Identification of TlpC, a novel 62 kDa MCP-like protein from *Bacillus subtilis*

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We report the sequence and characterization of the *Bacillus subtilis* *tlpC* gene. *tlpC* encodes a 61·8 kDa polypeptide (TlpC) which exhibits 30% amino acid identity with the *Escherichia coli* methyl-accepting chemotaxis proteins (MCPs) and 38% identity with *B. subtilis* MCPs within the C-terminal domain. The putative methylation sites parallel those of the *B. subtilis* MCPs, rather than those of the *E. coli* receptors. TlpC is methylated both *in vivo* and *in vitro* although the level of methylation is poor. In addition, the *E. coli* anti-Trg antibody is shown to cross-react with this membrane protein. Inactivation of the *tlpC* gene confirms that TlpC is not one of the previously characterized MCPs from *B. subtilis*. Capillary assays were performed using a variety of chemoeffectors, which included all 20 amino acids, several sugars, and several compounds previously classified as repellents. However, no chemotactic defect was observed for any of the chemoeffectors tested. We suggest that TlpC is similar to an evolutionary intermediate from which the major chemotactic transducers from *B. subtilis* arose.

Keywords: chemotaxis, *Bacillus subtilis*, MCP, DNA sequence, methylation

INTRODUCTION

In *Escherichia coli*, the methyl-accepting chemotaxis proteins (MCPs) (Kort *et al.*, 1975) are the receptors for attractants and repellents, and are subject to methyl-esterification on conserved glutamate side chains (Kleene *et al.*, 1977; Van der Werf & Koshland, 1977) located within their cytoplasmic domain. Binding of a repellent at the MCPs is believed to cause an increase in the rate of autophosphorylation of CheA, with subsequent increases in levels of CheY-P and CheB-P (Hess *et al.*, 1988; Ordal *et al.*, 1992). CheY-P binds to the switch to cause clockwise rotation of the flagella (Bourret *et al.*, 1990), whereas CheB-P demethylates the MCPs to restore the autophosphorylation of CheA to a prestimulus level (Lupas & Stock, 1989; Stewart *et al.*, 1990). Thus, the receptors play a dual role, one to facilitate excitation, which occurs upon the initial binding of attractants or repellents, and the other to bring about adaptation, which occurs upon the continued binding of a chemoeffector. Phosphorylation

reactions are involved in the excitation process, while methylation reactions are involved in adaptation.

MCPs are widespread among prokaryotes (Morgan *et al.*, 1993), and their presence in both eubacteria and archaea (Alam *et al.*, 1989; Yao & Spudich, 1993) is testimony to their ancient origin, which probably predates the separation of these two lines of descent. MCPs are also known to exist in the Gram-positive organism *Bacillus subtilis*. Biochemical evidence suggested that there were three MCPs, with molecular masses ranging from 77 to 97 kDa (Bedale *et al.*, 1988; Hanlon *et al.*, 1993). However, four *B. subtilis* genes encoding proteins homologous to *E. coli* MCPs have recently been cloned and characterized. These genes are located in three contiguous operons, and all have predicted molecular masses of 72 kDa. Interestingly, inactivation of two of these transducer-like proteins leads to no apparent loss of methylation and results in no observable defects in chemotaxis (Hanlon & Ordal, 1994).

Here we report the cloning, sequencing and characterization of a unique methyl-accepting protein from *B. subtilis*, designated TlpC (transducer-like protein). The corresponding gene was discovered in the course of a sequencing project seeking to uncover possible competence genes near the *comL* locus.

Abbreviation: MCP, methyl-accepting chemotaxis protein.

The GenBank accession number for the nucleotide sequence reported in this paper is X34005.

Table 1. Bacterial strains and plasmids used in the characterization of the *B. subtilis tlpC* gene

