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

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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-aminoacidic acid, L-Cys, and L-Val by the peptide synthetase δ-(L-α-amino acidyl)-L-cysteinyl-L-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 [hisD574 (rpsL3) supE44 supF58 lacY1 argF1] (34) and DH5α [strA186 lacM15 recA1 endA1 gyr96 thi-1 hsdR17 (rK-mK) supE44 thi-1 (rB-mB) supE44 thi-1 (rC-mC) supE44 thi-1 (rD-mD)] (80) were used as host strains. Plasmid pBSPsutB was used for cloning and sequencing in *E. coli*. Plasmid pGEM-T-easy (Promega) was used to clone PCR products. Plasmid pSB2 was a generous gift from H. Schwab, Technical University, Graz, Austria. DSM-Gist (Delft, The Netherlands) kindly provided the *Aspergillus nidulans* mutant of NRRL1951 (48). The construction of *P. chrysogenum* nr65 is described below. *A. nidulans* IG1 [sb3 prg69 pabaC1] (23) and SUL1p and SUL2p of *S. cerevisiae* (9, 21, 49) were added as indicated.

Where appropriate, sulfate salts were replaced by chloride salts, and methionine was added as a selection marker (2). Plasmid pBSsutA contains a 5.5-kb *pyr-4* fragment (Fig. 1) cloned into the PstI site of pBluescript II KS. Plasmid pBSsutB contains a 4.3-kb BamHI fragment cloned into the BamHI site of pBluescript II KS. Plasmid pBSsutB contains a 2.1-kb SalI fragment cloned into the SalI site of pBluescript II KS. Plasmid pBSsutB contains an internal 1.0-kb XhoI-SalI fragment of the SalI site cloned into the MCS of pBluescript II KS. Plasmid pBSsutB was constructed as follows. Plasmid pBSsutB was digested with EcoRV (in the multiple-cloning site) and HincII (28 bp upstream of the *sutA* ATG start codon), and a 737-bp EcoRV-HincII fragment containing part of the *sutB* promoter region was isolated (Fig. 1). This fragment was cloned into plasmid pBSputA from, from which the *sutA* promoter region was removed by digestion with BsiEI (49 bp upstream of the *sutA* ATG start codon), treatment with DNA polymerase (Klenow fragment), and digestion with EcoRV (in the multiple-cloning site). A genomic library of *P. chrysogenum* Q736 (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. *S. cerevisiae* strains were grown in YPG medium (23) or in 2% (w/v) glucose. *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 *nat-forw* (5′-ATT TCT TCC ATT AAG GAG TGC [GTG] [GAT] [GCT] [ATC] [TTC] [ATT] AA-3′) and *nat-rev* (5′-GAT TGC [GAT] [GCT] [TGC] [ATC] [TTC] AA-3′), and *sul- rev* (5′-GGA [GAT] [GCT] [TTC] [TGC] [ATC] [TTC] AA-3′), were designed to correspond to two stretches of amino acid residues present in CYS-14p of *N. crassa* (TYKV [VI][INE][TLK and EHAISKSFG]) (23) and SUL1p and SUL2p of *S. cerevisiae* (TYKV[VI][INE][TLK and EHAISKSFG]) (9, 21, 49). PCR was performed on chromosome P. chrysogenum DNA under standard conditions. PCR products of about 400 bp were isolated, treated with DNA polymerase (Klenow fragment), ligated into the Smal site of pBluescript II KS, and sequenced. Of the 20 clones...
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* sutA and sutB genes, and Northern analysis are indicated by the double-headed arrows. 

**FIG. 1.** Physical map of the genomic DNA fragments containing the *sutA* (A) and *sutB* (B) genes. Genomic DNA fragments present in phases 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 thick, black 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 intron in *sutA* were spliced out. The primers used to probe expression in the Northern analysis are indicated by the double-headed arrows.

A cotransformation was carried out with 5 μg of pBC1003 (which carries a phleomycin resistance marker [a gift from E. Fricelin, Biochemie GmbH]) mixed with 5 μg of pH1100sutB-XS. pH1100sutB-XS contains an internal 1.0-kb XhoI/SstI fragment of *pSU* (the multiple cloning site of *pSU*; see Fig. 1). Transformants were selected on medium containing 50 μg of phleomycin per ml in 25 ml of bottom agar overlayed with 20 ml of drug-free top agar into which transformed protoplasts had been mixed. Phleomycin-resistant transformants were 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 sulfate-limited medium in which all sulfate salts were replaced by chloride salts; this was followed by 16 h of growth. *P. chrysogenum* HP60 and *A. nidulans* mc145 were streaked on glucose-starved (at 25°C) on starter culture medium supplemented with 10 mM l-Met. The starter cultures were used to inoculate (at 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 sulfate-limited (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.

**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]) for *sutA* and by one intron (59 nt) 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 *sutA* is at exactly the same position as the second intron of *sutA* with respect to the amino acid sequence.

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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 MgSO₄, 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 pDBJB2, 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 pBSPsutB. When A. nidulans IG1 was cotransformed with pDBJB2 and pBSPsutB, 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 transport protein. 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
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 HP90 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 $2H^+\text{/SO}_4^{2-}$ 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 $\text{SO}_4^{2-}$) and the inhibition profile for SutB ($\text{S}_2\text{O}_5^{2-} \rightarrow \text{S}_2\text{O}_3^{2-} \sim \text{SO}_4^{2-}$) 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 $-2481$ (ATTGTACAAAT), $-1871$ (ATTACGTGTT), $-1513$ (GTGCCGTGAC), $-813$ (GTCACGTACC), and $-312$ (CTGACGTTCG) (sutA) and $-1516$ (ATGACGTGAT), $-983$ (ATTAGTGAAT), $-394$ (ACAACGTGGA), and $-231$ (ATT GGCCCAT) (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 AANTGTG 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 $\text{P-x-[Y]}\text{-[S]}\text{-[L]}\text{-[Y]}\text{-[STAG]}\text{-[2]}\text{-[x]}\text{-[4]}\text{-[LIVMF]}\text{-[2]}\text{-[Y]}\text{-[x]}\text{-[4]}\text{-[LIVMF]}\text{-[2]}\text{-[KR]}$ (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
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
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.

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