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Coexpression of the Long and Short Forms of CheA, the Chemotaxis Histidine Kinase, by Members of the Family *Enterobacteriaceae*

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CheA is the histidine protein kinase of a two-component signal transduction system required for bacterial chemotaxis. Motile cells of the enteric species *Escherichia coli* and *Salmonella typhimurium* synthesize two forms of CheA by utilizing in-frame initiation sites within the gene *cheA*. The full-length protein, $CheA_L$, plays an essential role in the chemotactic signaling pathway. In contrast, the function of the short form, $CheA_S$, remains elusive. Although $CheA_S$ lacks the histidine residue that becomes phosphorylated in $CheA_L$, it exhibits both kinase activity and the ability to interact with and enhance the activity of CheZ, a chemotaxis protein that accelerates dephosphorylation of the two-component response regulator CheY. To determine whether other members of the family *Enterobacteriaceae* express $CheA_S$ and CheZ, we analyzed immunoblots of proteins from clinical isolates of a variety of enteric species. All motile, chemotactic isolates that we tested coexpressed $CheA_L$, $CheA_S$, and CheZ. The only exceptions were closely related plant pathogens of the genus *Erwinia*, which expressed $CheA_L$ and CheZ but not $CheA_S$. We also analyzed nucleotide sequences of the *cheA* loci from isolates of *Serratia marcescens* and *Enterobacter cloacae*, demonstrating the presence of in-frame translation initiation sites similar to those observed in the *cheA* loci of *E. coli* and *S. typhimurium*. Since coexpression of $CheA_S$ and CheZ appears to be limited to motile, chemotactic enteric bacteria, we propose that $CheA_S$ may play an important role in chemotactic responses in some environmental niches encountered by enteric species.

The chemotaxis *cheA* gene of *Escherichia coli* and *Salmonella typhimurium* encodes two proteins, $CheA_L$ and $CheA_S$, which are translated in phase from two different initiation sites (17, 36, 38). Thus, $CheA_S$ is identical in primary sequence to $CheA_L$, except that it lacks the amino-terminal region containing the site of phosphorylation. $CheA_L$, the sensor in this two-component signal transduction pathway, is found in vitro as a dimer (10). It phosphorylates itself on a histidine residue located near its amino terminus (15). Phosphorylated $CheA_L$, in turn, donates its phosphate to the response regulators CheY, required for clockwise signal generation, and CheB, required for adaptation (reviewed in references 15, 19, and 40). $CheA_L$ homodimers form ternary complexes with CheW and chemoreceptors (11, 18). Within these complexes, $CheA_L$ autokinase activity is significantly accelerated relative to that of noncomplexed $CheA_L$ homodimers. Binding of ligand to the chemoreceptors within these ternary complexes greatly diminishes $CheA_L$ autophosphorylation (3, 4, 28). Although $CheA_S$ lacks the histidine residue that is phosphorylated, it retains the domains of $CheA_L$ that are required for kinase activity, ternary complex formation, and phosphotransfer to CheY and CheB (6, 16, 17, 27, 42). Evidence indicates that these domains function in $CheA_S$. In vitro, $CheA_S$ mediates receptor-modulated transphosphorylation of mutant $CheA_L$ proteins deficient in kinase activity or lacking carboxy-terminal segments required for ternary complex formation (41, 45, 46). In vivo, $CheA_S$ restores chemotactic ability to cells that express either kinase-deficient or truncated $CheA_L$ mutant proteins (45, 46). In addition to these interactions, $CheA_S$ binds to and enhances the activity of CheZ, a protein that accelerates CheY dephosphorylation (21, 22, 43).

Rationale. Despite the evidence that $CheA_S$ can perform numerous chemotaxis-associated activities, its specific role in chemotaxis remains unknown. Clearly, it is not essential for chemotaxis: cells of *E. coli* do not require $CheA_S$ to perform chemotaxis under standard laboratory conditions (33). Yet, these cells and those of the related enteric species *S. typhimurium* express $CheA_S$ at levels approximating those of $CheA_L$ (33, 43). Since cells of the nonenteric species tested thus far express only $CheA_L$ (9, 31), we suspected that coexpression of $CheA_L$ and $CheA_S$ might be a unique characteristic of the family *Enterobacteriaceae*. To test this hypothesis, we performed a survey using immunoblot analysis to identify which family members coexpress $CheA_L$ and $CheA_S$ and, due to the proposed $CheA_S$/CheZ relationship, extended this survey to include CheZ.

