Acta Biochim Biophys Sin (2010): 216–223 | © The Author 2010. Published by ABBS Editorial Office in association with Oxford University Press on behalf of the Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. DOI: 10.1093/abbs/gmq007.

Cloning and characterization of three *cheB* genes in *Leptospira interrogans*

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Motility and chemotaxis systems are critical for the virulence of leptospires. There were multiple copies of putative chemotaxis homologs located at leptospires large chromosome. CheB1 and CheB3 from *Leptospira interrogans* strain Lai are predicted to have a global CheB-like domain, but CheB2 is predicted to have a C-terminal effector domain only. In order to verify the function of three putative *cheB* genes, they were cloned into pQE31 vector and then expressed, respectively, in wild-type *Escherichia coli* strain RP437 and *cheB* defective strain RP4972. The results of swarming assays and the predicted ternary structures of CheB1 and CheB3 of *L. interrogans* strain Lai suggested that the absence of an N-terminal regulatory domain may be one of the reasons for the failure of CheB2 to complement an *E. coli cheB* mutant. Furthermore, CheB2 links solely to CheR1 and CheR3 in the interaction network of leptospires. Taken together, these results indicated that CheB2 may not function alone, and under certain physiological conditions, it may require CheB3 and CheR1 to function. The existence of multiple copies of chemotaxis gene homologs suggested that *L. interrogans* strain Lai might have a more complex chemosensory pathway.

Keywords *Leptospira interrogans*; chemotaxis; *cheB*

Received: October 18, 2009 Accepted: December 21, 2009

Introduction

Leptospirosis is a widely spread zoonosis of global concern and is caused by infection with pathogenic *Leptospira* species [1,2]. Infection causes flu-like episodes with severe renal and hepatic damage, such as hemorrhaging and jaundice. In more severe cases, massive pulmonary hemorrhages and even fatal sudden hemoptysis can occur. The disease is caused by corkscrew-shape bacteria of the genus *leptospira* [2].

In order to survive in the environment, bacteria need to be able to detect and move toward favorable conditions and away from unfavorable conditions. Chemotaxis and motility, which enable bacteria to sense and respond to numerous changes in their environment, are critical for the virulence of pathogenic leptospires [3,4]. Furthermore, chemotaxis enables bacteria to penetrate host tissue barriers during infection [5]. Chemotaxis relies on controlling the frequency of direction switching of flagellar rotation, which results in a net movement toward an attractant or away from a repellent [3,4]. Chemotaxis has been studied in great detail with the model organism *Escherichia coli* [6,7]. The chemotaxis system integrates environmental cues into a behavioral response using a dedicated signal transduction pathway. This pathway is composed of chemotaxis transducers, the histidine kinase CheA coupled to the chemotaxis transducers via docking protein CheW, the response regulator CheY, and the adaptation proteins CheB and CheR [8]. Homologous chemotaxis systems have been identified for distantly related bacteria and archaea [9,10].

Most or all of the common elements in the signaling process were involved in the generation and regulation of changes in the direction of flagellar rotation. CheB consists of an N-terminal regulatory domain and a C-terminal effector domain joined by a linker region. The methylesterase CheB is a member of a large and functionally diverse family of proteins known as response regulators. In the enteric bacteria *E. coli* and *Salmonella typhimurium*, CheB functions together with the methyltransferase CheR to control the level of receptor methylation [11]. The main function of the cheB product appears to be to regulate, through its methylesterase activity, the MCP methylation state of the cell [12]. And many studies indicated that CheB function is essential for sensory adaptation in several kinds of bacteria [12–15].

Analysis of the complete genomic sequence of *Leptospira interrogans* strain Lai suggested the presence of multiple copies of chemotaxis homologs located in its

chromosome (12 × *MCP*, 2 × *cheA*, 3 × *cheW*, 5 × *cheY*, 3 × *cheB*, 2 × *cheR*, 2 × *cheD*, 1 × *cheX*, but no *cheZ*) [16]. This implies that *L. interrogans* strain Lai employs and regulates a complex chemosensory pathway.

