Characterization of Two Genes Encoding Putative Cysteine Synthase Required for Cysteine Biosynthesis in *Schizosaccharomyces pombe*

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Cysteine synthase catalyzes the formation of cysteine from O-acetylserine, and is the key enzyme for de novo cysteine biosynthesis in *Schizosaccharomyces pombe*. An examination of the *S. pombe* database revealed that two gene products are predicted to encode proteins homologous to eukaryotic cysteine synthases. Disruption of one of these candidates, *cys1a*\(^+\) (SPBC36.04)\(^\dagger\), caused an obvious cysteine auxotrophy, while disruption of *cys1b*\(^+\) (SPAC3A12.17c) had no effect on the growth phenotype. Furthermore, overexpression of *cys1b*\(^+\) did not complement the cysteine auxotrophic phenotype of *cys1a* mutant cells. These results indicated that *cys1a*\(^+\), not *cys1b*\(^+\), primarily functions in the biosynthesis of cysteine in *S. pombe* cells. We constructed a bacterial-*S. pombe* shuttle vector containing *cys1a*\(^+\) as a selective marker gene. The combination of the cysteine auxotroph and new vector could be useful for the expression of a heterologous protein.

**Key words:** fission yeast; cysteine biosynthesis; cysteine synthase; marker gene

Cysteine is an essential component for all organisms. It plays a critical role in the structure, stability, and catalytic functions of many proteins. Fungi generate cysteine by a series of enzymatic reactions involving the assimilation of inorganic sulphur. The filamentous fungus *Aspergillus nidulans* has been reported to have the richest repertoire of sulphur metabolic options.\(^1,2\) It has two pathways for de novo cysteine biosynthesis, i.e. a cystathionine pathway and an O-acetylserine (OAS) pathway. In the cystathionine pathway, cysteine is synthesized from homocysteine via the intermediary formation of cystathionine and the final reaction is catalyzed by cystathionine \(\gamma\)-lyase (EC4.4.1.1) (Fig. 1-i, a). In the OAS pathway, on the other hand, cysteine is generated from serine via the intermediary formation of OAS and the final reaction is catalyzed by cysteine synthase (EC4.2.99.8) (Fig. 1-ii, b). In the budding yeast *Saccharomyces cerevisiae*, well studied in terms of sulphur metabolism, cysteine is predominantly synthesized via the cystathionine pathway\(^3\) although activities of enzymes involved in OAS pathway are detected in some strains\(^4,5\) (Fig. 1-i). Thus, a block of the cystathionine pathway due to a mutation of *CYS3/STR1* coding for cystathionine \(\gamma\)-lyase causes cysteine auxotrophy.\(^6,7\)

The fission yeast *Schizosaccharomyces pombe* has been reported to lack the cystathionine pathway and to synthesize cysteine only via the OAS pathway\(^8,9\) (Fig. 1-ii). Therefore it is expected that cysteine synthase is

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**Abbreviations:** OAS, O-acetyl serine; ORF, open reading frame; MCS, multi-cloning site; GFP, green fluorescent protein

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**Fig. 1.** Pathway of Cysteine Biosynthesis in *S. cerevisiae* (i) and *S. pombe* (ii).

Partially cited from Brzywczy et al.\(^9\) a, cystathionine \(\gamma\)-lyase; b, cysteine synthase. The OAS pathway and cystathionine pathway are respectively indicated with dotted squares.
required for de novo cysteine biosynthesis in this yeast. As a result of searching the *S. pombe* genomic database, we found that this yeast has two genes encoding a putative cysteine synthase. However, it has been unknown which gene (or both of them) is functional in the biosynthesis of cysteine. In this report, we characterized two fission yeast genes and showed that one of them is essential for the generation of cysteine and the other is not. Furthermore, we attempted to generate a new *S. pombe* transformation system using a cysteine auxotrophic strain obtained in this study.

**Materials and Methods**

**Strains and media.** The parent *S. pombe* strain ARCO39 (h−, leu1-32, ura4-C190T) was provided by Asahi Glass Co., Ltd (Yokohama, Japan). ARCO39 was derived from ATCC38399 (h−, leu1-32) and has a point mutation in *ura4* (C at nucleotide position 190 had changed to T). Yeast extract supplements (YES) medium and synthetic minimal medium (EMM) for *S. pombe* cells were used as described elsewhere.10) For the selection of mutant cells which lost *ura4*+ activity, 5′-fluoroorotic acid (FOA)-supplemented YNB medium (0.7% yeast nitrogen base, 2% glucose, 50 μg/ml uracil, 225 μg/ml each of leucine, adenine, histidine, and cysteine, and 0.1% FOA) was used. ARCO39 cells were transformed by the electroporation method as described previously.11) For transformation of *cyslaΔ* cells, another type of electroporation method12) and a lithium acetate method13) were also applied. The *Escherichia coli* strain used for all cloning procedures was XL1-blue (Stratagene, CA).