Immunoblot analysis of CheA expression in enteric bacteria. We generated affinity-purified polyclonal rabbit antisera raised against purified *E. coli* CheA by standard procedures (14). To demonstrate the specificity of this antibody, we grew motile cells of *E. coli* to mid-exponential phase (optical density at 600 nm [OD_{600}] = ~0.3) before harvesting them for immunoblot analysis (14). Wild-type cells of *E. coli* (strains K-12 and ATCC 25922, both obtained from the American Type Culture Collection, and RP437 [30]) yielded three prominent immunoreactive proteins (Fig. 1A). In each case, the two larger proteins corresponded to $CheA_L$ (apparent molecular mass = ~73 to 74 kDa) and $CheA_S$ (apparent molecular mass = ~62 to 64 kDa). In contrast, the smallest protein (apparent molecular mass = ~49 kDa) was not derived from *cheA*, since cells from which *cheA* (strain AJW1071 [46]) was deleted synthesized neither $CheA_L$ nor $CheA_S$ yet expressed this cross-reactive protein. Cells that possess the *cheA169Y*(Am) allele (strain

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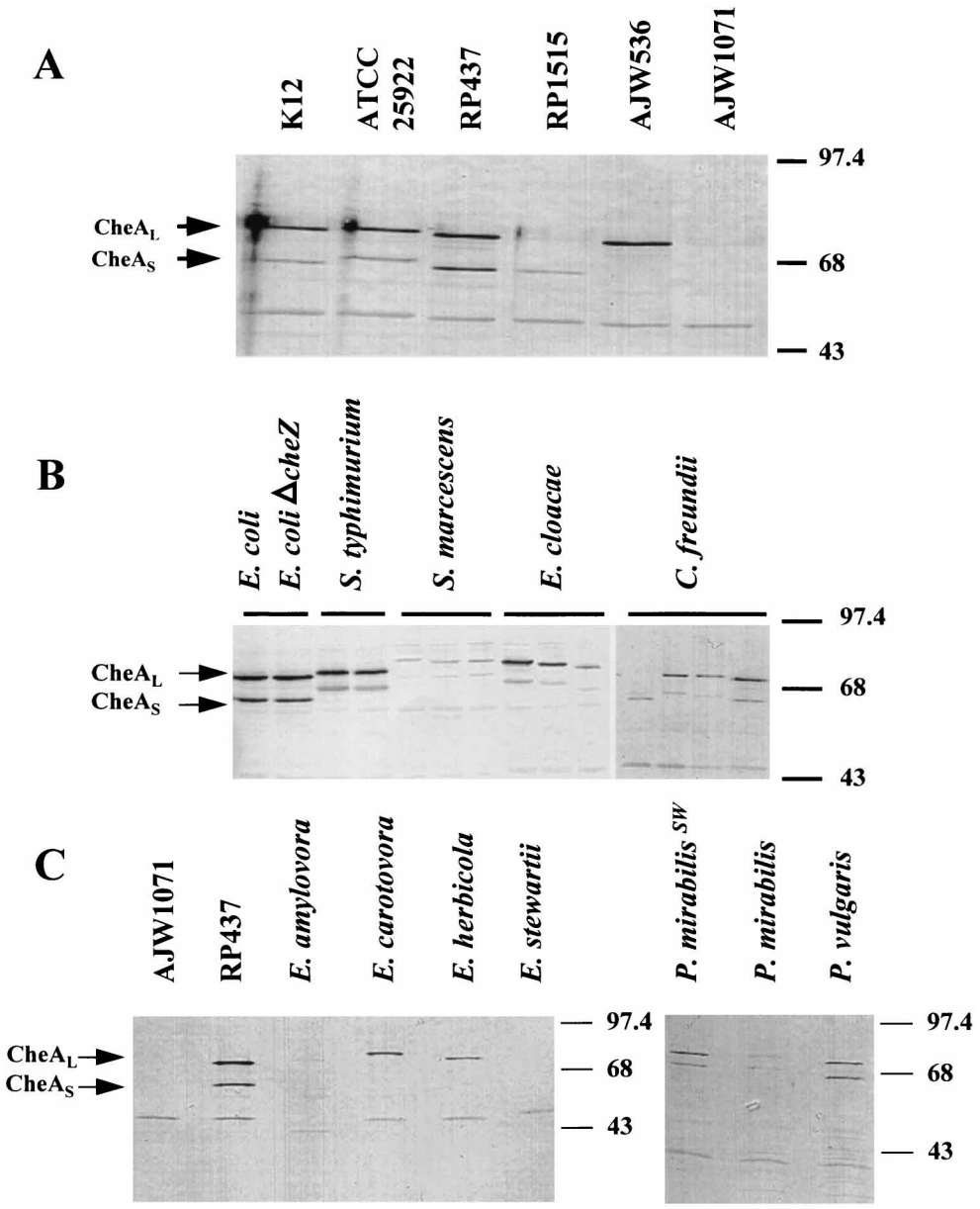

