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Published in:
Journal of Bacteriology

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
1999

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Citation for published version (APA):

van de Kamp, M., Pizzinini, E., Vos, A., van der Lende, T. R., Schuurs, T. A., Newbert, R. W., Turner, G., Konings, W. N., & Driessen, A. J. M. (1999). Sulfate Transport in *Penicillium chrysogenum*: Cloning and Characterization of the *sutA* and *sutB* Genes. *Journal of Bacteriology*, 181(23), 7228-7234.

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Sulfate Transport in *Penicillium chrysogenum*: Cloning and Characterization of the *sutA* and *sutB* Genes

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Received 8 July 1999/Accepted 19 September 1999

In industrial fermentations, *Penicillium chrysogenum* uses sulfate as the source of sulfur for the biosynthesis of penicillin. By a PCR-based approach, two genes, *sutA* and *sutB*, whose encoded products belong to the SulP superfamily of sulfate permeases were isolated. Transformation of a sulfate uptake-negative *sB3* mutant of *Aspergillus nidulans* with the *sutB* gene completely restored sulfate uptake activity. The *sutA* gene did not complement the *A. nidulans* *sB3* mutation, even when expressed under control of the *sutB* promoter. Expression of both *sutA* and *sutB* in *P. chrysogenum* is induced by growth under sulfur starvation conditions. However, *sutA* is expressed to a much lower level than is *sutB*. Disruption of *sutB* resulted in a loss of sulfate uptake ability. Overall, the results show that SutB is the major sulfate permease involved in sulfate uptake by *P. chrysogenum*.

The filamentous fungus *Penicillium chrysogenum* is well known for its ability to produce penicillin (5, 39, 57). Penicillin biosynthesis starts with the condensation of the amino acids L- α -aminoadipic acid, L-Cys, and L-Val by the peptide synthetase δ (L- α -aminoadipyl)-L-cysteinyl-D-valine synthetase. The three precursor amino acids are synthesized in the cell as part of the primary metabolism of the fungus. To accommodate to the high demand for sulfur to be assimilated and incorporated into penicillin by high-producing strains (46, 54), inorganic sulfate is added to the medium as the source of sulfur for the formation of Cys (15, 39).

The uptake of sulfate, the first step in the pathway, has been studied by using mycelium and isolated plasma membrane vesicles from *P. chrysogenum* (4, 10, 17, 18, 46, 56, 60). These experiments indicated that sulfate is actively transported across the plasma membrane via a sulfate/proton symport mechanism.

Sulfate uptake is an important point of regulation of the sulfur metabolism in fungi. In *Neurospora crassa*, sulfate uptake is subject to a mechanism called sulfur (metabolite) repression or regulation, involving the action of positively and negatively acting regulatory proteins on the expression of sulfate permease-encoding genes (22, 27, 32). A similar situation holds for *Aspergillus nidulans* (30, 35, 36) and *Saccharomyces cerevisiae* (9, 55). In contrast, little is known about the mechanism and regulation of sulfate uptake in *P. chrysogenum* despite its possible significance in penicillin biosynthesis. Therefore, we set out to investigate sulfate permease-encoding genes from *P. chrysogenum*. The data shows that *P. chrysogenum* has two genes, designated *sutA* and *sutB* (*sut* for “sulfate transporter”), that encode putative sulfate transporters. Whereas the function of SutA remains to be elucidated, SutB was shown to

be a functional sulfate transporter responsible for sulfate uptake in *P. chrysogenum* mycelium.

MATERIALS AND METHODS

Strains, plasmids, and libraries. *Escherichia coli* LE392 [*hdsR574* ($r_K^- m_K^+$) *supE44 supF58 lacY1 galK2 galT22 metB1 trpR55*] (34) and DH5 α [ϕ 80 Δ lacZ Δ M15 *recA1 endA1 gyrA96 thi-1 hdsR17* ($r_K^- m_K^+$) *supE44 relA1 deoR* Δ (*lacZYA-argF*)*U169*] (13) were used for phage handling and plasmid transformations, respectively. *P. chrysogenum* Wisconsin 54-1255 (Wis54-1255) has been described previously (8, 38). *P. chrysogenum* HP60 is a nicotinamide-requiring derivative of NRRL1951 (48). The construction of *P. chrysogenum* nr45 is described below. *A. nidulans* IG1 (*sB3 pyrG89 pabaA1*), carrying the *sB3* mutation (1, 52), was derived from a cross between strains G191 (2) and 0198 obtained from the Glasgow Stock Collection (J. Clutterbuck, University of Glasgow). Plasmid pBluescript II KS (Stratagene) was used for cloning and sequencing in *E. coli*. Plasmid pGEM-T-easy (Promega) was used to clone PCR products. Plasmid pDJB2 is an *A. nidulans* transformation vector carrying the *N. crassa* *pyr-4* gene as a selection marker (2). Plasmid pBSsutA contains a 5.5-kb *Pst*I fragment (Fig. 1) cloned into the *Pst*I site of pBluescript II KS. Plasmid pBSsutB contains a 4.3-kb *Bam*HI fragment cloned into the *Bam*HI site of pBluescript II KS. Plasmid pBSPsutB contains a 2.1-kb *Sal*I fragment cloned into the *Sal*I site of pBluescript II KS. Plasmid pBSsutB-XS contains an internal 1.0-kb *Xho*I-*Sal*I fragment of *sutB* cloned into the MCS of pBluescript II KS. Plasmid pBSPsutB-sutA was constructed as follows. Plasmid pBSsutB was digested with *Eco*RV (in the multiple-cloning site) and *Hinc*II (28 bp upstream of the *sutB* ATG start codon), and a 737-bp *Eco*RV-*Hinc*II fragment containing part of the *sutB* promoter region was isolated (Fig. 1). This fragment was cloned into plasmid pBSsutA, from which the *sutA* promoter region was removed by digestion with *Bsr*EII (49 bp upstream of the *sutA* ATG start codon), treatment with DNA polymerase (Klenow fragment), and digestion with *Eco*RV (in the multiple-cloning site). A genomic library of *P. chrysogenum* Q176 (38) DNA in phage λ -EMBL3a was a generous gift from H. Schwab, Technical University, Graz, Austria. DSM-Gist (Delft, The Netherlands) kindly provided the *P. chrysogenum* cDNA library.

