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Dual Chemotaxis Signaling Pathways in *Bacillus subtilis*: a σ^D-Dependent Gene Encodes a Novel Protein with Both CheW and CheY Homologous Domains

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The alternative sigma factor, σ^D, activates the expression of genes required for chemotaxis and motility in *Bacillus subtilis*, including those encoding flagellin, hook-associated proteins, and the motor proteins. The σ^D protein is encoded in a large operon which also encodes the structural proteins for the basal body and homologs of the enteric CheW, CheY, CheA, and CheB chemotaxis proteins. We report the identification and molecular characterization of a novel chemotaxis gene, *cheV*. The predicted CheV gene product contains an amino-terminal CheW homologous domain linked to a response regulator domain of the CheY family, suggesting that either or both of these functions are duplicated. Transcription of *cheV* initiates from a σ^D-dependent promoter element both in vivo and in vitro, and expression of a *cheV-lacZ* fusion is completely dependent on *sigD*. Expression is repressed by nonpolar mutations in structural genes for the basal body, *fliM* or *fliP*, indicating that *cheV* belongs to class III in the *B. subtilis* flagellar hierarchy. The *cheV* locus is monocistronic and is located at 123° on the *B. subtilis* genetic map near the previously defined *cheX* locus. A *cheV* mutant strain is motile but impaired in chemotaxis on swarm plates. Surprisingly, an insertion in the CheW homologous domain leads to a more severe defect than an insertion in the CheY homologous domain. The presence of dual pathways for chemotactic signal transduction is consistent with the residual signaling observed in previous studies of *cheW* mutants (D. W. Hanlon, L. Márques-Magaña, P. B. Carpenter, M. J. Chamberlin, and G. W. Ordal, J. Biol. Chem. 267:12055–12060, 1992).

The *Bacillus subtilis* alternative sigma factor, σ^D, activates the expression of a dozen or more operons required for chemotaxis and motility (for reviews, see references 14 and 31). The σ^D regulon is activated in late logarithmic growth during the transition to stationary phase (12, 26). This so-called transition state is a time of complex decision making (41). The cell senses the nature of available nutrients and responds by the activation of numerous secondary metabolic processes, including genetic competence, degradative enzyme synthesis, antibiotic synthesis, and motility. Under appropriate conditions, a dormant endospore can be produced.

Homologs of the *B. subtilis* σ^D protein (generically known as σ^{28} factors) have been described for a variety of gram-positive and gram-negative bacteria, including *Escherichia coli*, *Salmonella typhimurium*, *Pseudomonas aeruginosa*, *Vibrio parahaemolyticus*, and *Streptomyces coelicolor* (7, 14, 24, 39). With the interesting exception of *S. coelicolor* (7), these σ^{28} proteins all control expression of one or more flagellin genes. Sequence comparisons reveal that these σ factors form a closely related family of proteins known as the flagellar cluster (20). In addition, a great many other bacteria contain flagellin genes with promoter elements (P_{28}) which predict the presence of a corresponding σ^{28}-like regulatory factor (14). Thus, the σ^{28} family of regulatory proteins appears to be broadly distributed.

The extensive genetic characterization of the enterobacterial flagellar regulon provides us with our most complete picture of gene regulation by a member of the σ^{28} family of proteins (21). The genes required for flagellar synthesis are transcriptionally activated in a complex hierarchy. The *flhDC* master regulators are expressed under the influence of the cyclic AMP receptor protein and are essential for the transcription of the class II genes. One of the class II genes, *fliA*, encodes a σ factor (σ^F) homologous to *B. subtilis* σ^D (29). The σ^F protein, in turn, activates transcription of the class III flagellar and chemotaxis genes. The activity of σ^F is controlled by one of the class III genes, *flgM*, which encodes an anti-σ factor that prevents σ^F activity unless the products of all the class II genes are expressed and functional (11).

The flagellar regulon in *B. subtilis* is not as well characterized as that in the enterobacteria, but it appears to differ in several respects (5). Remarkably, most of the identified flagellar structural genes are in the large *flaA* operon, which spans at least 25 kb. These genes include those for the basal body structure, the σ^D protein, and homologs of the *E. coli* CheA, CheB, CheY, and CheW proteins (31). Peak expression of the *sigD* gene occurs at the end of logarithmic phase growth (19). The σ^D protein activates transcription of the flagellin gene (*hag*) (26), the *motAB* operon (27), an operon that encodes FlgM and FlgK homologs (31), genes for the methyl-accepting chemotaxis proteins (MCPs) (22, 31), the major autolysin (19), and an operon which encodes a hook-associated protein (*fliD*) (7a). Expression of the *hag* (flagellin) and the *fliD* genes is eliminated in strains containing disruptions in either the *fliM* or *fliP* gene (7a, 50), suggesting that *B. subtilis* flagellar genes can also be divided into classes II and III. It is not yet known, however, whether there is a master regulator for the *B. subtilis* flagellar regulon. In fact, a number of pleiotropic regulatory genes affect flagellar expression (1, 31, 34, 41), indicating that motility and chemotaxis are coregulated with many other transition state functions, including competence, induction of degradative enzymes and antibiotics, and perhaps sporulation.

