The *Bacillus subtilis* cysP gene encodes a novel sulphate permease related to the inorganic phosphate transporter (Pit) family

Maria Cecilia Mansilla and Diego de Mendoza

Sulphate permeases in the plasma membrane are responsible for uptake of environmental sulphate used in the sulphate assimilation pathway in bacteria and plants. Here it is reported that the ORF designated cysP, located on the *Bacillus subtilis* chromosome between cysH and five putative genes involved in sulphate assimilation, encodes a sulphate permease. cysP is able to complement *Escherichia coli* cysteine auxotrophs with mutations affecting either the membrane or periplasmic components of the sulphate-thiosulphate permease. Transport studies with cell suspensions of a cysA97 *E. coli* strain transformed with a plasmid expressing the *B. subtilis* cysP gene indicated that CysP catalyses sulphate uptake. Analysis of the primary sequence showed that CysP (354 amino acids, estimated molecular mass 24 kDa) is a hydrophobic protein which has 11 putative transmembrane helices. Sequence comparisons revealed that CysP, together with the phosphate permease of *Neurospora crassa*, Pho-4, and *E. coli* PitA, belongs to the family of related transporters, the inorganic phosphate transporter (Pit) family. Among the putative phosphate permeases, CysP shows a similar size and the same domain organization as the archaeal transporters as the archaean transporters. This is the first report of a sulphate permease in a Gram-positive organism.

**Keywords:** *Bacillus subtilis*, cysteine biosynthesis, sulphate, transport

INTRODUCTION

In many micro-organisms and higher plants sulphur metabolism is initiated by the uptake of sulphate from the environment. These organisms assimilate sulphate into cysteine, the first sulphur-containing amino acid, and various sulphur-containing secondary metabolites (Kredich, 1996). Thus, uptake of sulphate by cells is considered to be the key entry step of the sulphur cycle in nature. Because the sulphate transport system is involved in this initial step, it should play a central role in the regulation of the entire sulphur metabolism pathway by controlling the import of available sulphate.

Among bacterial sulphate transporters, those from *Escherichia coli* and *Salmonella typhimurium* are the best studied. These micro-organisms possess an ATP-binding cassette (ABC)-type sulphate-thiosulphate transport system (Higgins, 1992), which is controlled in parallel with cysteine biosynthetic enzymes and which is a part of the cysteine regulon; gene expression of this region requires sulphur limitation and a positive regulator, CysB, the product of the *cysB* gene (Kredich, 1996). Components of the sulphate-thiosulphate permease from *E. coli* and *S. typhimurium* are encoded by the contiguous genes *cysP, cysT*, *cysW* and *cysA*, and by the unlinked gene *sbp* (Kredich, 1996). The products of *cysT* and *cysW* span the membrane and form a channel for the passage of sulphate and related ions, *cysA* encodes a hydrophilic membrane-associated ATP-binding protein, and *sbp* and *cysP* encode the sulphate and thiosulphate periplasmic binding proteins, respectively (Hryniewicz et al., 1990; Sirko et al., 1990). Molecular analysis of sulphate transport in *Synechococcus* sp., a cyanobacterium, established a four-component membrane sulphate transport system, essentially similar to that of *E. coli* (Laudenbach & Grossman, 1991).

The *Neurospora crassa* cys-14+ gene, the first eukaryotic sulphate transporter gene to be cloned, encodes a protein of approximately 90 kDa with 12 putative hydrophobic membrane-spanning domains (Ketter et al., 1991). The CYS14 protein is localized within the plasma membrane fraction and its synthesis depends on sulphur depri-
Bacterial strains, plasmids and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. E. coli and B. subtilis strains were routinely grown in Luria–Bertani (LB) broth (Sambrook et al., 1989). The minimal medium used for E. coli was M9 (Miller, 1972) supplemented with 0.4% glucose, 2 mM MgSO₄, 0.001 mM CaCl₂ and 0.01% thiamine. For nutritional studies, MgCl₂ replaced MgSO₄, and glutathione (1 mM) or cysteine (1 mM) were used as sulphur sources. Antibiotics were added to media as follows: 100 µg ampicillin ml⁻¹, 5 µg chloramphenicol ml⁻¹. Spizizen salts (Spizizen, 1958), supplemented with 0.5% glucose and the required L-amino acids, was used as the minimal medium for B. subtilis. In nutritional studies, where different sulphur sources were tested, MgSO₄ and (NH₄)₂SO₄ were substituted by an equimolar amount of MgCl₂ and NH₄Cl, respectively.