Media and growth conditions. Manipulations with and growth of *E. coli* LE392 and DH5 α were performed by standard methods (43). *P. chrysogenum* and *A. nidulans* growth media and conditions have been described previously (2, 16, 17). Where appropriate, sulfate salts were replaced by chloride salts, and methionine was added as indicated.

Gene cloning and sequencing. Degenerate deoxyribonucleotide oligomers, designated *sut*-forw (5'-ACC TAC AAG GT[CT] [GA]T[CT] ATC [GA]A[CT] AC[TCA] CT[TGC] AA-3') and *sut*-rev (5'-CC GAA [GT]GA CTT [TG]GA [GA]AT [TG]GC [TGA]AT [AG]TG [TC]TC-3'), were designed to correspond to two stretches of amino acid residues present in CYS-14p of *N. crassa* (TYKV [VI][NE]TLK and EHAIKSKSFG) (23) and SUL1p and SUL2p of *S. cerevisiae* (TYKV[VI][NE]TLK and EHAIKSKSFG) (9, 21, 49). PCR was performed on chromosomal *P. chrysogenum* DNA under standard conditions. PCR products of about 400 bp were isolated, treated with DNA polymerase (Klenow fragment), ligated into the *Sma*I site of pBluescript II KS, and sequenced. Of the 20 clones

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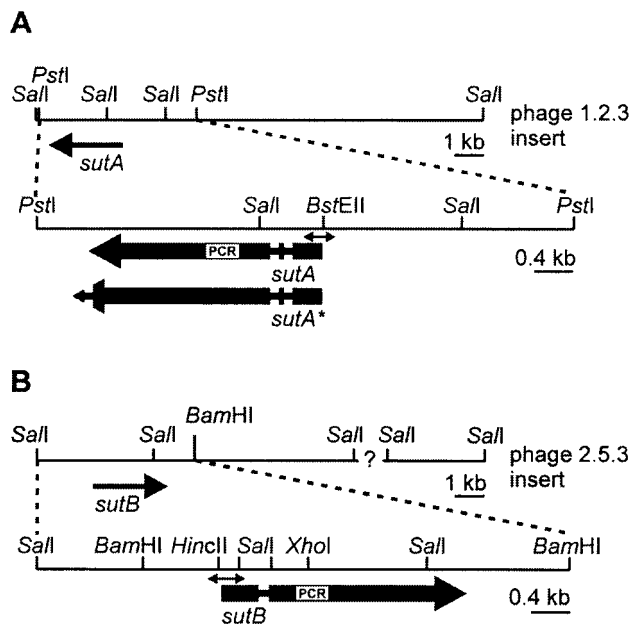


FIG. 1. Physical map of the genomic DNA fragments containing the *sutA* (A) and *sutB* (B) genes. Genomic DNA fragments present in phages 1.2.3 and 2.5.3 carrying the *sutA* and *sutB* gene are depicted. The *sutA* and *sutB* open reading frames are indicated by the large, thick arrows, in which the narrow regions represent introns. For *sutA*, two versions are depicted, designated *sutA* and *sutA**, the latter representing an extended open reading frame which would result if the last intron were spliced out. The sequences of the enlarged fragments (*PstI-PstI* for *sutA* and *SalI-BamHI* for *sutB*) are available in the GenBank database. The positions of the PCR fragments that were used to isolate the genes are indicated by the white boxes designated PCR. Regions that were used to probe expression in the Northern analysis are indicated by the double-headed arrows.

sequenced, two sets of 3 and 7 identical clones showed sequence similarity to known sulfate transporter-encoding genes but were different from each other. The PCR products were used to screen a genomic library of *P. chrysogenum* Q176 DNA in phage λ -EMBL3A by standard methods, resulting in the isolation of phages designated 1.2.3 and 2.5.3, which appeared to carry the *sutA* and *sutB* genes, respectively (Fig. 1). Further DNA manipulations to locate the genes on the phage-inserted DNA and to determine the nucleotide sequence were performed by standard methods (43).

cDNA analysis. cDNAs comprising the coding regions of *sutA* and *sutB* were isolated by PCR with a *P. chrysogenum* cDNA library and the primers *sutA*-forw (5'-CCG CAG GTG ACC CTC CAG ACT ACG-3') and *sutA*-rev (5'-GCT GGC CAA GAA CGG ATG CCC GCA-3') for *sutA* and *sutB*-forw (5'-CAG TTC CCA ATA CAC TCC CCG TGG-3') and *sutB*-rev (5'-CAG AGA GGT AGC AAG CAA TAG ATG-3') for *sutB*. PCR was performed with the Expand High Fidelity PCR system (Boehringer Mannheim) as specified by the manufacturer. Specific PCR products were cloned into the pGEM-T-easy vector (Promega) and completely sequenced. For *sutA*, a fragment encompassing a tentative third intron was amplified with the primers *sutA*-in-forw (5'-CTC ATG GCG GAG GAG GCA CCC ACA-3') and the aforementioned *sutA*-rev. To determine the sequences of the 5'- and 3'-untranslated regions of the *sutA* and *sutB* genes, primers which annealed in the coding regions but were directed outward of the genes were designed. For *sutA*, the primers *rev-sutA* (5'-GG GAT AAA GTC GAC TAT GAA GCC-3') and *forw-sutA* (5'-CTC ATG GCG GAG GAG GCA CCC ACA-3') were used, and for *sutB*, the primers *rev-sutB* (5'-GGG TGA TCT CGC GAA TCC AG-3') and *forw-sutB* (5'-CCG TCA TGT CGA CTC TTA CCG-3') were used. By using PCR (Expand Long Template PCR; Boehringer Mannheim), the cDNA library, and the primers mentioned above, the *sutA* and *sutB* flanking sequences as well as the vector backbone were amplified, treated with DNA polymerase (Klenow fragment), self-ligated, and sequenced.