Leptospira interrogans serovar Lai strains 017 and KH-1 move toward hemoglobin, movement that was related to their virulence [17]. The genetic approach to further studying the role of chemotaxis in *L. interrogans* virulence is hindered by the lack of adequate genetic systems and by their fastidious cultivation requirements. Bacterial chemotaxis has been studied extensively in *E. coli*, and this is arguably the best understood of all biological behaviors [6]. Data from our previous study suggested that CheW1 and CheW3 (but not CheW2) of *L. interrogans* strain Lai are able to substitute for the *E. coli* CheW proteins in the phosphorelay pathway and thus have an *in vivo* function analogous to that of *E. coli* CheW [18]. Thus, we proposed that *L. interrogans* and *E. coli* may have similar chemotaxis phosphorelay pathway effector mechanisms, although with some differences in their control by the signal terminator [19].

In this study, we used *in vivo* complementation to study the function of *L. interrogans* chemosensory genes. Homology was demonstrated among the three *L. interrogans cheB* genes and *E. coli cheB*. The mechanism of chemotaxis signaling in *L. interrogans* strain Lai was investigated. Finally, individual *L. interrogans* strain Lai *cheB* genes were tested for complementation of a *cheB*-deficient *E. coli* strain. In conclusion, we found that using *E. coli* as a surrogate host is an effective way to study the function of the *L. interrogans* chemosensory system.

Materials and Methods

Bacterial strains and plasmids

The bacterial strains and plasmids used in this study are shown in **Table 1**. *L. interrogans* strain Lai was cultured aerobically in liquid Ellinghausen–McCullough–Johnson–Harris medium [2] at 28°C. *Escherichia coli* strains were cultured aerobically in LB broth at 30°C. For solidification, bacto agar was added at 1.5%. Appropriate antibiotics were added when necessary (50 μg/ml streptomycin, 100 μg/ml ampicillin, and 25 μg/ml kanamycin).

Bioinformatics techniques

The amino acid sequences of all open reading frames (ORFs) in *L. interrogans* strain Lai were obtained from http://www.ncbi.nlm.nih.gov/sites/entrez?Db=genome&Cmd=ShowDetailView&TermToSearch=258. Multiple-sequence alignments were accomplished using Bioedit software. Domain prediction was done using Pfam software (http://pfam.sanger.ac.uk/). Predicted ternary structures were generated online (http://robetta.bakerlab.org/).

Construction of the recombinant plasmids

L. interrogans strain Lai was collected at a density of ~ 10^8 bacteria/ml. DNA fragments containing the ORFs of *cheB*1, *cheB*2, and *cheB*3 from *L. interrogans* strain Lai genomic DNA and *cheB* from *E. coli* strain K12 were amplified by PCR using oligonucleotides (**Table 2**). Construction of the recombinant plasmids was performed as described previously [19]. In this fashion, plasmids pQE31-cheB1(L), pQE31-cheB2(L), pQE31-cheB3(L), and pQE31-cheB(E) were constructed. Plasmids pRR48-cheBs, which express untagged CheB proteins, were constructed with the same cloning sites.

Expression of *L. interrogans* strain Lai *cheB* genes

The expression of recombinant plasmids was under the control of the *tac* promoter. This allows IPTG-induced expression of the desired protein with an N-terminal tag containing six histidine residues. Plasmids with inserts of the correct sequence were introduced into wild-type *E. coli* strain RP437 and the *cheB* defective strain RP4972 containing the compatible plasmid pREP4. Plasmid pREP4 has $lacI^q$ for negatively regulating expression from the *tac* promoter, thereby reducing 'leaky' expression. To test CheB1(L), CheB2(L), CheB3(L), and CheB(E) overproduction, 100 μl of overnight culture of RP437 (pREP4) containing the appropriate expression plasmid was added

Table 1 Bacterial strains and plasmids used in this study

Bacteria strains and plasmids	Description	Source
L. interrogans serovar strain Lai	*L. interrogans cterohaemorrhagiae* serovar lai strain Lai	Our lab
E. coli strain		
DH5α	-	Our lab
RP437	Wild type for chemotaxis, with pREP4	Dr J.S. Parkinson
RP4972	Δ*cheB*, with pREP4	Dr J.S. Parkinson
Plasmid		
pQE31	-	Qiagen, Hilden, Germany
pRR48	-	Dr J.S. Parkinson

Table 2 Primers employed for DNA amplification and molecular weight of expressed recombinant proteins