Strain or plasmid	Relevant genotype or description	Comment/reference
Strains		
<i>E. coli</i>		
TG1	M13 cloning host	Amersham
K38	Harbours pGP1-2	T7 expression host
JM101	<i>dam</i> ⁺ cloning host	Amersham
<i>B. subtilis</i>		
OI1085	Che ⁺ <i>bisH2 metC trpF7</i>	Ordal & Goldman (1976)
OI3054	OI1085(pDW41), Cm ^r	This work
Plasmids		
pBGSC6	Integration vector, Cm ^r	<i>Bacillus</i> Genetic Stock Center
pT7-6	<i>E. coli</i> expression plasmid, Ap ^r	Tabor & Richardson (1985)
pGP1-2	Contains gene encoding T7 polymerase, Km ^r	Tabor & Richardson (1985)
pGSM10	Contains <i>map62</i> gene within a 6 kb <i>SacI</i> - <i>EcoRI</i> DNA fragment, Ap ^r	This work
pDW35	1.9 kb <i>TaqI</i> fragment cloned into <i>AclI</i> site in pBluescriptSK ⁻ , Ap ^r	This work
pDW36	<i>XbaI</i> - <i>XbaI</i> fragment from pDW35 cloned into M13mp19	This work
pDW37	pDW36 with a <i>TaqI</i> site created via site-directed mutagenesis	This work
pDW38	1.7 kb <i>TaqI</i> fragment subcloned into <i>AclI</i> site in pBluescriptSK ⁻	This work
pDW39	1.7 kb <i>XbaI</i> - <i>SstI</i> fragment subcloned from pDW38 into <i>SaI</i> - <i>SstI</i> compatible sites in pT7-6	This work
pDW40	pDW39 containing a 400 bp <i>Bgl</i> II- <i>Bam</i> HI deletion	This work
pDW41	0.8 kb <i>Sph</i> I- <i>Bgl</i> II fragment subcloned into pBGSC6	This work

METHODS

Bacterial strains and plasmids. All bacterial strains and plasmids used in this study are described in Table 1.

DNA sequence and analysis. The 1.9 kb *TaqI* DNA fragment shown in Fig. 1 was sequenced on both strands to obtain an unambiguous consensus sequence. Sequencing was performed by the dideoxy chain termination method (Sanger *et al.*, 1977). Protein alignments were performed using the AALIGN program from DNASTAR.

Expression of TlpC. The 1.9 kb *TaqI* fragment containing *tlpC* was modified by site-directed mutagenesis to create an additional *TaqI* restriction site which was proximally located just upstream of the potential ribosome-binding site. The corresponding 1.7 kb *TaqI* fragment which was isolated from pDW37 did not contain the adjacent terminator or promoter regions which might have interfered with the expression analysis. This insert was ultimately subcloned into the pT7-6 expression plasmid and transformed into *E. coli* strain K38 for expression using the T7 expression system of Tabor & Richardson (1985). Plasmid pDW40, containing a 400 bp C-terminal deletion of the TlpC gene in pT7-6, was expressed in parallel. The protein samples were electrophoresed on 10% (w/v) gels and ³⁵S-labelled bands were visualized by autoradiography.

Mutagenesis of *tlpC*. A 0.8 kb *Sph*I-*Bgl*II fragment from *tlpC* was subcloned into pBGSC6, a plasmid which is unable to replicate in *B. subtilis*. The resulting plasmid, pDW41, was subsequently transformed into wild-type strain OI1085. A single

Campbell-like recombination event between homologous DNA resulted in the disruption of the *tlpC* gene, which was selected for by resistance to chloramphenicol. The integration event used to create mutant strain OI3054 was confirmed by Southern blot analysis.

Methylations. *In vivo* methylation experiments have been described previously (Ullah & Ordal, 1981). To visualize poorly methylated proteins, an extended methylation was performed for 10 min with 25 µCi (925 kBq) [*methyl*-³H]methionine. The procedure used to isolate membranes has been described elsewhere (Goldman & Ordal, 1984). *In vitro* methylation reactions were performed essentially as described by Goldman & Ordal (1984) with slight modification, which included an increase in the addition of *S*-adenosyl [*methyl*-³H]methionine to 20 µl. Reactions were incubated at room temperature for 3 h to achieve maximal labelling.

Other procedures. *E. coli* transformations were performed using the RbCl procedure of Hanahan (1985) and *B. subtilis* transformations were performed as described previously by Ordal *et al.* (1983). The immunoblot procedure has been described in detail elsewhere (Carpenter *et al.*, 1992). Capillary assays have also been described previously (Ordal & Goldman, 1975). Assays which were performed to examine sporulation (Rudner *et al.*, 1991) and competence (Ordal *et al.*, 1983) have been described. To examine protein secretion, cells from the wild type OI1085 and *tlpC* mutant strain OI3054 were grown in L-broth until early- and mid-exponential growth was reached (40 and 120 Klett units), as well as early-stationary phase

Fig. 1. Nucleotide and deduced amino acid sequence of *tlpC*. The predicted ribosome-binding site for the open reading frame corresponding to TlpC is underlined, and the stop codon is indicated with an asterisk. The putative promoter region is double underlined, and a predicted terminator from the upstream transcriptional unit is indicated by bold facing arrows. The location of relevant restriction sites which were used to construct the *tlpC* mutant are indicated above the DNA sequence.