Transformation of *A. nidulans*. *A. nidulans* IG1 was transformed as described by Ballance and Turner (2), using 2 μ g of pDJB2 mixed with 2 μ g of pBS*sutA*, pBS*sutB*, or pBS*psutAsutB*. Transformants were selected on minimal medium lacking uridine and uracil but supplemented with L-Met and *p*-aminobenzoic acid. After 5 days at 37°C, the transformants were tested for growth in the absence of methionine.

Construction of a *P. chrysogenum sutB* disruption mutant. *P. chrysogenum* HP60 was transformed as described by Bull et al. (6), except that protoplasts were prepared from mycelium grown for 36 h.

A cotransformation was carried out with 5 μ g of pBC1003 (which carries a phleomycin resistance marker [a gift from E. Friedlin, Biochemie GmbH]) mixed with 5 μ g of pBS*sutB*-XS. pBS*sutB*-XS contains an internal 1.0-kb *XhoI-SalI* fragment of *sutB* cloned into the multiple-cloning site of pBluescript II KS (Fig. 1). Transformants were selected on medium containing 50 μ g of phleomycin per ml in 25 ml of bottom agar overlaid with 20 ml of drug-free top agar into which transformed protoplasts had been mixed. Phleomycin-resistant transformants were tested for the ability to grow on sulfate as the sole sulfur source.

Sulfate uptake and expression studies. *A. nidulans* strains were grown aerobically at 37°C for 16 h on glucose-containing minimal medium in which all sulfate salts were replaced by chloride salts. As required, L-Met (0.25 or 5 mM) and/or MgSO₄ (0.1 or 2.0 mM) was added as the sulfur source(s). Where appropriate, uridine, uracil, and *p*-aminobenzoate were added to the medium at 10 mM, 20 mM, and 1 μ g/ml, respectively. *P. chrysogenum* Wis54-1255 was grown aerobically at 25°C on a sulfur-sufficient (≥ 5 to 10 mM sulfate) main culture medium with lactose as the C source. After 24 h, the medium was exchanged either for fresh original (S-rich) medium or for sulfurless medium in which all sulfate salts were replaced by chloride salts; this was followed by 16 h of growth. *P. chrysogenum* HP60 and nr45 were precultured overnight (at 25°C) on starter culture medium supplemented with 10 mM L-Met. The starter cultures were used to inoculate (at a 1:10 ratio) main culture medium with glucose as the C source, supplemented with 20 mM Met. After growth for 40 h, the medium was exchanged either for a medium containing 10 mM Met and normal sulfate levels (S-rich medium) or for a medium lacking Met and with all sulfate salts replaced by chloride salts (S starvation medium), and growth was continued for 4 h.

Mycelium for sulfate uptake studies was harvested by suction filtration, washed with ice-cold 0.9% NaCl, and resuspended in 50 mM potassium phosphate (pH 6.0) at approximately 10 ml/g (wet weight). After the mycelium had been cooled for at least 30 min on ice, it was aerated for 15 min at that temperature prior to the uptake experiments. After preincubation of the mycelium at 25°C for 3 min, sulfate (³⁵S]Na₂SO₄; specific activity, 10 to 20 mCi/mmol [ICN Pharmaceuticals]) was added to a final concentration of 10 mM. Samples were drawn and processed as described previously (17). To check the energy dependency of sulfate uptake, deenergization of the mycelium was performed with the protonophore carbonyl cyanide-*m*-chlorophenylhydrazone (10 mM final concentration), which was added at the start of the 3-min preincubation period. Dry weights of lyophilized samples were determined.

Mycelium for total-RNA isolation was harvested by suction filtration and immediately frozen in liquid nitrogen. The mycelium was ground in liquid nitrogen with a mortar and pestle, and RNA was isolated from the powdered mycelium with Trizol (Gibco BRL) as specified by the manufacturer. Electrophoresis and Northern blotting were carried out essentially as described previously (45). To ensure that hybridization was specific for *sutA* or *sutB*, probes for *sutA* and *sutB* were made from gene regions which have the lowest sequence identity (less than 40%) (Fig. 1) and hybridization was performed under stringent conditions. A *sutA*-specific fragment was amplified with primers *sutA*-N-forw (5'-CCG CAG GTG ACC CTC CAG ACT ACG-3') and *sutA*-N-rev (5'-GCC CCA GAT GTA ACG GCG AAC-3'), and a *sutB*-specific fragment was obtained with *sutB*-N-forw (5'-CAG TTC CCA ATA CAC TCC CCG TGG-3') and *sutB*-N-rev (5'-ATT GAG CAA GTA TCT GCC CAG-3'). For the constitutively expressed *actA* gene, an *actA* cDNA clone was isolated from a cDNA library of *P. chrysogenum* by PCR with primers ACT-1 (5'-CAG TCG AAG CGT GGT ATC CTC-3') and ACT-2 (5'-ACG TGG ATA CCG CCA GAC TCG-3') under standard conditions as specified by the manufacturer (Pharmacia Biotech). A DNA fragment to probe *pcbC* expression was kindly provided by DSM-Gist. Labeling was performed with an oligolabeling kit (Pharmacia Biotech), as specified by the manufacturer, and α -³²PdCTP.

