The Aspergillus nidulans cysA gene encodes a novel type of serine O-acetyltransferase which is homologous to homoserine O-acetyltransferases

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The Aspergillus nidulans cysA gene was cloned by functional complementation of the cysA1 mutation that impairs the synthesis of O-acetylserine. The molecular nature of cysA1 and cysA103 alleles was characterized; a nucleotide substitution and a frame shift were found in the former and a deletion mutation in the latter. The CYSA protein is 525 amino acids long and is encoded by an uninterrupted open reading frame. Expression of the cysA gene appears not to be regulated by sulfur, carbon and nitrogen sources. Protein sequence analysis reveals extensive similarity to homoserine O-acetyltransferases, particularly the bacterial ones, and no homology with known serine O-acetyltransferases. The authors propose that the CYSA protein is analogous to serine O-acetyltransferases, i.e. it catalyses the same reaction but has an independent evolutionary origin.

Keywords: A. nidulans, cysteine synthesis, analogous genes

INTRODUCTION

The synthesis of cysteine in Aspergillus nidulans, like that in many bacteria and plants, involves the acetylation of serine followed by its thiolation (Fig. 1, steps 1 and 2). If this pathway is impaired, the fungus can synthesize cysteine by an alternative pathway comprising homoserine γ-lyase (Fig. 1, steps 3 and 4) since homocysteine γ-lyase (Fig. 1, steps 5, 6 and 7). In consequence, two mutations, impairing both pathways, are required for cysteine auxotrophy. In addition, mutations affecting the main pathway, like cysA or cysB, suppress the mutations impairing cysteine γ-synthase and cystathionine β-lyase (Fig. 1, steps 3 and 4) since homocysteine synthesized from O-acetyhomoserine, the product of homoserine O-acetyltransferase (HATase) (Fig. 1, step 8), is utilized for both cysteine and methionine synthesis (Paszewski & Grabski, 1974, 1975).

cysA, cysD double mutants grow on cysteine as well as on O-acetyhomoserine (OAS); this differentiates them clearly from cysB, cysD double mutants, which require cysteine (both strains grow also on methionine). OAS also restores growth of cysA single mutants inhibited by γ-propargylglycine, which strongly blocks cystathionine γ-lyase (Fig. 1, step 7) (Piotrowska & Paszewski, 1986). Pieniążek et al. (1974) found no serine O-acetyltransferase (SATase) activity in the cysA1 strain. These data strongly suggest that OAS is an intermediate in the main cysteine-synthesizing pathway in A. nidulans.

In the present report, we describe the cloning and characterization of the A. nidulans cysA gene located on chromosome V (Cybis et al., 1988). The gene encodes a protein which is homologous to HATases, but not to any of the known SATases from bacteria and plants.

METHODS

Strains, media and growth conditions. The following strains of A. nidulans from our collection, which carry standard markers (see Clutterbuck, 1994) were used: cysA1, proA2 pabaA2, yA2; cysA1, cysD11, yA2, phenA2; cysB102, pyroA4, yA2; cysC103, choA1, chaA1; cysC103, cysD11, choA1, chaA1; cysD11, phenA2, yA2; metE31, micA2, biA1; sconB2,
nicA2, biA1; sconC3, pyroA4, ya2; metR1, pyroA4, biA1. pyroA4, ya2 was used as a reference wild-type strain. The genetic status of the cysC103 mutation was revised in the course of this work (see below). The sconB (Natorff et al., 1998) and sconC (Piotrowska et al., 1997; Natorff et al., 1993) genes encode sulfur regulatory proteins. The metR gene encodes a sulfur-specific transcriptional factor (Natorff et al., 1993).

