Mycoscience (2006) 47:98–105
DOI 10.1007/s10267-005-0278-0

SHORT COMMUNICATION

Minoru Ujita · Yosuke Katsuno · Kahori Suzuki
Kazumasa Sugiyama · Eiji Takeda · Eiji Yokoyama
Akira Hara

Molecular cloning and sequence analysis of the β-1,3-glucan synthase catalytic subunit gene from a medicinal fungus, *Cordyceps militaris*

Received: October 12, 2005 / Accepted: November 29, 2005

Abstract The entomopathogenic fungus *Cordyceps militaris* belongs to vegetable wasps and plant worms and is used as herbal medicine, but β-1,3-glucan biosynthesis has been poorly studied in *C. militaris*. The fungal *FKS1* gene encodes an integral membrane protein that is the catalytic subunit of β-1,3-glucan synthase. Here, we isolated cDNA clones encoding a full-length open reading frame of *C. militaris FKS1*. *Cordyceps militaris* Fks1 protein is a 1981 amino acid protein that shows significant similarity with other fungal Fks proteins. This study is the first report of molecular cloning of the β-1,3-glucan synthase catalytic subunit gene from vegetable wasps and plant worms.

Key words Catalytic subunit · cDNA cloning · *Cordyceps militaris* · β-1,3-Glucan synthase · Medicinal fungi

The medicinal mushroom *Cordyceps militaris* belongs to vegetable wasps and plant worms and is used as a tonic food and herbal medicine, but the molecular mechanisms of its pharmacological activities are not fully understood. A number of mushrooms contain biologically active β-glucans with antitumor and immunostimulating properties (Borchers et al. 1999; Wasser 2002). Mushroom β-glucans are widely sold as nutritional supplements and touted as beneficial for human health. It is known that lentinan, PSK (krestin), and schizophyllan show antitumor activity, which is beneficial in clinics when used in conjunction with chemotherapy. Moreover, mushroom β-glucans also seem to prevent oncogenesis and tumor metastasis. They do not attack tumor cells directly but exert their antitumor effects by stimulating immune responses in the host.

The fungal cell wall is mainly constituted of polysaccharides such as β-glucan and chitin, and among them β-1,3-glucan is the most prevalent. β-1,3-Glucans are synthesized from uridine 5′-diphosphate (UDP)-glucose by a membrane protein complex, β-1,3-glucan synthase. It seems that β-1,3-glucan biosynthesis occurs on the cytoplasmic side of the plasma membrane and β-1,3-glucan chains are extruded toward the periplasmic space. The β-1,3-glucan synthase complex has been shown to be composed of the catalytic subunit Fksp, a large molecular size polypeptide with transmembrane domains, and the regulatory subunit Rho1p, a small molecular size GTPase (Douglas et al. 1994; Mazur and Baginsky 1996; Beauvais et al. 2001). The *Saccharomyces cerevisiae FKS1* (*ScFKS1*) gene encodes a 215-kDa integral membrane protein (ScFks1p), and *FKS2* (*ScFKS2*), a homologue of *FKS1* encoding a 217-kDa integral membrane protein (ScFks2p), has also been cloned (Mazur et al. 1995). Simultaneous disruption of *ScFKS1* and *ScFKS2* is lethal, suggesting that ScFks1p and ScFks2p are alternative subunits with overlapping function.

β-1,3-Glucan biosynthesis has been studied at the molecular level almost exclusively in yeast and filamentous fungi, but poorly in mushrooms, especially vegetable wasps and plant worms. Here we report the cloning and sequencing of the *C. militaris* gene homologue of *FKS1*. This study is the first report of molecular cloning of the β-1,3-glucan synthase catalytic subunit gene from vegetable wasps and plant worms.

Based on the nucleotide sequences of the conserved regions of fungal *FKS* genes (*Coccidioides immitis*, *Cryptococcus neoformans*, *Paracoccidioides brasiliensis*, and *S. cerevisiae*), two degenerate primers, cmfks-5 (5′-CCYGA RTAYACYCTSCGYACYCGYATYTGG) and cmfks-3 (5′-AARCCSAGRTCRCGRCCYTTRCCRCAYTG), were designed. Polymerase chain reaction (PCR) was done using these primers and *C. militaris* cDNA as a template, and a 950-bp DNA fragment was obtained. The nucleotide sequence of the cloned genes was analyzed by dideoxy nucleotide sequencing. Based on the obtained nucleotide