In this report, we describe the cloning and characterization of a new chemotaxis gene, *cheV*, under control of the σ^D form of RNA polymerase. Transcriptional analyses suggest that this gene is in class III of the flagellar hierarchy. The CheV protein

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TABLE 1. *B. subtilis* strains

Strain	Genotype	Relevant characteristic(s)	Source or reference
CU1065	W168 *trpC2 attSP* β		Zahler
OI1085	*trpF7*(Am) *hisB*(Am) *met*	Che^+	Ordal
OI2737	OI1085 *cheW*::*cat*	$CheW^-$	13
OIB055	OI1085 *cheY*::*cat*	$CheY^-$	4
CB100	*trpC2 sigD*::pLM5	$SigD^-$	22
OI2537	*fliM*::*cat*	$FliM^-$	Ordal
OI2826	OI1085 *fliP*::*cat*	$FliP^-$	Ordal
HB4001	CU1065::pKF10	$CheV^+$	This work
HB4003	OI1085::pKF22	*cheV-lacZ* fusion	This work
HB4004	OI1085 *cheV*::*kan*	$CheV^-$	This work
HB4006	OI1085 *cheV*::*kan*	CheV (truncated)	This work
HB4009	OI2537::pKF22	$FliM^-$, *cheV-lacZ* fusion	This work
HB4010	OI2826::pKF22	$FliP^-$, *cheV-lacZ* fusion	This work
HB4011	CB100::pKF22	$SigD^-$, *cheV-lacZ* fusion	This work
HB4019	HB4004::pKF11	$CheV^+$	This work

TABLE 2. Plasmids

Plasmid	Description	Source or reference
pGW7	pBR322-based expression plasmid	38
pJH28-1	pGW7 containing 4.1-kb *Eco*RI insert from phage λgt11#1	This work
pJM114	Contains Kan^r cassette	33
pDEB8	Plasmid for integrating genes at *amy* (Cm^r)	6
pTKlac	Integrational *lacZ* fusion plasmid (Cm^r)	17
pAC7	Plasmid for integrating genes at *amy* (Kan^r)	T. Msadek
pGEM-*cat*	Integration vector for use in *B. subtilis* (Cm^r)	49
pKF1	pGEM-*cat* containing *Eco*RI-*Pst*I fragment from pJH28-1	This work
pKF2	$pBKSII^+$ containing 752-bp *Hin*dIII fragment from pKF1	This work
pKF3	$pBKSII^+$ containing 615-bp *Eco*RI-*Hin*dIII fragment from pKF1	This work
pKF10	pKF1 with deletion of *Bcl*I (position 330 in *cheV*) to *Pst*I	This work
pKF11	pDEB8 containing 1.5-kb *Eco*RI-*Bgl*II fragment of pJH28-1	This work
pKF13	$pBKSII^+$ containing *Eco*RI-*Pst*I fragment from pKF1	This work
pKF21	pTKlac containing *Eco*RI-*Bcl*I (at 330) from *cheV*	This work
pKF22	pKF21 containing Kan^r from pJM114 (as *Eco*RI-*Kpn*I fragment)	This work
pKF24	pKF2 containing Kan^r from pAC7 in *Bcl*I site (at 820)	This work
pKF25	pKF3 containing Kan^r from pAC7 in *Bcl*I site (at 330)	This work
pKF37	pET16x containing carboxyl-terminal 270 codons of *cheV*	This work

contains domains homologous to the CheW and CheY proteins encoded by the large *flaA* operon, suggesting that *B. subtilis* may have two partially overlapping chemotaxis-signaling pathways. This hypothesis is explored further in the accompanying manuscript (36).

MATERIALS AND METHODS

Bacterial strains and plasmids. All bacterial strains and plasmids used in this study are listed in Tables 1 and 2. The *cheV* gene was subcloned from phage λ (see below) to a pBR322-based plasmid, pGW7, to generate pJH28-1. The region of pJH28-1 containing the *cheV* locus was subcloned into pGEM-*cat* as a 1.5-kb *Eco*RI-to-*Pst*I fragment to generate pKF1. The 615-bp *Eco*RI-to-*Hin*dIII region containing the $P_{D\text{-}cheV}$ promoter and 5′ end of *cheV* was cloned into $pBKSII^+$ to generate pKF3. The adjacent 752-bp *Hin*dIII fragment containing the 3′ end of *cheV* was cloned into $pBKSII^+$ to generate pKF2.