Chromate resistance was tested on LB plates containing 10−2.5 mM K₂CrO₄ was added (Pardee et al., 1966).

Plasmid constructions. Plasmid preparations, restriction enzyme digests and agarose gel electrophoresis were carried out according to methods described by Sambrook et al. (1989). Competent E. coli cells were transformed with supercoiled plasmid DNA by using the calcium chloride procedure or by electroporation (Sambrook et al., 1989).

Expression analysis. Recombinant plasmids containing the appropriate DNA fragments under T7 promoter control were transformed into E. coli BL21(DE3). Induction of gene expression was done as described by Studier et al. (1990). Briefly, mid-exponential-phase cultures (1 ml) of BL21(DE3) carrying plasmids pBluescript II SK(+) or pBS181 were induced by adding 1 mM IPTG. After 10 min the cells received rifampicin (100 µg ml⁻¹) to inhibit mRNA synthesis from the E. coli chromosome. After further incubation for 1 h, they were labelled with 1 µCi [³⁵S]methionine [specific activity 1000 Ci mmol⁻¹ (37 TBq mmol⁻¹)]. After 10 min, the cells were harvested by centrifugation, washed twice with 10 mM Tris/HCl buffer (pH 7.4), and stored frozen until analysis. Cells were resuspended in 30 mM Tris/HCl buffer (pH 7.4) containing 10 mM MgCl₂ and 1 mM PMSF, and disrupted by sonication (four or five 10 s bursts) using a VibraCell Ultrasonic Processor (Sonics & Materials). The extracts were centrifuged at 190000 g for 1 h, and labelled proteins present in either soluble or membrane fractions were separated by SDS-PAGE and detected by autoradiography.

Assay of sulphate transport. Cultures of strain JM2314 carrying relevant plasmids were grown in M9 minimal medium supplemented with glutathione as sulphur source to exponential phase. Cells were collected, washed and then resuspended in Davis salts (Miller, 1972) and incubated for 5 min at 30 °C. The measurement of sulphate transport was performed by incubating at 30 °C a cell suspension containing 10⁸ cells ml⁻¹, 30 µg chloramphenicol ml⁻¹, 0.01 mM sodium sulphate and approximately 10⁶ c.p.m. ⁸⁵SO₄⁻ ml⁻¹ (1050 Ci mmol⁻¹). The incubation period was terminated by filtering the cell suspension through a 0.45 µm Millipore filter. The filters were washed with 5 ml Davis salts. Filters were
transferred to polyethylene vials containing 2 ml Optiphase 
*HiSafe 3* scintillation fluid (Wallac) and the radioactivity 
counted in an LKB Primo liquid scintillation counter. Uptake 
rates are expressed in nmol sulphate min\(^{-1}\) (g cellular 
protein\(^{-1}\)).