Gene	Primer sequence (5′ → 3′)	MW (kDa)
cheB1(L)	CGGGGTACCGATGATTCCAAATCCG (F)	38.7
	CCCAAGCTTTTAAATTCCTCTCTCT (R)	
cheB2(L)	CGCGGATCCTATGAACTACGAAGCAAT (F)	21.3
	CCCAAGCTTTCATCTTTGCTCCAGGT (R)	
cheB3(L)	CGCGGATCCCATGATTCAAGTTTTTAT (F)	38.0
	CCCAAGCTTCTAAAAATACTGAACCTC (R)	
cheB(E)	CGCGGATCCGATGAGCAAAATCAGGGTGT (F)	38.5
	AACTGCAGTTAAATACGTATCGCCTGTCC (R)	

MW, molecular weight.

into 5 ml of fresh LB. After 2 h at 30°C, IPTG was added to one tube (the induced sample) at a final concentration of 1 mM. The cultures were shaken at 30°C for 3 h. In order to confirm the correct expression of the fused proteins in RP4972, western blotting analysis was utilized as described previously [19]. In order to exclude the possibility that the histidine tag might interfere with CheB function, we also cloned *cheB* genes into pRR48 for expression of the cloned native proteins.

Escherichia coli motility assays

Swarming assays. *Escherichia coli* swarming assays were carried out using a modification of Wolfe's method [20]. Strains carrying the expression plasmids that tested positive for the production of induced proteins grew overnight in LB broth with streptomycin, ampicillin, and kanamycin at 30°C. A 5-μl of aliquot of the appropriate culture (10^6–10^7 cells) was inoculated onto the surface of an LB swarm plate containing streptomycin, ampicillin, kanamycin, 0.25% agar, and IPTG at 0, 1, 10, 100, or 200 μM near its center. The plates were incubated at 30°C in a humid environment. RP437 swarm sizes were measured after 8 h, and mutant strain swarm sizes were measured after 48 h. Three independent experiments were done and the corresponding swarm radii were averaged and plotted.

Swimming behavior assays. The free-swimming behavior of *E. coli* strains carrying expression plasmids was examined with a phase-contrast microscope. Cells grew overnight in tryptone broth (TB; 10 g/L tryptone, 5 g/L NaCl) with appropriate antibiotics. The stationary culture was diluted 1:100 in fresh TB with antibiotics and 10 μM IPTG, and incubated at 30°C for another hour before inverted phase-contrast microscopic examination. The observation starts when the cell appears in the view and finishes when the cell disappears from view. About 100 cells were detected at random. Each cell was observed for 10–15 s and its behavior was recorded. Three independent groups of cells were observed and the data averaged.

Escherichia coli growth assays. LB broth (15 ml) containing streptomycin, kanamycin, and ampicillin was inoculated with 150 μl of stationary phase overnight culture of RP437 (pREP4) containing an overexpression plasmid. Immediately after inoculation, 1 ml of the culture was withdrawn, and an OD_{600} reading was taken (time 0) in 1 cm cuvettes on a Pharmacia LKB Ultrospec II spectrophotometer zeroed with LB broth containing streptomycin, kanamycin, and ampicillin. The cultures were incubated at 30°C for 1 h, and IPTG was added to a final concentration of 0.1 mM. Then, 1 ml of samples was taken at 2, 3, 4, 6, and 8 h for quantification at OD_{600} [21].

Results

Multiple-sequence alignment of the amino acid sequences of three CheB-homolog from *L. interrogans* strain Lai and CheB of *E. coli* K12

A multiple-sequence alignment of three CheB homologs, from *L. interrogans* strain Lai, CheB of *E. coli* K12, and CheB of *S. typhimurium*, was accomplished using Bioedit software. The identity and similarity of CheB1(L), CheB2(L), and CheB3(L) with *E. coli* and *S. typhimurium* CheB were significant (39%, 29%, 47% and 58%, 46%, 67%, respectively; **Fig. 1**). The similarity of *L. interrogans* strain Lai CheB proteins to CheB of *E. coli* was determined using the plotSimilarity program of the GCG package. The window size was 10. Regions of high sequence similarity have higher scores than those with low similarity on an arbitrary scale. The dotted line is the average similarity score for the whole protein (**Fig. 2**). As shown in **Fig. 2**, we can see that in the region of residues 0–160, *L. interrogans* strain Lai CheB2 has much poorer homology to *E. coli* CheB than CheB1 or CheB3. All three CheBs have high homology to *E. coli* CheB in the region of 160–340.

