Functional and genetic characterization of *mcpC*, which encodes a third methyl-accepting chemotaxis protein in *Bacillus subtilis*

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A 3135 bp DNA segment downstream of the *spl* gene on the *Bacillus subtilis* chromosome was cloned and its nucleotide sequence determined. An open reading frame capable of encoding a putative protein of 654 amino acids with a calculated molecular mass of 72.1 kDa was identified. The deduced amino acid sequence was similar to the McpA and McpB proteins of *B. subtilis*. McpA and McpB encode different methyl-accepting chemotaxis proteins (MCPs). A mutant strain containing an antibiotic resistance DNA cassette inserted into the region containing the MCP-like reading frame suffered a complete loss of taxis to the amino acids cysteine, proline, threonine, glycine, serine, lysine, valine and arginine. The open reading frame was designated *mcpC*. The wild-type and an *mcpC* mutant strain were analysed for their content of methylated proteins and it was found that *mcpC* encodes a methylated membrane protein that has previously been designated H3. These results show that *mcpC* encodes a third MCP in *B. subtilis*. The transcription start site upstream of the *mcpC* gene was determined by primer extension analysis and it was found to be preceded by a potential promoter sequence that is recognized by the σ^D form of RNA polymerase. The level of β -galactosidase expressed from a transcriptional *mcpC-lacZ* fusion was increased threefold when cells entered the stationary phase. No β -galactosidase could be detected in a *sigD* genetic background.

Keywords: chemotaxis receptor protein, methylation, *mcpC* gene sequence, *mcpC* expression, σ^D -dependent expression

INTRODUCTION

Chemotaxis is a primitive behavioural system by which bacteria travel towards better environments as the result of modulating tendency to swim smoothly [anticlockwise (ACW) rotation of flagella] or tumble [clockwise (CW) rotation of flagella]. Rotational direction is governed by the operation of a two-component system, involving an autophosphorylating kinase, CheA, and a response regulator, CheY (Bischoff & Ordal, 1991; Fuhrer & Ordal, 1991). When *Bacillus subtilis* encounters increasing attractant concentration, more attractant will bind to the receptors (methyl-accepting chemotaxis

proteins; MCPs) to activate CheA and produce more CheY-P, which will bind to switches at the base of the flagella to cause ACW rotation (excitation). When the bacteria head, by chance, towards lower attractant concentrations, attractant leaves the receptors and CW rotation ensues. Adaptation, to return the ratio of ACW/CW rotation to normal (random) even with the stimulus still present, occurs with release of methanol from methylesterified receptors. This involves the methylesterase CheB, which is also phosphorylated by CheA-P, and thereby activated (Kirsch *et al.*, 1993a). The methyltransferase CheR, using S-adenosyl-methionine, puts methyl groups onto the receptors (Kirsch *et al.*, 1993b). At least two other proteins, not found in the Gram-negative *Escherichia coli*, participate in the reactions of excitation and adaptation – CheC and CheD – and they are also thought to interact with the receptors (Rosario & Ordal, 1996). Finally, *B. subtilis* has two

Abbreviation: MCP, methyl-accepting chemotaxis protein.

The EMBL accession number for the nucleotide sequence reported in this paper is X97385.

other proteins which may help couple signals from the receptors to CheA – CheW and CheV (Hanlon *et al.*, 1992; Rosario *et al.*, 1994). Thus, a number of proteins – CheA, CheB, CheR, CheC, CheD, CheW and CheV – apparently bind to the receptors and might be expected to cause sequence conservation among the receptors to maintain functional protein–protein interactions.

The receptors themselves are methylesterified on selected glutamate side chains. Most of the receptors from a variety of organisms have a similar three-dimensional geometry – an N-terminal region between two membrane-spanning regions, where interactions with attractants or repellents are thought to occur, and a C-terminal region where interactions with the chemotaxis proteins mediating sensory transduction are thought to occur (Le Moual & Koshland, 1996). Thus, all receptors known contain a region where CheW and CheA are thought to interact. In *B. subtilis*, two MCPs, McpA and McpB, have been identified. McpA mediates taxis to glucose and α -methyl glucosides and McpB mediates all taxis to asparagine, but only some taxis to aspartate, histidine and glutamine (Hanlon & Ordal, 1994). In the four gene cluster containing *mcpA* and *mcpB*, two other genes, *tlpA* and *tlpB*, are located. They encode proteins with extensive homology to both McpA and McpB but their function is unknown. A third transducer-like protein, TlpC, encoded by a gene not coupled to the *mcpA* gene cluster only shows similarity to McpA and McpB in the C-terminal region. TlpC mutants have normal chemotaxis phenotype (Hanlon *et al.*, 1994). One feature that has proven unique among four of the receptors of *B. subtilis* thus far studied – McpA, McpB, TlpA and TlpB – is extensive amino acid sequence conservation in the N-terminal part. This is believed to reflect interactions with binding proteins, perhaps similar to the maltose-binding proteins of *E. coli* (and also the galactose- and ribose-binding proteins of *E. coli*; Kossmann *et al.*, 1988), which bind their respective ligands and then bind to the methylated receptors.