**Disruption of cysla*Δ*/cyslb*Δ*. cysla*Δ* or cyslb*Δ* was disrupted using *ura4*+14) as a selective marker. The construction of disruptants of the target genes is shown in Fig. 2-ii. For the disruption of *cysla*+, the 1.1-kb DNA fragments including a part of the open reading frame (ORF) of *cysla* were amplified from wild-type *S. pombe* genomic DNA and subcloned into pGEM-T (Promega, WI). A 0.84-kb EcoRI-KpnI fragment was eliminated from the ORF and a *ura4*+ cassette was inserted (Fig. 2-ii, upper). For the disruption of *cyslb*+, 1.9-kb fragments including the full-length ORF of *cyslb* were amplified from wild-type *S. pombe* genomic DNA and subcloned into pGEM-T Easy (Promega). A 0.98-kb EcoRV-HindIII fragment was eliminated from the ORF and an *ura4*+ cassette was inserted (Fig. 2-ii, lower). Linearized DNA fragments carrying the disrupted gene were used to transform the parent strain ARCO39 and stable *ura4*+ transformants were selected. Genomic disruption of the target gene was confirmed by PCR. Mutant cells were subsequently plated on FOA-supplemented YNB media to select for loss of *ura4*+ activity. Primers for PCR were as follows. *cysla*: 5′-TCATTCTATGTGCTTTTCACCTCATCCTCC-3′ and 5′-GGATCGGCCACGAAAGCAACAGCAGACC-3′, cyslb*: 5′-CGATTGAATGG-AATGAAGGGGTAG-GC-3′ and 5′-GATTATTTCTGATATTAGACAGAG-CG-3′.

**Fig. 2. Alignment of Deduced Amino Acid Sequences from *A. nidulans* cysB, cysla*, and cyslb* (i) and Schematic Representation of Disruption of *cysla*+ or *cyslb*+ (ii).**

i) The deduced amino acid sequences of *cysla*+ and *cyslb*+ had 66% and 44% identities with that of *A. nidulans* cysB. Areas homologous to *A. nidulans* cysB are indicated with dotted boxes. Partial deduced amino acid sequences from *A. nidulans* cysB (GenBank accession No. U19195), cysla+, and cyslb+ are aligned. Identical amino acids with *A. nidulans* cysB are shown in black boxes. Two lysine residues responsible for pyridoxal 5′-phosphate cofactor binding are indicated with asterisks. ii) *cysla*+ (upper) or cyslb+ (lower) was disrupted using *ura4*+ as a marker gene. The ORF of the target gene is shown with a black arrow. The *ura4*+ cassette is shown with an open arrow.

**Reverse transcribed-polymerase chain reaction (RT-PCR).** Total RNA was prepared from ARCO39, *cyslaΔ*, or *cyslbΔ* cells at a logarithmic phase in YES medium using an RNaseasy Mini kit (QIAGEN, Japan). First-strand cDNA was prepared with RevarTraAce (TOYOBO, Japan) using the oligo (dT)20 primer. Total RNA was treated with RNase free DNase I (QIAGEN) before use. PCR was done for 25 cycles using ExTaq (TaKaRa, Japan) and his3+ expression was used as an internal control.15) Primers for PCR were as follows.
cys1a: 5'-CTTACT-GTCTCAGAAACCATGG-TCC-3' and 5'-GCTCAATATCTTCTGTATGG-TGGCTC-3', cys1b: 5'-GCTCATTGTCAGAGATGAAC-CTCCAGCTCCC-3' and 5'-GTGACTAAGTGAGACATAAGCTAGGAGGC-3', cys1b: 5'-GCTACATTATGAA-CTCATTAAAGGCTC-3' and 5'-TCTGTGC-AACGTCATACGCCATGC-3'.