GenBank accession numbers. The *sutA* and *sutB* sequences can be found in the GenBank database with accession no. AF163975 and AF163974, respectively.

RESULTS

Cloning of the *sutA* and *sutB* genes. Two putative sulfate transporter-encoding genes, *sutA* and *sutB*, were cloned from *P. chrysogenum* genomic DNA by a PCR-based approach as described in Materials and Methods. The nucleotide sequences of a 5.5-kb region encompassing the *sutA* gene and of a 5.8-kb region encompassing the *sutB* gene were determined. The *sutA* and *sutB* genes encode single polypeptides of 746 and 842 amino acid residues, respectively, with predicted molecular masses of 81.5 kDa (SutA) and 91.9 kDa (SutB). cDNA analysis showed that the coding regions are interrupted by two introns (63 and 60 nucleotides (nt) [nt 260 to 322 and 469 to 528]) for *sutA* and by one intron (59 nt [nt 457 to 515]) for *sutB*. These regions fit the intron consensus sequence 5'-GTN NGT.....CT[GA]AC...YAG-3' (numbering is relative to the ATG start codon). The intron in *sutB* is at exactly the same position as the second intron of *sutA* with respect to the amino

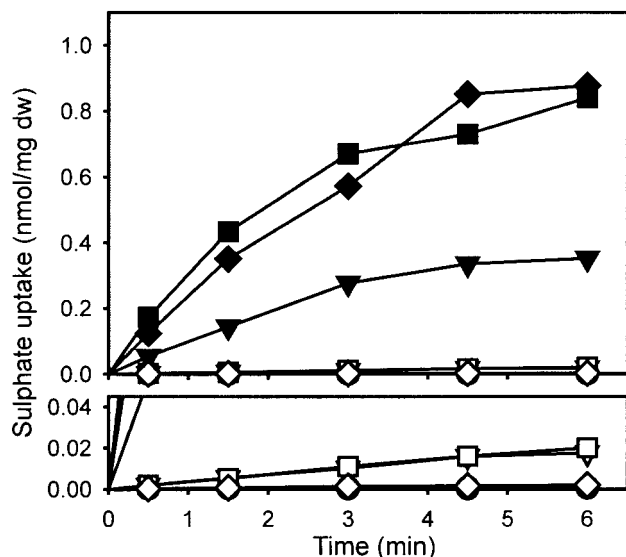


FIG. 2. Sulfate uptake by four different strains under high-sulfate (5 mM Met and 2 mM MgSO_4) or low-sulfate (0.25 mM Met) conditions. Shown are the wild-type strain *A. nidulans* R21 (high, \diamond ; low, \blacklozenge), the sulfate uptake-deficient strain *A. nidulans* IG1 (high, \circ ; low, \bullet), carrying the mutant *sB3* allele of the sulfate permease gene *sB*, and strains M27 (high, ∇ ; low, \blacktriangledown) and M63 (high, \square ; low, \blacksquare), both of which are *A. nidulans* IG1 strains complemented with the *P. chrysogenum* *sutB* gene. The lower panel is a partial magnification of the upper panel to indicate that some residual sulfate uptake can be detected under high-sulfate conditions. dw, dry weight.

acid sequence. The *sutA* gene was suspected to contain an additional intron in the 3' region (nt 2279 to 2332 [5'-GTC AGAN₂₈CTGAAN₁₂TAG-3']). Splicing out of this putative intron would extend the amino acid sequence identity between SutA and SutB (see below). Therefore, three independently isolated cDNAs were analyzed, with special attention paid to this region. From none of these cDNAs was the suspected intron spliced out. Furthermore, a fragment encompassing the putative intron was amplified by PCR with the cDNA library. One major band was detected, with the suspected intron not spliced out. A very faint band was detected (<5% abundance) from which the intron was putatively spliced out. cDNA analysis showed that the 5' untranslated regions are ≥ 60 nt (*sutA*) and ≥ 171 nt (*sutB*). Sequences directly upstream of the transcribed but untranslated regions of *sutA* and *sutB* are particularly CT rich and contain TATA- and CCAAT-like sequences that may be involved in transcription. cDNA analysis showed that the 3' untranslated regions are 342 nt (*sutA*) and 423 nt (*sutB*), not including the poly(A) tails.

Genetic complementation of the *A. nidulans* *sB3* mutant. To investigate the function of the proteins encoded by the *sutA* and *sutB* genes, their ability to complement the *sB3* (sulfate permease) mutation of *A. nidulans* was tested by cotransformation with pDJ2 and plasmids pBSsutA or pBSsutB, using the *pyr-4* gene of pDJ2 as a selectable marker. Plasmids pBSsutA and pBSsutB contain about 2.5 and 0.8 kb of the respective promoter regions (Fig. 1). Of 50 Pyr^+ transformants, 14 showed complementation of the *sB3* mutation by the *sutB* gene from their ability to grow on a medium with sulfate as the sole sulfur source. Of these 14 clones, 2, named M27 and M63, were used for sulfate uptake studies. Strains M27 and M63, as well as the parental *sB3* mutant strain IG1 and strain R21 (wild type for *sB*), were grown for 16 h on an S-poor medium containing 0.25 mM Met as the sole S source. After being harvested, mycelium was resuspended in phosphate

buffer and sulfate uptake was studied. Strains M27 and M63 showed uptake levels comparable to that of R21, whereas sulfate uptake by IG1 was undetectably low (Fig. 2). When the strains were grown on S-rich medium containing 5 mM Met and 2 mM MgSO_4 , sulfate uptake by R21 was repressed more than 500-fold, while sulfate uptake of the *sutB*⁺ strains M27 and M63 was repressed approximately 50-fold (Fig. 2).