**RESULTS AND DISCUSSION**

**The ylnA gene encodes a sulphate transporter**

The ylnA ORF from *B. subtilis* shows homology with phosphate permeases (Mansilla & de Mendoza, 1997) but no significant homology with proteins involved in sulphate uptake (see below). However, we have found that ylnA is transcriptionally induced by sulphate starvation, together with cysH and other genes involved in cysteine biosynthesis (unpublished results), suggesting that its gene product is a sulphate permease. To test the possibility that the ylnA gene product is involved in sulphate transport, we tried complementation of different classes of *E. coli* mutants unable to transport sulphate and thus to use sulphate as a sole sulphur source. Mutants affected in cysT as well as the uncharacterized cysA97 mutant are cysteine auxotrophs and they are unable to utilize sulphate as a sole sulphur source (Kredich, 1996). Strains carrying a single mutation in cysP or sbp are able to utilize both sulphate and thiosulphate as a sole sulphur source, whilst the inactivation of both genes leads to cysteine auxotrophy resulting from the block in the transport of both ions (Sirko *et al.*, 1995).

Complementation analysis was performed with plasmid pBS170 (which contains cysH, ylnA and the 5’ portion of ylnB, Table 1) and its derivatives: pBS181 (which includes a complete copy of ylnA), pBS184 (containing ylnA lacking the first 60 bp of the ORF), pBS188 (which includes ylnA without the last 140 bp of the ORF) and pBS190, a derivative of pBS181 possessing a frameshift mutation in ylnA (Table 1). As shown in Fig. 2, only plasmids pBS170 and pBS181, containing a complete functional copy of ylnA, restored the capacity of the ylnA mutants to grow in minimal medium with sulphate as a sulphur source. These data strongly suggested that ylnA encodes a protein involved in sulphate transport. Moreover, the protein encoded by this gene should have a substrate recognition site since ylnA is able to bypass the requirement of *E. coli* for the sbp and cysP genes, which encode the sulphate and thiosulphate periplasmic binding proteins (Sirko *et al.*, 1995).

To directly prove that ylnA encodes a sulphate transporter we tested whether plasmid pBS181 could restore

**Table 1. Bacterial strains and plasmids used in this study**

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristic(s)</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bacillus subtilis</strong></td>
<td>trpC2 pheA1</td>
<td>Laboratory stock</td>
</tr>
<tr>
<td><strong>Escherichia coli</strong></td>
<td><em>supE44 thi-1 ΔlacU169(q80dlacZAM15) endA1 recA1 bsdR17 gyrA96 relA1</em></td>
<td>Laboratory stock</td>
</tr>
<tr>
<td>DH5α</td>
<td><em>ompT rpsL mT pLysS</em></td>
<td>Laboratory stock</td>
</tr>
<tr>
<td>EC2402</td>
<td>cysA97</td>
<td>Sirko <em>et al.</em> (1995)</td>
</tr>
<tr>
<td>BL21(DE3)</td>
<td>sbp cysP</td>
<td>CGSC*</td>
</tr>
<tr>
<td>EC2256</td>
<td>F′ araD319 ΔlacU169 rpsL thi fla trp6 cysT329::lac imm* (1990)</td>
<td>Hryniewicz et al. (1990)</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td><strong>E. coli</strong> cloning vector, Amp(^\text{R})</td>
<td>Stratagene</td>
</tr>
<tr>
<td>pBluescript II SK(+)</td>
<td>3·4 kb EcoRI–EcoRI fragment of chromosomal DNA of JH642 cloned into pBluescript II SK(+)</td>
<td>Mansilla &amp; de Mendoza (1997)</td>
</tr>
<tr>
<td>pBS170</td>
<td>1·98 kb BglII–HindIII fragment of pBS170 cloned into pBluescript II KS(+) digested with BamHI/HindIII</td>
<td>This study</td>
</tr>
<tr>
<td>pBS181</td>
<td>2·2 kb PstI–PstI fragment of pBS170 (extending from the PstI site in ylnA to the PstI site in the pBS170 polynucleotides) cloned into pBluescript II KS(+) digested with PstI</td>
<td>This study</td>
</tr>
<tr>
<td>pBS184</td>
<td>1·32 kb BglII–CiaI fragment of pBS170 cloned into pBluescript II SK(+) digested with BamHI/CiaI</td>
<td>This study</td>
</tr>
<tr>
<td>pBS188</td>
<td>pBS181 digested with PstI, the protruding ends digested with Klenow DNA polymerase and the fragment religated</td>
<td>This study</td>
</tr>
</tbody>
</table>