In this report, we describe the identification of a new MCP in *B. subtilis* that is encoded by the gene *mcpC*. It is demonstrated that McpC is involved in chemotaxis and that *mcpC* expression is dependent on the *sigD* gene product.

METHODS

Bacterial strains, plasmids and media. The bacterial strains and plasmids used in this work are listed in Table 1. *B. subtilis* was grown in Spizizen minimal salt medium (Saxild & Nygaard, 1987) supplemented with 1 μ g thiamin hydrochloride ml⁻¹, 50 μ g L-tryptophan ml⁻¹ and with 0.4% of the stated carbon source. Additional amino acids required by auxotrophic strains were added at 50 μ g ml⁻¹ concentrations. L broth (Difco) was used as a rich medium for both *E. coli* and *B. subtilis*. Culturing of cells was performed at 37 °C. For selection of antibiotic resistance the following concentrations were used: ampicillin, 100 μ g ml⁻¹; chloramphenicol, 5 μ g ml⁻¹; neomycin, 5 μ g ml⁻¹; erythromycin, 150 μ g ml⁻¹ (*E. coli*); and erythromycin and lincomycin, 1 and 25 μ g ml⁻¹, respectively (*B. subtilis*).

DNA manipulations and genetic techniques. Chromosomal DNA from *B. subtilis* was isolated as described previously (Saxild & Nygaard, 1987). Plasmid DNA was isolated by the alkaline/SDS method (Birnboim & Doly, 1979). Transformation of *E. coli* (Hanahan, 1983) and *B. subtilis* (Boylan *et al.*, 1972) was performed as described previously. Treatment of DNA with restriction enzymes, T4 DNA ligase and the Klenow fragment of DNA polymerase was performed as recommended by the supplier (Gibco-BRL). For Southern blot analysis, DNA was blotted onto a nitrocellulose membrane (GeneScreenTM; NEN Research Products). The DIG DNA Labelling and Detection kit (Boehringer Mannheim) was used for colorimetric detection of the hybridization products. The recipe supplied by the manufacturer was followed.

DNA sequencing. DNA sequences were obtained by the chain-termination reaction method with dideoxyribonucleotides as described by Sanger *et al.* (1977). All sequencing analysis was done on the double-stranded plasmid DNA templates pCW10 and pBOEC335.

Isolation of RNA and primer extension analysis. Total RNA from *B. subtilis* was isolated using a previously described method (Saxild *et al.*, 1995). The primer extension analysis using SuperScript RNaseH⁻ reverse transcriptase (Gibco-BRL) has also been described previously by Saxild *et al.* (1995). The radiolabelled cDNA products were separated on a 6% (w/v) polyacrylamide sequencing gel and visualized by autoradiography.

In vivo methylation assay and PAGE. The strains OI1085, OI3202, OI3280 and OI3282 were grown to 180 Klett units in L broth with antibiotic where appropriate and washed three times in chemotaxis buffer (Ordal & Goldman, 1975) containing chloramphenicol (250 μ g ml⁻¹). The cells were then resuspended in protoplast buffer (Ullah & Ordal, 1981) containing 250 μ g chloramphenicol ml⁻¹ at an OD₆₀₀ of 1.0 (Pharmacia-LKB Ultrospec III spectrophotometer). Lysozyme was added to a final concentration of 1 mg ml⁻¹, and the cells were incubated at 37 °C with gentle shaking for 20 min. Methylation was initiated by the addition of 50 μ Ci [*methyl*-³H]methionine ml⁻¹ (1.85 $\times$ 10⁶ Bq ml⁻¹) (6.0 $\times$ 10⁻⁷ M). Incubation was continued for an additional 15 min and samples were frozen in a dry ice/acetone bath. Protoplasts were recovered by centrifugation at 4 °C and resuspended in 100 μ l 2 $\times$ SDS/EDTA solubilizer buffer (Hanlon & Ordal, 1994). SDS-PAGE was performed by the method of Laemmli (1970). Samples were solubilized in 2 $\times$ SDS/EDTA solubilizer buffer by boiling for 7 min and applied to 10% SDS-PAGE gels and run at 25 mA for 3 h. The labelled proteins were then visualized by fluorography by the method of Laskey & Mills (1975).