Plasmid constructions. pAL-cys1a+ and pAL-cys1b+ were constructed by integrating full-length cys1a+ or cys1b+ into the multi-cloning site (MCS) of pAL-SK (+). pAL was a gift from Dr. C. Shimoda (Osaka City Univ., Japan). To construct pAL-cys1a+, a 1.75-kb fragment of cysla+ including the full-length ORF was amplified from wild-type S. pombe genomic DNA and subcloned into pGEM-T Easy. The amplification of cysla+ was done using the upstream primer described in "Disruption of cysla+/cys1b+" and a newly designed downstream primer: 5'-GTATCTCCAGCATAATTAGGAC-TTGCTC-3'. For pAL-cys1b+, a 1.89-kb cys1b+ fragment integrated into pGEM-T Easy as described in "Disruption of cysla+/cys1b+" was used. cysla+ or cys1b+ fragments were released by digesting with NotI. pGEM-T Easy contains two NotI sites flanking the TA cloning site. Then the cysla+ or cys1b+ fragment with NotI ends was inserted into the NotI site of the MCS of pAL-SK (+).

The bacterial S. pombe shuttle vectors containing cys1a+ as a selective marker were constructed by replacing LEU2 of the preexisting vector pAL-SK (+) or pART1[16] with cys1a+. The constructs are shown in Fig. 5-i. The sequence of pAL or pART1 except the LEU2 marker area was amplified by inverse PCR with primers carrying a NotI site. These PCR products were digested with NotI and self-ligated. Then, the 1.75-kb NotI fragment of cysla+ described above was inserted into the NotI site of the plasmid lacking a marker gene. The NotI site inside the MCS of pAL had been abolished by blunting. These new vectors were designated pAC and pARTC, respectively.

For the expression of green fluorescent protein (GFP), pARTC-GFP was constructed. A 0.7-kb GFP gene fragment derived from pEGFP-N1 (CLONTECH) was inserted into the BamHI site inside the MCS of pARTC.

Microscopic observation. For confocal microscopy, cells were observed on an Olympus BX-60 fluorescence microscope using a U-MGFP filter set (Olympus, Tokyo, Japan). Images were collected with a Sensys Cooled CCD Camera using a MetaMorph (Roper Scientific, San Diego, CA), and were saved as Adobe Photoshop files on a Macintosh G4 computer.

Results and Discussion

Characterization of two fission yeast genes encoding a putative cysteine synthase

We characterized two S. pombe genes predicted to participate in the final step of cysteine biosynthesis, namely cysteine synthase genes. SPBC36.04 and SPAC3A12.17c were found as a result of searching the Sanger Centre Fission Yeast Genome Sequencing Project (www.sanger.ac.uk/Projects/S_pombe) for a S. pombe homologue of the Aspergillus nidulans cysteine synthase gene (cysB). We used the amino acid sequence of the A. nidulans cysB product[17] for the search because cysB had been reported to function sufficiently well to generate cysteine.[18] Provisionally, SPBC36.04 was designated cys1a+ and SPAC3A12.17c was designated cys1b+. The deduced amino acid sequence of cysla+ and cys1b+ had 66% and 44% identity with that of A. nidulans cysB, respectively. Both of them had two lysine residues which bind the pyridoxal 5'-phosphate cofactor,[18] which are highly conserved among cysteine synthases of plants and bacteria (Fig. 2-i).

First, we generated cys1a and cys1b disruptants, and checked their growth phenotypes. The cys1aΔ cells grew more slowly than the parent ARC039 cells. They showed 1.2-times longer doubling time than that of parent cells in YES liquid media and 1.4 times in EMM supplemented with 2 mM cysteine. The cellular concentration of cys1aΔ was about 30% less than that of parent cells when the growth curve reached a plateau (data not shown). Furthermore, cys1bΔ cells could not grow on cysteine-free media, nor use OAS, a substrate of cysteine synthase (Fig. 3-i). In contrast, the cys1bΔ cells grew the same as parent ARC039 cells on all types of media tested. These results indicate that cys1aΔ cells lost all or almost all activity of cysteine synthase while cys1bΔ cells retained sufficient enzymatic activity to generate cysteine. To assess whether cys1b+ functions in cysteine biosynthesis or not, we did a complementary examination. As shown in Fig. 3-ii, overexpression of cys1b+ could not restore a defect of cysteine synthesis in cys1aΔ cells. Then we did RT-PCR to check the expression of cysla+ and cys1b+ in the parent ARC039, cys1aΔ, and cys1bΔ strain. In the wild-type (ARC039) strain, both cys1a+ and cys1b+ specific amplification was detected (Fig. 4). The specific amplification of cysla+ or cys1b+ was not detected in the respective mutant strain, indicating that cys1a+ or cys1b+ is completely knocked out in these strains. Taken together, these results suggest that cysla+ predominantly functions as a cysteine synthase gene and that cys1b+ has no function in the cysteine biosynthesis, at least under normal growth conditions, although the transcript is produced. The role of cys1b+ is unexplained in this study. Efforts are underway to express the cys1b+ product in E. coli cells to confirm the cysteine synthase activity. However, the possibility that cys1b+ participates in the cysteine synthesis cannot be excluded because the transcription of cys1b+, not only cysla+, increases in response to an oxidative stress.[19] Cysteine is an essential component of glutathione, which has important role in the response to oxidative stress. Therefore, up-regulated cys1b+ expression may assist
the cysteine synthesis required in the response to environmental stresses.