To create a *cheV-lacZ* fusion, the *Eco*RI-to-*Bcl*I (293-bp) fragment of *cheV* was cloned from pKF1 into the integrational vector, pTKlac (17), to generate plasmid pKF21. To allow selection of integrants by kanamycin resistance, a cassette from plasmid pJM114 (33) was removed as an *Eco*RI-to-*Kpn*I fragment and cloned into pKF21 to generate pKF22. In this transcriptional fusion vector, *lacZ* expression initiates from the *spoVG* ribosome-binding site under control of the $P_{D\text{-}cheV}$ promoter.

To create gene disruptions in the amino-terminal and carboxyl-terminal domains of CheV, a kanamycin resistance cassette was inserted into either of the two *Bcl*I sites. For the amino-terminal insertion, the kanamycin resistance gene was removed from plasmid pAC7 as a *Bgl*II-to-*Bam*HI fragment and cloned into the unique *Bcl*I site of plasmid pKF3 to generate pKF25 (Kan^r in the opposite orientation from that of *cheV*). For the carboxyl-terminal insertion, the same Kan^r gene was cloned into the unique *Bcl*I site of plasmid pKF2 (Table 2) to generate pKF24 (Kan^r in the same orientation as that of *cheV*).

For complementation studies, the entire *cheV* gene was removed from pJH28-1 as a 1.5-kb *Eco*RI-to-*Bgl*II fragment and cloned into plasmid pDEB8 (6) to generate pKF11. In this plasmid, the *cheV* gene is flanked by sequences from the amylase locus to allow for double crossover gene replacement into *amyE*. Recombinants were screened for an inability to degrade starch on plates.

For expression of a truncated *cheV* gene in *E. coli*, a 1.3-kb *Bsp*HI-to-*Bam*HI fragment from pKF13 was subcloned into the overexpression plasmid pET16x (Novagen, Madison, Wis.) after digestion with *Nco*I and *Bam*HI to generate plasmid pKF37. This plasmid allows expression of a 270-amino-acid carboxyl-terminal fragment of CheV.

Isolation of the *cheV* gene. The DNA segment containing the *cheV* gene was originally cloned from a λgt11 library (43) during screens aimed at the isolation of the *sigD* gene (15, 16). Approximately 8×10^4 λgt11 phage plaques were screened with mouse anti-σ^D antibodies, and five clones expressing cross-reactive proteins were identified. All five phage contained overlapping genomic segments varying in size from 2.3 to 4.1 kb. The region common to all five inserts begins in the carboxyl-terminal region of the *cheV* gene and extends about 2 kb downstream. The reason for the original isolation of these five phage with anti-σ^D antisera is unknown.

The largest phage insert (λgt11#1) contained adjacent 0.75- and 2.8-kb *Hin*dIII fragments. Southern hybridization analysis performed on isolated *B. subtilis* W168 chromosomal DNA verified that this region was isolated without gross alteration (data not shown). Approximately 3 kb of DNA sequence from this region of λgt11#1 was determined. To confirm that this sequence was not altered by propagation in phage λ, we have

recloned the *cheV* region from *B. subtilis* CU1065 DNA by vector rescue and sequenced the recovered chromosomal DNA by using modified T7 DNA polymerase (Sequenase) and the Sequenase kit (U.S. Biochemical). First, plasmid pKF1 was digested by *Bcl*I and *Pst*I, treated with the Klenow fragment of DNA polymerase I, and ligated to generate a deletion derivative, pKF10, which retains 297 bp of *B. subtilis* DNA between an *Eco*RI site and the modified *Bcl*I site in *cheV*. Purified chromosomal DNA from strain HB4001(CU1065::pKF10) was treated with either *Nco*I or *Sph*I and recircularized to allow recovery of upstream or downstream linked DNA, respectively.

Primer extension mapping. RNA was isolated from CU1065 and CB100 at time T_{-1}, T_0, and T_1 by a method previously described (12), except that the cells were extracted three times with hot phenol immediately after sonication and RQ1 DNase I (ProMega Biotech) was substituted for proteinase K-treated DNase I. Time T_0 is defined by the intercept of the linear portion of a logarithmic growth plot with the line representing stationary phase and occurs about 20 min after deviation from log phase growth. Time T_{-1} and T_1 are 60 min before and after T_0, respectively. Primer extension analysis of in vivo RNA samples was performed as described by Kuroda and Sekiguchi (19). The 17-mer oligonucleotide primer 1 (5′-GCATTTTCA CCCACGCC-3′) was labeled with [γ-^{32}P]ATP and polynucleotide kinase (37). Labeled primer 1 (2 pmol) was added to RNA (80 to 100 μg) and precipitated with ethanol. The pellet was resuspended in 20 μl of hybridization buffer (60 mM NaCl, 50 mM Tris-HCl [pH 8.0], 10 mM dithiothreitol), heated to 90°C for 3 min, and cooled slowly to 40°C. Thirty microliters of an extension solution (60 mM NaCl, 50 mM Tris-HCl [pH 8.0], 13 mM dithiothreitol, 10 mM $MgCl_2$, 7.5 mM deoxynucleoside triphosphates, 10 U of avian myeloblastosis virus reverse transcriptase) was added, and the reaction mixture was incubated at 37°C for 60 min. Nucleic acids were then extracted with phenol-$CHCl_3$, precipitated with ethanol, resuspended in 10 μl of 0.1 M NaOH, and heated at 95°C for 3 min. Samples were separated by electrophoresis on a 7 M urea–6% polyacrylamide denaturing gel. Double-stranded pKF1 was sequenced by using the identical primer, and the reaction products were electrophoresed adjacent to the primer extension products.

