axis indicates the increased bias toward smooth-swimming behavior (counterclockwise [CCW] rotation). Wt, wild type.

Considering the phenotype of MGS55, the mutant *cheZ* library was screened on the basis of the assumption that mutant strains carrying defective (loss-of-function) CheZ proteins would swarm on motility plates better than MGS55 in the absence of IPTG, since they should have a reduced smooth-swimming bias, resulting in chemotactic behavior that is closer to that of the wild type (Fig. 1B, part a). However, mutant strains which swarm less than MGS55 could carry defective

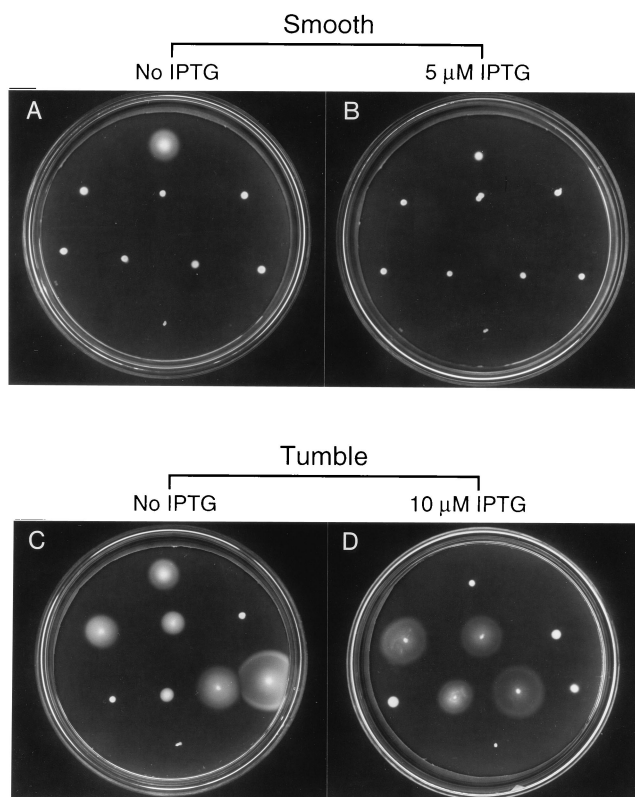


FIG. 2. Swarming patterns of smooth-swimming and tumbling *cheZ* mutants. Colonies of presumed smooth-swimming- or tumbling-biased *cheZ* mutants were stabbed onto motility plates, and their swarming behavior in the absence or presence of IPTG was observed. The swarming pattern of wild-type positive control strain MGS55 is in the upper part of each plate; its smooth-swimming bias is increased when expression of CheZ is induced by supplementing the plate with IPTG, leading to a loss of swarming capacity. The negative control strain MGS12 is represented in the lower part of each plate. (A and B) Swarming patterns of smooth-swimming-biased *cheZ* mutants (gain-of-function). There is no improvement in the swarming capacity of these mutants when IPTG is added to the plate because of their basal strong bias toward smooth-swimming behavior. (C and D) Swarming patterns of tumbling-biased *cheZ* mutants (loss-of-function). Tumbling *cheZ* mutants showed three different kind of swarming patterns as a result of the different degrees of bias toward tumbling behavior. Mutants slightly tumbling biased swarm more than wild-type control MGS55 (top) in the absence of IPTG (e.g., CheZ134EK mutant [second row from top, extreme right]); after IPTG induction, the diameter of the colony will be reduced because of the increased smooth swimming behavior. Mutants with medium tumbling bias show comparable or less swarming ability than that of MGS55 without IPTG (e.g., CheZ65AV mutant [top row, center]) and increased swarming capacity when IPTG is added. Finally, mutants with a strong tumbling bias appeared as nonswarming colonies on the plate in the absence of IPTG (e.g., CheZ117FS and CheZ115RP mutants [second row from top, middle left and extreme left]) and either started swarming upon induction (CheZ117FS) or were unable to form swarming patterns even with a very high level of IPTG (CheZ115RP) because of the incapacity to recover the strong tumbling bias.