TCGATTTCTGAAACTATGTTCACTCACCACGCAAAACCTTGAATAGCATCACACAACCGGCTGAAGATCAGGCGGGTTTATTTTCTAAAAATAAACTTTAAACCCAAAAACCCGAT	120

<u>AAGTAATATGACCTGCAAAATCGTAAAGGAGGAATTATGTTGATAAAACGATTAAAGATGAAGCTAGGAACATAAAATCTTTGCTCGTTTTCGTGGTGAATCTTTGTTTTCGGCATCA</u>	240
M L I K R F K M K L G T K I L C L V F V I L L F S A S	28
GTGGCACTGTGATGCTTAAGGAAATTCAGAAAGCATGAAACAAATGGCAACTGAAAAGCGAAAGGTGATCTCGCTTTAAGCAGCACTTATATTGATGATGTCATGTCAGGCGACTGG	360
V G T V M L K E I T E S M K Q M A T E X A K G D L A L S S T Y I D D V M S G D W	68
CAGGTGAAAAACAATAGCTCTATAAAGGCAACACAAATCAATGGAATGAAGATATTGTTGATCTGCTTGGTGAAGACAGGTGATACCATTACCATTTTCCAAGGCGATACTCGT	480
Q V K N N K L Y K S Q T Q I N G N E D I V D L L G E K T G D T I T I F Q G D T R	108
GTTCACCAATGTCTGAAAAATGGCGAAAGAGCGTGGGACCCAGGCTTCTTCTGAAGTTATTGCCGCTGTTTTAAAGAAAGGAAAGCGTTTTTACGGACAAGCAGATGTAGCAGGT	600
V A T N V M K N G E R A V G T Q A S S E V I A A V L K K G K R F Y G Q A D V A G	148
<u>SphI</u>	
TCTTCTTATCAGACTGCATACATGCCGTTAAAAAGATCAAAATGGGAACATCATCGCATGCTGTATACCGGAGCTAATCAAAGTATTTTAGCATCACTTACACAATCACTTTTCACTCAA	720
S S Y Q T A Y M P L K D Q N G N I I G M L Y T G A N Q S I L A S L T Q S L F T Q	188
TTGCTATCGTTCTTGTATCGTCATTATGGTATCAGTCATCCTTGTCTTGGTTTTTACCAGAAAAATCAACAAGCGCTGAACGCTTTAAAAAGCGCTTTTGAAGTGCAGGAAACGGA	840
F A I V L V I V I M V S V I L V L V F T R K I N K R L N A L K S A F E S A G N G	228
GATATGACAATAGAGGTGTGAGACAAAACCGGTGACGAGCTTTTCAAGTAAAGCTCTATTATAATGAAGATGAGAATGAAGCTGAACGATACCATAACAACCGTACAACATCAGCCCTT	960
D M T I E V S D K T G D E L S E L S V Y Y N K M R M K L N D T I Q T V Q Q S A L	268
CAGCTTGCCTCAGCCTCTCAGCAGCTCTCAGCGGAGCAGAGGAAACAAATCAAGCTTCAGAAAAATCACAGAAGCTGTACAGCAGATTGCAATGGAGCCCAAGATCAGATCACCGGA	1080
Q L A S A S Q Q L S A G A E E T N Q A S E K I T E A V Q Q I A N G A Q D Q I T R	308
ATTGAAAATAGTAAAGCTCATTAAAAACAAGCTTCAGCAGACATCCGGATATTTCTGCAAACTACTGCCGCTATCGCTGACAAAGGGCAGCTCGCTCAATCAAAAGCAGACATTGGACAA	1200
: E N S E S S L K Q A S A D I R D : S A N T A A I A D K G Q L A Q S K A D I G Q	348
AAGGAAATTGCAATGTACAGGCGCAATGGATGCGATTCAACAGTCAATTGAGAAAAGCGGAGAAATCATACCCAGTTAGACGGCGCTCTAAACAAATTGAGCAAAATTTTATCGGTC	1320
K E I A N V Q A Q M D A I H Q S I Q K S G E I I H Q L D G R S K Q I E Q I L S V	388
ATCACCCAAATTGCTGATCAGACAAACCTGCTTGGCTGAATGCGAGATTGAAGCTGCCAGAGCCGGTGAACAAGGAAAGGCTTTGCTGTTGCTGATGAAGTACGGAATTAGCA	1440
I T Q I A D Q T N L L A L N A A I E A A R A G E Q G K G F A V V A D E V R K L A	428
<u>BglII</u>	
GAAGAGTCTCAACAGTCTCGGGGAGATTTCAAACTGATCATAGAAATACAAAAGGATATGAACCGGTCTGCAAGATCTGTTGAGCATGTGAAAACAGAGCTGCGAGAAGGAGTCACC	1560
E E S Q Q S A G Q I S K L I I E I Q K D M N R S A R S V E H V K T E A A E G V T	466
ATGATCCAGCGCACCAGAGATGCATTAAAGGAAATGCGAGCTGCAACAGGTGAAATCAGCGCGAGATTAGTGATTTATCAGCATCTGTAACAAACATTTACGCCAGTGACATCAAAAT	1680
M I Q R T R D A F K E I A A A T G E I S A E I S D L S A S V T N I S A S A H Q I	508
AATGATTCCTTTGCGAGCAATACGGCCGACATTAAAGAACTACTAAAAATACCGGCAAGCGCTGCATTACAGAGGAGCAATTTGCTGCAATGGAGGAGATTACCGCAGCTTCTGAA	1800
N D S F A A N T A D I K E S T K N T R Q A A A L T E E Q F A A M E E : T A A S E	548
ACACTTTCTCAATTAGCTGAGGAGCTTACCGGTATCATAAGCCAAATTTAAATGATTAAATCAAGCCAAACCGGCTGATGACTTACTATCATTATTGTAATTTGGCAATCGTATTTTGTCTG	1920
T L S Q L A E E L T G I I A Q F K M I N Q A E N G *	573
AAAACAAATATTTGCA	1936