Predicted ternary structure of CheB1 and CheB3 of *L. interrogans* strain Lai

The predicted ternary structure of CheB1 and CheB3 of *L. interrogans* indicated that the three conserved aspartate

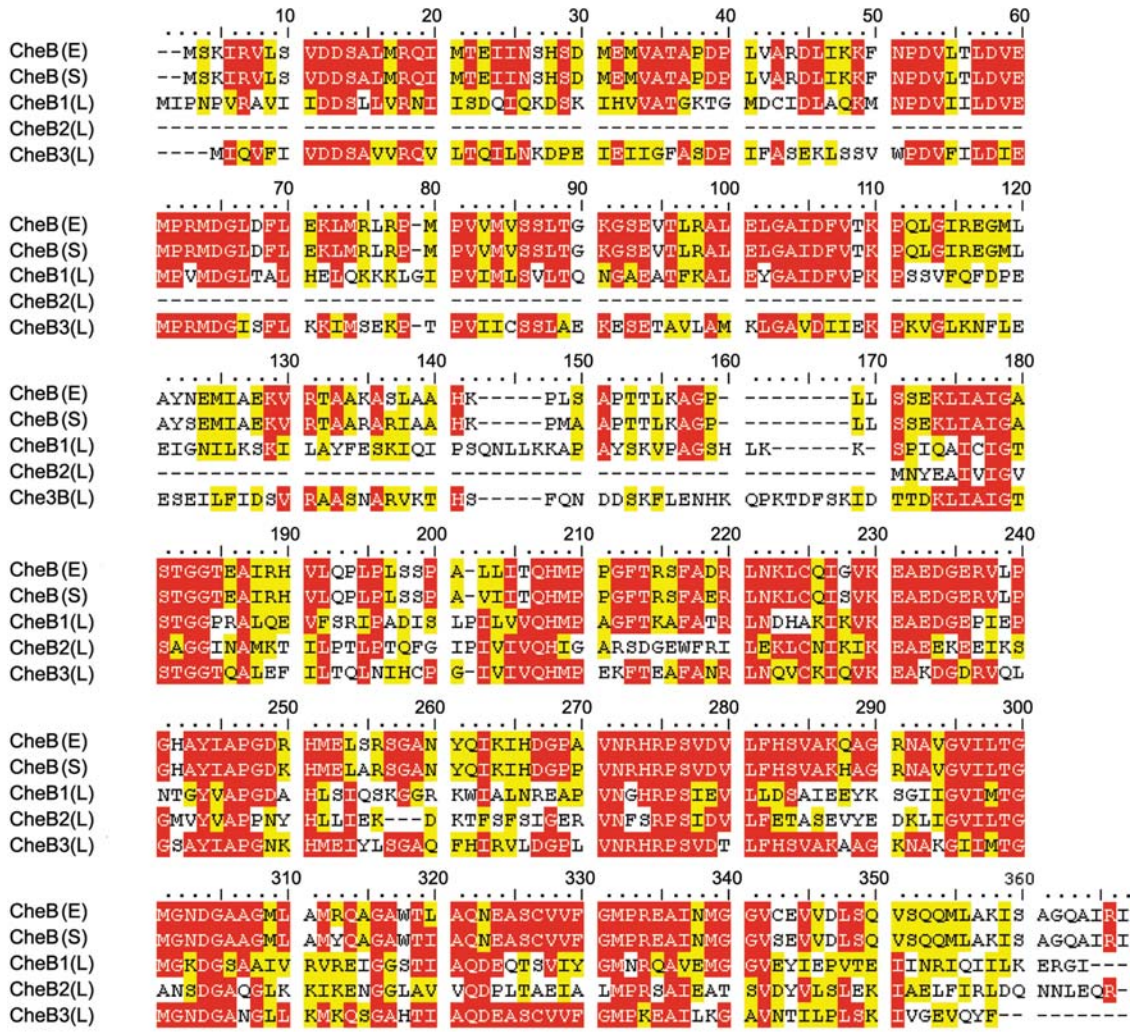

Figure 1 Multiple-sequence alignment of three CheB homologs from *L. interrogans* strain Lai, CheB of *E. coli* K12, and *S. typhimurium* Identical amino acid residues are shaded in red and conserved amino acid residues are shaded in yellow. The alignment was accomplished using Bioedit software. Gaps introduced to maximize the similarity alignment are indicated by dashes. The bacterial abbreviations used are: CheB(E), *E. coli* K12 CheB; CheB(S), CheB of *S. typhimurium*; CheB(L), CheB of *L. interrogans* serovar strain Lai.