Strains were grown in minimal medium (MM), containing 2 mM sulfate, as described previously (Paszewski & Grabski, 1973) or in MM–S (minimal minus sulfur medium) with sulfates substituted with corresponding chlorides. These media were supplemented according to the auxotrophic requirements of the strains employed. The complete medium was according to Cove (1966). The cultures were grown with shaking (200 r.p.m.) at 37°C for 12–16 h. Mycelia were harvested by filtration, blotted on filter paper and used, depending on the experiment, for cell-free extracts, protoplast preparation or nucleic acid isolation.

To test the effect of carbon catabolite repression and nitrogen metabolite repression on cysA gene expression, the wild-type mycelia were grown in media containing either sulfate (0.25 mM or 2 mM) or methionine (5 mM) and non-repressing carbon and nitrogen sources (0.1% fructose, 5 mM urea) for 8 h at 37°C. The cultures were then supplemented with different combinations of carbon and nitrogen sources as follows: 0.1% fructose, 5 mM urea (non-repressed); 1% glucose, 5 mM urea (carbon repressed); 0.1% fructose, 20 mM ammonium tartrate (nitrogen repressed); 1% glucose, 20 mM ammonium tartrate (carbon and nitrogen repressed) for 2 h at 37°C (Dzikowska et al., 1999; Gonzalez et al., 1997).

Escherichia coli XL-1 Blue (Stratagene) was used for cloning and plasmid propagation. E. coli strains were grown in standard media, LB, 2×TY and NZY (Sambrook et al., 1989), as required. Antibiotics were added in the following concentrations: ampicillin 50 μg ml⁻¹, kanamycin 50 μg ml⁻¹, tetracycline 35 μg ml⁻¹.

Plasmids, gene libraries and synthetic primers. The cloning vectors were pBluescript II KS and SK (Stratagene) and pUC19 (Yanisch-Perron et al., 1985). The pHELP1 plasmid (Gems et al., 1991), supplied by J. Clutterbuck (University of Glasgow, UK), contains the AMA1 sequence, which enables autonomous replication. Co-transformation with this plasmid results in up to 200-fold increase of transformation efficiency (Gems & Clutterbuck, 1993). The pCI9R plasmid, containing a fragment of the A. nidulans γ-actin gene (Fidel et al., 1988), was obtained from R. Bradshaw (Massey University, Palmerston North, New Zealand). The A. nidulans chromosome-specific cosmid pWE15 and pLORIST2 gene libraries (Brody et al., 1991) and the A. nidulans iZAPII 24 h developmental cDNA library, constructed by R. Aramayo, were obtained from the Fungal Genetics Stock Center, Kansas City, KS, USA.

The following oligonucleotides were used as primers for PCR and primer extension: CYSCATG, 5′-GGAATTCAT-GAGTCCGCTGAAAGGCCTCCTC-3′ [384 + extension]; CYSCNFGLG, 5′-TCCCCGCCTTATCGTCTTGGCCTTGTAGTTCTTGATACAAATTGTTGCGA-3′ [1958 + C + extension]; MGPWR3, 5′-GATGGCGGTAGGAGGAGGACG-3′ [196(C)]; MGPW4, 5′-GGTTCACGGGACTTTCATGCGACG-3′ [372(C)]; ANAK1032, 5′-GTTGATGAGGCGACAGTCCA-3′ [1032]; ANAK1669, 5′-TACCAGGC-TTCCAGACCA-3′ [1669(C)]. The numbers in square brackets correspond to the first nucleotide (in bold) from the 5′ end of the primer which hybridizes with the template sequences found in the GenBank. (C) designates the complementary strand; ‘extension’ denotes nucleotide stretches added for various purposes, e. g. cloning sites.

DNA isolation. A. nidulans DNA was isolated by grinding mycelia frozen in liquid nitrogen in a mortar as described by Yelton et al. (1984). Plasmid DNA from E. coli was isolated by alkaline lysis (Sambrook et al., 1989) or using a QIAGEN Plasmid Kit.