(Fig. 1B, parts b and c, loss-of-function) or enhanced (Fig. 1B, gain-of-function) CheZ, e.g., they would be biased toward tumbling or extremely smooth-swimming behavior. In fact, both kinds of mutants should exhibit similar sizes of swarming colonies (diameter smaller than that of MGS55). In this category of mutants, it was necessary to analyze the swimming behavior of the strains in liquid cultures under a microscope to distinguish between those with smooth-swimming or tumbling behavior.

We screened ~300 miniswarm plates (~15,000 colonies), selecting for colonies that swarmed more than the control strain MGS55 (tumbling) or less than strain MGS55 (smooth swimming or tumbling) in the absence of IPTG.

In vivo analysis of smooth-swimming or tumbling *cheZ* mutant strains. Seven hundred ninety-two clones were isolated from the miniswarm plates as candidates for smooth-swimming or tumbling mutants. Bacteria were stabbed into motility agar containing different concentrations of IPTG to distinguish between strains carrying gain- or loss-of-function CheZ protein. Smooth-swimming mutant strains appeared as nonswarming colonies in the absence of IPTG (Fig. 2A), and no improvement in the swarming capacity (diameter of the colony) was observed when expression of CheZ was induced by adding IPTG (Fig. 2B). This swarming pattern is due to the smooth-swimming bias of gain-of-function *cheZ* mutant strains as confirmed by observing their swimming behavior in liquid culture (data not shown).

cheZ mutants with the tumbling phenotype could be classified as having three different kinds of swarming patterns due to different degrees of bias toward tumbling (Fig. 2C and D). Mutants with a slight tumbling bias (defined as mild in Fig. 3) swarm more than the wild-type control strain MGS55 in the absence of IPTG (Fig. 1B, part a), while after induction, the diameter of the colony becomes smaller because of increased smooth swimming due to the overexpression of the partially functional mutant CheZ (Fig. 2C and D, e.g., CheZ134EK mutant). Mutants with a medium bias toward tumbling (Fig. 3, medium) show comparable or less swarming ability than that of MGS55 without IPTG (Fig. 1B, part b) and increased swarming capacity when IPTG is added (Fig. 2C and D, e.g., CheZ65AV mutant). Finally, mutants with a strong bias toward tumbling behavior (Fig. 3, strong) appeared as nonswarming colonies on plates in the absence of IPTG (Fig. 1B, part c) and either started swarming upon induction or were unable to form swarming patterns even at very high levels of IPTG (Fig. 2C and D, e.g., CheZ117FS or CheZ115RP mutant). Thirty clones each of the strains with smooth-swimming or tumbling behavior were chosen for further investigation, and their respective *cheZ* mutant genes were sequenced.

CheZ gain- and loss-of-function mutants map to separate domains of the CheZ protein. Among the 60 sequenced clones, we found that 6 of the smooth-swimming strains and 13 of the tumbling strains carried single point mutations in the *cheZ* gene. The remaining clones had double or triple mutations and were not considered further. An additional 13 gain-of-function mutants were derived from a previously screened library of CheZ suppressors of mutant CheY23ND (18). These CheZ mutants were transformed into a wild-type *cheY* background and found to determine smooth-swimming behavior in the absence of IPTG induction and therefore to be gain-of-function mutants.

The changes resulting in gain-of-function mutations are found on the CheZ protein only at the N terminus (residues 17 to 54) and at the C terminus (residues 152 to 170), defining two clusters, which are shown in Fig. 3. Interestingly, mutations accounting for loss of function of CheZ are distributed mainly in the central region of the protein, separated from the two clusters of the gain-of-function mutations. Also shown in Fig. 3 is the degree of in vivo impairment of the swarming and swimming ability for the loss-of-function CheZ mutants. All gain-of-function mutants appeared in vivo as strongly smooth-swimming biased and therefore not significantly different from each other in swarming or swimming behavior (see also Fig. 2A and B). Single point mutation phenotypes were confirmed by reintroducing the mutated *cheZ* genes into a $\Delta cheZ$ strain to ensure that the phenotypes observed were not due to mutations elsewhere in the chromosome.