M. Ujita (✉) · Y. Katsuno · K. Suzuki · K. Sugiyama · E. Takeda · A. Hara
Laboratory of Biological Chemistry, Department of Applied Biological Chemistry, Faculty of Agriculture, Meijo University, 1-501 Shiogamaguchi, Tempaku-ku, Nagoya 468-8502, Japan
Tel. +81-52-838-2449; Fax +81-52-835-7450
e-mail: ujita@ccmfs.meijo-u.ac.jp

E. Yokoyama
The Agricultural High-Tech Research Center, Meijo University, Nagoya, Japan

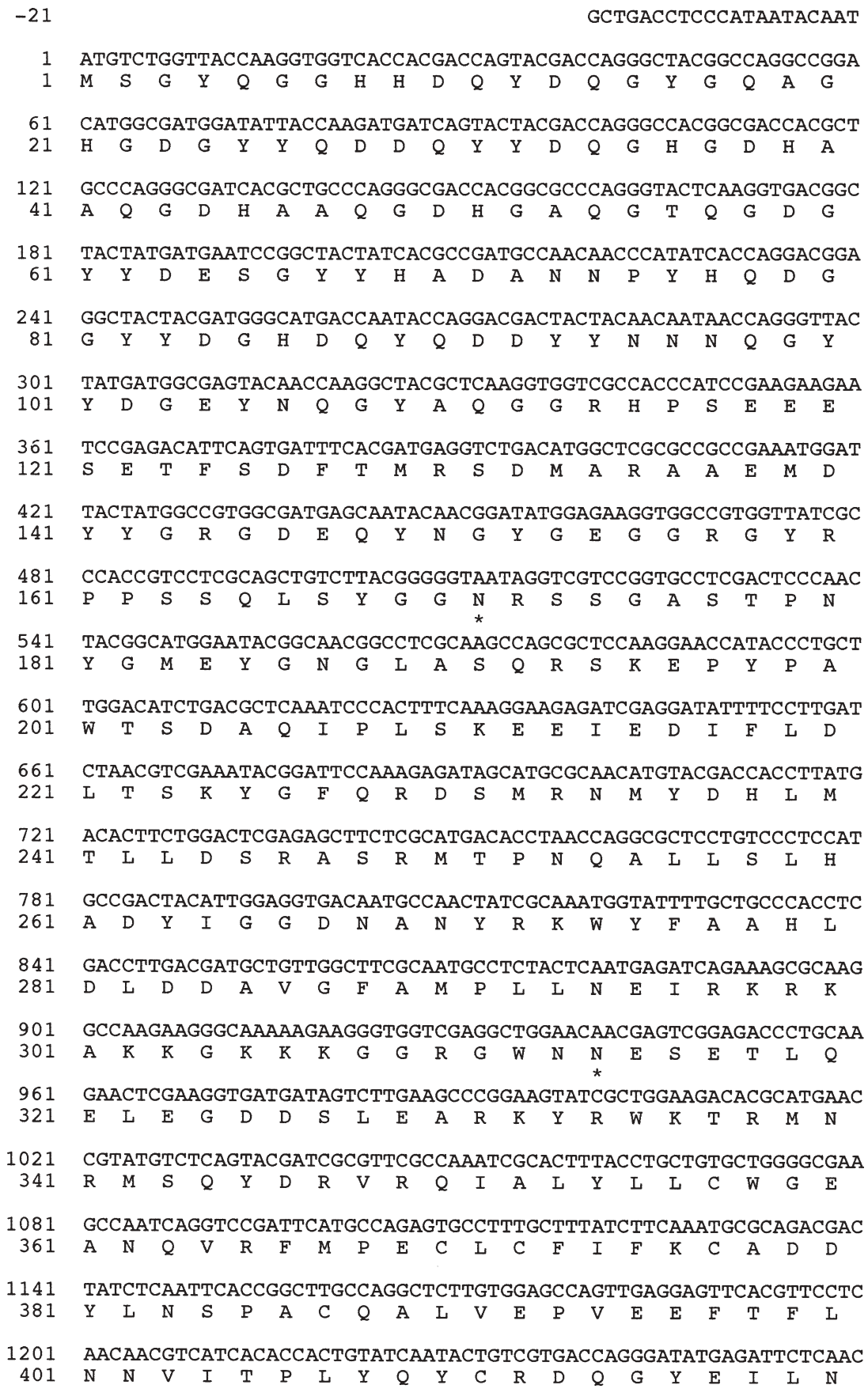

Fig. 1. Nucleotide sequence of *Cordyceps militaris FKS1* and deduced amino acid sequence. The predicted transmembrane domains are *double underlined* and *numbered*. The region corresponding to the putative cytoplasmic domain is *underlined*. The CmFks1p domains 1 and 2 are indicated by *dashed underlines*. The termination codon and potential *N*-glycosylation sites are denoted by *asterisks*