residues (corresponding with the residues Asp10, 11, and 56 in *E. coli* and *S. typhimurium*) are oriented toward and contributed to an acidic active site cluster (**Fig. 3**). In addition, the conserved Ser164, His190, and Asp286 homologous residues are all located in a cleft of the *L. interrogans* CheB1 and CheB3, which formed by loops at the carboxy ends of β-strands cβ1, cβ2, and cβ7 (corresponding with the residues Ser164, His190, and Asp286 in *E. coli* and *S. typhimurium*; **Fig. 3**).

Effects of the expression of *L. interrogans cheB* genes on the swarming phenotypes of wild-type RP437 and *cheB*-deficient *E. coli* strain RP4972

Escherichia coli wild-type strain RP437 (pREP4) and *cheB* null strain RP4972 containing the pREP4 plasmid expressing *lacI*^{q} were transformed with plasmids expressing amino terminal-tagged cheB1(L), cheB2(L), and cheB3(L) or with pQE31 and cheB(E) as a control. Expression of these fused proteins was confirmed by western blotting analysis. The effects of expressing the three N-terminal His-tagged CheB proteins in *cheB*-deficient *E. coli* strain RP4972 are shown in **Fig. 4**. CheB1 and CheB3 restored swarming to RP4972, giving the biggest bands at an IPTG concentration of 10 μM. For CheB2, no significant increase in swarm size compared with that of the negative control (RP4972), containing only pQE31, was seen at any IPTG concentration. Expression of native (untagged) *cheB*2 also failed to restore swarming to RP4972. The swarming of RP437 was inhibited by high expression of CheB1 and CheB3, but to a lesser extent by CheB2 (**Fig. 5**). The inhibitory effect was IPTG concentration-dependent. In each case, there was no significant effect on the growth rate of RP437.

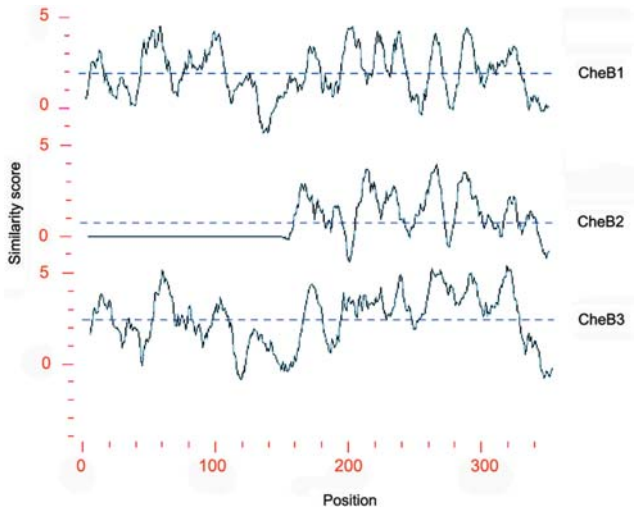

Figure 2 Comparison of the similarity of the *L. interrogans* strain Lai CheB proteins to *E. coli* CheB The plots were generated using the PlotSimilarity program (Chinese Academy of Sciences, GCG package). The window size was 10. Regions of high sequence similarity have higher scores than those with low similarity on an arbitrary scale. The dashed line is the average similarity score for the whole protein. In the region of amino acids 0–160, *L. interrogans* strain Lai CheB2 has much poorer homology to *E. coli* CheB than CheB1 or CheB3. It was also found that all three CheBs have high homology to *E. coli* CheB at region of amino acids 160–340.