Analysis of in vivo phenotypes of strains with *cheZ* deletions. We constructed a series of deletions in the *cheZ* gene. Six

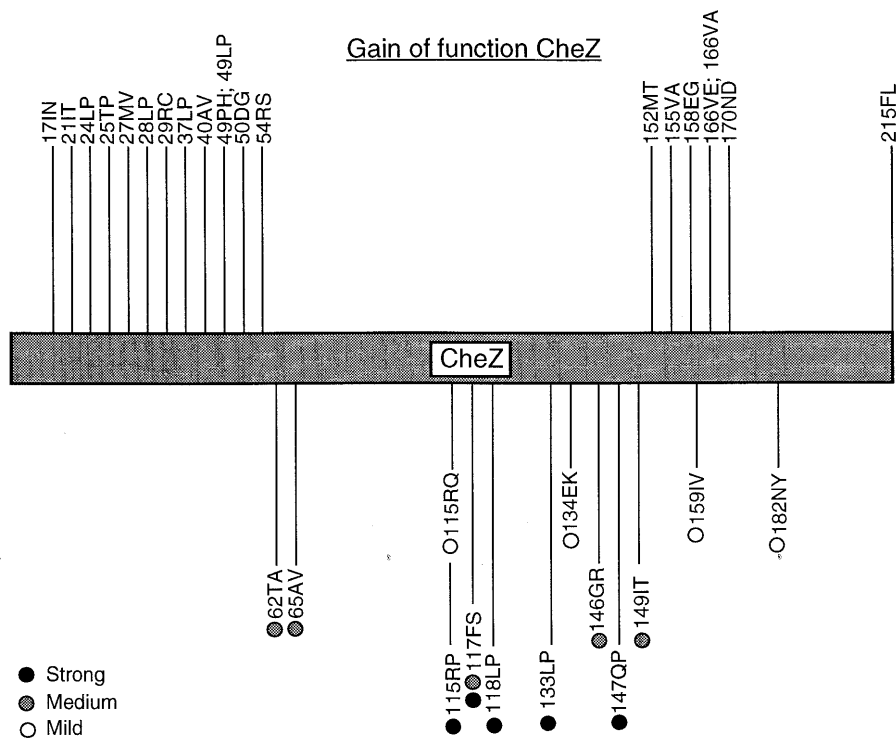


FIG. 3. Distribution of gain- and loss-of-function mutants on the CheZ protein. Mutations generating CheZ gain-of-function proteins define clusters at the N and C termini of the protein. The lengths of the vertical lines correspond to the strength of the in vivo behavior of the strains. All gain-of-function mutants appeared in vivo as strongly smooth-swimming biased and therefore not significantly different from each other in swarming or swimming behavior. Mutations accounting for loss-of-function CheZ are distributed mainly in the central region of the protein, separated from the two clusters of the activated mutations. The patterns of the circles together with the length of the vertical lines indicate the degree of in vivo impairment (i.e., tumbling) for the loss-of-function CheZ mutants.

deletions at the N terminus, three at the C terminus, and three at both the N and C termini were obtained by PCR with different sets of priming oligonucleotides (Fig. 4A). The swarming behavior of representative *cheZ* deletion strains is shown in Fig. 4B. The phenotypes of the strains with the remaining deletions are omitted from the figure since they were found to be similar to those shown (see the legend to Fig. 4B).

All of the deletions we analyzed altered the swarming pattern from that of the wild-type strain. When 17 amino acid residues were deleted either at the N or C terminus (yielding mutant proteins 18–215 and 1–198), the swarming capacities of the two resulting strains were similar and only slightly different from that of strains carrying wild-type CheZ. We observed that while deletion of 17 amino acids at the C terminus produces a truncated protein which appears to be stable, the 17-amino-acid N-terminal truncation induces instability of the resulting protein. Deletion of either 17 (CheZ 18–215) or 34 (CheZ 35–215) amino acids at the N terminus results in mutants with similar swarming patterns. The same was true for the deletion of 17 or 34 amino acids at the C terminus (CheZ 1–198 or 1–182, respectively).