Capillary assay. Strains were streaked out on TBAB (Difco) plates (with antibiotic where appropriate) and grown overnight at 30 °C. Single colonies were resuspended in 2 ml TBR (1%, w/v, Bactotryptone; 0.5% NaCl) and diluted 1:50 in minimal medium containing glycerol as the carbon source (20 mM). Cultures were grown for 4 h and washed in chemotaxis buffer. Cells were finally resuspended in chemotaxis buffer to an OD₆₀₀ of 0.001 and tested for taxis to all 20 amino acids. Capillary assays were performed as previously described (Ordal *et al.*, 1977).

β -Galactosidase assay. Cells for enzyme measurement were harvested in mid-exponential growth phase or in early stationary phase and homogenized by sonication in 30 mM phosphate buffer (pH 7.5), 1 mM EDTA and 1 mM DTT. Cell debris was removed by centrifugation. β -Galactosidase was measured according to the method of Miller (1972). All the

Table 1. Bacterial strains and plasmids

Strain/plasmid		Characteristics	Source/reference
<i>B. subtilis</i>			
HH1	<i>trpC2</i>		168; C. Anagnostopoulos, INRA, Jouy-en-Josas, France
HH71	<i>trpC2 purL8 prg-71::lacZ (cat)</i>		Saxild <i>et al.</i> (1994)
HH226	<i>trpC2 amyE::mcpC-lacZ; Neo^R</i>		Transformation of HH1 by pHH1008 digested with <i>XhoI</i>
HH247	<i>trpC2 sigD::pLM5 (cat)</i>		1A716; <i>Bacillus</i> Genetic Stock Center, Columbus, OH, USA
JM6	<i>trpC2 mcpC::pBOEA335 (cat); Cm^R</i>		Transformation of HH1 by pBOEA335
JM9	<i>trpC2 mcpC::erm</i>		Transformation of HH1 by pBOEB(<i>erm</i>)335 selecting <i>Er^R</i> ; transformants screened for <i>Cm^S</i>
JM10	<i>trpC2 mcpC::erm sigD::pLM5 (cat); Cm^R</i>		Transformation of JM9 by DNA from HH247
OI1085	<i>trpF7 hisH2 metC</i>		Ordal & Goldman (1975)
OI3202	<i>trpF7 hisH2 metC Δ(mcpB-tlpA-mcpA-tlpB) ΔtlpC</i>		G. W. Ordal
OI3280	<i>trpF7 hisH2 metC mcpC::erm; Er^R</i>		Transformation of OI1085 by DNA from JM9
OI3281	<i>trpF7 hisH2 metC Δ(mcpB-tlpA-mcpA-tlpB) ΔtlpC mcpC::erm; Er^R</i>		Transformation of OI3202 by DNA from JM9
<i>E. coli</i>			
MC1061	F ⁻ <i>araD139 Δ(ara-leu)7696 galE15 galK16 Δ(lac)X74 rpsL (Str^R) hsdR2 (r⁻ m⁻) mcrA mcrB</i>		Stratagene
Plasmids			
pKS(+)	Ap ^R		Stratagene
pDG268neo	Ap ^R (<i>E. coli</i>), Neo ^R (<i>B. subtilis</i>); vector used for integration of transcriptional <i>lacZ</i> fusions into the <i>amyE</i> gene of <i>B. subtilis</i>		Saxild <i>et al.</i> (1995)
pBOE335	Ap ^R (<i>E. coli</i>), Cm ^R (<i>B. subtilis</i>); pUC19 containing the <i>cat</i> gene cloned into the <i>KasI</i> site		Saxild <i>et al.</i> (1996)
pHH1008	0.8 kb <i>HindIII</i> fragment from pCW10 ligated to pGD268neo digested with <i>HindIII</i> ; used for integration of a transcriptional fusion of <i>mcpC</i> to <i>lacZ</i> into the <i>amyE</i> gene		This work
pHH1017	Ap ^R (<i>E. coli</i>), <i>Er^R</i> (<i>E. coli</i> and <i>B. subtilis</i>); 1.1 kb <i>EcoRI</i> fragment containing the <i>erm</i> gene ligated to pKS(+) digested with <i>EcoRI</i>		Saxild <i>et al.</i> (1996)
pCW1	pSGMU38 derivative (Saxild <i>et al.</i> , 1994) containing 2.5 kb <i>B. subtilis</i> DNA rescued from chromosomal DNA from strain HH71		This work
pCW10	2.5 kb <i>SphI-EcoRI</i> DNA fragment from pCW1 ligated to pKS(+) that has been digested with <i>EcoRV</i> and <i>EcoRI</i> ; the <i>SphI</i> site of the insert was blunt-ended with Klenow DNA polymerase prior to ligation		This work
pBOEA335	1.2 kb <i>HindIII</i> fragment from pCW10 containing an internal part of <i>mcpC</i> ligated to pBOE335 digested with <i>HindIII</i> ; the direction of transcription of the <i>mcpC</i> segment is in parallel with that of the <i>lacZ</i> α fragment of pBOE335		This work
pBOEB335	1.2 kb <i>HindIII</i> fragment from pCW10 containing an internal part of <i>mcpC</i> ligated to pBOE335 digested with <i>HindIII</i> ; the direction of transcription of the <i>mcpC</i> segment is opposite of that of the <i>lacZ</i> α fragment of pBOE335		This work
pBOEB(<i>erm</i>)335	1.1 kb <i>SmaI-EcoRV</i> fragment from pHH1017 containing the <i>erm</i> gene cloned into the unique <i>EcoRV</i> site in the 1.2 kb <i>HindIII</i> <i>mcpC</i> insert in pBOEB335		This work
pBOEC335	pBOE335 derivative containing a 2.0 kb <i>HindIII-BglII</i> fragment rescued from chromosomal DNA from strain JM6		This work