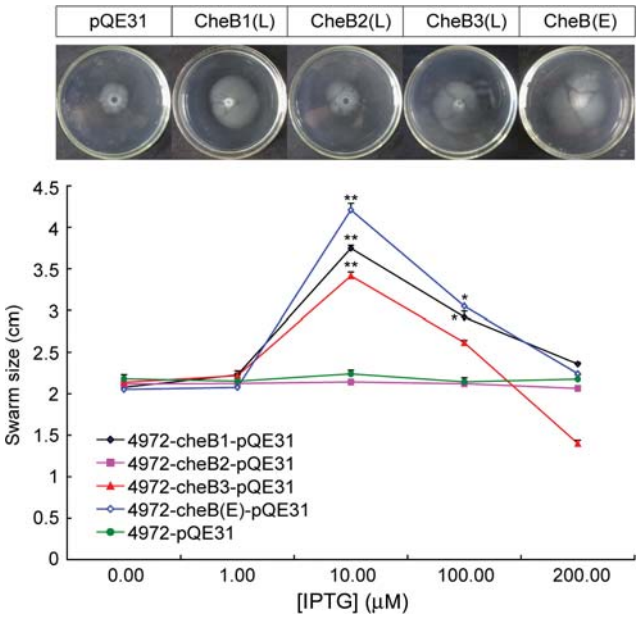

Figure 4 Effects of expressing the *L. interrogans* strain Lai *cheB* genes in *E. coli* RP4972 The incubation time is 48 h and three independent experiments were done. The corresponding swarm radius was shown as the mean ± SD. Examples of swarm plates containing 10 μM IPTG are shown (*$P < 0.05$ and **$P < 0.01$).

Effects of the expression of *L. interrogans cheB* genes on the tumbling frequency of wild-type RP437 and *cheB*-deficient *E. coli* strain RP4972

Phase-contrast microscopy was used to test the effects of expressing *L. interrogans* strain Lai *cheB* genes on the swimming behavior of *E. coli* strains. The expression of CheB1 and CheB3 in RP4972 caused a marked reduction in tumbling frequency, whereas CheB2 expression resulted in only a slight reduction in tumbling. The wild-type strain (RP437) had a random distribution of runs and tumbles. However, expression of *cheB1* and *cheB3* at levels that

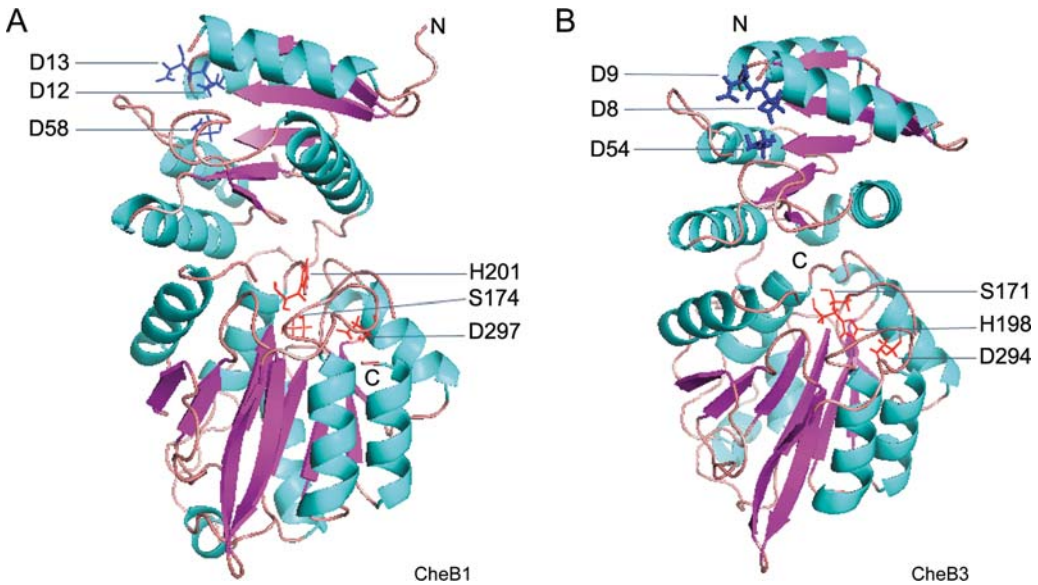

Figure 3 Predicted tertiary structure of (A) CheB1 and (B) CheB3 of *L. interrogans* strain Lai The blue sticks indicate the conserved aspartate cluster (corresponding to residues Asp10, Asp11, and Asp56 in *E. coli* and *S. typhimurium*) among CheB homologs. The red sticks indicate the active site residues located in a cleft formed by loops at the carboxyl ends of β-strands (corresponding to residues Ser164, His190, and Asp286 in *E. coli* and *S. typhimurium*) among CheB homologs. The structures were generated online (http://robetta.bakerlab.org/).