To map the in vitro transcription start site, supercoiled pKF1 was used as a template for in vitro transcription by reconstituted σ^D holoenzyme as described previously (16). Purified core RNA polymerase (0.5 μg) was added to 2.5 μg of template with and without 0.5 μg of purified σ^D protein in transcription buffer (40 mM Tris [pH 8.0], 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 400 μM deoxynucleoside triphosphates). Reaction mixtures were incubated at 37°C for 15 min, and the nucleic acids were precipitated with ethanol. Two picomoles of labeled primer 1 was added, and the primer extension reactions were carried out and analyzed as described above.

Chemotaxis assays. Chemotactic proficiency was judged by the rate of swarm formation on soft agar plates (0.3% agar). Tryptone and minimal medium swarm plates containing mannitol as an attractant were prepared as described previously (32). Cells were spotted with a toothpick, and swarm formation was allowed to proceed for 8 to 24 h at 37°C.

Genetic techniques. Transformation of *B. subtilis* was performed by using an improved, one-step method developed by F. Kunst and coworkers (18). Briefly, this procedure involves the growth of cells in modified competence medium (MC medium) (a glucose minimal medium supplemented with 0.1% casein hydrolysate and 0.2% potassium glutamate) until 60 to 90 min after T_0. DNA is added, and incubation at 37°C is continued for 60 min prior to selection of transformants.

The *cheV* gene was mapped by hybridization of labeled oligonucleotide probe 1 to a nitrocellulose filter containing an ordered collection of *B. subtilis* chromosomal DNA cloned into yeast artificial chromosomes (2). Strong hybridization was found to clone F3-containing DNA from the *ptsI* locus (118°) to near *ctaD* at 127° on the genetic map. Since hybridization was not detected to the two overlapping clones (15-115 and 15-112), we conclude that *cheV* is located between *pts* and *ctaD* near 123°. This position is consistent with the results of phage PBS1-mediated transduction with a collection of mapped Tn*917* (MLS^r)-containing strains as recipients (44). The Cm^r marker in strain HB4001 was found to be linked by transduction with the MLS^r markers of strain CU4152 (map position 126°) and strain CU4151 (map position 121°).

β-Galactosidase assays. Cells were grown in 2× YT (37), and 1-ml samples were removed at the indicated time points. Assays for β-galactosidase were performed as described previously (8).

Nucleotide sequence accession numbers. The sequence of this region together with 1.5 kb of downstream flanking DNA has been submitted to the EMBL and GenBank data bases under accession no. Z29584 and U05345, respectively.

RESULTS

Sequence of the *cheV* gene. In experiments aimed at the cloning of the structural gene for the σ^D polypeptide, five independent λgt11 phage that expressed proteins reactive with polyclonal anti-σ^D antibodies were identified (see Materials and Methods). However, DNA sequence analysis failed to reveal any plausible candidates for the *sigD* gene, which was subsequently isolated by using a degenerate oligonucleotide probe (15). Comparison of the predicted polypeptides encoded by the cloned *B. subtilis* DNA with computer data base sequences revealed the presence of a putative gene, *cheV*, homologous to both of the enterobacterial chemotaxis proteins, CheW and CheY. The major *B. subtilis* flagellar operon, *flaA*, also encodes homologs of CheW and CheY (4, 13, 15, 31), suggesting that either or both of these functions may be duplicated in *B. subtilis*. A schematic of the *cheV* region of the chromosome is shown in Fig. 1.

The sequence of 1,681 bp of *B. subtilis* DNA is presented in Fig. 2. Immediately upstream of the *cheV* gene is a candidate promoter element for recognition by the σ^D form of RNA polymerase (see below). The predicted *cheV* gene initiates with a UUG start codon, which is relatively common in *B. subtilis* (45). The putative ribosome-binding site (UAGCGAGGGA) matches the 3′ end of the *B. subtilis* 16S rRNA at the indicated seven positions and is predicted to lead to efficient translation (45).