Fig. 1. *Continued*

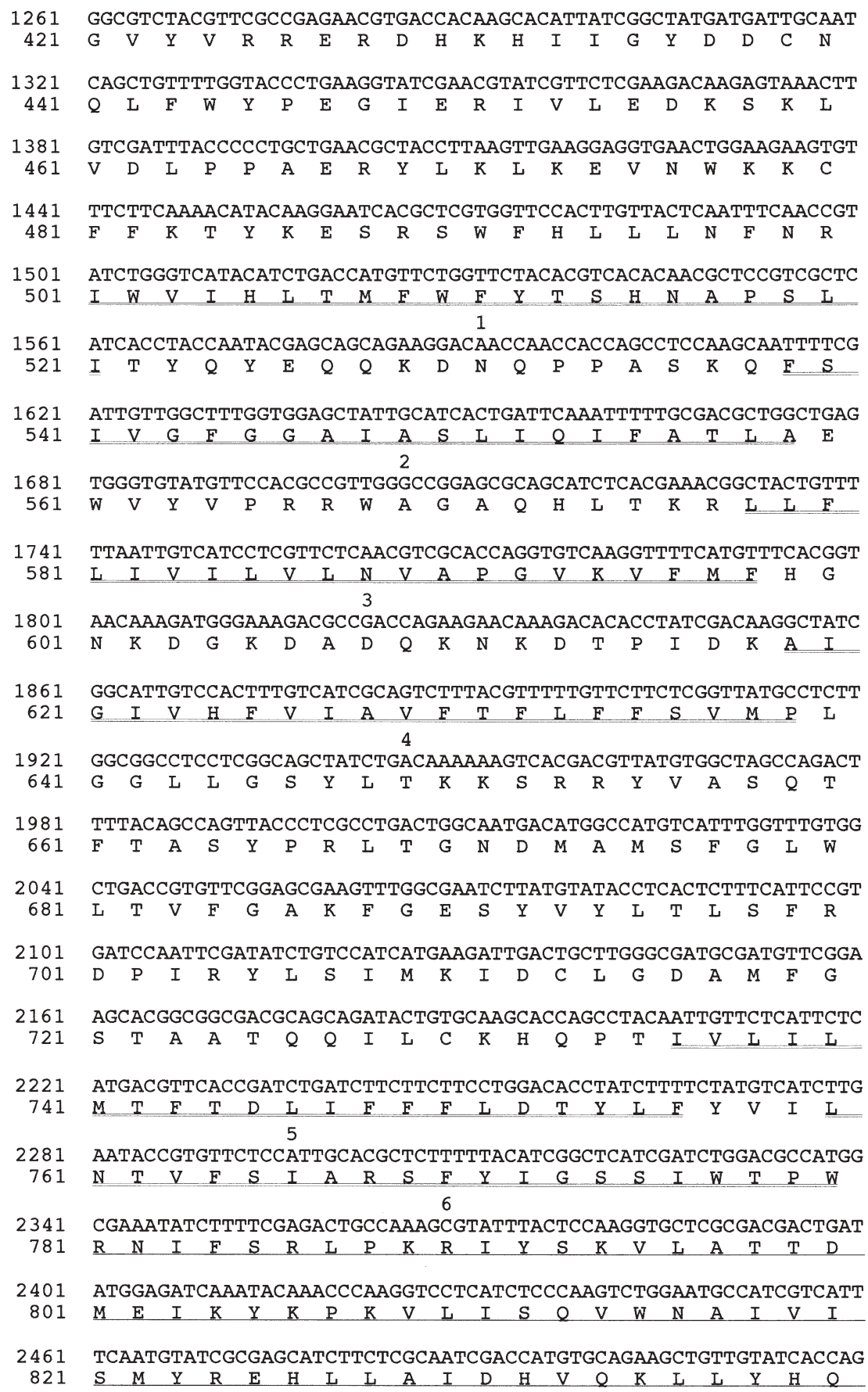

Fig. 1. *Continued*

```
2521  GTGCCCTCTGAGCAGGAAGGCAAACGGACATTACGCGCTCCGACCTTCTTCGTTTCCCAG
 841  V  P  S  E  Q  E  G  K  R  T  L  R  A  P  T  F  F  V  S  Q

2581  GAAGACCACTCGTTCAAAACAGAATTCTTTCCCAGCCACAGTGAAGCTGAGCGGCGCATC
 861  E  D  H  S  F  K  T  E  F  F  P  S  H  S  E  A  E  R  R  I

2641  TCATTCTTTGCCCAGTCTCTCTCCACACCTATTCCCGAACCCGTCCCTGTCGATAACATG
 881  S  F  F  A  Q  S  L  S  T  P  I  P  E  P  V  P  V  D  N  M

2701  CCTACGTTCACAGTCATGATCCCCCATTACAGTGAAAAGATTCTCCTCTCTCTGCGAGAA
 901  P  T  F  T  V  M  I  P  H  Y  S  E  K  I  L  L  S  L  R  E

      Domain 1
2761  ATCATTCGTGAAGACGAGCCCTACTCCCGTGTGACACTCCTGGAATACCTGAAGCAGCTT
 921  I  I  R  E  D  E  P  Y  S  R  V  T  L  L  E  Y  L  K  Q  L

2821  CATCCGCATGAATGGGAATGCTTTGTCAAGGACACAAAGATTTTGGCAGATGAAACAGCT
 941  H  P  H  E  W  E  C  F  V  K  D  T  K  I  L  A  D  E  T  A

2881  CAGATGAATGGCGAACCAGAGAAGAGCGAAAAAGACACAGCGAAGAGCAAAATCGACGAT
 961  Q  M  N  G  E  P  E  K  S  E  K  D  T  A  K  S  K  I  D  D

2941  TTGCCTTTTTACTGCATTGGTTTCAAGTCTTCTGCCCCGGAGTATACTCTTCGCACACGT
 981  L  P  F  Y  C  I  G  F  K  S  S  A  P  E  Y  T  L  R  T  R

3001  ATTTGGGCCTCTTTGCGTTCGCAAACTTTATATCGCACAGTGTCTGGCTTTATGAACTAC
1001  I  W  A  S  L  R  S  Q  T  L  Y  R  T  V  S  G  F  M  N  Y

                                  Domain 2                        *
3061  AGTCGTGCCATCAAGCTCCTGTATCGTGTCGAGAATCCCGAGGTTGTTCAGATGTTTGGT
1021  S  R  A  I  K  L  L  Y  R  V  E  N  P  E  V  V  Q  M  F  G