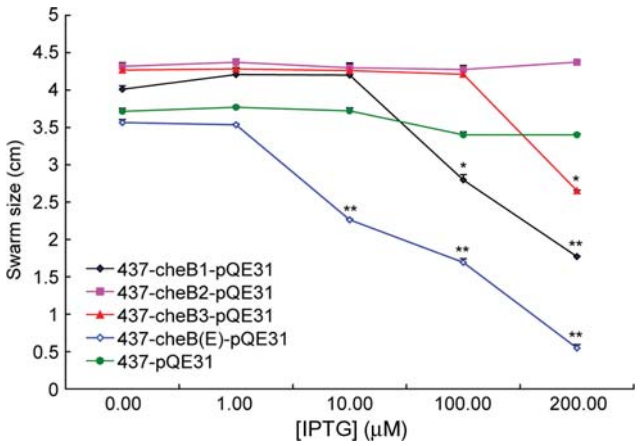

Figure 5 Effect of expression of the *L. interrogans* strain Lai *cheB* genes in *E. coli* RP437 The incubation time is 8 h and three independent experiments were done. The corresponding swarm radius was shown as the mean ± SD (*$P < 0.05$ and **$P < 0.01$).

inhibited the swarm size of wild-type *E. coli* RP437 resulted in a high smooth-swimming behavior, and *cheB2* had the least effect on the behavior of RP437 (**Fig. 5**).

Discussion

In our previous study, we predicted protein–protein interactions of *L. interrogans* strain Lai chemotaxis proteins by four computational methods that calculate the functional linkages. This prediction of the interaction network of *L. interrogans* strain Lai chemotaxis and motility associated proteins demonstrated that the 11 MCPs are likely to link to each other and that CheB2 links solely to CheR1 and CheB3, suggesting that CheB2 may not function alone but under certain physiological conditions may require CheB3 and CheR1 for function [18].

Multiple amino acid sequence alignment of the three CheB homologs from *L. interrogans* strain Lai [CheB1(L), CheB2(L), and CheB3(L)] shows >29% identity and >46% similarity with *E. coli* K12 CheB. CheB1(L) and CheB3(L) from *L. interrogans* strain Lai are predicted to have a global CheB-like domain, but CheB2(L) is predicted to have a C-terminal effector domain only. A similarity plot comparing the amino acid sequences of *L. interrogans* strain Lai CheBs with *E. coli* CheB (**Fig. 2**) shows that in the region of residues 0–160, CheB2 has much poorer homology to *E. coli* CheB than either *L. interrogans* CheB1 or CheB3.

To further investigate the function of these genes, we expressed them in the *cheB* mutant strain RP4972 and in the corresponding wild-type *E. coli* strain RP437. Our results indicate that *cheB1* and *cheB3* can complement the defective swarming phenotype of the *cheB* mutant *E. coli* RP4972, whereas cheB2 cannot (**Fig. 4**). Overexpression of *cheB1* and *cheB3* in wild-type *E. coli* RP437 greatly inhibited its swarming. However, this inhibitory effect was considerably less when *cheB2* was overexpressed (**Fig. 5**). Thus, we conclude that *L. interrogans* strain Lai CheB1 and CheB3 can play roles in the phosphorelay pathway of *E. coli*. However, the lack of a swarm ring indicated that *L. interrogans* strain Lai CheB1 and CheB3 did not completely restore chemotaxis to the *E. coli* strains. The methylesterase CheB is a member of a large and functionally diverse family of proteins known as response regulators [11]. The high-energy phosphoryl group of phospho-CheA can be transferred to two proteins: the response regulator CheY, which when phosphorylated interacts with the flagellar motor causing clockwise flagellar rotation, and the methylesterase CheB, which when phosphorylated is activated to demethylate the receptors, thereby attenuating the response [22,23]. This phosphorylation network is very complex and defects in any aspect of the signal transduction network will lead to a change in the bacterial phenotype.