*Strain obtained from the *Escherichia coli* Genetic Stock Center.
leads to reduced accumulation of chromium (Cervantes et al., 1990; Nies & Silver, 1989). Since the genome of E. coli does not encode ChrA homologues, resistance to chromate in this micro-organism has been associated with a deficiency in sulphate uptake. We tested whether plasmids containing B. subtilis cysP could confer chromate sensitivity to E. coli strains unable to transport sulphate. The result of this experiment showed that plasmids pBS170 and pBS181 conferred sensitivity to chromate to the cysT, cysP sbp and cysA97 mutants (Fig. 2), indicating that the cysP gene product can mediate the transport of chromate, as does the E. coli sulphate transporter system.

We have found that sulphate is able to relieve the toxicity of chromate in cysP+ B. subtilis. In fact, strain JH642 is resistant to chromate in LB medium or in minimal media supplemented with 1 mM sulphate (data not shown). However, in minimal media supplemented with 1 mM glutathione as a sole sulphur source, the growth of strain JH642 is inhibited by chromate (data not shown). The toxicity of chromate in this case is relieved by the addition of 1 mM sulphate, thus indicating that sulphate by itself leads to reduced accumulation of chromate (data not shown). These results agree with the suggestion that the B. subtilis ChrA homologues YwrA and YwrB could catalyse a chromate/sulphate antiport, exchanging sulphate for the toxic accumulated chromate (Nies et al., 1998).

**Expression of cysP in E. coli**

To analyse the protein encoded by cysP, we used plasmid pBS181 containing cysP under the control of the T7 promoter (Table 1). This plasmid was transformed into E. coli BL21(λDE3). Induction of gene expression was done according to the method of Studier et al. (1990). Expression of the DNA insert containing cysP in pBS181 resulted in the detection of a protein of 24 kDa (Fig. 4, lane 2). As described in the Introduction, the molecular mass calculated from the deduced primary sequence of CysP (YlnA) is 42.3 kDa, larger than the molecular mass of 24 kDa estimated by the mobility of the cysP gene product. Such an aberrant migration on SDS-PAGE is

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**Table 1. Growth of E. coli mutants deficient in sulphate transport in the presence of different ylnA region plasmids.**

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>SO$_4^{2-}$ uptake</th>
<th>CrO$_4^{2-}$ uptake</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cysA97 cysT cysP sbp</td>
<td>cysA97 cysT cysP sbp</td>
</tr>
<tr>
<td>pBS170</td>
<td>+ + +</td>
<td>− − −</td>
</tr>
<tr>
<td>pBS181</td>
<td>+ + +</td>
<td>− − −</td>
</tr>
<tr>
<td>pBS184</td>
<td>− − −</td>
<td>+ + +</td>
</tr>
<tr>
<td>pBS188</td>
<td>− − −</td>
<td>+ + +</td>
</tr>
<tr>
<td>pBS190</td>
<td>− − −</td>
<td>+ + +</td>
</tr>
</tbody>
</table>

**Fig. 2.** Growth of E. coli mutants deficient in sulphate transport in the presence of different ylnA region plasmids. Strains JM2314 (cysA97), EC2256 (cysT) and EC2402 (cysP sbp) transformed with the appropriate plasmid were tested for growth in minimal medium supplemented with 1 mM MgSO$_4$ as a sulphur source or in LB medium with 0.25 mM K$_2$CrO$_4$. −, No growth; +, good growth.