In contrast to the results for *sutB*, of 50 tested Pyr^+ transformants cotransformed with pBSsutA and pDJ2, none showed complementation of the *sB3* phenotype. To circumvent the possibility that *sutA* did not complement the *A. nidulans* IG1 *sB3* mutation because of low expression of the *sutA* gene (see below), the 2.5-kb promoter region of *sutA* present in pBSsutA was replaced by the 0.8-kb promoter region upstream of *sutB* on pBSsutB, yielding plasmid pBSPsutBsutA. When *A. nidulans* IG1 was cotransformed with pDJ2 and pBSPsutBsutA, none of the tested Pyr^+ clones showed complementation of the *sB3* mutation as judged by their ability to grow on a medium with sulfate as the sole sulfur source. Northern analysis showed that in some of these transformants the *sutA* gene was expressed under the control of the *sutB* promoter during growth on S-poor medium. One of these clones (designated BA2) was used for sulfate uptake studies. After growth for 16 h on an S-poor medium containing 0.25 mM Met as the sole S source, sulfate uptake was measured, but not detected (not shown). These data demonstrate that SutB is a sulfate transporter. The function of SutA remains to be elucidated.

Expression of *sutA* and *sutB*, and disruption of *sutB* in *P. chrysogenum*. Northern analysis with *P. chrysogenum* Wis54-1255 showed that transcription of *sutA* and *sutB* was almost completely repressed when the strain was grown (for 40 h) under S-sufficient conditions (i.e., normal levels of sulfate) on main culture medium with lactose as the C source. When the mycelium was starved for sulfur for 16 h, after 24 h of growth on main culture medium, expression of both *sutA* and *sutB* was induced. However, expression of *sutB* was an order of magnitude stronger than that of *sutA* (Fig. 3A). The level of *sutA* and *sutB* expression corresponded to sulfate uptake by mycelium grown under S-rich and S starvation conditions (Fig. 3B).

To study the function of *sutB* and *sutA* in *P. chrysogenum*, the *sutB* gene of *P. chrysogenum* HP60 was disrupted by homologous integration of an internal fragment of *sutB* following

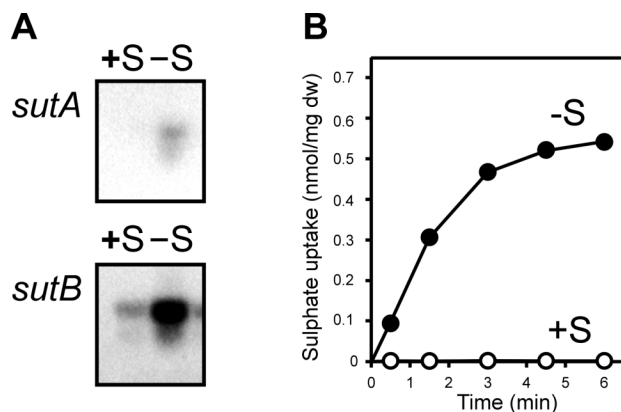


FIG. 3. Northern blots showing the expression of *sutA* and *sutB* in *P. chrysogenum* Wis54-1255 (equal amounts of RNA were loaded [not shown]) (A) and corresponding sulfate uptake levels (B). Wis54-1255 was grown for 24 h in a sulfur-sufficient medium with lactose as the C source, after which the medium was exchanged either for fresh medium of the same composition (+S) or for fresh medium in which all sulfate salts were replaced by chloride salts (-S), and growth was continued for 16 h. dw, dry weight.

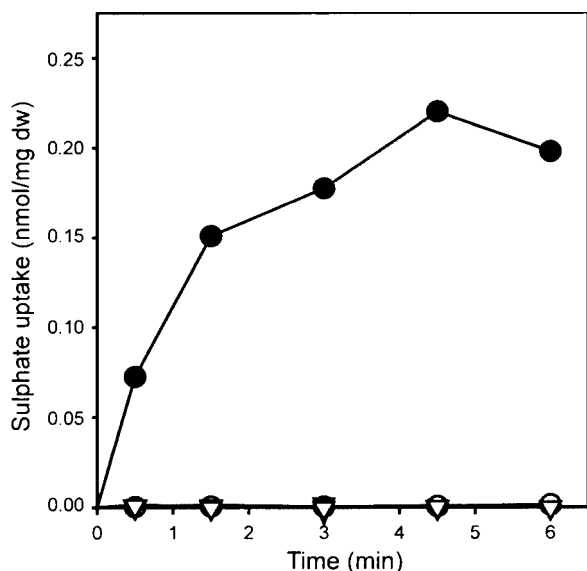


FIG. 4. Sulphate uptake by *P. chrysogenum* HP60 (○, ●) (wild type) and nr45 (▽, ▼) (*sutB* disruptant). The strains were grown for 40 h in a sulfur-sufficient medium with glucose as the C source supplemented with 20 mM Met. Subsequently, growth was allowed for 4 h on fresh medium of the same composition supplemented with 10 mM Met (open symbols) or on fresh medium without Met in which all sulfate salts were replaced by chloride salts (solid symbols). dw, dry weight.