Following the termination codon of the *cheV* gene is a factor-independent terminator. This sequence could function to terminate transcription from either direction as, in both cases, a string of U residues is encoded following a hairpin structure. Indeed, this bidirectional terminator has been previously characterized during in vitro studies of factor-independent termination by *E. coli* RNA polymerase and is equally efficient in either orientation (35). It is likely that this inverted repeat element functions as a bidirectional terminator in vivo since a convergent gene (*orfB*) terminates very close to the downstream boundary of the repeat (Fig. 1). The predicted OrfB gene product is a 142-amino-acid polypeptide with no significant similarity to other proteins in the GenBank data

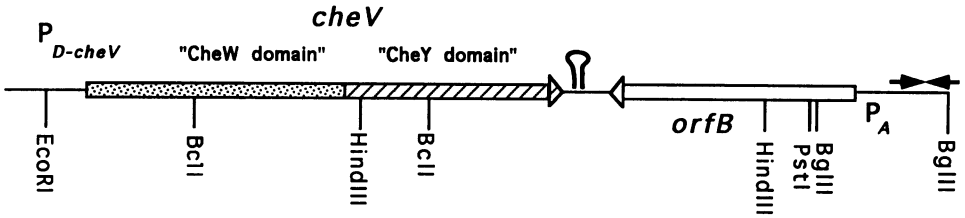

FIG. 1. Schematic of *cheV* region. The convergent *cheV* and *orfB* genes are illustrated, together with restriction sites mentioned in the text. The hairpin structure represents the bidirectional terminator between the two open reading frames, and the arrows upstream of *orfB* indicate an inverted repeat element.

base. The *orfB* gene may be transcribed from the putative σ^A-dependent promoter element indicated in Fig. 2.

Sequence of the CheV protein. The amino-terminal region of the predicted CheV protein is 27% identical to the *E. coli* CheW protein. This degree of similarity is somewhat greater than that between CheV and the other sequenced *B. subtilis* CheW homolog (25%) or the related FrzA protein of *Myxococcus xanthus* (21%) (13, 23). A sequence comparison of the *E. coli* and *B. subtilis* CheW proteins with the amino-terminal domain of CheV is presented in Fig. 3A. Apart from known CheW homologs, no significant similarity was detected between the amino-terminal portion of CheV and other proteins in the GenBank data base.

The carboxyl-terminal domain of the CheV protein is 26% identical to the *E. coli* CheY protein (Fig. 3B). As was observed for the CheW homologous region, this is somewhat greater than the identity between CheV and the *B. subtilis* CheY protein (24%) or the related PilG protein from *P. aeruginosa* (25%) (4, 9). Interestingly, this domain of CheV is most closely related to the *M. xanthus* FrzE protein (35% identity), which is also a two-domain chemotaxis protein containing, in this case, CheA and CheY homologous portions (25).

CheY homologous domains are found in a large number of regulatory proteins, and significant similarity was observed between CheV and many of these response regulator proteins. The CheV protein contains all of the most highly conserved residues of response regulators, including the two acidic residues which form a Mg(II)-binding pocket (CheV residues E-181 and D-182), the aspartate which is phosphorylated (CheV D-235), and the conserved active site lysine (CheV K-287) (46). When aligned with the *E. coli* CheY protein (28), the carboxyl-terminal region of CheV contains a 9-amino-acid insertion in the region between α-helix 2 and β-strand 3. This region of CheY appears to be conformationally flexible as judged from a comparison of the *E. coli* CheY and *S. typhimurium* CheY crystal structures (47).

To verify that the *cheW* and *cheY* homologous portions of the *cheV* gene could be cotranslated in vivo, we cloned the carboxyl-terminal 270 codons of the *cheV* open reading frame into a T7 RNA polymerase-driven overexpression plasmid, pET16x (42) (see Methods and Materials). This allowed expression from an internal methionine codon and was designed to overcome possible problems with trying to express protein to a high level in *E. coli* from a UUG start codon. Protein gel analysis of strain BL21/DE3 (pKF37) reveals the accumulation of an approximately 30-kDa product upon IPTG (isopropyl-β-D-thiogalactopyranoside) induction, consistent with the predicted 31.0-kDa size of the truncated CheV product (data not shown). This demonstrates that this gene is translated in vivo to yield a protein containing both the CheW and CheY homologous portions.

Expression of *cheV* by the σ^D form of RNA polymerase. To determine if the proposed σ^D-dependent promoter element ($P_{D\text{-}cheV}$) was used in vivo, RNA was isolated during late logarithmic phase and transition phase from wild-type and *sigD* mutant cells and analyzed by primer extension analysis (Fig. 4). A single major transcript, which initiated at the site indicated in Fig. 2, was detected. This mRNA is most abundant in cells entering the transition phase, as has been reported for other σ^D-dependent transcripts (12, 26), and is not present in cells containing a disruption of the *sigD* gene (data not shown).

To determine if σ^D RNA polymerase was sufficient to direct transcription from $P_{D\text{-}cheV}$, purified σ^D protein was added to *B. subtilis* core RNA polymerase and used to transcribe a supercoiled plasmid template containing the *cheV* gene. The reconstituted σ^D RNA polymerase initiated transcription at the identical start site as detected in vivo, and production of this RNA product required both core enzyme and the σ^D protein (Fig. 4 and data not shown).