FIG. 1. Immunoblot analysis with *E. coli* CheA antibody after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10% polyacrylamide). (A) Lysates were prepared from *E. coli* laboratory strains (K-12, RP437, RP1515, AJW536, and AJW1071) and a human clinical isolate of *E. coli* (ATCC 25922). Cells were grown with aeration in tryptone broth (TB) (1% [wt/vol] tryptone, 0.5% [wt/vol] NaCl) at 31°C to an optical density at 600 nm of ~0.3 before being harvested. (B) Lysates were prepared from wild-type or Δ*cheZ* laboratory strains of *E. coli* (RP437 and RP1616, respectively [18]), from the wild-type laboratory *S. typhimurium* strain LT-2 (kindly provided by K. Hughes, University of Washington, Seattle), and from motile, chemotactic clinical isolates of *S. typhimurium*, *S. marcescens*, *E. cloacae*, or *C. freundii*. The first isolate of *C. freundii* was motile but nonchemotactic. Cells were grown as described for panel A. (C) Lysates were prepared from an *E. coli* strain from which *cheA* was deleted and one with wild-type *cheA* (laboratory strains AJW1071 and RP437, respectively), from plant pathogens of the genus *Erwinia* or from clinical isolates of human pathogens of the genus *Proteus*. Cells of *Proteus* spp. were handled as described for panel A, except that differentiated swarmer cells of *P. mirabilis* (SW) were grown on Luria-Bertani (TB supplemented with 0.5% [wt/vol] yeast extract) agar medium at 37°C until they covered most of the agar surface (2). Cells of *Erwinia* spp. were grown as described for panel A, except that PCG medium (1% [wt/vol] peptone, 0.1% [wt/vol] Casamino Acids, 0.2% [vol/vol] D-glucose) was substituted for TB. In panels B and C, note the presence of two cross-reactive proteins: one, which migrates slightly faster than CheA$_S$, elicits a rather faint signal; a second, which possesses an apparent molecular mass of ~50 kDa, elicits a relatively strong signal. In each panel, the arrows at the left of the blot point to the positions of *E. coli* CheA$_L$ and CheA$_S$ proteins and at the right of the blot the molecular mass standards are labeled in kilodaltons.

RP1515 [36]) cannot synthesize CheA$_L$ because of a UAG termination codon located between the CheA$_L$ and CheA$_S$ translation initiation sites. They synthesized the 62-kDa CheA protein but not the 73-kDa protein. In contrast, cells that possess the *cheA98ML* allele (strain AJW536 [23]) cannot synthesize CheA$_S$ due to a mutation that interrupts the CheA$_S$ translation initiation site. They synthesized the 73-kDa CheA protein but not the 62-kDa protein. Whereas the inability to complete CheA$_L$ translation resulted in reduced steady state levels of CheA$_S$, the inability to express CheA$_S$ exerted no effect upon the levels of CheA$_L$.