Transformation of A. nidulans and cloning of the cysA gene. Preparation of protoplasts from the cysAI, cysDI1, choAI, chaAI strain and subsequent transformation were performed as described by Stenko et al. (1998) except for the amount of Novozyme 224 (5–6 mg ml⁻¹) and helixate (2.5 mg ml⁻¹) used. The protoplasts were co-transformed to the wild-type phenotype (Cys⁺) with DNA isolated from subpools of chromosome V cosmid clones and the pHELP1 plasmid. The subpool that produced Cys⁺ transformants was further subdivided in order to identify the cosmid able to rescue the cysAI mutation. To identify the region of the cloned cosmid complementing the mutation we used the ‘instant gene bank’ method of Gems et al. (1994). The method is based on the observation that co-transformation of a strain with the restricted pHELP1 plasmid and transforming DNA leads to in vivo ligation of DNA fragments with the vector. This method allows the selection of transformants harbouring plasmids carrying required DNA.
fragments, which can be then rescued into *E. coli* (Ballance & Turner, 1985). The *A. nidulans* insert which reversed auxotrophy was used as a probe to screen the cosmids digests. All the digests were transferred to a membrane and after Southern hybridization (Sambrook et al., 1989) the smallest transforming restriction fragment was cloned into a sequencing vector and subsequently checked for complementation of the cysA1 mutation. This last step was necessary to sequence the original copy of cysA, excluding any rearrangements caused by *in vivo* ligation in *A. nidulans*.

A cysA cDNA copy was isolated from the iZAPII library according to the Stratagene protocol using a digoxigenin-labelled probe (Boehringer Mannheim Kit).

**PCR reactions.** PCR reactions were performed in an Air Thermocycler (Idaho Technology) according to the supplier’s instructions. The Promega Taq DNA polymerase was used. The cycling conditions were: 94 °C/20 s + (94 °C/1 s, 50–58 °C/1 s, 72 °C/20–35 s) × 30 + 72 °C/30 s. At least three independent clones of each allele were sequenced on both strands.

**DNA sequencing.** DNA sequence was determined using an ALF automatic sequencer (Pharmacia) or an ABI310 sequencer (Perkin Elmer).

**Mapping of the 5’ and 3’ cysA mRNA termini.** The cysA transcription initiation site was mapped by primer extension analysis. MGPR3 or MGPR4 oligonucleotides were hybridized to total RNA isolated from the wild-type strain grown in minimal medium. The primer was then extended with 200 units M-MLV reverse transcriptase (Promega) at 42 °C for 2 h, using [α-32P]dCTP according to the Gibco-BRL protocol. The reaction products were fractionated by 8% denaturing gel electrophoresis in parallel with DNA sequencing reactions initiated with the same primer. The 3’ end of cysA mRNA was determined by sequencing cDNA clones of the cysA gene.

**RNA isolation and Northern analysis.** Total RNA was isolated using TRI Reagent (Molecular Research Center) according to the manufacturer’s instructions (Chomczynski, 1993). A sample containing 20 µg total RNA was glyoxylated at 50 °C for 1 h followed by fractionation in 1% agarose and overnight capillary transfer to a nylon membrane (Hybond-N, Amersham). Filters were prehybridized for 1 h at 65 °C. DNA probes were α-32P-labelled by random priming with a DNA labelling kit (MBI Fermentas). Hybridization was carried out overnight at 65 °C. Filters were washed for 1 h with 2× SSC, 0.1% SDS at 50 °C and then with 1× SSC, 0.1% SDS. Standard autoradiography with intensifying screens was performed at −80 °C. All the procedures were according to Sambrook *et al.* (1989).

**Computer sequence analysis.** Computer analysis of DNA and protein sequences was performed using GENEPRO 4.20 (Riverside Scientific), GCG software (version 8.1) (Genetics Computer Group) on a Silicon Graphics Challenge computer, ALLALL (Computational Biochemistry Research Group Server) (Gonnet, 1994), PSORT II (Nakai & Kanehisa, 1992), BOXSHADE 3.21, PRODOM 99.1 and BLAST (Altschul et al., 1990) using the GenBank, EMBL, PIR (R), PDB and SWISS-PROT databases.