cotransformation with a phleomycin resistance vector. Of 100 phleomycin-resistant transformants tested, only one, nr45, failed to grow normally on sulfate as the sole sulfur source. This strain, which grew normally on medium supplemented with methionine, also showed resistance to selenate, indicative of a lesion in an early step in sulfate assimilation (1). DNA extracted from this strain and probed with pBSsutB-XS confirmed the disruption of *sutB* by homologous integration of pBSsutB-XS (data not shown). Sulfate uptake by strain HP60 grown for 44 h on an S-rich main culture medium (containing normal amounts of sulfate salts and 10 to 20 mM Met) was completely repressed compared to that by strain HP60 grown for 40 h under S-sufficient conditions and then starved for 4 h on S-less main culture medium (no sulfate salts, no L-Met) (Fig. 4). No sulfate uptake was detected for the *sutB* disruptant strain nr45, grown either under S-rich or under S starvation conditions (Fig. 4). These data demonstrate that SutB is the major sulfate permease involved in sulfate uptake by *P. chrysogenum*.

DISCUSSION

We have cloned two *P. chrysogenum* genes, *sutA* and *sutB*, one of which, *sutB*, encodes a functional sulfate permease. *sutB* complements the *sB3* mutation of *A. nidulans*, and disruption of *sutB* in *P. chrysogenum* abolishes sulfate uptake. SutB appears to be the major sulfate permease present during mycelial growth and should therefore be located at the plasma membrane. SutB probably represents the high-affinity high-capacity $2\text{H}^+/\text{SO}_4^{2-}$ symport system, which has been kinetically characterized by Hillenga et al. (17). In line with this, both the K_m for SutB (20 to 30 mM for SO_4^{2-}) and the inhibition profile for SutB ($\text{S}_2\text{O}_3^{2-} > \text{S}_2\text{O}_5^{2-} \sim \text{SO}_3^{2-}$) after expression of the *sutB* gene in the *A. nidulans* *sB3* strain (57a), resemble the characteristics of the *P. chrysogenum* system in mycelium (4, 17, 60). The physiological function of SutA remains unclear. Expression of SutA in mycelium is low and is not enhanced in the

early growth stages (57b), unlike the situation indicated for *N. crassa* *cys-13* (31). It is unlikely that SutA represents a low-affinity system, since *P. chrysogenum* nr45 (*sutB* disruptant) does not grow on a medium with high sulfate concentrations without methionine. *sutA* may encode a thiosulfate, tetrathionate, or sulfite transporter (56) or may function as a sulfate transporter in the vacuolar membrane (18).

SutA seems to be truncated at its C terminus in comparison to SutB. Although the *sutA* genomic sequence suggests that an intron (nt 2279 to 2332 with respect to the ATG start codon) runs over the stop codon, cDNA analysis showed that it is not removed from the mRNA. If this intron were spliced out, SutA would be extended by 54 amino acid residues, which is very similar to the C terminus of SutB (Fig. 5) and to the C termini of SUL1p and SUL2p from *S. cerevisiae* (9, 49). It will be interesting to see whether (physiological) conditions exist that facilitate the removal of the putative intron from the primary transcript, resulting in SutA proteins with greater similarity to SutB and other sulfate transporters.

Expression of both *sutA* and *sutB* is induced when *P. chrysogenum* is grown under S starvation conditions (Fig. 3 and 4). Also, in *A. nidulans*, SutB-mediated sulfate uptake is subject to sulfur regulation (Fig. 2). S regulation is a well-documented phenomenon in *N. crassa*, *A. nidulans*, and *S. cerevisiae* (32, 55). For *sutB*, the 800 nt upstream of the start codon that are present on pBSsutB are almost completely sufficient for S regulation in *A. nidulans* (Fig. 2 and 4). In *N. crassa*, S regulation is positively mediated by the DNA-binding protein CYS-3p (22, 23, 26, 27, 32). Recently, a positively acting CYS-3p homologue has been found in *A. nidulans* (37). No CYS-3p homologue has been reported for *P. chrysogenum*. Sequences that weakly resemble the CYS-3p binding-site consensus ATGR YRYCAT (26, 27) are present upstream of *sutA* and *sutB* at positions -2481 (ATTGTACAAT), -1871 (ATTACGTGTT), -1513 (GTCGCGTGAC), -813 (GTCACGTACC), and -312 (CTGACGTTTCG) (*sutA*) and -1516 (ATGACGTGAT), -983 (ATTATGTAAT), -394 (ACAACGTGGA), and -231 (ATTGCGCCAT) (*sutB*) with respect to the ATG start codon. Other sequences in the *sutA* and *sutB* promoter regions resemble the consensus binding site TCACGTG, which is recognized in *S. cerevisiae* by the Cbf1p-Met4p-Met28p complex (24, 25, 55) (*sutA*, positions -2209, -2105, -1870, -1512, -904, and -812; *sutB*, positions -1909, and -1515 [note that some of these sites are part of putative CYS3p homologue-binding sites]), or the consensus binding site AAANTGTG of the positive regulators Met31p and Met32p (3) (*sutA*, positions -2129, -1600, and -1000; *sutB*, positions -1483, -1475, and -876). A possible function for these *cis*-acting elements and their proposed *trans*-acting binding factors remains to be investigated.

