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

MART VAN DE KAMP,1 ENRICA PIZZININI,1 ARNOLD VOS,1 TED R. VAN DER LENDE,1 THEO A. SCHUURS,1 ROGER W. NEWBERT,2† GEOFFREY TURNER,2 WIL N. KONINGS,1 AND ARNOLD J. M. DRIESSEN1*

Department of Molecular Microbiology, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, 9751 NN Haren, The Netherlands,1 and Department of Molecular Biology and Biotechnology, Krebs Institute for Biomolecular Research, University of Sheffield, Sheffield S10 2TN, United Kingdom2

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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, t-Cys, and t-Val by the peptide synthetase δ(1-α-aminoacidyl)-l-cysteyl-t-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 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.

Sulfur 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 [Δ(lacZΔM15 lacY1 proAB galU galK rpsL22 thi-1 hsdR17 (rK- mK-) supE44 recA1 deoR1 araD139 (lac-proAB) F’ lacZΔM15 lacY1 proAB galU galK rpsL22 thi-1 hsdR17 (rK- mK-) supE44 recA1 deoR EII (49 bp upstream of the ATG start codon), treatment with DNA polymerase (Klenow fragment), subcloning into the MCS of pBluescript II KS (Stratagene) for cloning and sequencing in *E. coli*. Plasmid pGEM-T-Easy (Promega) was used to clone PCR products. Plasmid pDB2 is an *A. nidulans* transformation vector carrying the *ncr* prr-1 gene as a selection marker (2). Plasmid pBStsA contains a 5.5-kb Prl fragment (Fig. 1) cloned into the Prl site of pBluescript II KS. Plasmid pBSTsB contains a 4.3-kb BamHI fragment cloned into the BamHI site of pBluescript II KS. Plasmid pBPSntB contains a 2.1-kb SstII fragment cloned into the SstI site of pBluescript II KS. Plasmid pBSTsB-NS contains an internal 1.0-kb XhoI-SstI fragment of *sutA* cloned into the MCS of pBluescript II KS. Plasmid pBPStsA was constructed as follows. Plasmid pBSTsB was digested with EcoRI in the (multiple-cloning site) and HincII (28 bp upstream of the *sutA* ATG start codon), and a 737-bp EcoRI-HincII fragment containing part of the *sutA* promoter region was isolated (Fig. 1). This fragment was cloned into plasmid pBStsA, from which the *sutA* promoter region was removed by digestion with BsrEI (49 bp upstream of the *sutA* ATG start codon), treatment with DNA polymerase (Klenow fragment), and digestion with EcoRI 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-Gst (Delft, The Netherlands) kindly provided the *P. chrysogenum* cDNA library.

Media and growth conditions. Manipulations with and growth of *E. coli* LE392 and DH5α 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 *nur-tor* (5′-ACC TAC AAG GT[C/T] [GA][A]/CT [AG][A]/CT AAC][TCA] [TTG][A]-3′) and *nur-ev* (5′-GG AA [G/T][GG] [GC][T][G]-3′), were designed to correspond to two stretches of amino acid residues present in CY5-14p of *N. crassa* (TYYK [VI][I][NE][I][LE][K] and EHAII[S/K/S]FG) (23) and SUL1p and SUL2p of *S. cerevisiae* (TYYK[V]/[I][NE][I][LE][I] and EHAII[S/K/S]FG) (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 SmaI site of pBluescript II KS, and sequenced. Of the 20 clones

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* Corresponding author. Mailing address: Department of Molecular Microbiology, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, P.O. Box 14, 9750 AA Haren, The Netherlands. Phone: 31-50-3621580. Fax: 31-50-3632154. E-mail: A.J.M.Driessen@BIOL.RUG.NL.
† Present address: Synpac Pharmaceuticals, Cambois, Bedlington, Northumberland NE2 7DB, United Kingdom.