Protein structure predictions were obtained with the position-specific iterative BLAST algorithm (PSI-BLAST) (Altschul et al., 1997) and FFAS, a proprietary profile–profile alignment program, developed at the authors’ laboratory (Rychlewski et al., 2000), followed by homology modelling using MODELLER (Sali & Blundell, 1994). In both prediction algorithms, the prediction reliability is expressed as an *e*-value, which can be interpreted as the probability that the prediction is false. *e*-values of 0.001 and less are normally associated with very reliable predictions (Altschul *et al.*, 1997; Rychlewski *et al.*, 2000).

**RESULTS**

**Isolation of cysA clones**

Since the cysA1 mutant is a prototroph we used a double mutant cysA1, cysD11, having both pathways of cysteine synthesis impaired, for the cysA gene cloning. This strain was transformed with a chromosome V specific library. As the cysA gene is located on this chromosome and the cysD gene on chromosome II, prototrophic transformants could only arise by acquisition of the cysA gene. This led to the identification of one cosmid, W05C10, which complemented the cysA1 mutation. This cosmid contained an *A. nidulans* insert of about 43 kb [data not shown]. Applying the ‘instant gene bank’ procedure (see Methods) we co-transformed the mutant strain with pHFIII linearized with BamHI and the W05C10 cosmid digested with EcoRI. Cys+ transformants were selected. The transforming recombined plasmids were rescued in *E. coli*. One of them, containing an 8 kb *A. nidulans* insert, was used as a probe to identify the shortest transforming fragment of the cosmid digests. It turned out to be a 3.9 kb EcoRI–HindIII fragment which was cloned into pBluescript KS.

To identify the cDNA copy of the cysA gene, about 105 phage clones from the cDNA library were screened by plaque hybridization with the digoxigenin-labelled *Bam*HI–HindIII fragment from the 5’ region of the cysA gene. Five positive plaques were found. Three of them, giving the strongest hybridization signals, were purified and amplified. pBluescript SK(−) plasmids containing cDNA inserts were excised *in vivo* according to the Stratagene protocol. Only one cDNA insert extended (6 bp) beyond the putative translation initiation codon.

**Nucleotide sequence analysis**

The 3.9 kb EcoRI–HindIII fragment was sequenced on both strands (GenBank accession number AF029885). DNA sequence analysis revealed one major open reading frame corresponding to the cysA gene. This gene has no introns as shown by cDNA sequencing. The main transcription start point, determined by 5’ extension, is positioned at −161 from the putative ATG codon assumed to be the start point of translation. Although there is another in-frame ATG located 45 nt upstream from the former one, we stress that the cDNA we have isolated, which contains only 6 nt upstream from the initiation codon, complements both the cysA1 and cysA103 (deletion) mutations. This shows that the translation product beginning with the downstream ATG is functional. Upstream from the transcription start site there is a putative CAAT-box (−71). Furthermore, at a typical distance (−34) from the transcription start point there is a CT-rich region which has
Fig. 2. For legend see facing page.
been noted in other fungal genes at, or near, transcription start sites. In the promoter region many putative CREA and AREA binding sites were found. The UAG translational termination codon is followed by a purine (A), which is typical for eukaryotic genes (UNKles, 1992). The putative polyadenylation signal (AUAA) is located 10 nt after the stop codon. Sequencing of cDNA indicated polyadenylation sites 28 nt and 35 nt downstream from the UAG.