Fig. 1. For legend see facing page.

(180 Klett units). Samples were collected and the cells were separated from the supernatant. The proteins in the supernatant were immediately precipitated on ice with 7% (w/v) TCA for 15 min. Following centrifugation and an acetone/ethanol wash, the protein pellets were resuspended in 1× SDS solubilizer (62 mM Tris pH 6.8, 2% SDS, 10% glycerol, 5% β -mercaptoethanol). Equivalent amounts of protein were boiled for 5 min and electrophoresed by SDS-PAGE on 10% (w/v) polyacrylamide gels. Secreted proteins were detected by silver stain.

RESULTS

Nucleotide sequence and analysis of *tlpC*

DNA sequence analysis of a 1.9 kb *TaqI* fragment reveals a large open reading frame (ORF) which encodes a polypeptide of 573 amino acids, with a predicted molecular mass of 61.8 kDa (Fig. 1). A putative ribosome-binding site (RBS) is located only four nucleotides upstream of the start codon ATG and seven nucleotides upstream of the alternative start codon TTG and displays strong similarity to the consensus RBS sequence (TAAGGAGG) of *B. subtilis*. This spacing is shorter than the reported optimal length of 8–9 nucleotides, and might therefore be expected to reduce the translational yield of this protein (Vellanoweth & Rabinowitz, 1992). The upstream nucleotides encompass a putative σ^D consensus promoter element which closely resembles the consensus sequence of CTAAA...N₁₆...CCGATAT (Mirel *et al.*, 1992). Although an obvious transcriptional terminator is not located after this gene product, designated TlpC, it is very likely that this gene is monocistronic since the immediately adjacent *nucA* gene is specific for competence (Vosman *et al.*, 1988). Because the transcription of genes involved with the physiological process of competence does not coincide with the observed expression of TlpC, it follows that the transcriptional regulation of *tlpC* must be unique.

In order to verify that this proposed ORF encodes a protein of the predicted size, a 1.7 kb *TaqI* fragment containing the entire *tlpC* gene which was deleted for the adjacent transcriptional elements was subcloned from pDW38 into the *E. coli* expression plasmid pT7-6. The resulting plasmid pDW39 was then introduced into *E. coli* strain K38. Expression of the subcloned DNA produced a radiolabelled protein of 63 kDa, which is in good agreement with the predicted molecular mass (Fig. 2, lane 1). However, several lower molecular mass bands of strong intensity were co-expressed. To demonstrate that the 63 kDa radiolabelled band corresponds to the expression of *tlpC*, a 400 bp C-terminal deletion of the *tlpC* gene was constructed. Expression of the resulting fragment produced the expected shift of 15 kDa in the expression profile (Fig. 2, lane 2). It is suspected that these lower molecular mass bands result from degradation or additional internal start sites that are recognized in *E. coli*.