The primer extension mapping data suggest that $P_{D\text{-}cheV}$ is responsible for transcription of the *cheV* gene but do not rule out contributions from other promoter elements. To determine if *cheV* expression is dependent on σ^D in vivo, we engineered a transcriptional fusion between *cheV* and the *E. coli lacZ* gene (plasmid pKF22). The level of β-galactosidase synthesis was then determined as a function of the growth state in several genetic backgrounds. Expression of *cheV-lacZ* is completely dependent on *sigD*, as judged by the lack of β-galactosidase production in strain HB4011. In addition, nonpolar insertion mutations in either *fliM* or *fliP* reduced *cheV-lacZ* expression by 3- or 50-fold, respectively. Mutations in these basal body structural genes are known to inhibit transcription of the flagellin gene by 100-fold and prevent expression of other σ^D-dependent functions (50). These results are consistent with the hypothesis that *B. subtilis*, like *E. coli*, contains an anti-σ factor which acts to repress class III genes, such as *cheV*, unless the hook-basal body structure is intact and functional.

Genetic mapping of the *cheV* gene. The map position of *cheV* was determined by a combination of phage-mediated transduction and hybridization of a radiolabeled probe to a nitrocellulose filter containing a genomic yeast artificial chromosome library of *B. subtilis* DNA (2) (see Materials and Methods). These data indicate that *cheV* is located near 123° on the *B. subtilis* genetic map (yeast artificial chromosome clone F3) between the *ptsI* and *ctaD* loci.

These data place the *cheV* locus close to the σ^D-dependent *motAB* operon (27) and the *cheX* chemotaxis locus (51). The *cheX* locus was defined by a Tn*917lacZ* insertion as a locus required for chemotaxis to sugars. Unlike *cheV*, however, *cheX* does not depend on *sigD* for expression (51). To determine if *cheV* is allelic to *cheX*, we tested for linkage by genetic transformation. These two loci are not linked, suggesting that