In order to analyse the mutated form we cloned the cysA1 allele and found four mutations, all except one located at the 3' end of the gene. In the case of the cysA allele, three independent PCR products were sequenced on both strands. The wild-type allele PCR products obtained with the same polymerase confirmed the previously established genomic and cDNA sequences. Three of the mutated sites are clustered. Two of the four mutations are substitutions (A784C, T1547G) and two are insertions (1516A and 1545T [the numbers represent the nucleotides situated upstream of the insertion]). These changes in DNA sequence lead to an amino acid substitution (S262R) and frameshifts (the first at position 506), in the latter case causing premature protein chain termination at amino acid 518.

Protein sequence analysis and structure predictions

The cysA gene encodes a peptide of 525 amino acids with a calculated molecular mass of 57 kDa. Contrary to expectations from the cysA1 phenotype the CYSA protein exhibited a very high degree of identity to the family of SATases, not to HATases. The protein shows a high degree of identity (52–57%) to the CYSA homologue, the SATase from Schizosaccharomyces pombe (Fig. 2) and a lower but significant identity (23–29%) to other HATases, like protein from Schizosaccharomyces pombe with a high degree of identity (52–57%) to the CYSA homologue, the SATase from Schizosaccharomyces pombe (Fig. 2) and a lower but significant identity (23–29%) to other HATases. The CYSA protein shows a very high degree of identity to the SATase family (Vaara, 1992) and its absence in CYSA. The CYSA protein is clearly not a typical HATase. As seen in Fig. 2, the main distinctive feature of this protein is the lack of the 70 amino acid loop in the C-terminal half found in classical eukaryotic HATases.

Regulation of cysA expression

cysA expression was assayed in the wild-type, sconB2, sconC3 and metR1 strains under repressing and derepressing conditions (i.e. high and low levels of sulfur source). Total RNA was isolated and Northern blots were prepared and probed with an β-32P-labelled fragment of the cysA gene amplified with CYSCATG and CYSCNFLG primers. As a control, the same blots were hybridized with an β-32P-labelled fragment of the γ-actin gene amplified with ANAKT1032 and ANAKT1669 primers. No significant difference in the level of the cysA transcript was found in mycelia grown in the presence of a high or low level of sulfur source (Fig. 3).

The cysA transcript did not show any regulation by nitrogen or carbon source regardless of the sulfur source used in the experiment (data not shown).

Revised status of the cysC103 mutation

The cysC103 as well as cysB102 mutations were originally isolated as suppressors of the metB3 mutation (Ayling, 1969) and later found to impair the main pathway of cysteine synthesis (Paszewski & Grabski, 1975). Since the cysteine-requiring double mutant cysC103, cysD11 grew well on cysteine but weakly and with some delay on OAS, it was suggested that the mutation interfered in the conversion of OAS to cysteine and represents a separate locus named cysC. Its biochemical nature remained unknown (Paszewski et al., 1994).

In the present work we have transformed the cysC103, cysD11 mutant with the cysA gene to Cys+ phenotype, finding that this gene complements the cysC103 mut-
Fig. 3. Analysis of cysA expression in the wild-type and sulfur regulatory mutant strains grown in various sulfur conditions (Met, methionine). Total RNA (20 µg) was subjected to electrophoresis, blotted to a nylon membrane and hybridized with 32P-labelled DNA probes.

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<th>Wild-type</th>
<th>sconB2</th>
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<tr>
<td>Met (mM)</td>
<td>–</td>
<td>0·25</td>
<td>5</td>
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<td>Sulfate (mM)</td>
<td>0·1</td>
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Fig. 4. (a) Southern analysis of DNAs from the wild-type (pyroA4, yA2) (1) and cysA103 (cysA103, chaA1, chaA1) (2) strains digested with BamHI and hybridized with a cysA probe. Hybridization with a cysB probe was used as a control. (b) Analysis of cysA expression in the cysA103 (cysA103, chaA1, chaA1) and cysD11 (cysD11, phenA2, yA2) strains grown on different sulfur sources (Met, methionine). Experimental conditions as in Fig. 3.