Fig. 3. Growth Phenotypes of cys1a and cys1b Mutant Strains (i) and Overexpression of cys1a<sup>+</sup> or cys1b<sup>+</sup> in the Cysteine Auxotrophic (cys1aΔ) Strain.  
<i>i) </i>Parent strain ARC039, cys1aΔ, and cys1bΔ were cultivated on cysteine-free EMM (upper) or 2 mM OAS-supplemented EMM (lower) for 48 hours at 30°C.  
<i>ii) </i>Full-length cys1a<sup>+</sup> or cys1b<sup>+</sup> was integrated into the multi-cloning site of pAL-SK (+) and used for transformation of cys1aΔ cells. Transformants were cultivated on cysteine-free medium for 48 hours at 30°C. Strains: 1, ARC039; 2, cys1aΔ; 3, cys1bΔ; 4, cys1aΔ+pAL-cys1a<sup>+</sup>; 5, cys1aΔ+pAL-cys1b<sup>+</sup>.

Fig. 4. RT-PCR Analysis in S. pombe Strain ARC039, cys1aΔ and cys1bΔ.  
PCR was done for 25 cycles using 1st strand cDNA as a template prepared from total RNA. RNAs were prepared from each strain in logarithmic phase in YES media.

Development of a S. pombe transformation system using cys1a<sup>+</sup> as a selective marker

<i>S. pombe</i> is a useful eukaryotic model for investigations in cell biology and molecular-genetic techniques have been developed for this yeast. However, there remains a need for more selectable marker genes for molecular genetic manipulation. In this study, we therefore tried to use cys1a<sup>+</sup> as a new selectable marker gene. We developed a new transformation system using a combination of cys1a<sup>+</sup> and the cysteine auxotrophic (cys1aΔ) strain obtained in this study. New bacterial-S. pombe shuttle vectors containing cys1a<sup>+</sup> as a selective marker were constructed as described in Materials and Methods. These vectors have a backbone of high copy plasmid pAL-SK (+) or pART1 and a cys1a<sup>+</sup> area containing its own promoter and were designated pAC and pARTC, respectively (Fig. 5-i). Both of these vectors were confirmed to complement the cysteine auxotrophy of cys1aΔ cells (Fig. 5-ii). To evaluate the new transformation system, we did heterologous protein
(GFP) expression using a combination of the cys1Δ strain and a new vector pARTC. cys1Δ cells were transformed with pARTC-GFP (described in Materials and Methods) by the electroporation method including dithiothreitol (DTT) pretreatment and plated onto cysteine-free EMM. After 7–10 days incubation at 30 °C, colonies of transformants were obtained. Although the transformation efficiency was less than $2 \times 10^6$ colonies per $1 \mu g$ plasmid, all transformants tested were confirmed to produce GFP (Fig. 5-iii). To improve the transformation efficiency, we tested two other methods, the electroporation method without DTT pretreatment and the lithium acetate method. The transformation efficiency was considerably improved (about $8 \times 10^2$ colonies per $\mu g$ plasmid) when the electroporation method without DTT pretreatment was used. However, the efficiency was dramatically decreased when cryopreserved competent cells were used (data not shown). On the other hand, we could not obtain any transformant by the lithium acetate method. These results suggest that cys1Δ cells are sensitive to chemical and cold stresses. In these cells, the supply of cysteine may be not enough for the production of glutathione, essential for adaptation to various environmental stresses. The infirmity of cys1Δ cells may be related to low efficiency in this new transformation system. Although it may not be appropriate for studies which require a high transformation efficiency, this new transformation system will be useful in genetic studies of S. pombe, such as vector expression.

In conclusion, we identified the S. pombe cysteine synthase gene required for de novo cysteine biosynthesis. The result also supported a previous study indicating that S. pombe lacks the cystathionine pathway and generates cysteine only via the OAS pathway. Furthermore, we developed a new S. pombe transformation system using a cysteine auxotrophic strain obtained in this study. In spite of a relatively low efficiency, this new transformation system will be useful for molecular genetic studies in S. pombe, in which more selectable markers are needed.

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