To determine whether other members of the family *Entero-*

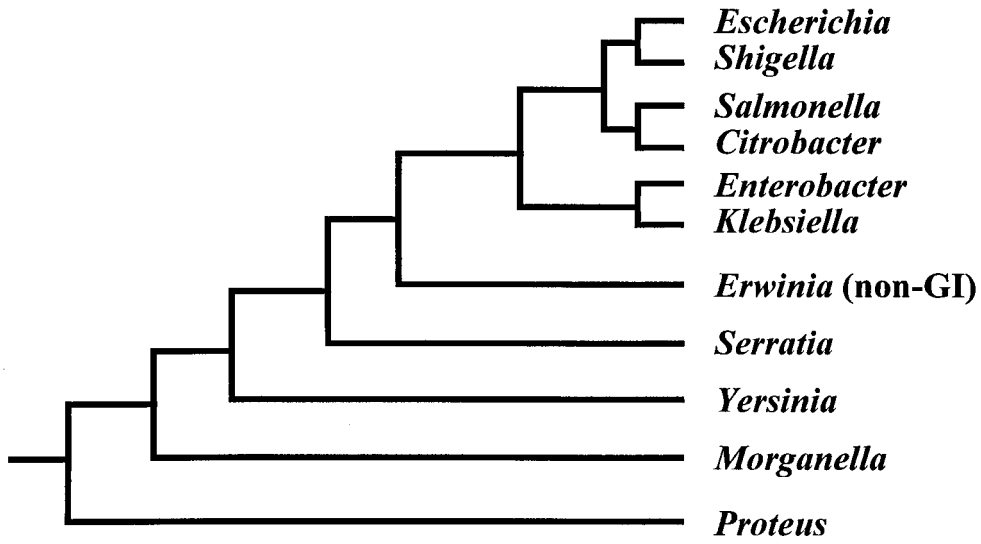

FIG. 2. Relatedness among the family *Enterobacteriaceae* (7). All genera are associated with the GI tract, except where noted.

bacteriaceae synthesize both $CheA_L$ and $CheA_S$, we performed immunoblot analyses on lysates from a variety of primary clinical isolates of enteric species (kindly provided by Roberta Carey, Clinical Microbiology Laboratory, Loyola University Chicago Hospital). Cells of chemotactic clinical isolates of *S. typhimurium*, *Serratia marcescens*, *Enterobacter cloacae*, and *Citrobacter freundii* synthesized two prominent immunoreactive proteins similar in size to *E. coli* $CheA_L$ and $CheA_S$. One motile, but nonchemotactic, isolate of *C. freundii* synthesized only $CheA_S$ (Fig. 1B). Chemotactic isolates of *Enterobacter aerogenes*, *Morganella morganii* (data not shown), and *Yersinia enterocolitica* (25) also synthesized both CheA proteins. Isolates of the nonmotile species *Klebsiella oxytoca*, *K. pneumoniae*, *Shigella flexneri* (data not shown), and *Yersinia pestis* (25) synthesized neither CheA protein.

As in *E. coli*, the two CheA proteins varied in size among isolates of the same species. Interestingly, the difference in the apparent sizes of the $CheA_L$ and $CheA_S$ proteins synthesized by *E. coli* (~9 kDa), *E. cloacae* (~8 kDa), and *C. freundii* (~7 kDa) strains was greater than the difference in apparent sizes of the $CheA_L$ and $CheA_S$ proteins synthesized by *S. typhimurium* (~5.5 kDa) and *S. marcescens* (~5.5 kDa).

To determine whether coexpression of $CheA_L$ and $CheA_S$ is characteristic of the entire family *Enterobacteriaceae* or only of those species that inhabit the lower gastrointestinal (GI) tract of vertebrates, we performed immunoblot analyses on cell lysates from a variety of plant pathogens of the closely related genus *Erwinia* (kindly provided by D. Coplin, Ohio State University) and from the more distantly related opportunistic human pathogens of the genus *Proteus* (Fig. 2). Like *E. coli* RP437, *P. mirabilis* and *P. vulgaris* coexpressed two immunoreactive bands (Fig. 1C). Furthermore, differentiated swarmer cells of *P. mirabilis* synthesized significantly more of both proteins than did undifferentiated swimmer cells. In contrast, *E. amylovora*, *E. carotovora*, and *E. herbicola* appeared to synthesize a single immunoreactive band of about 72 to 74 kDa. Since these cells are chemotactic, it is likely that this band corresponds to $CheA_L$. The nonmotile *E. stewartii* synthesized no detectable CheA protein.