CheB has a two-domain architecture, with an N-terminal regulatory domain homologous to CheY and a C-terminal effector domain with amidase/esterase activity [24]. Phosphorylation of the N-terminal domain of the intact protein also results in enhanced methylesterase activity [25,26] that is significantly higher than that of the isolated C-terminal domain [27]. Thus, Djordjevic and Stock [24] considered that the N-terminal domain plays dual regulatory roles, functioning to inhibit methylesterase activity when unphosphorylated and to stimulate activity when phosphorylated. In unphosphorylated CheB, the N-terminal domain packs against the active site of the C-terminal domain and thus inhibits methylesterase activity by directly restricting access to the active site [11]. Therefore, we conclude that the absence of the N-terminal regulatory domain may be one of the reasons for the failure of *L. interrogans* CheB2 to complement the *E. coli cheB* mutant.

CheB belongs to the class of serine hydrolases that contains active site catalytic triads consisting of serine, histidine, and aspartate residues. In the catalytic domain of CheB, the active site residues, Ser164, His190, and Asp286, are located in a cleft formed by loops at the carboxyl ends of β-strands, cβ1, cβ2, and cβ7 (β-strands within the C-terminal domain) [11]. In CheB, phosphoryl transfer from the histidine kinase CheA occurs at Asp-56, which is located atop strand β3 in the N-terminal domain. The phosphoryl transfer reaction is magnesium dependent and in the structure of Mg^{2+}-CheY, the active site residues, Asp12, Asp13, and Asp57, coordinate the essential metal cation [28]. Multiple-sequence alignment of the amino acid sequences of CheB1 and CheB3 from *L. interrogans* and *S. typhimurium* showed that most of the conserved sites in *S. typhimurium* CheB protein that determine its chemotaxis signaling were found in CheB1 and CheB3 of *L. interrogans* (**Fig. 1**). The predicted tertiary structure of CheB1

and CheB3 of *L. interrogans* indicated that the three conserved aspartate residues and conserved Ser164, His190, and Asp286 homologous residues probably have the similar function as that in *E. coli* CheB protein (**Fig. 3**).

Taken together, these data suggest that CheB1 and CheB3 of *L. interrogans* strain Lai are able to substitute for the *E. coli* CheB proteins in chemoreceptor methylation and thus have an *in vivo* function analogous to *E. coli* CheB function. Again, the lack of swarm ring indicated that *L. interrogans* strain Lai CheB1 and CheB3 could not restore chemotaxis completely to the *E. coli* strains. The existence of multiple copies of the chemotaxis proteins suggested that the *L. interrogans* strain Lai might have a complex chemosensory pathway. Our studies demonstrating by complementation that *L. interrogans* CheB1 and CheB3 act differently from CheB2 define one aspect of that complexity. Although the precise reason for the difference in the complementation of CheB2 remains unknown, the absence of the N-terminal regulatory domain maybe one of the reasons for the failure of CheB2 to complement an *E. coli cheB* mutant. In addition, based on our previous study that predicted protein–protein interactions of *L. interrogans* strain Lai chemotaxis proteins, we propose that CheB2 may not function alone, but under certain physiological conditions, it may require CheB3 and CheR1 for function [18]. It is important to note that multiple chemotaxis operons and gene homologs have been found in many bacterial genomes, including *Borrelia burgdorferi* [29], *Treponema pallidum* [30], *Sinorhizobium meliloti* [31], and *Rhodobacter sphaeroides* [21]. Our study demonstrates the feasibility of combining *in vivo* and *in silico* approaches to study the function of multiple-copy chemotaxis proteins.

Acknowledgements

We are extremely grateful to Dr Sandy Parkinson at the University of Utah for kindly providing the *E. coli* strains.

Funding

This work was supported by the grants from the National Natural Science Foundation of China (30830002, 30670102, and 30770820), the National Key Program for Infectious Diseases of China (2008ZX10004-002, 2008ZX10004-009, and 2009ZX10004-712), Program of Shanghai Subject Chief Scientist (09XD1402700), and the National High Technology Research and Development Program of China.

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