3121  GGAAACTCAGAAAAGTTGGAACGAGAACTGGAGAGAATGGCTCGACGCAAGTTCAAGCTC
1041  G  N  S  E  K  L  E  R  E  L  E  R  M  A  R  R  K  F  K  L

3181  GTTGTGTCTATGCAACGCTATTCCAAATTCAAGAAGGAAGAGATGGAAAATGCCGAATTT
1061  V  V  S  M  Q  R  Y  S  K  F  K  K  E  E  M  E  N  A  E  F

3241  TTACTGCGCGCCTATCCGGATCTTCAAATTGCTTACCTGGATGAGGAGCCTCCTCTAGCA
1081  L  L  R  A  Y  P  D  L  Q  I  A  Y  L  D  E  E  P  P  L  A

3301  GAAGGCGAGGAACCTCGCCTGTACTCCGCACTTATCGACGGTCACTCAGAACTCATGGAG
1101  E  G  E  E  P  R  L  Y  S  A  L  I  D  G  H  S  E  L  M  E

3361  AATGGTATGAGGCGACCAAAGTTCCGTGTCCAGCTCTCTGGTAACCCGGTATTGGGTGAT
1121  N  G  M  R  R  P  K  F  R  V  Q  L  S  G  N  P  V  L  G  D

3421  GGTAAATCCGACAACCAGAATCACGCCATCATCTTTTACCGCGGTGAATACATTCAGCTC
1141  G  K  S  D  N  Q  N  H  A  I  I  F  Y  R  G  E  Y  I  Q  L

3481  ATCGACGCCAACCAAGACAACTATCTGGAAGAATGCCTGAAGATTCGCAGTGTCCTCGCA
1161  I  D  A  N  Q  D  N  Y  L  E  E  C  L  K  I  R  S  V  L  A

3541  GAGTTCGGGGAAATGAAGCCAGACAACCATTCCCCTTACACACCCGGAGTCAAGAACGAT
1181  E  F  G  E  M  K  P  D  N  H  S  P  Y  T  P  G  V  K  N  D

3601  GTGCACACTCCAGTTGCTATTCTTGGTGCTCGCGAGTACATTTTCTCGGAGAATATTGGT
1201  V  H  T  P  V  A  I  L  G  A  R  E  Y  I  F  S  E  N  I  G

3661  ATTCTCGGTGATGTTGCTGCTGGAAAAGAACAAACATTTGGTACCCTCTTTGCCCGTACT
1221  I  L  G  D  V  A  A  G  K  E  Q  T  F  G  T  L  F  A  R  T

3721  ATGGCACAGGTTGGTGGCAAACTTCACTACGGCCATCCTGATTTCCTAAACGGTATCTTC
1241  M  A  Q  V  G  G  K  L  H  Y  G  H  P  D  F  L  N  G  I  F
```

