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Development and Characterization of Expression Vectors for the Riboflavin Overproducing Fungus *Eremothecium ashbyii* using *Ashbya gossypii* Genes

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Abstract

Eremothecium ashbyii is a filamentous hemi-ascomycete fungus and a natural overproducer of riboflavin. The present study was undertaken to characterize the molecular tools constructed for the genetic manipulation of this organism based on plasmids constructed for the related organisms *A. gossypii* and *S. cerevisiae* using two candidate genes. The candidate gene, *SPR3* homolog of *S. cerevisiae,* is known to play a role in cytokinesis in *S. cerevisiae*. This gene was chosen to aid in future studies on the regulation of septation and its role in the excretion of riboflavin in *E. ashbyii*, as yeast cytokinesis is analogous to the septation of filamentous fungi. The second candidate gene was the *S. cerevisiae RAD*14 homolog, which is known to play a key role in the nucleotide excision repair pathway. Reporter plasmids, constructed previously in a preliminary study with the *AgSPR*3-like gene and the *AgRAD*14-like gene fused to the *Lac*Z reporter gene, were used in this study. These plasmids were characterized by sequencing followed by homology searches. While the former revealed homology to the *S. cerevisiae* septin protein family, *SPR*3 gene, and the *Neurospora crassa CDC*12 gene involved in cell cycle regulation, the latter showed homology to the *S. cerevisiae HOG*I gene involved in the osmotic stress response.

Keywords – Cytokinesis – *HOG1* – NER pathway – Septation – *SPR*3

Introduction

Eremothecium ashbyii is a filamentous hemi-ascomycete fungus with a genome similar to the yeast *Saccharomyces cerevisiae*. It is known for the natural overproduction of riboflavin (Zhang et al. 2021), thus making it industrially important. Riboflavin, also known as vitamin B_2, is important for cells. It forms the main component of flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN), which function as coenzymes for many oxidoreductases (Zhang et al. 2021). It is an important ingredient in multivitamin formulations and is also used as a food additive and in the formulation of animal feed supplements.

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Morphological studies of *E. ashbyii* show the presence of slimy colonies. The mycelium is found to be coenocytic at the earliest stage, which eventually divides into plurinucleate segments of unequal length by forming callose plugs. Swelling of the segments leads to the division of nuclei, causing condensation of the sporoplasm around the nuclei. Ascospores, usually 8–16 per ascus, are hyaline and smooth. Sporulation is correlated with riboflavin production (Pujari & Chandra 2001). Non-sporulating *E. ashbyii* produces less riboflavin (Zhang et al. 2021). It has been shown that *E. ashbyii* accumulates lipids in its mycelia before riboflavin overproduction (Vijayalakshmi et al. 2010). The amount of lipid accumulated in mycelia decreases as riboflavin production increases. Monitoring of the lipid accumulation was done with the fluorescent probe Nile blue (Vijayalakshmi et al. 2003). The lipid accumulation was correlated with extracellular lipase activity and also with the production of riboflavin (Vijayalakshmi et al. 2010). Riboflavin is transported out of the overproducer's cytoplasm rather than being stored in the cell.

In *E. ashbyii*, one of the mechanisms of countering riboflavin-induced toxicity is through the modulation of membrane fluidity by incorporating a large percentage of unsaturated fatty acids, and notably oleic acids, in the membrane phospholipids (Vijayalakshmi et al. 2010). Preliminary studies have shown that morphological changes are produced as a first line of defense (Sampath & Vijayalakshmi 2018). Another line of adaptation to stress is the change in membrane fluidity as a result of alterations in the composition of the membrane phospholipids (Vijayalakshmi et al. 2010). Regarding riboflavin overproduction by *E. ashbyii*, a question of relevance to be addressed is whether changes in membrane fluidity alone are enough to allow the riboflavin to be excreted. Cellular processes such as vesicle trafficking, cytoskeletal organization, and regulation are vital to riboflavin production and excretion. Septation is an important process that plays a role in the organism's development. Hence, the proteins involved in septation and cytoskeletal organization are good candidates for researching the role of the cytoskeleton and septation in riboflavin production and excretion in *E. ashbyii.* The *SPR*3 gene is known to play a role in cytokinesis in *S. cerevisiae* (Alexander & Michelle 2019). The cytokinesis process in yeast is analogous to the process of septation in filamentous fungi. Hence, this *SPR3* gene homolog was chosen to aid future studies on the regulation of septation and its role in riboflavin excretion in *E. ashbyii*.