Immunoblot analysis of CheZ in enteric bacteria. Since experimental evidence indicates that $CheA_S$ and CheZ interact specifically (21, 22, 43), we surmised that CheZ, like $CheA_S$, is a component of the chemotactic signal transduction pathway shared by most, if not all, motile enteric bacteria. To test this hypothesis, we performed immunoblot analyses using an *E. coli* CheZ antibody (kindly provided by H. Wang and P. Matsumura, University of Illinois at Chicago) on lysates of several enteric species (Fig. 3A). Like *E. coli* and *S. typhimurium*, chemotactic clinical isolates of *S. marcescens* and *E. cloacae* synthesized an immunoreactive protein with a molecular mass that approximates that of *E. coli* CheZ. Cells of *P. mirabilis*, *P. vulgaris*, and the chemotactic *Erwinia* spp. also expressed this protein (Fig. 3B).

Sequence analysis of the 5′ end of enteric *cheA* genes. To determine whether the two immunoreactive proteins observed in cell lysates from motile enteric bacteria were indeed $CheA_L$ and $CheA_S$, we examined the nucleotide sequence of the 5′ portion of the *cheA* gene derived from each of several enteric species. To do so, we used two primers to amplify the region of *cheA* that would include translation initiation sites of both $CheA_L$ and $CheA_S$. Since one primer must bind to sequences upstream of *cheA*, we designed an oligonucleotide (forward primer F1.1) that would prime within the 3′ region of *motB*, the gene located immediately upstream of *cheA* in the *mocha* operon of *E. coli* and *S. typhimurium* (Fig. 4). To identify the most conserved sequences within this region, we aligned the *motB* sequences from *E. coli* (37), *S. typhimurium* (39), and *B. subtilis* (26). To ensure that the PCR products included all sequences encompassing the $CheA_S$ initiation codon, we designed a reverse primer (R1) complementary to a sequence that encodes a portion of the highly conserved CheA transmitter domain located about 550 bp 3′ of the $CheA_S$ initiation codon. For this purpose, we aligned *cheA* sequences from *E. coli* (17), *S. typhimurium* (38), *B. subtilis* (9), *Caulobacter crescentus* (1), and *Pseudomonas aeruginosa* (29).

We isolated chromosomal DNA as described previously (24). We performed PCR reactions in an Omn-E Thermal Cycler (Hybaid, Middlesex, United Kingdom) beginning with a 5-min denaturation step at 94°C and a 3-min "hot start" addition of *Taq* polymerase at 80°C, followed by 30 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 52°C, and extension for 1 min at 72°C. We completed the amplification with a 5-min extension step at 72°C, purified the resultant fragments by passage through Wizard PCR Prep columns

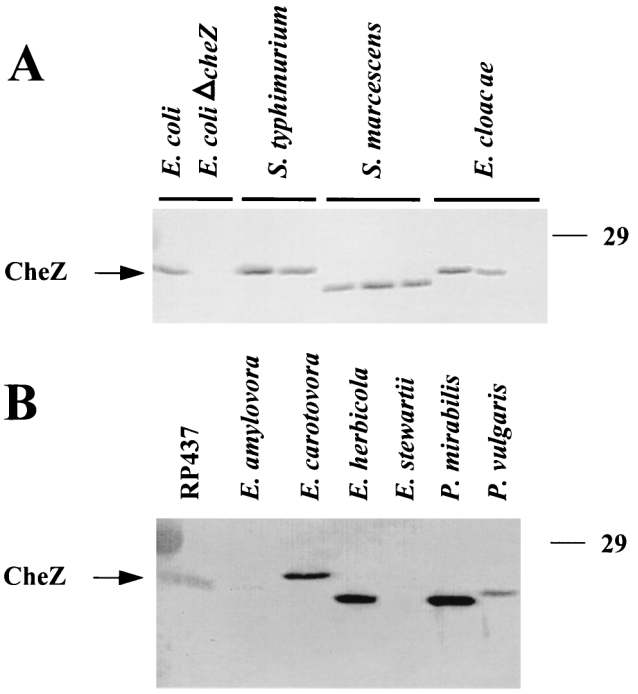

FIG. 3. Immunoblot analysis with *E. coli* CheZ antibody after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (15% polyacrylamide). (A) Lysates were prepared from wild-type or Δ*cheZ* laboratory strains of *E. coli* (RP437 and RP1616, respectively), from the wild-type laboratory *S. typhimurium* strain LT-2 or from motile clinical isolates of *S. typhimurium*, *S. marcescens*, and *E. cloacae*. Cells were grown as described for Fig. 1A. Note the very faint signal from the third *E. cloacae* isolate (lane 10), a result consistent with that observed with anti-CheA antibody (Fig. 1B, lane 10). Molecular mass standards are labeled in kilodaltons to the right of the blot. (B) Lysates were prepared from wild-type *E. coli* (laboratory strain RP437), from plant pathogens of the genus *Erwinia*, or from clinical isolates of human pathogens of the genus *Proteus*. Cells were grown as described for Fig. 1C. Note the very faint signal from cells of *E. amylovora* (lane 2), a result consistent with the low CheA expression observed for that isolate (Fig. 1C, lane 3). Molecular mass standards are labeled in kilodaltons to the right of the blot.