[illegible]

Fig. 1. Nucleotide sequence of a 3.1 kb region downstream of *spl* containing the *mcpC* and *orf1* genes. The deduced amino acid sequences are shown above (*mcpC*) or beneath (*orf1*) the nucleotide sequence. The -35 and -10 elements of the σ^D -dependent promoter upstream of *mcpC* are boxed. The position of the transcription start site of the *mcpC* gene is shown by a bent arrow. The -35 and -10 elements of a putative σ^A -dependent promoter upstream of *orf1* are indicated by asterisks beneath the sequence. Potential ribosome-binding sites in front of the reading frames are indicated by lines beneath the sequence. Potential factor-independent transcription terminator structures downstream of *spl* and *mcpC* are indicated (>>><<<). Only relevant restriction sites are indicated.

determinations were repeated at least three times. One unit of specific enzyme activity was defined as 1 nmol substrate converted min^{-1} (mg protein^{-1}). Total protein was determined by the Lowry method.

Cloning of the chromosomal region containing *mcpC*. DNA flanking the pSGMU38 insertion point in strain HH71 [*prg-71::lacZ* (pSGMU38)] (Saxild *et al.*, 1994) was isolated using the standard plasmid rescue technique (Youngman *et al.*, 1984). DNA from strain HH71 was digested with *Sph*I, ligated and subsequently transformed into *E. coli* strain MC1061 by selection for Ap^R . A plasmid (pCW1) containing a 2.5 kb insert of *B. subtilis* DNA was isolated from one of the transformants. pCW1 was digested with *Sph*I, and the DNA was blunt-ended with the Klenow fragment of DNA polymerase and subsequently cleaved with *Eco*RI. *Eco*RI cuts the plasmid in the sequence containing the *spoIIAA* RBS that has been inserted in front of the *lacZ* gene of pSGMU38 (Errington, 1986). The 2.5 kb fragment was isolated from an agarose gel and ligated to *Eco*RV- and *Eco*RI-cleaved pBlue-script. The ligation mixture was transformed into MC1061 by selection for Ap^R . A plasmid containing the 2.5 kb insert was named pCW10. pCW10 was digested with *Hind*III and a 1.2 kb fragment of *B. subtilis* DNA was isolated and ligated to *Hind*III-cleaved pBOE335. After transformation of the ligation mixture into MC1061, a plasmid (pBOEA335) was isolated that contained the insert oriented so that the direction of transcription of the *mcpC* segment encoded by the 1.2 kb *Hind*III fragment was in parallel with the direction of transcription of the *lacZ* α fragment of pBOE335. Strain HH1 was transformed with pBOEA335 by selection for Cm^R and since the plasmid cannot replicate in *B. subtilis* it was integrated into the chromosome in the *mcpC* locus by homologous recombination. By using a plasmid rescue procedure similar to the one described above, DNA flanking the pBOEA335 insertion point was isolated. DNA from strain JM6 (*mcpC::pBOEA335*) was digested with *Bam*HI and *Bgl*II, ligated and transformed into MC1061 by selection for Ap^R . A plasmid (pBOEC335) containing 0.8 kb *B. subtilis* DNA in addition to the 1.2 kb *Hind*III fragment was isolated from one of the transformants.