Further deletions generated a marked defect in chemotactic behavior: deletion of 67 amino acid residues at the N or C terminus (CheZ 68–215 or 1–148, respectively) completely abolished chemotaxis. SDS gel analysis of whole-cell extract suggests that while the C-terminal deletions did not alter the stability of the CheZ protein, all of the N-terminal deletions resulted in reduced stability (data not shown).

In vitro analysis of CheZ mutants. We chose several CheZ gain- or loss-of-function mutants for in vitro assay. Mutant proteins were purified and assayed for their ability to dephosphorylate wild-type CheY. The intracellular levels of the CheZ

protein were measured for each clone and found to be comparable in all of the strains (see Materials and Methods).

Gain-of-function mutants CheZ166VE, -170ND, -28LP, and -24LP showed increased dephosphorylation activity on wild-type phospho-CheY (Fig. 5A) (see reference 18). However, mutant CheZ29RC, which confers to cells a bias toward smooth-swimming behavior in vivo, seems to be slightly defective in dephosphorylating phospho-CheY compared with wild-type CheZ (Fig. 5). The strongest gain-of-function mutant was found to be CheZ24LP, which dephosphorylates wild-type phospho-CheY ~4.6 times more efficiently than wild-type CheZ does (Fig. 5).

All of the loss-of-function mutants showed reduced dephosphorylation activity on phospho-CheY compared with wild-type CheZ; these reduced activities ranged from ~2.0-fold less for CheZ134EK to ~8.0-fold less for CheZ117FS (Fig. 5). Interestingly, the in vitro activity of these loss-of-function mutants was correlated with their in vivo behavior. For example, CheZ134EK and CheZ115RQ mutants showed a less-pronounced tumbling bias in vivo than mutants CheZ62TA, -65AV, and -149IT and were found to be less defective in dephosphorylating CheY. On the contrary, the CheZ117FS mutant, which has a fairly strong tumbling bias, was the most impaired for phospho-CheY dephosphorylation.

DISCUSSION

Mutations encoded by the *cheZ* gene generating smooth-swimming or tumbling phenotypes map in different clusters on the CheZ protein. We have isolated smooth-swimming or tumbling CheZ mutants (gain or loss of function) on the basis of their swarming and swimming behavior. The analysis of several