(Promega), ligated the purified fragments into the vector pGEM-T (Promega), and transformed the ligation mixtures into strain AJW399, a Δ*cheA* derivative of strain JM107 (47). We identified recombinant plasmids containing *cheA* nucleotide sequences by colony lift hybridization using the BioTrace NT Binding Matrices protocol (Gelman Sciences, Ann Arbor, Mich.). All filters were incubated for approximately 16 h at 45°C and probed with a 516-bp *Nde*I-*Sal*I fragment from the 5′ region of the *E. coli cheA* gene labeled by incubation with T4 polynucleotide kinase and [γ-^{32}P]ATP (32).

As predicted, these primers amplified a product of about 1.0 kb from chromosomal DNA isolated from wild-type *E. coli* cells but not from cells from which *cheA* was deleted. They also amplified products of about 1.0 kb from *S. typhimurium*, *S. marcescens*, *P. mirabilis*, and one isolate of *E. cloacae*. In contrast, they amplified smaller products, which ranged from about 0.6 kb to about 0.9 kb, from *E. herbicola*, *P. vulgaris*, *C. freundii*, and a second isolate of *E. cloacae* (data not shown).

Subsequently, we cloned the 1.0-kb products obtained from *S. marcescens* and one of the *E. cloacae* isolates as well as a 0.9-kb product from the second *E. cloacae* isolate into plasmid pGEM-T for DNA sequence analysis (Fig. 5). The first 414 nucleotides of *cheA* from *E. coli*, *S. typhimurium*, *E. cloacae*, and *S. marcescens* shared considerable identity. *E. cloacae* and *S. marcescens* were most similar (87% identity), whereas *E. coli* and *S. marcescens* were least similar (75% identity). All four species shared several features associated with translation. These include (i) the ribosome binding site (RBS) centered 8 bp upstream of the GTG that encodes the translation initiation codon for $CheA_L$ in *E. coli* and *S. typhimurium* (17, 38); (ii) the RBS centered 7 bp upstream of the ATG that encodes the translation initiation codon for $CheA_S$ in *E. coli* (17); and (iii) the RBS centered 5 bp upstream of the ATG that is reported to encode the translation initiation codon for $CheA_S$ in *S. typhimurium* (39).

Coexpression of $CheA_S$ and CheZ is limited to enteric bacteria. Intriguingly, no one has yet reported the coexpression of $CheA_L$ and $CheA_S$ from motile, chemotactic species of nonenteric bacteria. No internal translation initiation sites are apparent within the *cheA* genes from *B. subtilis* (9), *Rhizobium meliloti* (13), *Rhodobacter sphaeroides* (44), *Halobacterium salinarium* (31), *Listeria monocytogenes* (8), *Caulobacter crescentus* (1), and *Pseudomonas aeruginosa* (29). Additionally, *cheA* expression studies reveal that motile cells of *H. salinarium* and *B. subtilis* express only the full-length CheA protein (9, 31). In contrast, a wide variety of motile, chemotactic members of the family *Enterobacteriaceae* that are commensal or pathogenic in the lower GI tract of vertebrates coexpressed $CheA_L$ and $CheA_S$. Under the conditions we tested, species of *Erwinia* expressed only the larger of these two proteins. Since these plant pathogens are rarely associated with human colonization or disease (35), we suspect that $CheA_S$ plays some role under conditions encountered within the predominately anaerobic environment of the lower GI tract.