Homology to known proteins

The amino acid sequence of this predicted protein exhibited homology to the MCPs from *E. coli* (Bollinger *et al.*, 1984; Krikos *et al.*, 1983; Russo & Koshland, 1983;

Boyd *et al.*, 1983), as well as to chemotactic transducer proteins from other organisms, including FrzCD from *Myxococcus xanthus* (McCleary *et al.*, 1990), McpA from *Caulobacter crescentus* (Alley *et al.*, 1992), HtrI from *Halobacterium halobium* (Yao & Spudich, 1993) and, more strikingly, MCPs from *B. subtilis*. A hydropathy profile of TlpC revealed two hydrophobic segments in the N-terminal region of TlpC. These segments are characteristic of transmembrane regions and their location is typical of MCPs (Fig. 3). On comparing various MCPs, the region of high conservation was found to be localized within the C-terminal domain (Fig. 4), with no detectable homology in the N-terminal region. An amino acid alignment between TlpC and a representative *E. coli* transducer Tar showed 30.0% identity in a 297 amino acid overlap (Fig. 4). The similarity between TlpC and *B. subtilis* McpA was higher (38.3% identity) and extended through a 358 amino acid overlap. This similarity is not as extensive as within previously characterized MCPs, which exhibit an overall 57–64% amino acid identity among them that includes the N-terminal, extra-membrane region (Hanlon & Ordal, 1994; Fig. 4). Moreover, there are two regions of TlpC that are much more similar to McpA from *B. subtilis* than to Tar from *E. coli* (see underlined residues in Fig. 4).

The putative methylation sites also reflect a greater similarity with the *B. subtilis* MCPs, compared with *E. coli* transducers (Fig. 4). Three of the four predicted sites appear to be conserved, while none are in the locations found in Tar (Fig. 4). By contrast, the molecular mass of TlpC is similar to that of the *E. coli* MCPs, which range from 58 to 60 kDa (Taylor & Lengeler, 1990), and considerably less than the 72 kDa observed for the four MCP or MCP-type genes sequenced thus far from *B. subtilis* (Hanlon & Ordal, 1994).

Interestingly, a search of the TlpC protein sequence against sequences in the GenBank also identified significant homology to HlyB of *Vibrio cholerae*, with the two proteins sharing 23.8% amino acid identity which extends the entire length of the two proteins (data not shown). HlyB from *V. cholerae* is a 60.3 kDa putative outer membrane associated protein which is required for the secretion of haemolysin (Aim & Manning, 1990).

Characterization of a null mutant in *tlpC*

In order to examine the role of TlpC in *B. subtilis*, a mutation was created in the *tlpC* ORF using an integration plasmid pDW41, which contains an *SphI*–*Bgl*II DNA fragment that is internal to the *tlpC* gene. Transformation and homologous recombination into wild type strain OI1085 resulted in the chromosomal disruption of *tlpC* to create mutant strain OI3054. The fidelity of the gene disruption was confirmed by Southern blot analysis (data not shown).

A standard *in vivo* methylation reaction which was performed on the wild-type and corresponding mutant strains did not detect any visible differences in the methylation profiles. However, under conditions requiring an extended methylation with an increased amount of

Fig. 2. SDS-PAGE analysis following expression of TlpC. The autoradiogram shows expression of *B. subtilis* TlpC in *E. coli* strain K38. Lane 1, pDW39; lane 2, pDW40 containing a 400 bp C-terminal deletion of the *tlpC* gene. Molecular mass standards are indicated.

Fig. 3. Hydropathy profile of TlpC. The algorithm of Kyte and Doolittle was used with a window span of 19. Hydropathy values are shown on the vertical axis. The dashed line shows the threshold hydropathy value for known membrane-spanning segments of the *Rhodobacter viridis* photosynthetic reaction centre L subunit. Vertical lines represent increments of 20 amino acids.

[methyl-³H]methionine, a noticeably methylated protein migrating at approximately 63 kDa on SDS-PAGE was present in wild type strain OI1085 but not in OI3054 (Fig. 5). As observed from the fluorogram, this methylated protein does not correspond to the major MCPs of *B. subtilis*, which are grossly overexposed. An *in vitro* methylation was subsequently performed which also demonstrated that this membrane-bound protein was able to be methylated, albeit poorly (data not shown). In addition, it was determined that the chemotactic methyltransferase, CheR, was required for the methylation of this previously uncharacterized TlpC protein, since this protein is unmethylated in a methyltransferase mutant strain (data not shown).