Construction of an *mcpC* mutant. A 1.1 kb *Eco*RV-*Sma*I DNA fragment containing *erm* which encodes erythromycin resistance was isolated from pHH1017 (Saxild *et al.*, 1996) and ligated to pBOEA335 that was cleaved in the unique *Eco*RV site located in the 1.2 kb *Hind*III *B. subtilis* insert. The ligation mixture was transformed into MC1061 by selection for Er^R . From one of the transformants a plasmid [pBOEA(*erm*)335] with the *erm* gene inserted was isolated. The direction of transcription of the *erm* gene cloned in pBOEA(*erm*)335 was the opposite of that of the *mcpC* gene. pBOEA(*erm*)335 was transformed into HH1 by selection for Er^R . The expected double-recombination event (strain JM3) was confirmed by Southern blot analysis. Strain JM3 has the *erm* gene inserted in the unique *Eco*RV site in *mcpC*.

Construction of an *mcpC-lacZ* fusion. The 0.8 kb *Hind*III fragment containing the *mcpC* transcription start site was isolated from pCW10 and ligated to pDG268neo digested with *Hind*III. The ligation mixture was transformed into MC1061 by selection for Ap^R . The orientation of the insert in plasmid pHH1008 was so that the directions of transcription of *mcpC* and *lacZ* were in parallel. The plasmid was linearized with *Xho*I and transformed into HH1 by selection for Neo^R . pHH1008 was integrated into the *amyE* locus of HH1 by a

double crossing-over event (Saxild *et al.*, 1996), resulting in strain HH226 (*amyE::mcpC-lacZ*).

RESULTS

Nucleotide and deduced amino acid sequences of the *orf1* and *mcpC* genes and products

A 3135 bp nucleotide sequence of the *B. subtilis* chromosome present in plasmids pCW10 and pBOEC335 was determined and is shown in Fig. 1. Nucleotides 1–396 have previously been sequenced and comprise the 3'-end of the *spl* gene (Fajardo-Cavazos *et al.*, 1993). The previously published sequence (Fajardo-Cavazos *et al.*, 1993) lacks a G residue at position 312 in the sequence shown in Fig. 1. Addition of a G residue creates a *Not*I restriction site. We found a *Not*I site that maps close to the *Sph*I site in the 3'-end of the *spl* gene that is present in pCW10. *prg-71* (the original *mcpC* allele; Saxild *et al.*, 1994) is located exactly between *metC* and *proAB* on the *B. subtilis* linkage map (Saxild *et al.*, 1994) and according to previously published results (Itaya, 1993) only one *Not*I site has been identified in this region. We therefore suggest that the *Not*I site located downstream of *spl* is a candidate for the *Not*I site located at 1375 kb on the physical map of the *B. subtilis* chromosome (Itaya, 1993).

Two divergently orientated open reading frames were located downstream of *spl*. The reading frames encode putative proteins of 226 aa (30.2 kDa) and 654 aa (72.1 kDa), respectively. Using the BLAST algorithm (Altschul *et al.*, 1990), homology searches were performed to obtain information about the possible function of the two open reading frames. The homology search did not reveal information about the function of the 226 aa open reading frame (*orf1*). However, the 654 aa reading frame was found to be approximately 27% identical to the McpA and McpB proteins from *B. subtilis*. McpA and McpB are different MCPs involved in the chemotactic response to carbohydrates and amino acids, respectively (Hanlon & Ordal, 1994). Based on this information and on the functional analysis presented later, we assigned the designation *mcpC* to the 654 aa open reading frame. The *B. subtilis* genome sequencing project has not given this reading frame a systematic 'y'-designation, but has instead adopted the *mcpC* designation suggested by us. The nucleotide sequence downstream of *mcpC* contains a potential factor-independent transcription terminator structure (Fig. 1). Further downstream of *mcpC* the 5'-end of a new reading frame was located. The deduced amino acid sequence (43 aa in total) showed 40–46% identical amino acids to the N-terminal end of 6-phosphogluconate dehydrogenase from several prokaryotic organisms. The possible function of the two reading frames lying up- and downstream of *mcpC* was not investigated further.