Hydropathy analysis (28, 29, 47, 58) of SutA and SutB shows a pattern typical for a polytopic membrane protein, with 14 putative hydrophobic transmembrane (TM) helices in the N-terminal part of the protein followed by a long C-terminal extension (Fig. 5A). The overall sequence identity of the SutA and SutB proteins is 66% (Fig. 5A). Both proteins show significant homology to eukaryotic sulfate permeases from fungi, plants, and animals (data not shown). These proteins are clustered, together with a number of prokaryotic proteins, in the so-called SulP superfamily of sulfate permeases, which belongs to the class of secondary transporters (40, 42). These proteins all contain a motif which has become known as the sulfate permease signature. Originally this motif was defined as P-x-Y-[GS]-L-Y-[STAG](2)-x(4)-[LIVMF](2)-Y-x(3)-[GSTA](2)-S-[KR](44, 50), and it runs over the TM helix 3 (depicted in Fig. 5B). This motif is present in both SutA and SutB. However, a database search with this motif fails to recognize many

A

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SutA ---MAETISSRVGRAFAKLLHIDLGVTPPV-VTDETGT-----YSYYEHEPTVGDWLRGHMP
SutB MFQKWGEANSNIQGLAKVLGIKLAYRDP LGATGETVTRGESAFSMGTVDTSYNEPEPTSIDWIREITP

SutA TTPLVRR YIWGLEPFLHWIGYINVOQLIGD LVAGITVGA VVIPOGMAYAE LAKLPPEYGLYSSEFMGVLIY
SutB SGAQLGR YLLNLFPPFLTWIGNYNLTWLGDI LVAGITVGA VVPOGMAYANL AGLPVOYGLYSSEFMGVLIY

SutA WFFATSKD LTIGPVAVMSTLIGSII IRVQAVHPEI PPPVLASALAI ICGIVSEFLGLLR LGFI VDFIPLP
SutB WFFATSKD LTIGPVAVMSTLTGTIV TEVQDIY PDYPAHLIASALAVI CGGIVLVMGLLR IGFI VDFIPLP

SutA AITAFMTGSAINVCAGQVKT VLGEKAHFSTRGATYKII IDTLKHLPSAQMD AAMGLTALAMLY IGIR SACN
SutB AISAFMTGSALSICSGQVP TMLGETADFSTRGATYEVI INTLKYLP TSTLDAAMGV TACAMLYI IRSVCT

SutA YGTKKKPHKAKL FEFLSTLR TAFVLLY TMISAA VNLHRRN NPFAKLLGNV PRGFKAAGV PKIDV PIKA
SutB YAARKQPARAKM WFEVSTLR IVFVILFY TMISAA TNLHRKDN PAFKLLGSV PRGFQQA AVPTMDA KIKA

SutA FVSELP VAIVLLIEHIAIS SKS FGRVNNY TIDPSQEF TAIGISNLLGPFLGAY PATGSE SRTAI KAKCGV
SutB FVGE LPAAVVLLIEHIAIS SKS FGRVNNY TIDPSQEF VAIGVSNLLGPFLGGYPATGSE SRTAI KSKAGV

SutA RTPLAG VVTAIVVLLAIYALPALEFF YIPKSSLSAVII HAVGDLVTP PRITYQ FWRVSPID ALIFLMGVIV
SutB RTPLAG VITAVVLLAIYALPALEFF YIPKASLAGVI IHAVGDLITP PNTVYQ FWRVSP LDALIFFIGVLV

SutA IIFSTI ETGIYCTISVSLAVLLER LARARGQ FLGYIQVH SVVGDHILN ----- ASKGDSNTE FGDGTGMP
SutB IVFSSI ENGIYCTVCVAVAVLLFR VAKARGQ FLGRVTI HSVVGDHLLDGDGKYGS FGTNKTP SDDEEH HH

SutA RRIYLP TEHEGGTNPRI KIHQPAPGV FIYRVSEGF NYPNANHY TDHLVSHI FKETR RTNSQAWETT GGDRP
SutB RTIFLPLN HTDGSNPDIE VEQPLPGIF IYRFAEGFNYPNANHY TDSLVA NI FKNTR RTNPNTYK NRGDRP

SutA WNDPGP TRAERKVLMAEE APTSPLPTLR AIILDFAAVNNVDV TSVQNLIDVRN QLDRWAS PDSVQWHFAH
SutB WNDAGP RRGK----- EGSDDDSHL PLLQAVILDFSSVNNVDV TSIQNLIDVRN QLDMYAS PRTVQWHFAH

SutA IHN RWTKRALAAAGFG FPAISDRSGPRC QRSVFNVAESLDGGLSGS IDQCRVVG SEDWS--- KDLEAGLK
SutB INN RWTKRGLAAAGFG YPTPVASDGFHRW KPIF SVAE-IEGSASAAAHAEI IANQRD QSQKPSDIES SGFK
SutA* SDRSGPRC QRSVFNVAESLDGGLSGSIDQCRVVG ----- ASS

SutA V - - - - - + + + + -
SutB SDSNTTARE TDGIETASE DSEVIREDKFNREIT DSKAYQRRPKVALVQGMNRP FFHIDLT-- SALQSAVA
SutA* SVMGVSEDDI -VVQAAETDAKVNTHRRRGRSG ----- ISTGMAVVDGINRPF FFHVDLTTTSALKSALA

SutB NSSDEIP HIE
SutA* DS

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putative and experimentally proven sulfate permeases, including SutB (Fig. 5B). Therefore, a new motif is proposed with the sequence D-[LIVFM](2)-[GAS]-G-[ILV]-x(7)-[PL]-x(15, 16)-[GS]-L-[YWFIL], which starts at TM helix 2 and runs into TM helix 3 (depicted in Fig. 5B). This motif is both complete and

specific in the recognition of (putative) sulfate permeases in sequence databases.