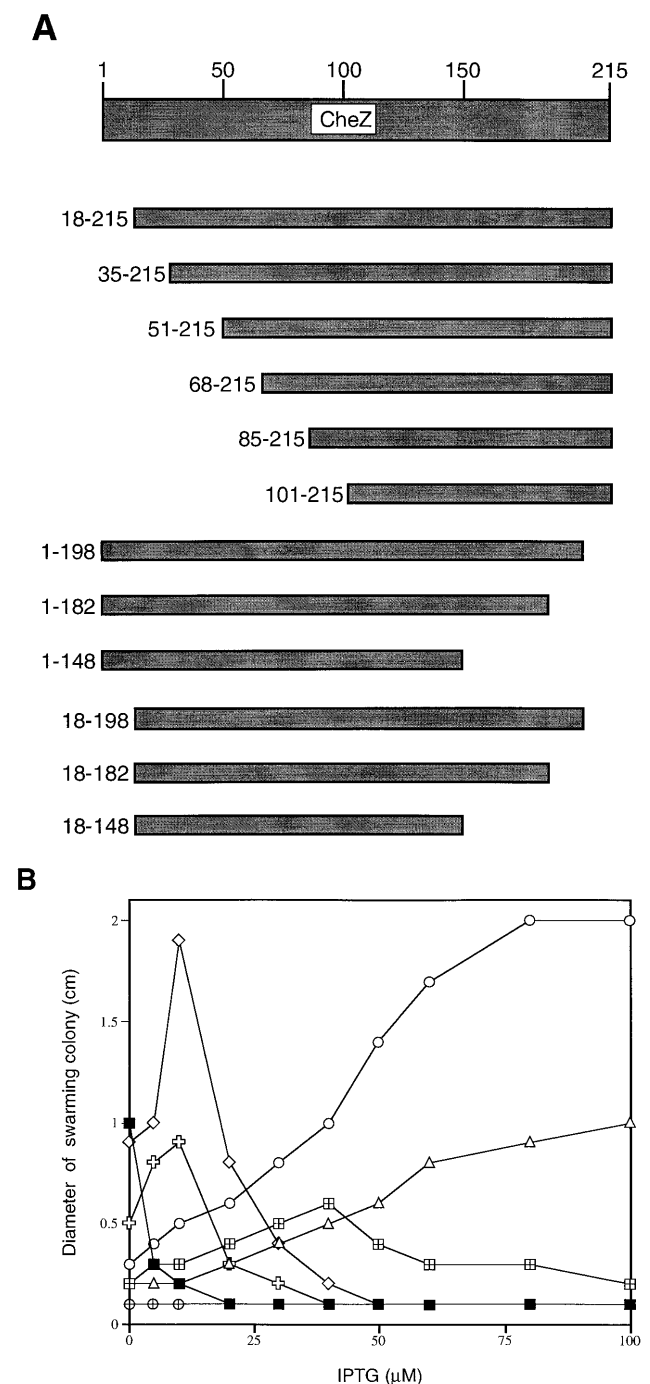


FIG. 4. Graphic representation and in vivo behavior of CheZ deletions. (A) Twelve truncated CheZ proteins were obtained by PCR with different sets of priming oligonucleotides. (B) Swarming patterns of *cheZ* deletion mutants were determined after induction of CheZ expression with increasing amounts of IPTG. Some CheZ deletion mutants had similar swarming patterns, and they are represented by the same symbol. Symbols: ■, MGS55 control strain; ◇, CheZ 18-215 and 35-215; ⊕, CheZ 1-198 and 1-182; ○, CheZ 51-215; △, CheZ 18-198 and 18-182; ⊞, CheZ 101-215 and 85-215; ⊕, CheZ 68-215, 1-148, and 18-148.

gain- or loss-of-function CheZ mutants, measured as the ability to dephosphorylate radiolabelled CheY, demonstrates that the degree of enhancement or impairment of their activity in vitro was consistent with their in vivo phenotype. Among the ana-

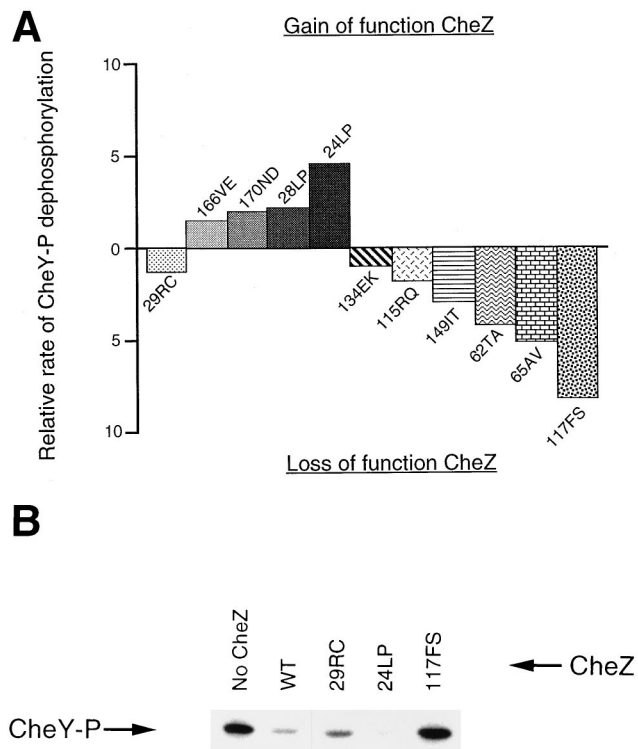


FIG. 5. Dephosphorylation activity of gain- and loss-of-function CheZ mutants on phospho-CheY. (A) The relative rate of phospho-CheY dephosphorylation is represented for the gain- and loss-of-function CheZ mutants. Rates are calculated by assuming that the wild-type CheZ activity on phospho-CheY is equal to 0. Each value is calculated as a mean of three experiments; the standard deviation was <8%. (B) CheY was phosphorylated with [32 P]acetyl phosphate in the absence or presence of wild-type or CheZ mutant proteins. Reaction products were separated by SDS-PAGE, and the amount of recovered phospho-CheY was quantified with a PhosphorImager (Molecular Dynamics). CheY-P, phosphorylated CheY.