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A cotransformation was carried out with 5 μg of pBC1003 (which carries a phleomycin resistance marker [a gift from E. Friedlin, Biochemie GmbH]) mixed with 5 μg of pBSsutB-XS. pBSsutB-XS contains an internal 1.0-kb XhoI-SstI fragment of pBluescript II (Fig. 1). Transformants were selected on medium containing 50 μg 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 selected on the sole sulfate as the sole sulfur source. 

Sulfate uptake and expression studies. A. nidulans strains were grown aero-

ically 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 MgSO₄ (0.1 or 2.0 mM) sulfur sources were added. After 5 days at 37°C, the transformants were tested for growth in the absence of sulfate. Growth was recorded after 5 days at 37°C. All transformants grew well in the absence of sulfate. Two sets of 3 and 7 identical clones showed sequence similarity to the wild-type gene. Specific PCR products were cloned into the pGEM-T-easy vector (Promega). The PCR products were used to screen a genomic library of P. chrysogenum. The nucleotide sequences of the enlarged fragments (SacI and HindIII) were used to probe expression positions of the PCR fragments that were used to isolate the genes. The nucleotide sequences of the 5′- and 3′-end fragments of the amplified genes were determined. The primers used for the 5′-end amplification were ACT-2 (5′-ACG TGG ATA CCG CCA GAC TCG-3′) and ACT-1 (5′-GCT TTC CCA ATA CAC TCC CCG TGG-3′) and for the 3′-end amplification were 5′-CCG TCA TGT CGA CCA GCC CCC A-3′ and 5′-CGG TG A CGA CTC AGG-3′. Northern blotting was carried out essentially as described previously (45).

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 non-coding 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 sutN-forw (5′-CCG CAG GTG ACC CTC CAG ACG-3′) and sutN-rev (5′-GCT GCC GAA CGA GTG ACC ACG AAC-3′), and a sutB-specific fragment was obtained with 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 sutA. PCR products were cloned into the P. chrysogenum genomic library by PCR with primers ACT-1 (5′-CAG TCC ATG CAG GTG ATC CTC-3′) and ACT-2 (5′-ACG TGG ATA CCG CCA GCC CAC TGG-3′) under standard conditions as specified by the manufacturer (Pharmacia Biotech). A DNA probe was produced by nick-translation with [α-32P]dCTP. Labelling was performed with an oligolabelling kit (Pharmacia Biotech), as specified by the manufacturer, and [α-32P]dCTP.

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

cloning of the sutA and sutB genes...
acid sequence. The sutA gene was suspected to contain an additional intron in the 3' region (nt 2279 to 2332 [5'-GTC AGAN3CTGAAN5'TAG-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 pDJB2 and plasmids pBSsutA or pBSsutB, using the pyr-4 gene of pDJB2 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 undetectable low (Fig. 2). When the strains were grown on an 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 pDJB2, 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 pBSputA. When A. nidulans IG1 was cotransformed with pDJB2 and pBSputA, 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 an 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
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 selenite, 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 HP90 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-deficient 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⁺/SO₄²⁻ symport system, which has been kinetically characterized by Hillenga et al. (17). In line with this, both the Kₘ for SutB (20 to 30 mM for SO₄²⁻) and the inhibition profile for SutB (S₂O₃²⁻ > S₂O₅²⁻ > SO₄²⁻) 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 sulfate 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 (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 (ATTGTACAA), −1871 (ATTACGTGTT), −1513 (GTCGCTTGAC), −813 (GTCACTGACC), and −312 (GTCGCCGCT) (sutA) and −1516 (ATGACGTGTC) (sutB), −983 (ATTATGTAAT), −394 (ACAAACGTGGA), and −231 (ATT GCGCCAT) (sutB) with respect to the ATG start codon. Other sequences in the sutA and sutB promoter regions resemble the consensus binding site TCACTGTG, which is recognized in S. cerevisiae by the Cbf1p-Met4p-Met28p complex (24, 25, 55) (sutA, positions −2209, −2105, −1870, −1512, −924, 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 AAAANTGTG 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-[S]-G-[L]-Y-[TAG][2-x][Y-x]-[LIVMF][2-Y-x][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
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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