Excess endogenous riboflavin is known to cause photo-induced damage similar to UV damage, causing DNA, RNA, and protein damage, whereas exogenous riboflavin protects the spores (Silva et al. 2019; Sugimoto et al. 2010). Organisms subjected to UV treatment start producing riboflavin at an early stage, accounting for the importance of maintaining their genomic integrity. Though a change in membrane fluidity and composition of membrane phospholipids has been implicated in countering riboflavin toxicity (Vijayalakshmi et al. 2010), the above findings point to the possible existence of another pathway triggered by UV exposure. UV-induced damage is mostly repaired by the RAD proteins of the Nucleotide Excision Repair (NER) mechanism (Perego et al. 2000) and is probably active during the overproduction of riboflavin. The genome of *E. ashbyii* is similar to that of *S. cerevisiae* (Prillinger et al. 1997). The NER pathway has been characterized in detail in *S. cerevisiae*, in which several proteins like *RAD*1, *RAD*10, and *RAD*14 have been shown to play a role. The NER may probably be functioning in *E. ashbyii* to combat riboflavin toxicity, thus maintaining the genome stability of the organism. Hence, the *S. cerevisiae RAD* 14 homologue was chosen with a view to aiding future studies on the role of the NER pathway in combating riboflavin-induced stress in *E. ashbyii*.

Molecular studies have been successfully undertaken in *A. gossypii*, and many vectors have been constructed for use in *A. gossypii* (Wright & Philippsen 1991, Steiner et al. 1995, Wendland et al. 2000). Additionally, the genome of *A. gossypii* has been completely sequenced (Dietrich et al. 2004), making genetic studies feasible in this organism. However, no genomic studies have been carried out so far on *E. ashbyii*. In another study, vectors constructed in *A. gossypii* were used to successfully transform *E. ashbyii* (Manoj & Vijayalakshmi, unpublished results). Hence, in this study, the vectors were constructed using *A. gossypii* genes to aid in future studies on *E. ashbyii*.

Materials and Methods*:*

Organism and culture conditions

Eremothecium ashbyii MTCC 366 (also known as NRRL 1363) was cultured on Potato Dextrose Agar slants and plates (PDA g/l, Potato infusion-200, Dextrose-20, Agar-20 pH 5.6± 0.2) and cultures were maintained at 25 °C. Subculturing was done at 15-day intervals on PDA slants to maintain the culture.

Chemicals

All chemicals used were of analytical grade from local sources.

DNA manipulations

All DNA manipulations were carried out according to Sambrook & Russell (2001) using the *Escherichia coli* DH5-α strain as the host (Hanahan 1983). Before cloning, the sequences of the PCR-amplified fragments were verified by sequencing. Sequencing was done by Eurofins (Bangalore, India). The proper orientation of insertion was verified by restriction analysis. Competent cells were prepared, and the ligated DNA mixture was mixed with competent cells and transformed by the heat shock method. Transformed colonies were selected by the blue/white screening method (Green & Sambrook 2012).

Plasmids and constructs

Plasmids used in the present study are listed in Table 1. PCR primers used are listed in Table 2.

Plasmid isolation

Plasmid isolation was carried out using the alkaline lysis method. Single transformed colonies were grown overnight in 10 ml of LB broth containing ampicillin (50 µg/ml). A 1.5 ml of LB broth was centrifuged at 10,000 rpm for 2 min at 4 °C. A 100 µl of the re-suspension buffer (50 mM glucose, 10 mM EDTA, 25 mM TrisHCl, pH 8.0) was added to the pellet and mixed properly.

A white precipitate was formed, indicating the presence of chromosomal DNA when 200 µl of lysis solution (0.2N NaOH, 1% SDS) was added to the above mixture. Finally, 150 µl of neutralizing solution (of composition per 100 ml: 60 ml of 5M potassium acetate, 11.5 ml of glacial acetic acid and 28.5 ml of deionized water) was added to the solution. After the addition, centrifugation was done at 10,000 rpm for 10 minutes at 4 °C. To the supernatant, an equal amount of phenol: chloroform: isoamyl alcohol (25:24:1) was added. The top aqueous layer was collected after centrifuging at 10,000 rpm for 2 minutes. Then, 1 ml of 100% ethanol was added to the top aqueous layer and collected. Centrifugation was done at 10,000 rpm for 5 minutes at -4 °C, and the pellet was collected. To the pellet, 1 ml of ice-cold 70% ethanol was added and centrifuged at 10,000 rpm for 2 minutes. The collected pellet was air-dried and re-suspended with 100 µl TE buffer (Sambrook & Russell 2001).