Fig. 1. *Continued*

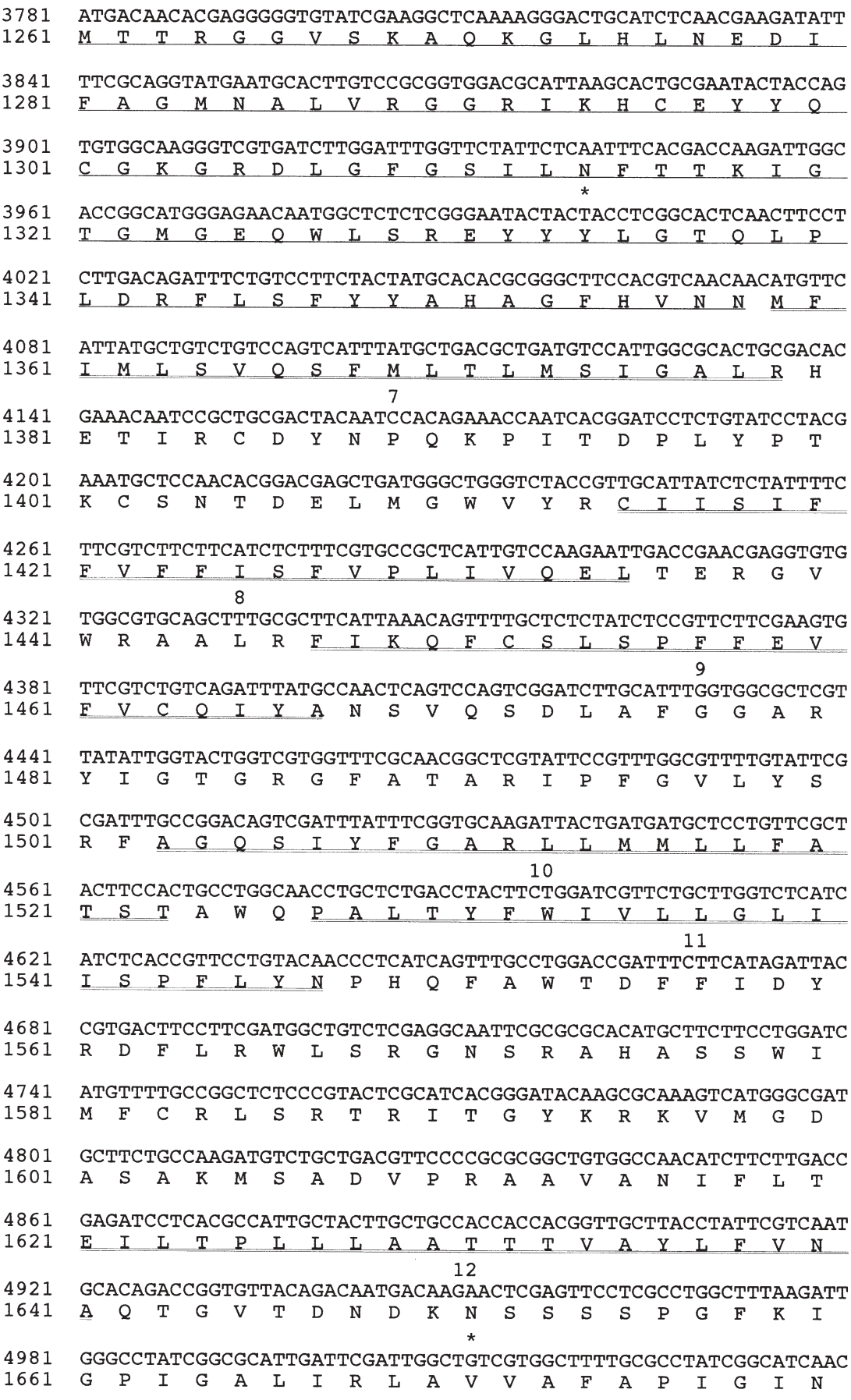

Fig. 1. *Continued*

```
5041  GCCGGCGTTCTTGCAGCCATGTTTGGAATGGCTTGTTGTATGGGACCAGTTCTGAACATG
1681  A  G  V  L  A  A  M  F  G  M  A  C  C  M  G  P  V  L  N  M
                                   13
5101  TGTTGCAAGAAGTTCGGGCCTGTTCTCGCTGGTATCGCCCACGGCGCGGCTGCAGTCTTC
1701  C  C  K  K  F  G  P  V  L  A  G  I  A  H  G  A  A  A  V  F
5161  ATGATTATCTTCTTCGAAGTTATGTATGTTTTGGAAGGATTCAACTTCGCCAGAGCTCTT
1721  M  I  I  F  F  E  V  M  Y  V  L  E  G  F  N  F  A  R  A  L
      14
5221  GCGGGTATCATCGCCGCCATGTGCATCCAGCGTTTCATTTTTAAGCTCATTGTGTCACTG
1741  A  G  I  I  A  A  M  C  I  Q  R  F  I  F  K  L  I  V  S  L
5281  GCGTTGACCAGAGAGTTCAAGACTGATCAGTCCAACATTGCCTTCTGGAACGGAAAATGG
1761  A  L  T  R  E  F  K  T  D  Q  S  N  I  A  F  W  N  G  K  W
5341  TACTCAATGGGCTGGCACTCAGTATCCCAACCGGCCCGAGAGTTTTTATGCAAGATTACG
1781  Y  S  M  G  W  H  S  V  S  Q  P  A  R  E  F  L  C  K  I  T
5401  GAACTCAGCATGTTCTCTGCTGATTTCATTCTGGGACATTGGATTTTATTCATGATGGCT
1801  E  L  S  M  F  S  A  D  F  I  L  G  H  W  I  L  F  M  M  A
                                                  15
5461  CCGTTGATTTTGATCCCGCAGATTGATAAGATCCATTCCATGATGCTCTTCTGGTTGCTG
1821  P  L  I  L  I  P  Q  I  D  K  I  H  S  M  M  L  F  W  L  L
5521  CCTAGTCGTCAGATTCGCCCACCCATCTACTCCATGAAGCAGTCAAAGCTTCGGCGTAGA
1841  P  S  R  Q  I  R  P  P  I  Y  S  M  K  Q  S  K  L  R  R  R
5581  CGTGTTATTCGATTTGCCATTCTTTACTTTGTTCTCTTCATCATTTTCCTGGCGCTCGTT
1861  R  V  I  R  F  A  I  L  Y  F  V  L  F  I  I  F  L  A  L  V
                                                  16
5641  GTTGCTCCAGGCGTCATTGGCAAGAAGTTCCTTGGCGATACAATCTTCAAAGCTCTGGAT
1881  V  A  P  G  V  I  G  K  K  F  L  G  D  T  I  F  K  A  L  D
5701  AATGGCAACGGCGGCCCCGCCAATTTGCACCTTCTGCAGCCGTGGGGACTGGACAACAAC
1901  N  G  N  G  G  P  A  N  L  H  L  L  Q  P  W  G  L  D  N  N
                                                              *