An immunoblot analysis of membranes prepared from

strains OI1085 and OI3054 was performed using the *E. coli* anti-Trg antibody, which has been shown to cross-react with the *B. subtilis* MCPs (Nowlin *et al.*, 1985). The results presented in Fig. 6 demonstrate that the *E. coli* antibody cross-reacts with TlpC in wild-type membranes, while no cross-reactivity is observed in membranes prepared from the mutant strain OI3054. Cross-reactivity is also observed with the H3 MCP in both strains, which has an apparent molecular mass of 76 kDa on SDS-polyacrylamide gels. Although the *E. coli* Trg antibody cross-reacts with the other *B. subtilis* transducers, the recognition is substantially weaker than observed with H3 and often difficult to detect by immunoblot analysis. The results obtained thus far indicate that TlpC is a methylated integral membrane protein with strong homology to chemotactic transducers from other organisms, but is not one of the previously identified MCPs that have been biochemically characterized in *B. subtilis*.

All 20 amino acids and many sugars are attractants for *B. subtilis*, while a diverse array of compounds serve as repellents (Ordal & Goldman, 1976). A broad-based survey was undertaken to determine whether TlpC was involved in chemotaxis. Capillary assays were performed on the OI3054 mutant strain to quantify chemotaxis towards all 20 amino acids, and a variety of sugars which included glucose, fructose, maltose, mannose, methyl α -glucoside, sorbitol and sucrose. All amino acids were initially surveyed at previously determined apparent K_D concentrations (Ordal *et al.*, 1979), while sugars were assayed at concentrations generating maximal accumulations in a capillary assay ('peak concentrations') (Ordal *et al.*, 1979). Several repellents which included butyrate, 2,6-dibromophenol, indole, quinacrine and sodium cyanide were also examined (data not shown) (Ordal & Goldman, 1976). The results from these experiments demonstrated that the OI3054 mutant strain was chemotactically wild-type toward all of the compounds tested, suggesting that TlpC may not be involved in chemotaxis.

The lack of an observed chemotactic phenotype prompted us to explore the possibility that the TlpC protein was somehow involved in another cellular process which utilizes the TlpC receptor to recognize an appropriate stimulus and cytoplasmically transduce the signal received by this protein using a two component regulatory system homologous to CheA and CheY. Several assays were therefore performed to examine the processes of protein secretion, competence, and sporulation in *B. subtilis*. The inactivation of the *tlpC* gene produced no observable defects for any of these processes.

DISCUSSION

This paper reports the nucleotide sequence of the *tlpC* gene, which encodes a predicted 61.8 kDa polypeptide, in agreement with results obtained using a T7 expression system in *E. coli*. The amino acid sequence of TlpC suggests that this *B. subtilis* protein should be involved in the chemotactic signal transduction process, based upon its strong homology to chemotactic transducers from other organisms, including the MCPs from *E. coli* (Tar,