A hydropathy profile (Kyte & Doolittle, 1982) of the McpC sequence revealed two hydrophobic regions

Table 2. Capillary assays of the wild-type and an McpC mutant strain

Values are expressed as number of bacteria in the capillary tube.

Amino acid	Wild-type (OI1085)	McpC mutant (OI3280)	Percentage reduction	Receptor class
Cysteine	1608 ± 438	0 ± 44	100	A
Proline	1122 ± 206	5 ± 13	99	A
Threonine	1861 ± 684	5 ± 5	99	A
Glycine	7459 ± 1363	24 ± 36	99	A
Serine	813 ± 184	7 ± 7	99	A
Lysine	1237 ± 306	18 ± 16	99	A
Valine	1028 ± 322	17 ± 4	98	A
Arginine	1061 ± 351	22 ± 31	98	A
Alanine	1452 ± 268	116 ± 85	92	B
Tyrosine	1576 ± 289	132 ± 6	92	B
Glutamate	2080 ± 230	225 ± 96	89	B
Isoleucine	4930 ± 299	621 ± 75	87	B
Methionine	1410 ± 87	295 ± 30	79	B
Tryptophan	3324 ± 762	727 ± 67	78	B
Phenylalanine	1661 ± 656	460 ± 110	72	B
Leucine	2248 ± 293	684 ± 14	70	B
Aspartate	1398 ± 297	908 ± 126	35	B
Glutamine	2437 ± 396	1602 ± 257	34	B
Histidine	1189 ± 479	873 ± 192	27	B
Asparagine	2850 ± 89	3694 ± 308	0	C

characteristic of membrane-spanning segments – one segment includes amino acids 1–20 and the other amino acids 280–300 (data not shown). The location of the two membrane-spanning segments in the N-terminal part of the McpC protein is very similar to the location of the hydrophobic segments found in the McpA, McpB, TlpA, TlpB and TlpC proteins (Hanlon *et al.*, 1994; Hanlon & Ordal, 1994). It is apparent that McpC is related to McpB and McpA, in that there is some similarity in both the N-terminal (between the two putative membrane-spanning regions) and the C-terminal (following the second putative membrane-spanning region) regions, but much less than McpA and McpB have for each other (the percentage identity between McpB and McpA and between McpB and McpC is 60.5% and 27%, respectively). Furthermore, all three MCPs are of the same size. The regions of enhanced similarity appear to be the following (numbered on the basis of the McpC sequence): 1–29, 135–162, 243–264, 299–398, 463–536 and 590–638. The first three are thought to be in the N-terminal region, the fourth from the beginning of the second membrane-spanning region into the C-terminal region, and the last two in the C-terminal part. Using the consensus sequence derived from the alignment of methylation sites of several methylated proteins constructed by Le Moual & Koshland (1996), we located potential methylation sites at amino acid positions 364, 385, 616 and 623 in the McpC sequence. The potential methylation sites at positions 364 and 623 in McpC are located at similar positions in McpA and McpB (Hanlon

& Ordal, 1994), whereas the other two are located at different positions.

Characterization of a mutant defective in *mcpC*

To test the function of the McpC protein, the *mcpC* gene was disrupted by insertion of an erythromycin antibiotic resistance DNA cassette (*erm* gene) as described in Methods. Since the deduced amino acid sequence of McpC shows similarity to the McpA and McpB sequences, strain OI3280 (*mcpC::erm*) was tested in a capillary assay for its chemotactic response to all 20 amino acids. As shown in Table 2, the taxis to all amino acids except for asparagine (receptor class C in Table 2) was more or less reduced in strain OI3280 when compared to the wild-type strain. An almost total loss of taxis to cysteine, proline, glycine, serine, lysine, valine and arginine was observed (receptor class A in Table 2) while the response to the remaining 11 amino acids was reduced to 80–20% of the levels found in the wild-type (receptor class B in Table 2). In previous experiments, we found that *mcpB* mutants are completely defective in asparagine taxis and partially defective in glutamine, aspartate and histidine taxis (Hanlon & Ordal, 1994). To confirm that glutamine, aspartate, histidine and possibly also glutamate taxes are mediated by McpB and McpC, we constructed a double mutant and carried out capillary assays. There was no taxis to any of these amino acids, as expected (data not shown). The double