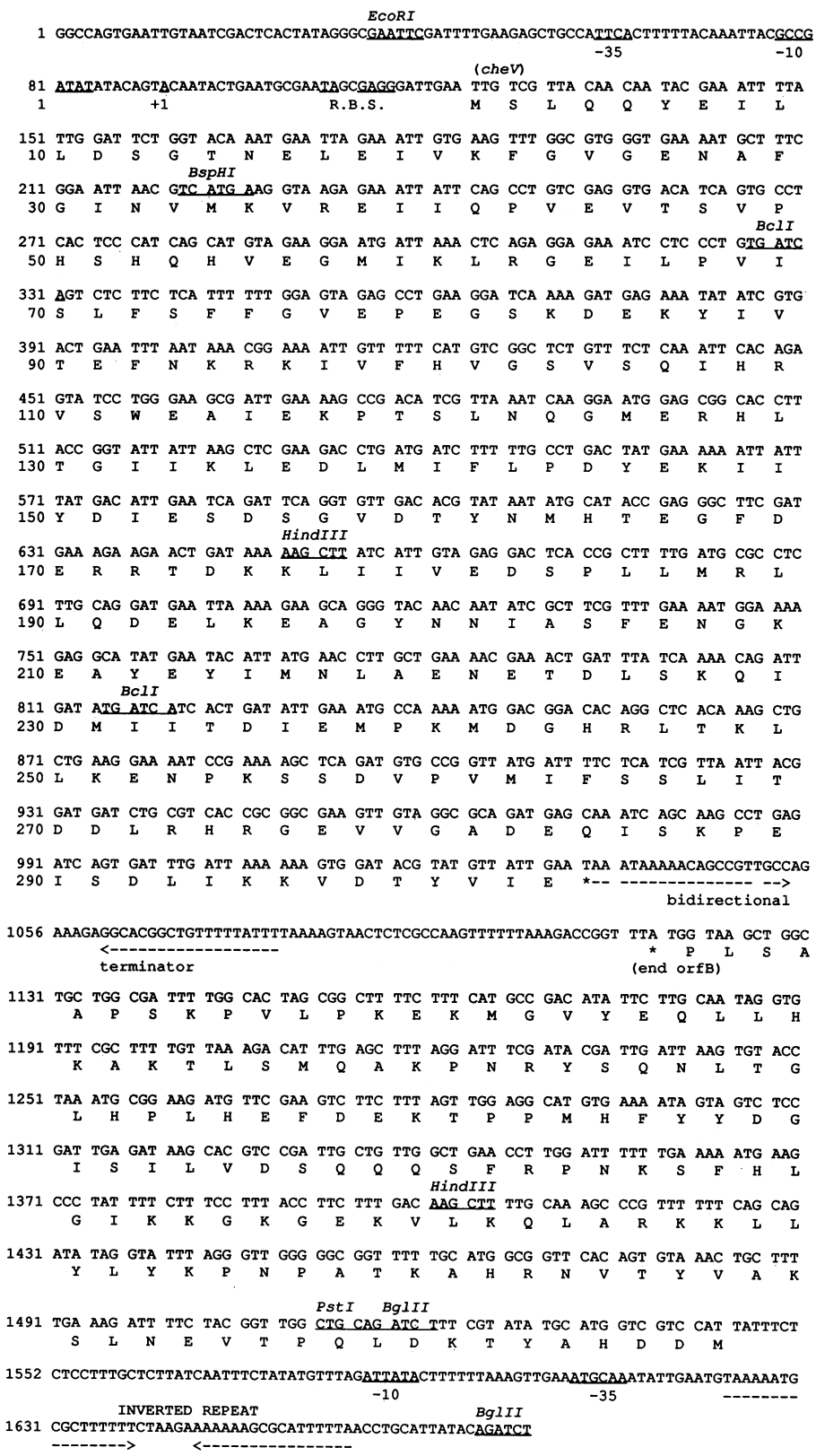

FIG. 2. DNA sequence including *cheV* and *orfB*. The σ^D-dependent promoter for *cheV* is underlined, and the +1 transcription start site is indicated. The putative promoter for *orfB* and the adjacent inverted repeat element are indicated. The numbers on the left indicate the position in the nucleotide sequence (top) and CheV amino acid sequence (bottom).

FIG. 3. Protein alignments of selected CheW and CheY proteins. (A) The CheW homologous region of CheV is aligned to the *E. coli* CheW protein (Eco) and *B. subtilis* CheW. For this analysis, conservative substitutions were defined as those within the groups: A and G; D, E, N, and Q; K and R; S and T; I, L, M, and V; and F, Y, and W. (B) The CheY homologous region of CheV was aligned with *E. coli* CheY and *B. subtilis* CheY and regions of similarity indicated in panel A. Active site residues are indicated by • and the site of phosphorylation is indicated by *. Positions at which all three proteins are identical or conservatively substituted are enclosed in boxes. The last four residues of the amino-terminal portion of CheV are underlined to indicate the region of overlap with the carboxyl-terminal segment in panel B.

they are separated by at least several kilobases (data not shown).

Genetic analysis of *cheV*. To determine if the predicted CheV protein was important for chemotaxis, gene disruptions (plasmids pKF24 and pKF25) were transferred to the chromosome, and the resulting strains were analyzed for their ability to swarm on semisolid agar plates (Fig. 5). Insertion of the kanamycin gene into the 5′-proximal *BclI* site of the *cheV* gene reduced the swarm diameter by 50% on both minimal medium and tryptone swarm plates. This defect could be complemented by insertion of the *cheV* gene into the amylase locus. Surprisingly, an insertion in the second *BclI* site had only a slight effect on swarm size (data not shown, but see the accompanying paper [36]). These results suggest that the CheW and CheY homologous regions of the CheV polypeptide function independently and that the truncated polypeptide produced by disruption of the CheY homologous domain is still at least partially functional. A more detailed analysis of the chemotaxis defect in the *cheV* mutant strain and studies of the epistasis between *cheV*, *cheW*, and *cheY* are presented in the accompanying paper (36).

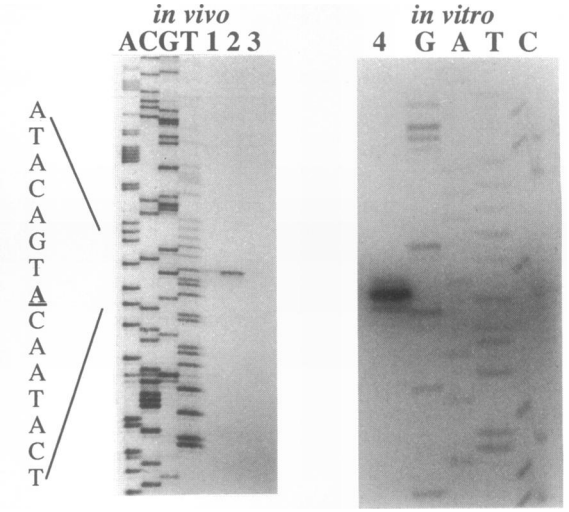

FIG. 4. Primer extension analysis of $P_{D\text{-}cheV}$ promoter. In vivo and in vitro RNA samples were prepared as described in Materials and Methods and analyzed by in vitro primer extension. The sequence at the left is the noncoding (top) strand and is complementary to that in the DNA sequencing ladders. The underlined A residue is the primary in vivo and in vitro start site for transcription. Lanes: 1, 2, and 3, reaction products with RNA isolated from *B. subtilis* at T_{-1}, T_0, and T_{+1}, respectively; 4, primer extension products from a reaction with RNA from an in vitro transcription reaction mixture containing reconstituted σ^D holoenzyme.