**Fig. 3.** Uptake of $^{35}$SO$_4^{2-}$ by plasmid-carrying strains. Sulphate transport was measured as described in Methods. Each datum point is the mean of three separate experiments with a mean error in transport values of less than 5%. ●, JM2314 (pBluescript II SK(+)); ▼, JM2314 (pBS181).

the capacity to transport sulphate to the E. coli cysA97 strain JM2314. To this end, cultures of strain JM2314 carrying plasmids pBluescript II SK(+) or pBS181 were assayed. In whole-cell transport assays, strain JM2314 bearing pBS181 showed $^{35}$SO$_4^{2-}$ transport activity, whereas no measurable accumulation of radioactive sulphate was observed in strain JM2314 transformed with pBluescript II SK(+) (Fig. 3). This experiment clearly demonstrates that the B. subtilis ylnA gene product possesses sulphate permease activity. Thus, we have renamed this gene cysP, the letter P denoting a permease.

**Chromate sensitivity in E. coli and B. subtilis**

In *Pseudomonas aeruginosa* and *Alcaligenes eutrophus*, chromate is accumulated by sulphate uptake systems, and expression of ChrA (chromate resistance protein A) leads to reduced accumulation of chromium (Cervantes et al., 1990; Nies & Silver, 1989). Since the genome of *E. coli* does not encode ChrA homologues, resistance to chromate in this micro-organism has been associated with a deficiency in sulphate uptake. We tested whether plasmids containing *B. subtilis* cysP could confer chromate sensitivity to *E. coli* strains unable to transport sulphate. The result of this experiment showed that plasmids pBS170 and pBS181 conferred sensitivity to chromate to the cysT, cysP sbp and cysA97 mutants (Fig. 2), indicating that the cysP gene product can mediate the transport of chromate, as does the *E. coli* sulphate transporter system.

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membrane-associated proteins. Moreover, a putative segments (TMSs) (Fig. 5), resembling those of other a hydrophobic peptide with 10–12 transmembrane terminded as described by Kyte & Doolittle (1982), shows The hydropathy profile of the product of cysP 36 (Buchel et al, 1987). To analyse if indeed CysP is localized in the membrane fraction, strain BL21(DE3) carrying plasmid pBS181 was labelled with [35S]methionine. Cells were disrupted by sonicaton, the extracts centrifuged and labelled proteins present either in the soluble or membrane fractions subjected to SDS-PAGE. Autoradiography of the gel showed that the bulk of the 24 kDa protein encoded by cysP is localized in the membrane fraction (data not shown).

**Predicted structure of CysP**

The hydropathy profile of the product of cysP, determined as described by Kyte & Doolittle (1982), shows a hydrophobic peptide with 10–12 transmembrane segments (TMSs) (Fig. 5), resembling those of other membrane-associated proteins. Moreover, a putative cleavable N-terminal signal peptide, characteristic of membrane-bound proteins, was determined in CysP using the program PSORT (Nakai, 1996).

Charge bias analysis of membrane protein topology, performed using the program TOPRED (von Heijne, 1992) was used to predict the most likely putative TMSs of CysP. This analysis revealed that the polypeptide would contain 11 TMSs, where the C terminus is cytoplasmic and the N terminus is on the outside surface of the membrane. According to this model of CysP, several basic amino acids residues, such as lysine and arginine, would be asymmetrically distributed on both sides of the membrane. We suggest that CysP contains 20 basic amino acids located in the cytoplasmic loops of the protein, whilst the loops located on the outside face of the membrane contain two positively charged amino acids. This charge distribution is in agreement with the positive-inside rule (von Heijne, 1992). Nevertheless, the actual number of TMSs should be determined experimentally.

Analysis of the amino acid sequence of the cysP gene product using the BLAST algorithm (Altschul et al., 1997) revealed that this protein is composed of two homologous domains, called D1 and D2, possessing 69% similarity. Domain D1 extends from residue 1 to 150 and contains five TMSs (TMS 1–TMS 5). Domain D2 extends from residue 163 to 313 and possesses TMS 6–TMS 10. This topological organization of the transporter suggests that CysP might have arisen by a tandem internal gene duplication event.