Fig. 2. *In vivo* protein methylation. Cells were labelled with [*methyl*- ^3H]methionine for 15 min and methylated membrane proteins were separated by SDS-PAGE and visualized by fluorography as described in Methods. Lanes: 1, wild-type strain OI1085; 2, *mcpC* mutant strain OI3280; 3, deletion strain OI3202 (*mcpA mcpB tlpA tlpB tlpC*); 4, strain OI3281, which is an *mcpC* mutant derivative of strain OI3202.

mutant was also tested in capillary assays for taxis to five other class B amino acids (tryptophan, methionine, isoleucine, phenylalanine and leucine) and levels identical to the ones found for the *McpC* mutant (Table 2) were observed (data not shown).

The wild-type strain OI1085 and different deletion mutant derivatives were labelled with [*methyl*- ^3H]methionine and analysed for their content of methylated membrane proteins as described in the Methods section. Four radiolabelled protein bands were visible in the 76–97 kDa region of the methylation gel (Fig. 2). The upper band was designated H1 and has previously been shown to be the protein encoded by *mcpA*. The two middle bands were designated H2 and are possibly *TlpA* and *TlpB* since they are missing in a strain deleted for *mcpA*, *mcpB*, *tlpA* and *tlpB* (Fig. 2). Strains OI3280 (*mcpC::erm*) and OI3281 (lacking all known MCPs) lack the lower part of the lowest band, designated H3 (Fig. 2) (the upper part of which is *McpB*; Hanlon & Ordal, 1994). This observation strongly indicates that *mcpC* encodes a methylated membrane protein corresponding to part of the H3 band.

Mapping of the *mcpC* transcription start site

Two oligonucleotides which are complementary to nucleotides 933–948 (primer 1) and 1006–1024 (primer 2) in the *mcpC* sequence (Fig. 1) were used in two primer extension reactions containing RNA isolated from HH1 cells harvested in the late-exponential growth phase. As illustrated in Fig. 3, primer 2 was extended to a T residue which is complementary to the A residue no. 892 (A_{892}) in the *mcpC* sequence. The same result was obtained with primer 1 (data not shown). Immediately upstream of the A_{892} position we located a sequence that resembles the recognition sequence for the σ^D form of RNA polymerase (857-TTCAT-16_{nt}-CCGATAT-884; Gilman *et al.*, 1981).

Fig. 3. Mapping of the *mcpC* transcription start point by primer extension analysis. Total RNA was isolated from strain HH1 harvested in the late-exponential growth phase. First-strand cDNA synthesis was performed as described in Methods. The DNA primer (primer 2) is complementary to nucleotides 1024–1006 (Fig. 1). The most likely transcription start point (A_{892}) is indicated by a bent arrow. The emphasized sequence is given as the complementary sequence of the one shown in the sequencing ladder.

Expression of *mcpC*

Strains HH226 (*amyE::mcpC-lacZ*) and MJ10 (*amyE::mcpC-lacZ sigD::cat*) were grown in minimal medium and the level of β -galactosidase was determined. The *mcpC* expression started to increase in the late-exponential growth phase and reached a maximum during the early-stationary phase (Fig. 4). An identical regulatory pattern was observed when strain HH226 was grown in rich medium (data not shown). No β -galactosidase activity could be detected in the *sigD* genetic background and this strongly indicated that *mcpC* expression is dependent on the *sigD*-encoded sigma factor (Fig. 4). The influence of growth on different carbon sources on the expression of *mcpC* was analysed in HH226 cells grown on glucose, glycerol or succinate. No significant

Fig. 4. Effect of the growth phase on the expression of an *mcpC-lacZ* fusion. □, ■, OD₄₅₀; △, ▲, β-galactosidase activity. Open symbols, data from strain HH226 (*mcpC-lacZ*); filled symbols, data from strain JM10 (*mcpC-lacZ sigD*). β-Galactosidase activity was measured as described in Methods.

changes in the expression level or in the growth-phase-dependent variation were observed (data not shown). Different mutations were introduced into strain HH226 to characterize the stationary phase signal that is responsible for the increase in *mcpC* expression. Mutations in the transition state regulator gene *abrB* or in the sporulation initiation control gene *spo0A* did not affect the *mcpC* expression (data not shown). Furthermore, purine and pyrimidine starvation of purine and pyrimidine auxotrophic derivatives of strain HH226, respectively, resulted in an increase in *mcpC* expression that was identical to the one observed when the wild-type strain enters the stationary phase as illustrated in Fig. 4 (data not shown). Therefore, we conclude that under the conditions tested we have not been able to identify a specific genetic factor or physiological signal that is responsible for the induction of *mcpC* expression.