According to the topology model of the whole SulP family, based on hydropathy profile analysis (data not shown), the N termini of both SutA and SutB are located in the cytosol. The

B

	TM2	TM3	TM4	
SUTA	WLI GD LVAGITVGVAVVVPQGMAYAE LAKLPPE YGLYSSFMGVLIYWFFATS KDIT TIGPVAVMS-141-746			
SUTB	WLW GD LVAGITVGVAVVVPQGMAYANLAGL PVQ YGLYSSFMGVLIYWFFATS KDIT TIGPVAVMS-158-842			
CYS14	WLL GD FIAGVTVGVFVVVPQGMAYAKLANL AP EYGLYTSFVGVFLYWFATS KDIT TIGAVAVMS-137-788			
SUL1	WGY AD LVAGITVGCVLVPQSMSYAQIASL SPE YGLYSSFIGAFIYSLFATS KDVC IGPVAVMS-182-859			
SUL2	WFT AD LIAGITIGCVLVPQSMSYAQVATL PAQ YGLYSSFIGAYSYSFFATS KDVC IGPVAVMS-198-893			
HVST1	KFK GD LVAGLTIASLCIPQDIGYAKLANLQ PHV GLYSSFVPLIYALMGSS RDLA IGPVAVMS-158-660			
AST56	LFF KD LMAGLTLASLCIPQSIGYANLAGL DPE YGLYTSVVPPLIYSTMGTS RRLA IGPVAVMS-149-658			
DTDST	NIL GD VMSGLIVGILLVPSIAYSLLAGQ EPV YGLYTSFFASTIYFLLGTS RHSV GIFGVLC-169-739			

←-----Old motif-----→
 ←-----New motif-----→

Old PROSITE-motif: P-x-Y-[GS]-L-Y-[STAG](2)-x(4)-[LIVMFY](3)-x(3)-[GSTA](2)-S-[KR]
New PROSITE-motif: D-[LIVMF](2)-[GAS]-G-[ILV]-x(7)-[PL]-x(4)-[YFIL]-x(10,11)-[GS]-L-[YWFIL].

FIG. 5. (A) Alignment of *SutA* and *SutB* amino acid sequences. Indicated are identical residues (!); charges (+, -), predicted protein kinase C phosphorylation sites ([STX][RK]) (59), and predicted N glycosylation sites (NX[STX], with X¹P) (12, 33) which are present in both *SutA* and *SutB*; predicted transmembrane helices (black background); and predicted extracellular loops (bold type). Also shown is the alignment of an extended version of *SutA*, named *SutA** (see Fig. 1 and Discussion). Alignments were made with ClustalX (20), and hydropathy profile analysis was done with MemGen version 4.08 (28, 29). (B) Alignment of *SutA* and *SutB* amino acid sequences with sequences of some examples of the *SulP* family of sulfate permeases whose function has been proven experimentally (except *SutA*). Shown are the regions around the old sulfate permease motif (44) and the region around the proposed new sulfate permease motif. Alignments were made with the ClustalX program (20). SUTA, *P. chrysogenum* *SutA* (accession no. AF163975) (this work); SUTB, *P. chrysogenum* *SutB* (AF163974) (this work); CYS14, *N. crassa* *Cys14* (BP23622) (19, 31); SUL1, *S. cerevisiae* *Sul1* (P38359) (9, 49); SUL2, *S. cerevisiae* *Sul2* (S64926 and Z73264) (YLR092w) (9); HVST1, *Hordeum vulgare* *HVST1* (U52867) (51); AST56, *Arabidopsis thaliana* *Ast56* (AB012047 and S74246) (53); DTDST, *Homo sapiens* *DTDST* (P50443) (14, 41). The old motif fails to recognize *SutB*, *Sul2*, and *HVST1*.

C-terminal domain of both systems is predicted to be located in the cytosol as well, in line with topology data for the human *DRA*-encoded sulfate transporter (7). Previously published models were based on alignments of a small number of eukaryotic sulfate permeases (see e.g., references 9, 11, 50, and 51) and predicted 12 or fewer TM helices. However, a hydropathy profile based on the 50 presently available sequences (not shown) predicts 14 TM helices for most eukaryotic sulfate permeases and 13 TM helices for the prokaryotic sulfate permeases. The predicted TM helix 1 appears to be present in a subset of eukaryotic sulfate permeases, including *SutA* and *SutB* (*P. chrysogenum*), *SUL1p*, *SUL2p*, and *SULXp* (*S. cerevisiae*), *CYS-14p* (*N. crassa*), and some plant, nematode, and mammalian sulfate permeases, but it is lacking in other eukaryotic sulfate permeases and in all prokaryotic permeases. The current model predicts the presence of TM helices 13 and 14, whereas in most previous models a single TM helix was predicted. However, the previously proposed topology models disobey the so-called positive-inside rule (14, 47, 50, 58), while the prediction of TM helix 14 yields a topology with a charge distribution which is in better agreement with the positive-inside rule, as seen in Fig. 5.

Summarizing, *P. chrysogenum* contains two genes, designated *sutA* and *sutB*, that encode putative sulfate transporters. *SutB* is the system responsible for sulfate uptake in mycelium of *P. chrysogenum*, whereas the role of *SutA* remains to be determined. Future studies will address the regulation and expression of these systems in relation to the high demand for sulfur during penicillin biosynthesis.

ACKNOWLEDGMENTS

This project was supported by the EC, Eurofung Cell Factory-RTD4CY96-0535.

We thank DSM-Gist. (Delft, the Netherlands) for determining the nucleotide sequences and providing the *P. chrysogenum* cDNA library and the *pcbC* probe, and we thank J. S. Lolkema and D.-J. Slotboom for discussions with respect to the *SutA* and *SutB* topology model.

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