5761  AACACTGAAGGCAAGACTGAAACTGGCACCAAGGCTGGTGGTGCTGATGCATCTGCCACA
1921  N  T  E  G  K  T  E  T  G  T  K  A  G  G  A  D  A  S  A  T
5821  GATGCCTCGAAGCTGCGTCTTATTCTAAGCTTGAAACGCCACACGCATCGATTTGAAAAG
1941  D  A  S  K  L  R  L  I  L  S  L  K  R  H  T  H  R  F  E  K
5881  CCACCATGTTCTGGGAAATCACAAACCATTGTACCGATTATTAGATTGTTTCTGTTATTA
1961  P  P  C  S  G  K  S  Q  T  I  V  P  I  I  R  L  F  L  L  L
5941  TTTTGATCCATTTTGTCTCGGATAGGGTTAATACAGAAGTTTTGGTTGG
1981  F  *
```

sequences, several primers were designed for 5′/3′ rapid amplification of cDNA ends (RACE). RACE was done using a 5′/3′ RACE kit (Takara, Otsu, Japan). Sequence comparisons to databases were performed using the BLAST program (National Center for Biotechnology Information). Sequence analysis was performed with the Genetyx program (Genetyx, Tokyo, Japan). Hydropathy analysis was performed by the method of Sipos and von Heijne (1993).

Here, we isolated a single *FKS* homologue that we named *CmFKS1*. An open reading frame of 5943 bp was identified in the complete sequence (Fig. 1). The deduced sequence of 1981 amino acids shows 72% identity to *P. brasiliensis* Fks1p, 71% to FksAp from *Aspergillus nidulans*, 70% to Fksp from *Aspergillus fumigatus*, 63% to *Yarrowia lipolytica* Fks1p, 62% to Fks1p and Fks2p from *S. cerevisiae*, 61% to *Candida albicans* Fks1p, and 60% to *C. neoformans* Fks1p. Sequence analysis showed that *C. militaris* Fks1p (CmFks1p) is an integral membrane protein very similar to other Fksps in yeast and filamentous fungi. Similar to *S. cerevisiae* Fks1p (ScFks1p), CmFks1p possesses 16 potential transmembrane domains with a relatively large hydrophilic domain in the middle of the protein. The region of CmFks1p most homologous to ScFks1p is the large hydrophilic domain of 578 amino acids that is predicted to be a cytoplasmic domain. This domain is 84%