Similarly, with the exception of *P. aeruginosa* (20), no CheZ homologs have been reported outside the *Enterobacteriaceae*. Thus, it seems that coexpression of $CheA_S$ and CheZ might

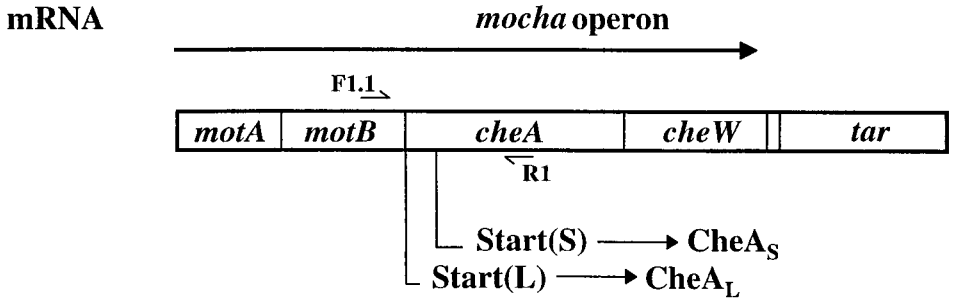

FIG. 4. Schematic diagram of the *mocha* operon of *E. coli*. Start(L) and Start(S) represent the translation initiation sites for $CheA_L$ and $CheA_S$, respectively. Arrows represent the PCR primers F1.1 and R1, based, respectively, on a conserved region in the 3′ end of the *motB* genes from *E. coli*, *S. typhimurium*, and *Bacillus subtilis* and a conserved region in the 5′ end of the *cheA* genes from *E. coli*, *S. typhimurium*, *Caulobacter crescentus*, and *Pseudomonas aeruginosa.*