DISCUSSION

The *B. subtilis* σ^D protein is an alternative σ factor which directs expression of class III flagellar and chemotaxis genes (14, 15, 22, 26, 27, 31, 51). Preliminary indications suggest that the flagellar hierarchy in *B. subtilis* is analogous to that in the enteric bacteria. In both systems, a functionally complete basal

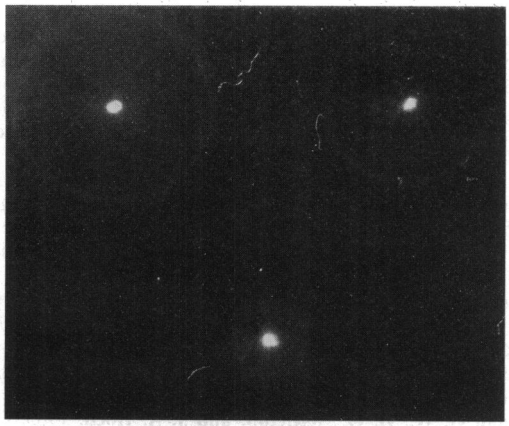

FIG. 5. Swarm plate analysis. Wild type (OI1085 [upper left]), a *cheV* mutant (HB4004 [bottom]), and a complemented *cheV* mutant (HB4019 [upper right]) were spotted on minimal medium-mannitol soft agar plates and allowed to swarm for 8 h.

body and hook structure, encoded by class II genes, is necessary for high level transcription of class III (late) genes from σ^{28}-dependent promoter elements (31). In enterobacteria, this coupling involves a specific anti-σ^{28} factor encoded by the *flgM* gene (30). The observation that *B. subtilis* also contains an *flgM* gene suggests that the mechanism of morphological coupling in the two systems has been conserved (31).

In this study, we have cloned and sequenced a new chemotaxis gene, *cheV*, from *B. subtilis*. The *cheV* gene is expressed from a σ^{D}-dependent promoter element both in vitro and in vivo. The *cheV* gene appears to be monocistronic, as it is followed by a factor-independent terminator which is predicted to terminate the *cheV* transcript and a convergent transcript from a downstream gene. Most of the other known *che* genes in *B. subtilis* are under σ^{A} control. These include *cheW*, *cheY*, *cheB*, and *cheA* homologs cotranscribed with *sigD* as part of the 25-kb *flaA* locus (5, 31).

In addition to CheV, the σ^{D} protein is known to be essential for transcription of flagellin (15, 26), the MotA and MotB motor proteins (27), hook-associated proteins (7a), the FlgM protein (31), and the MCPs (22). In addition, σ^{D} contributes to the expression of the major autolysin (19).

The predicted *cheV* gene product represents a novel type of chemotaxis protein which contains domains homologous to both CheW and CheY. CheW functions to physically bridge the CheA-autophosphorylating protein kinase to the MCP (10). In the presence of appropriate chemical signals, the CheA protein donates a phosphoryl group from a conserved histidine residue to an acceptor aspartate of the CheY protein (40). The phospho-CheY protein then interacts with the switch proteins at the base of the flagellum to determine the direction of rotation (48). Although the basic pathway of information flow appears to be similar in both *E. coli* and *B. subtilis*, there are also significant differences. For example, phospho-CheY acts as a tumble generator in *E. coli* but stimulates smooth swimming in *B. subtilis* (5, 31).

Previous studies of *B. subtilis* chemotaxis genes had indicated that *cheW* null mutants were still capable of chemotactic signaling, although with reduced sensitivity (13). This led to the suggestion that CheW in *B. subtilis* was not essential for coupling chemosensory information to the output signal alteration in the direction of flagellar rotation. The observation that *B. subtilis* has a second protein containing a CheW homologous domain suggests that a CheW function may in fact be essential for chemotaxis, as observed for the *E. coli* system. The studies described in the accompanying paper demonstrate that this is in fact the case; *B. subtilis* strains lacking both CheV and CheW fail to chemotax (36). For reasons not yet understood, both CheV and CheW are required for optimal chemotaxis. These two homologs may interact with different subsets of MCPs or be active at different times. Our transcriptional analyses indicate that CheV is expressed as a late gene with peak expression in early stationary phase. The other *che* genes are cotranscribed as part of the *flaA* locus, which is maximally expressed near the end of logarithmic phase growth (19).

The presence of a CheY homologous domain in the CheV polypeptide is intriguing. If CheV functions as a coupling factor to link MCPs to CheA, it seems unlikely that the CheY domain can also interact with switch proteins. Indeed, an insertion mutation in the CheY homologous domain of CheV displays only a modest defect in chemotaxis, whereas null mutants in *cheY* lack chemotactic signaling and tumble incessantly (4). In addition, *cheY* mutants altered in the phosphoacceptor site are signaling deficient, suggesting that the phosphotransfer mechanism observed in *E. coli* is also present in *B. subtilis* (3). The CheY homologous domain of CheV could play a regulatory role in the chemotaxis system by (i) helping to recruit CheA kinase to the MCPs, (ii) serving as a competitive inhibitor of CheY phosphorylation, or (iii) serving as a substrate for another protein kinase. Since response regulator domains of the CheY type are found in a large number of two-component systems, there is no a priori reason to assume that the CheY domain of CheV interacts with CheA rather than with another cellular protein kinase.

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