**Phylogenetic relationship**

A comparison of the primary structure of CysP with protein sequences in the databases revealed a high level of similarity to those of several phosphate permeases of both prokaryotic and eukaryotic origin. However, no significant homology was found between CysP and proteins belonging to the SulP family. Among the putative phosphate permeases, CysP shows a similar size and the same domain organization as the archaeal transporters (Table 2). The CysP gene product of *B. subtilis* showed a high level of similarity (67–72%) to the putative phosphate transporters of *Pyrococcus horikoshii*, *Archaeoglobus fulgidus* and *Methanobacterium thermoautotrophicum*. We also found that *Neurospora crassa* Pho-4 and *E. coli* PitA proteins show significant homology with D1 in the N-terminal region and with D2 in the C-terminal region (Table 2). Both proteins have a central region which is not homologous to CysP, which accounts for the difference in size, since both Pho-4 and PitA are larger than CysP (Mann et al., 1989; Sofia et al., 1994).

All the proteins that show significant homology with *B. subtilis* CysP belong to the inorganic phosphate transporter (Pit) family (Saier et al., 1999). Functionally characterized members of this family appear to catalyse inorganic phosphate uptake, either by proton or sodium symport (Versaw & Metzenberg, 1995; Martinez & Persson, 1998).
Table 2. Amino acid identity and similarity of the CysP domains of *B. subtilis* to corresponding proteins of other organisms

<table>
<thead>
<tr>
<th>Organism</th>
<th>Name or description</th>
<th>GenBank accession no.</th>
<th>Size (no. of amino acids)</th>
<th>Domain D1*</th>
<th>Domain D2*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Archaebacterium fulgidus</td>
<td>Putative phosphate permease</td>
<td>AAB89449</td>
<td>333</td>
<td>35 71</td>
<td>31 67</td>
</tr>
<tr>
<td>Pyrococcus borkhii</td>
<td>Putative sodium-dependent phosphate permease</td>
<td>BAA30231</td>
<td>318</td>
<td>33 69</td>
<td>30 72</td>
</tr>
<tr>
<td>Methanobacterium thermoautotrophicum</td>
<td>Sodium-dependent phosphate permease</td>
<td>AAB86351</td>
<td>326</td>
<td>33 69</td>
<td>28 68</td>
</tr>
<tr>
<td>Haemophilus influenzae</td>
<td>Putative phosphate permease</td>
<td>P45268</td>
<td>420</td>
<td>31 69</td>
<td>31 69</td>
</tr>
<tr>
<td>Neurospora crassa</td>
<td>Pho4, phosphate-repressible phosphate permease</td>
<td>P15710</td>
<td>590</td>
<td>25 67</td>
<td>21 68</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>PitA, low-affinity inorganic phosphate permease</td>
<td>P37308</td>
<td>499</td>
<td>23 66</td>
<td>23 59</td>
</tr>
<tr>
<td>Bacillus subtilis</td>
<td>YkAB, putative phosphate permease</td>
<td>CAA05564</td>
<td>328</td>
<td>25 66</td>
<td>23 60</td>
</tr>
</tbody>
</table>

*Domain D1 of CysP extends from residue 1 to 150 and domain D2 extends from residue 163 to 313. Alignments were created with the CLUSTAL method (Thompson et al., 1994). I, identity; S, similarity.

Concluding remarks

The results presented here show that the cysP gene product is a membrane-bound protein with a gel electrophoresis migration corresponding to an apparent molecular mass of 24 kDa. The fact that cysP is able to restore the capacity to transport sulphate to the cysA97 strain JM2314 and to relieve cysteine auxotrophy of mutants affected in both the periplasmic and membrane strain JM2314 and to relieve cysteine auxotrophy of organisms demonstrates that its gene product is a sulphate transport sulphate; thus, the assumed function of the uncharacterized members of the Pit family as putative phosphate transporters should be reconsidered.

ACKNOWLEDGEMENTS

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REFERENCES


Identification of a gene encoding a novel protein involved in repressible phosphate permease of *Neurospora crassa*.