<i>B. subtilis</i> McpA	IRSITTPKQLVGSSKRISSEGLDTETIDIRSKDELGELGKSFNNMASSLR
<i>B. subtilis</i> McpB	VSITKPLKRLVQSSKTISRGLDTETIEIHSKDELGELGESFNEMGQSLR
<i>B. subtilis</i> TlpC	TRKINRLNALKSAPESAGNDMTIEVSDKTDGDELSVYNNKMRMLN
<i>E. coli</i> Tar	RRMLLTPLAKIIAHIREIAGGNLANTLTI
<i>B. subtilis</i> McpA	SLIHAIQDSVDNVAASSEELTASAAQTSKATEHITLAEIQFSNGNEKQNE
<i>B. subtilis</i> McpB	SLISAIQDSVNNVAASSEQLTASAGQTSKATEHITMAIEQFSNGNEEQSR
<i>B. subtilis</i> TlpC	DTIQTVQSSALQLASASQQLSAGAEETNQASEKITEAVQQIANGAHDQIT
<i>E. coli</i> Tar	DGRSEMGLAQSVSHMQRSRLTDTVTHVREGSDAIYAGTREIAAGNTDLSS
<i>B. subtilis</i> McpA	NIETAAEHYQMNDGLTNMAQASEVITDSSVQSTIEASEGGKLVHQTVGQ
<i>B. subtilis</i> McpB	KVESSSHQNLNLMNEGLQQVVSQTSSDITKASIQSTIEAGTGEKFVQQTVGQ
<i>B. subtilis</i> TlpC	RIENSSSLKQASADIRDISANTAAIADKGLAQSKADIQKKEIANVQVQ
<i>E. coli</i> Tar	RTEQQASALEETAASMEQL---TATVKQNADNARQASQLAQASDTAQ-H
<i>B. subtilis</i> McpA	MNVIDKSVKAEQVVRGLETKSKDITNLRVINGIADQTNLLALNAAIEA
<i>B. subtilis</i> McpB	MNSINQSVQQAEEAVVKGLEGKSKDITSILRVINGIADQTNLLALNAAIEA
<i>B. subtilis</i> TlpC	MDAIHQSIQKSGEIIHQLDGRSKQIEQLSVITQIADQTNLLALNAAIEA
<i>E. coli</i> Tar	GGKVVDGVVKT---MHEIADSSKKIADIISVIDGIAFQTNILALNAAVEA
<i>B. subtilis</i> McpA	ARAGEYGRGFSVVAEEVRKLAQVSADSAKEIEGLIIEIVKEINTSLGMFQ
<i>B. subtilis</i> McpB	ARAGESGRGFSVVAEEVRKLAQVSADSAKEIEKLIQEIIVAEIDTSLHMFQ
<i>B. subtilis</i> TlpC	ARAGEQKGFAVVAEEVRKLAESQSSAQSKLIIIEIQKDMNRSARSVE
<i>E. coli</i> Tar	ARAGEQGRGFAVVAEEVRNLAASRAQAQAEIKALIEDSVSRVDTGSVLVE
<i>B. subtilis</i> McpA	SVNQEVQTLGLDITDKTEMSFKRISSEMTNQIAGELQNMSTVQQLSSASSEE
<i>B. subtilis</i> McpB	EVNQEVQSGLVVTDNTKESFQSI FSMNTNEIAGKLQTMNSTVQQLSDRSQH
<i>B. subtilis</i> TlpC	HVKTEAAEGVTMIQRTTRDAFKEIAAATGEISAEISDLISASVTNISASAHQ
<i>E. coli</i> Tar	SAGETMNNIVNAVTRVTDIMGEIASASDEQSRGIDQVALAVSEMDRVTTQ
<i>B. subtilis</i> McpA	VSGASEHIASISKESSAHIQDIAASAEQQLASMEIESSSAETLSSMAEEL
<i>B. subtilis</i> McpB	VSAAVSGIADVSKESSASIQDIAASAEQQLASMEIESSSATTLAQMAEEL
<i>B. subtilis</i> TlpC	INDSFAANTADIKESTKNTRQAAALTEEQFAAMEEITAASETLSQAEEL
<i>E. coli</i> Tar	NASLVQESAAAAAALAEQASRLTQAVSAFRLAA-SPLTNKPTPSRPAE
<i>B. subtilis</i> McpA	RDMTKRFKIE
<i>B. subtilis</i> McpB	RDLTQKQKIE
<i>B. subtilis</i> TlpC	TGIISQFKMINQAENG
<i>E. coli</i> Tar	QPPAQPRLRIAEQDPNWETP

Fig. 4. Amino acid alignment of the C-terminal region of *B. subtilis* TlpC protein with the C-terminal regions of the *E. coli* transducer Tar and the *B. subtilis* transducers McpA and McpB. The C-terminal region is defined to be the segment of protein following the second membrane-spanning region. Alignment was performed using the AALIGN program from DNASTAR. Identical amino acid residues between TlpC and other proteins are in bold. Solid triangles below and the asterisks above the amino acid sequence denote the reported methylation sites of the *E. coli* transducers and the predicted methylation sites of TlpC, respectively. The asterisk in parenthesis indicates the predicted site in McpA and McpB that is not present in TlpC. The underlining indicates regions where the overall similarity of TlpC is considerably greater to McpA than to Tar.

Fig. 5. *In vivo* protein methylation. Methylations were performed for 10 min with 25 μ l [methyl- 3 H]methionine per sample. Lane 1, wild-type strain OI1085; lane 2, *tlpC* mutant OI3054. The arrowhead indicates the position of TlpC.

Fig. 6. Immunoblot analysis. The cross-reacting *E. coli* anti-Trg antibody was used to detect TlpC among total membrane proteins. Lane 1, OI1085; lane 2, OI3054. The arrowhead indicates the position of TlpC.

Tap, Trg and Tsr (Bollinger *et al.*, 1984; Krikos *et al.*, 1983; Russo & Koshland, 1983; Boyd *et al.*, 1983), FrzCD from *M. xanthus* (McCleary *et al.*, 1990), MCPA from *C. crescentus* (Alley *et al.*, 1992), HtrI from *H. halobium* (Yao & Spudich, 1993), and the MCPs (McpA and McpB) and transducer-like proteins (TlpA and TlpB) from *B. subtilis* (Hanlon & Ordal, 1994). In addition, the hydropathy profile predicts two hydrophobic membrane spanning regions for TlpC, which is biochemically supported by its partition in the membrane fraction. Based upon the *E. coli* model, it would appear that these transmembrane regions

separate two domains of the protein: an N-terminal extra-membrane binding region, which might interact with extracellular effectors, and a C-terminal domain, which would be responsible for interacting with several chemotaxis proteins to cytoplasmically transduce the signal received by the receptor and ultimately control the swimming behaviour of the bacterium.