lyzed CheZ proteins, only one of the mutants with a bias towards smooth swimming, i.e., CheZ29RC, which presumably would have enhanced CheZ activity, was found to dephosphorylate phospho-CheY less than the wild-type CheZ did. The apparent discrepancy between the in vivo phenotype and the in vitro activity of this mutant (CheZ29RC) could be explained by arguing that some of the activated mutants may be altered in their interaction with another component of the chemotactic machinery (e.g., FliM), which results in stronger dephosphorylation of CheY.

The distribution of the mutations seems to define different regions on the CheZ protein responsible for the gain or loss of function. *cheZ* mutations that produce strains with smooth-swimming behavior, resulting from CheZ mutant proteins with enhanced activity, were clustered mainly at the N and C termini. On the contrary, mutations that produce tumbling-biased strains, and are therefore impaired in protein functionality, mapped mainly in the central portion of CheZ.

Mutations in the central domain of CheZ impair protein function. Although we are not able to assign a specific role to the two domains defined by the mutations, our data suggest different functions for these domains. It is possible that the central region of CheZ represents a functional domain which is involved primarily in the dephosphorylation of CheY. Another possibility is that mutations in those particular residues alter the correct folding of the CheZ protein. Support for this hy-

pothesis can be found in the observation that the most inactive CheZ mutants carried mutations converting specific residues to a proline, which often alters the protein folding (e.g., the 115RP mutation is stronger than the 115RQ mutation).

Gain-of-function mutations map at the termini of the CheZ protein. Mutations mapping in the N- and C-terminal portions of the protein (gain-of-function mutations) may enhance the capacity of CheZ to interact with CheY, therefore improving its dephosphorylation activity and consequently causing smooth-swimming behavior. Support for this hypothesis comes from a previous report in which some of the gain-of-function CheZ mutants (e.g., CheZ24LP, -28LP, -170ND, and -166VE) were shown to also be suppressors of the CheY23ND mutant (18). CheY23ND was found to be defective in binding to CheZ, whereas the isolated suppressors showed restored binding to the mutant CheY proteins. The observation that CheZ suppressors of CheY23ND also showed enhanced activity on wild-type CheY suggests the possibility that gain-of-function CheZ mutants will bind to wild-type CheY more efficiently than wild-type CheZ will. However, our attempts to measure possible variations in the affinity between activated CheZ mutants and wild-type CheY failed to show any significant differences (18a). A possible reason may be the limited sensitivity of our binding assay.

Gel analysis suggests that the C-terminal deletions did not alter the stability of the CheZ protein, whereas all of the N-terminal deletions may have reduced the stability. This observation complicates the interpretation of the results obtained with the N-terminal deletions, since it is not possible to establish whether the observed behaviors are due to the reduced stability of the truncated CheZ or to the deletion of a specific functional domain. Deletion of the N-terminal 17 amino acids had little effect on the activity of the protein. Removing the first 17 amino acids of the CheZ protein may increase the instability of the protein. Support for this hypothesis can also be found in the observation that none of the gain- or loss-of-function mutants mapped in the first 17 residues of CheZ, as if mutations in that region do not significantly affect the catalytic function.

Deletion of 67 amino acids at the C terminus of CheZ did not significantly alter protein stability yet gave the most impaired phenotype. Interestingly, this deletion overlaps with the domain that, when mutagenized, appears to determine smooth-swimming behavior *in vivo* and increased activity *in vitro*. Some of these gain-of-function mutants were found to be suppressors of the CheY23ND mutation and showed restored binding to CheY23ND (18). These data suggest that the deletion of the last 67 amino acids of the protein may alter the ability of the CheZ protein to bind to CheY.

The resolution of the three-dimensional structure of CheZ will allow a greater understanding of the effect of CheZ mutations reported in the present work.

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ADDENDUM IN PROOF

A recent paper (Y. Blat and M. Eisenbach, *Biochemistry* **35**:5679–5683, 1996) also describes CheZ domain structure.

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