Fig. 2. Domains 1 and 2 are conserved regions between CmFks1p and BcsAp. Identical amino acids are denoted by *asterisks*; + indicates similar amino acids. CmFks1p, *C. militaris* Fks1p; BcsAp, catalytic subunit of cellulose synthase from *Acetobacter xylinium*

Domain 1

```
CmFks1p   890 PIPEPVPVDNMPTFTVMIPHYSEKI 914
              *+* *  **+ **  + ** * *++
BcsAp     138 PLPLPDNVDDWPTVDIFIPTYDEQL 162
```

Domain 2

```
CmFks1p   997 LRTRIWASLRSQTLYRTVSGFMNYSRAIKLLYRVENP 1033
              **  + + * ++ *   +   * +** +  +++***
BcsAp     350 LRIPVASGLATERLTTHIGQRMRWARGMIQIFRVDNP  386
```

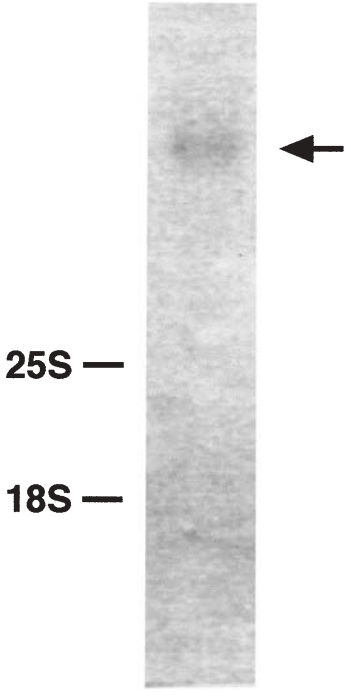

Fig. 3. Northern blot analysis of *C. militaris FKS1* transcript. Northern blot of total RNA isolated from *C. militaris* and probed with the 1.7-kb *CmFKS1* cDNA fragment. *Arrow* indicates the position of the *CmFKS1* mRNA band. Positions of the 25S and 18S rRNA markers are indicated

identical to the corresponding region of ScFks1p and is a candidate for the location of the catalytic site. As reported with other Fksps, CmFks1p does not contain the proposed UDP-glucose binding motif QXXRW, but it has regions with homology to BcsAp, the catalytic subunit of cellulose synthase from *Acetobacter xylinium* (Kelly et al. 1996). These specific domains (domains 1 and 2, Fig. 2) are located in the hydrophilic central region, as indicated in Fig. 1. Because cellulose synthase catalyzes the formation of β-1,4-glucan using UDP-glucose as a substrate, these conserved domains may be involved in UDP-glucose binding. Similar to ScFks1p, CmFks1p has six potential *N*-glycosylation sites. *CmFKS1*, the gene encoding a catalytic subunit Fks1p of β-1,3-glucan synthase in *C. militaris*, was studied for the first time in vegetable wasps and plant worms. The complete nucleotide sequence of *CmFKS1* has been submitted to DDBJ/EMBL/GenBank under accession number AB219141.

To analyze the *CmFKS1* gene expression, Northern blot analysis was done as described previously (Ujita et al. 1995). *Cordyceps militaris* was grown in a potato dextrose broth medium (Difco) at 25°C. At the late logarithmic phase of the growth, the hyphae were harvested by centrifugation. For total RNA isolation, an RNeasy kit (Qiagen) was employed. Thirty micrograms of total RNA from *C. militaris* was electrophoresed in a denaturing 2.2 M formaldehyde-1.0% agarose gel and transferred as described previously (Ujita et al. 1995). A 1.7-kb cDNA fragment corresponding to nucleotide positions 2998–4668 in Fig. 1 was labeled using DIG DNA labeling and detection kit (Roche) and used as a probe. Detection of the hybridized probe was done according to the manufacturer's instructions. Northern blot analysis revealed that *C. militaris FKS1* mRNA of approximately 9 kb was expressed in hyphae (Fig. 3). A single transcript was detected as observed with *S. cerevisiae* (Mazur et al. 1995), *A. nidulans* (Kelly et al. 1996), *C. albicans* (Mio et al. 1997), *C. neoformans* (Thompson et al. 1999), and *Y. lipolytica* (Leon et al. 2002) (Fig. 3).