The initial prediction that the TlpC protein would be involved in chemotaxis stems from the observed immunogenic cross-reactivity of this protein with the *E. coli* Trg antibody, in addition to the ability of TlpC to become methylated both *in vivo* and *in vitro*, although at a much reduced level compared to the previously biochemically characterized MCPs (Goldman & Ordal, 1984; Goldman *et al.*, 1982; Hanlon & Ordal, 1994). Furthermore, the transcription of this gene appears to be regulated by the σ^D form of RNA polymerase, in agreement with the regulation of the major MCPs from *B. subtilis* (Marquez *et al.*, 1990; Hanlon & Ordal, 1994). However, an examination of the chemotactic ability of this mutant strain, as examined by capillary assays, reveals that it is apparently wild-type to all chemoeffectors tested including amino acids, sugars and repellents (Ordal & Goldman, 1976).

It is still possible, however, that TlpC mediates taxis for chemoeffectors whose taxis is also mediated by another MCP. For example, a mutant in *mcpA* shows greatly reduced but not eliminated taxis to glucose and methyl α -glucoside, while a mutant in *mcpB* shows appreciable taxis towards aspartate, histidine and glutamine. Only taxis toward asparagine is completely eliminated in the *mcpB* mutant (Hanlon & Ordal, 1994). Thus, other MCPs must

also mediate these taxes. Only after the remaining MCP genes have been cloned and inactivated will it be possible to create a strain containing only *tlpC*. Only then should it be possible to investigate what taxes, if any, TlpC mediates. It also may be that we have not tested the appropriate chemoeffector for this receptor. However, based upon previous studies which demonstrate that all amino acids and many sugars are attractants (Ordal *et al.*, 1977, 1979), while several compounds serve as repellents (Ordal & Goldman, 1976), it seems peculiar that this potential chemotactic receptor would not recognize any of these chemoeffectors tested. Finally, it is possible that TlpC might carry out other non-chemotactic functions.

Recently, we have cloned, sequenced, and characterized four genes encoding proteins of 72 kDa, which are homologous to chemotactic transducers from other organisms. Two of these genes encode major MCPs of *B. subtilis*, based upon the loss of methylated bands visualized on SDS-polyacrylamide gels in the corresponding mutant strains. In addition, these transducers are responsible for recognizing various sugar or amino acid attractants. However, inactivation of the other two transducer-like proteins (TlpA and TlpB) had no apparent effect upon the MCP methylation profiles. Furthermore, no chemotactic defect was observed in the absence of these transducer-like proteins for any amino acid, sugar or repellent tested (Hanlon & Ordal, 1994). Thus, TlpC is not the only MCP-type protein whose function is obscure.

It is noteworthy that TlpC has three of the four predicted methylation sites of McpA and McpB (and TlpA and TlpB) but none of the sites identified in Tar. Furthermore, TlpC has additional regions of similarity with McpA and McpB that are not found in Tar (Fig. 4). This suggests that other, unique, proteins might interact with the *B. subtilis* MCPs, proteins that are not found in *E. coli*. Three candidates have been identified thus far [CheC, CheD (M. Rosario, J. Kirby & G. Ordal, unpublished) and CheV (Frederick & Helman, 1994; Rosario *et al.*, 1991)].

These similarities also indicate TlpC's closer relationship to the *B. subtilis* MCPs, compared to the *E. coli* MCPs. However, there is no detectable similarity between TlpC and the N-terminal regions of McpA, McpB, TlpA and TlpB, which are very similar to each other. This similarity among the last four proteins is believed to reflect the requirement to bind similar members of a family of chemoeffector/binding protein complexes rather than the chemoeffectors directly (Hanlon & Ordal, 1994). The lack of similarity between TlpC and the other proteins, along with its smaller molecular mass, indicates that it performs other functions.

The fact that TlpC is similar in size to the *E. coli* MCPs leads to speculation about the possible evolutionary divergence between the *B. subtilis* and *E. coli* transducers. It is conceivable that TlpC is closely related to the ancestor of the *B. subtilis* transducers which are involved in chemotaxis and has itself descended from an *E. coli*-like transducer. After the evolutionary lines that gave rise to *B. subtilis* and *E. coli* diverged, the ancestor of TlpC may

have given rise to the larger chemotactic transducers of *B. subtilis*.

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