DISCUSSION

In this report, we describe a novel gene, *mcpC*, that is involved in the chemotactic behaviour of *B. subtilis*. Zuberi *et al.* (1990) have previously screened the *B. subtilis* chromosome for genes involved in chemotaxis and motility and it was suggested that at least two transcription units (*cheX* and a *mot* locus) were located near *ptsI*. In addition, Fredrick & Helmann (1994) have located *cheV* in the same region. *mcpC* is also placed close to *ptsI* (*ptsI* is located immediately upstream of *spl*), but neither the phenotype of an *mcpC* mutant nor the nucleotide sequence of the *mcpC* gene corresponded to the data reported for the chemotaxis and motility loci previously assigned to this part of the chromosome. This indicates that the region immediately downstream of *spl* contains at least four genes involved in chemotaxis and motility, namely *mcpC*, *cheX*, *cheV* and a *mot* gene.

It is clear that McpC plays a major role in chemotaxis in *B. subtilis*. It is required for taxis to the amino acids cysteine, proline, threonine, glycine, serine, lysine, valine

and arginine and for some others – aspartate, glutamine, histidine and glutamate – McpC and McpB are the two receptors either of which can mediate the taxis. Since an *mcpC mcpB* double mutant has not lost taxis to tryptophan, methionine, isoleucine, phenylalanine and leucine, other uncharacterized MCPs must be present in *B. subtilis*. As hypothesized previously, taxis to amino acids in *B. subtilis* is probably not mediated by binding of the amino acid to the receptor directly, like serine binding to Tsr in *E. coli* (Hedblom & Adler, 1980), but instead an amino acid/binding-protein complex probably binds the receptor, as for maltose taxis in *E. coli* (Kossmann *et al.*, 1988). The great structural variety in the amino acids whose taxis are mediated through McpC is further testimony in support of this hypothesis.

There are selected places in both the N-terminal and C-terminal regions where McpC is more similar to McpB and McpA. There is reason to believe that there is a family of binding proteins that mediates the taxis to the respective amino acids. This supposition is supported by the fact that both McpB and McpC each can mediate taxis to aspartate, histidine and glutamine. In the N-terminal part, we imagine the regions of conserved amino acids are where the binding proteins come into contact with the respective MCPs. In the C-terminal part, we imagine conserved regions are where the proteins that mediate the excitatory and adaptational reactions – CheA, CheW, CheV, CheR, CheB, CheC and CheD – come into contact with the receptor. This type of information should eventually prove useful in investigating how each of the chemotaxis proteins come into contact with the receptors.

Based on nucleotide sequence data it has been suggested that *mcpA*, *mcpB*, *tlpA*, *tlpB* and *tlpC* are all expressed from promoters recognized by the σ^D form of RNA polymerase (Hanlon *et al.*, 1994; Hanlon & Ordal, 1994). In this report, we show experimentally that *mcpC* expression is dependent on the *sigD* gene product. The transcription start site was located only 14 nucleotides upstream of the initiation codon. This is a surprisingly short leader sequence. The level of expression of *mcpC* was increased in the late-exponential growth phase and was maximal in the early-stationary phase. The same growth-phase-dependent pattern has been reported for the cellular level of SigD (Ordal *et al.*, 1993). Based on the fact that the control of expression of *sigD* and *mcpC* is independent of global regulators such as AbrB and SpoA (Márquez-Magana *et al.*, 1994), we suggest that *mcpC* expression may be strictly correlated with the level of SigD. The original reported *mcpC* allele (*prg-71::lacZ*) showed an increase in expression when cells entered the stationary phase as a consequence of purine starvation (Saxild *et al.*, 1994). From the data presented here, we conclude that growth arrest in general and not a specific purine starvation signal is responsible for the induction of *mcpC* expression.

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