The cell wall β-glucan of yeast, filamentous fungi, and mushrooms is essential for their shape and viability, and some fungal β-glucans show antitumor activity by activating immune systems. It has been shown that the maitake mushroom (*Grifola frondosa*) β-glucan exhibits strong anticancer activity by increasing immunocompetent cell activity (Kodama et al. 2002). *Grifola frondosa* β-glucan has been reported to exert its antitumor effect in tumor-bearing mice by enhancing the immune system through activation of macrophages, T cells, and natural killer (NK) cells, which are cytotoxic for tumor cells and play an important role in regulating immune responses. *Grifola frondosa* β-glucan also appears to repress cancer progression (Kodama et al. 2003). Furthermore, the biosynthetic pathways of β-glucans are possible targets for the design of novel chemotherapeutics against human pathogens such as fungi, because mammals are not thought to possess β-glucan synthases. Although β-1,3-glucan synthesis has been intensively studied, the biosynthetic pathway is not fully understood. Molecular cloning of the *CmFKS1* gene might lead to the elucidation of the biosynthetic pathway of *C. militaris* β-glucans that are used as a tonic food and herbal medicine and integrate the pharmacological activities of vegetable wasps and plant worms with basic science.

Acknowledgments This work was supported in part by a Grant-in-Aid (16780076 to M.U.) from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and by The Agricultural High-Tech Research Center of Meijo University, under the project Environmental Control Through the Functions of Microorganisms.

References

Beauvais A, Bruneau JM, Mol PC, Buitrago MJ, Legrand R, Latge JP (2001) Glucan synthase complex of *Aspergillus fumigatus*. J Bacteriol 183:2273–2279

Borchers AT, Stern JS, Hackman RM, Keen CL, Gershwin ME (1999) Mushrooms, tumors, and immunity. Proc Soc Exp Biol Med 221:281–293

Douglas CM, Foor F, Marrinan JA, Morin N, Nielsen JB, Dahl AM, Mazur P, Baginsky W, Li W, El-Sherbeini M, Clemas JA, Mandala SM, Frommer BR, Kurtz MB (1994) The *Saccharomyces cerevisiae FKS1 (ETG1)* gene encodes an integral membrane protein which is a subunit of 1,3-β-D-glucan synthase. Proc Natl Acad Sci USA 91:12907–12911

Kelly R, Register E, Hsu MJ, Kurtz M, Nielsen J (1996) Isolation of a gene involved in 1,3-β-glucan synthesis in *Aspergillus nidulans* and purification of the corresponding protein. J Bacteriol 178:4381–4391

Kodama N, Komuta K, Nanba H (2002) Can maitake MD-fraction aid cancer patients? Altern Med Rev 7:236–239

Kodama N, Komuta K, Nanba H (2003) Effect of Maitake (*Grifola frondosa*) D-Fraction on the activation of NK cells in cancer patients. J Med Food 6:371–377

Leon M, Sentandreu R, Zueco J (2002) A single *FKS* homologue in *Yarrowia lipolytica* is essential for viability. Yeast 19:1003–1014

Mazur P, Baginsky W (1996) *In vitro* activity of 1,3-β-D-glucan synthase requires the GTP-binding protein Rho1. J Biol Chem 271:14604–14609

Mazur P, Morin N, Baginsky W, El-Sherbeini M, Clemas JA, Nielsen JB, Foor F (1995) Differential expression and function of two homologous subunits of yeast 1,3-β-D-glucan synthase. Mol Cell Biol 15:5671–5681

Mio T, Adachi-Shimizu M, Tachibana Y, Tabuchi H, Inoue SB, Yabe T, Yamada-Okabe T, Arisawa M, Watanabe T, Yamada-Okabe H (1997) Cloning of the *Candida albicans* homolog of *Saccharomyces cerevisiae GSC1*/*FKS1* and its involvement in β-1,3-glucan synthesis. J Bacteriol 179:4096–4105

Sipos L, von Heijne G (1993) Predicting the topology of eukaryotic membrane proteins. Eur J Biochem 213:1333–1340

Thompson JR, Douglas CM, Li W, Jue CK, Pramanik B, Yuan X, Rude TH, Toffaletti DL, Perfect JR, Kurtz M (1999) A glucan synthase *FKS1* homolog in *Cryptococcus neoformans* is single copy and encodes an essential function. J Bacteriol 181:444–453

Ujita M, Shinomura T, Kimata K (1995) Molecular cloning of the mouse osteoglycin-encoding gene. Gene (Amst) 158:237–240

Wasser SP (2002) Medicinal mushrooms as a source of antitumor and immunomodulating polysaccharides. Appl Microbiol Biotechnol 60:258–274