DISCUSSION

The cysA gene of *A. nidulans* was cloned by functional complementation of the cysA1 mutation, and analysed. Since cysA mutants respond to OAS, which appears to be an intermediate in the main pathway of cysteine synthesis, it was expected that the gene would encode a SATase. It turned out, however, that the CYSA protein is highly similar to HATases and not to any of 22 bacterial and plant SATases whose sequences are available in databases. All SATases represent a group of homologous proteins. The gene was found mutated in the cysA1 and deleted in the cysA103 (formerly cysC103) strain, indicating that the cloned gene is not a suppressor of these mutations. There is a possibility that the CYSA protein does not function as a SATase itself but is its activator, which happens to be similar to HATase. This seems unlikely since the search in the *A. nidulans* genome for sequences potentially encoding SATases (SATases from watermelon and *E. coli* were used as references), kindly performed at our request by Cereon Genomics, LLC, Cambridge, MA, USA, did not reveal such a sequence (personal communication). Therefore, both the nutritional and molecular data strongly suggest that cysA encodes a HATase-like SATase. This conclusion is supported by the fact that *A. nidulans* possesses a bona fide HATase encoded by metE (GenBank accession number AF162658). metE mutations render strains OAH dependent, which indi-
cates that CYSA does not have a HATase activity, at least in vivo.

Interestingly, Schiz. pombe possesses a protein (GenBank accession number CAB53733), described as HATase-like, which is highly similar to CYSA. We believe that it also functions as a SATase. The two proteins appear to be phylogenetically closely related (Fig. 2) and, having overall homology with HATases, they are clearly closer to the bacterial than the eukaryotic (Fig. 2) and, having overall homology with HATases, proteins appear to be phylogenetically closely related.

Interestingly, Schiz. pombe also has a HATase encoded by met2, homologous to the A. nidulans METE. These two enzymes are typical eukaryotic HATases.

It would be worthwhile to characterize the CYSA protein biochemically. Since the SATase activity measured by acetylation of \(^{14}\)C-serine observed in earlier experiments by Pienta\'z\'ek et al. (1974) was extremely low in the wild-type strain (but totally missing in cysA1), we have attempted to apply the spectrophotometric assay used for bacteria and plants in order to characterize the enzyme biochemically. Unfortunately this did not work, even though we observed SATase activity in control assays with bacterial and plant extracts (data not shown). Possibly, because of differences between the CYSA protein and classical SATases, different assay conditions and purification procedures are required.

The cysA gene, similarly to cysB and metG (Topczewski et al., 1997; Sienko & Paszewski, 1999) appears not to be transcriptionally regulated. Since mutations in this gene lead to a derepression of some enzymes of sulfur metabolism (Paszewski & Grabski, 1975), OAS does not seem to play the role of an inducer in this metabolism as found in enterobacteria (Kredich, 1992) and as postulated in plants (Neuenschwander et al., 1991; Smith et al., 1997) and some Saccharomyces cerevisiae strains which have SATase activity (Ono et al., 1996). In addition, the cysA gene was found not to be regulated by carbon or nitrogen sources, although some putative AREA and CREA binding sites are found in the cysA promoter region.

Since the HATase and SATase structures are markedly different, it can be concluded that CYSA is analogous, but not homologous, to SATases. Such proteins of independent evolutionary origin catalysing the same reaction are not uncommon, but are relatively rare (Galperin et al., 1998). Most likely the cysA gene has differentiated from an ancestor gene encoding a HATase of bacterial type which acquired a novel function. In this connection it is worth noting that O-acetylsereine thiolase, encoded by cysB (Topczewski et al., 1997), is also closely related to bacterial and not to its eukaryotic counterparts. In addition, both genes have a very low frequency of 'optimal' codons: 0 for cysA and 0.4 for cysB, respectively (Lloyd & Sharp, 1991; Nakamura et al., 1999). It is therefore tempting to speculate that the cysA and cysB genes are both of prokaryotic origin, especially as the proteins they encode are likely to be localized in the mitochondrion.

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