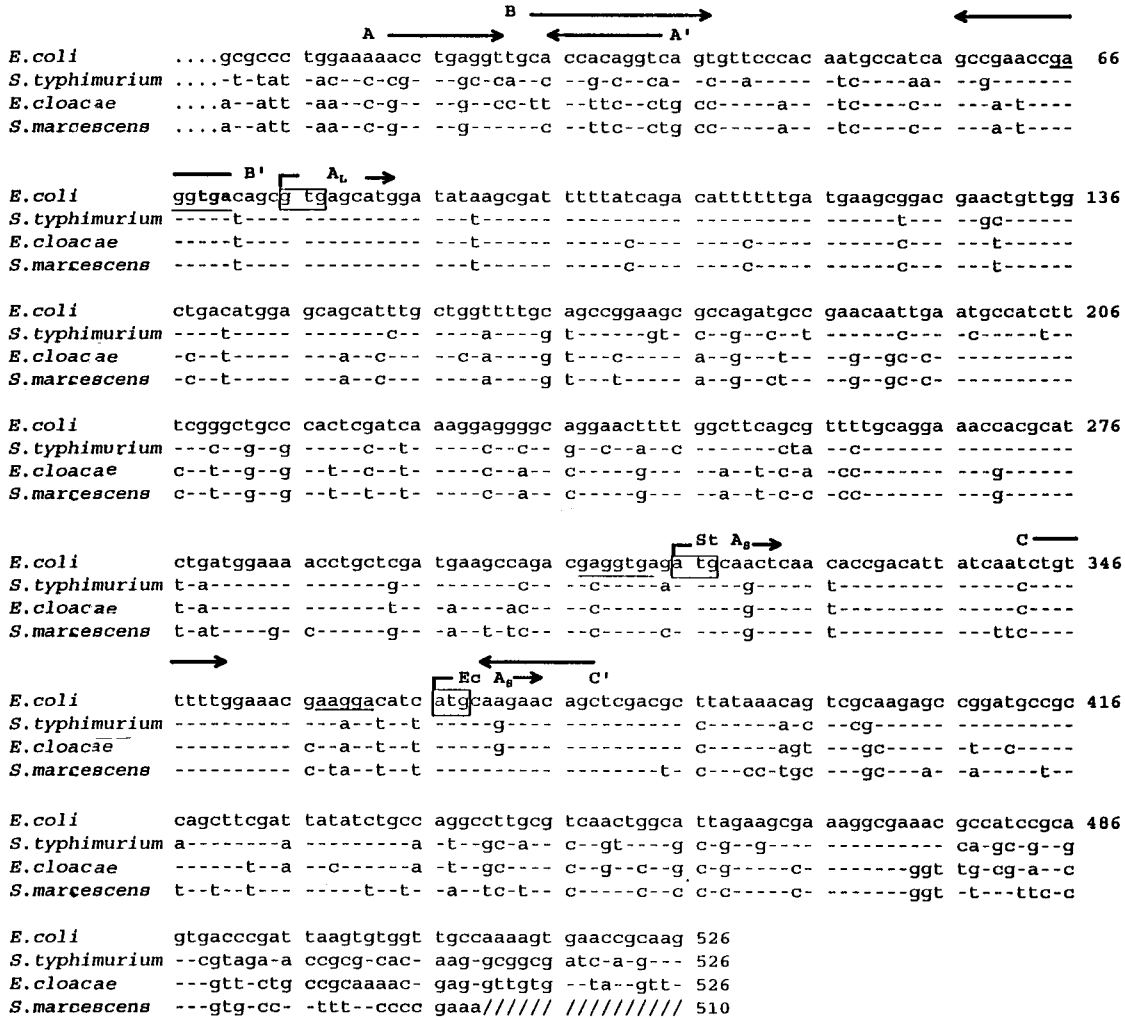

FIG. 5. Comparison of nucleotide sequences spanning the 3′ MotB-5′ CheA region. *E. coli* and *S. typhimurium* sequences have been reported previously (16, 36, 39). We sequenced a *S. typhimurium* clinical isolate: within this region, its sequence was identical to the sequence published for strain LT-2. We also sequenced two independent clinical isolates of *E. cloacae*, one of which yielded a 1.0-kb PCR product and one which yielded a 0.9-kb product: within the region shown, their sequences were identical. The *S. marcescens* sequence was obtained from two independent PCR products of a single clinical isolate. Double-stranded DNAs were sequenced by the chain termination method of Sanger et al. (34) with Sequenase, version 2.0 (U.S. Biochemicals, Cleveland, Ohio) or with a *Taq*-DyeDeoxy Terminator Cycle Sequencing kit developed by Applied Biosystems Inc. (Perkin-Elmer Corp., Foster City, Calif.). DNA sequence analysis was performed on both strands with an ABI Prism 377 automated DNA sequencer. To align nucleotide sequences, the Genetics Computer Group program Pileup (12) was used with a gap weight of 3.0 and a gap length weight of 0.1. Only differences from the published *E. coli* sequence are shown. The putative Shine-Dalgarno sequences are underlined, and verified or putative translation initiation codons are enclosed in boxes. The TGA that encodes the termination codon of *motB* is in boldface type. Boldface arrows above the sequence represent inverted repeats predicted to form mRNA secondary structures (16). Slashes represent missing nucleotides. Numbering of nucleotides is arbitrary.

only occur in the enteric bacteria. Since $CheA_S$ regulates the activity of CheZ, their codistribution strengthens the argument that their interaction plays an important role in vivo (21, 22, 43).

Alternative $CheA_S$ translation initiation sites. The difference in electrophoretic mobility between $CheA_L$ and $CheA_S$ in *E. coli* is greater than that in *S. typhimurium*. The initiation site proposed for *S. typhimurium* $CheA_S$ (38) does not correspond to the initiation site reported for *E. coli* $CheA_S$ (17, 33). Our observations support this hypothesis. It seems that in each species there are two internal initiation sites, each of which can potentially act to direct translation of a short version of CheA. Seemingly, *S. typhimurium* uses the upstream site while *E. coli* uses the downstream one. On the basis of immunoblot analyses, we conclude that *S. marcescens* uses the upstream site while *E. cloacae* and *C. freundii* use the downstream one. Although the apparent lack of a Shine-Dalgarno sequence explains why the downstream site would not initiate translation in *S. marcescens*, there exist no obvious features that can explain why *E. coli* and *E. cloacae* seemingly use the downstream site while *S. typhimurium* preferentially uses the upstream one.

To reconcile the observation that *E. coli* does not require $CheA_S$ for chemotaxis under typical aerobic laboratory conditions (33) with the fact that it synthesizes $CheA_L$ and $CheA_S$ in approximately equivalent amounts (33, 43), it has been suggested that $CheA_S$ might be an evolutionary relic (33). Although it no longer possesses a physiological role, the argument goes, it continues to be expressed because of constraints placed on the nucleotide sequence by the amino acid sequence of $CheA_L$. In light of our observation that enteric bacteria utilize two different internal sequences to initiate translation of $CheA_S$, it seems unlikely that such constraints exist.

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