Table 1. List of Plasmids used in the present study and their properties

Plasmids	Properties	Source
Plasmid BB	*Amp*^{r}, *Gen*^{r}A derivative of pSV 2 in which the *RIB* 3 gene is excluded and restriction sites for integration of RAD 14 are included**.**	Sampath & Vijayalakshmi (2009), unpublished thesis work.
Plasmid D	*Amp*^{r}, *Gen*^{r}A derivative of pSV 2 and contains the *lacZ* reporter gene from pTS 24 **(**Schlösser, 2007**)**, under the control of the *SPR* 3 promoter	Vinod & Vijayalakshmi (2009), unpublished thesis work.

Restriction digestion

Isolated plasmid DNA was subjected to restriction digestion as follows: 2 µl of each plasmid DNA was taken separately, and 1 µl of the 10X tango buffer (ThermoFisher) was added to each sample. To this mixture, 2 µl of restriction enzyme *BamH1* (ThermoFisher) was added, and the samples were incubated in the water bath at 37 ˚C overnight. Later, the reaction mix was electrophoresed on a 1 % agarose gel.

Table 2. List of primers used in PCR

Primer Sequence *(5'-3')*	**Template**
Sam1 *RAD 14* (Promoter + ORF) **Forward*:*** ATG CTG GGG CCC ATG GCG CTG ACC GCA GAG Reverse: ATG CTG GCT AGC CTA CAA CAT TAT TTC TTC TAT CTC TAT C	*A. gossypii* genomic DNA
Sam2 *RAD 14* (Promoter) **Forward*:*** CAT GGG CCC TTA CTA GCG TGA CCC CCG GG Reverse: ATC GCT AGC GAA CAG ACA GGA GCC TAT GTA G	*A. gossypii* genomic DNA
Vin1 – *SPR3* (Promoter + ORF) Forward**:** ATT CGG GCC CCG TCA GAG TGC ACA AGC TCG Reverse**:** ATT CGC TAG CCT TGC TGC TCA TGC TGC CC	*A. gossypii* genomic DNA
Vin2 – *SPR3* (Promoter) Forward**:** ACT TGG GCC CCG TCA GAG TGC ACA AGC TCG Reverse**:** ACT TGC TAG CCT CCG TCC TTC TTA TTC TGT TTG	*A. gossypii* genomic DNA

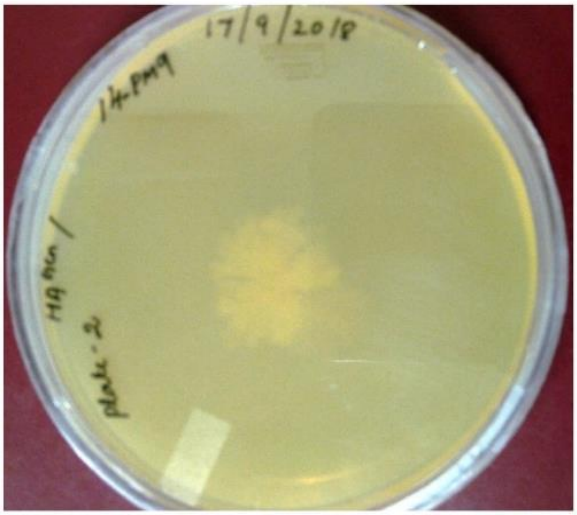
Sequence for *Apa* I - GGG CCC
Sequence for *Nhe* I - GCT AGC

Result

The plasmids were constructed using *A. gossypii* genes. Homologous recombination has been reported as the main mechanism for DNA integration in *A. gossypii* (Steiner et al. 1995). Since *A. gossypii* vectors bearing *A. gossypii* genes have been experimentally proven to successfully transform *E. ashbyii* (Fig. 1) (Manoj & Vijayalakshmi, unpublished results), it is quite possible that homologous recombination may prove to be the main mechanism for transformation in *E. ashbyii* as well. Therefore, we used *A. gossypii* genes to construct vectors for *E. ashbyii*.

The plasmid D is a derivative of the original integrative plasmid pSV 2 (Vijayalakshmi et al. 2003), which contains the *GFP* gene under the control of the *RIB* 3 promoter and also specific loci for the targeted integration of the plasmid into the *A. gossypii* genome. In this plasmid, the *RIB* 3 promoter was replaced by the *A. gossypii SPR 3* gene/promoter homolog, the Lac Z gene replaced the GFP gene, and the whole fragment was cloned into the *Bgl* II restriction site of plasmid pSV 2. The cloning was verified by linearizing the plasmids using *BamH1* digestion.

Plate showing *E.ashbyii* transformed with RIB3p GFP bearing Plasmid (pSV-2)



*RIB*3p GFP Expressing cells in Transformant (right panel) Control: wild type *E. ashbyii* (left panel)

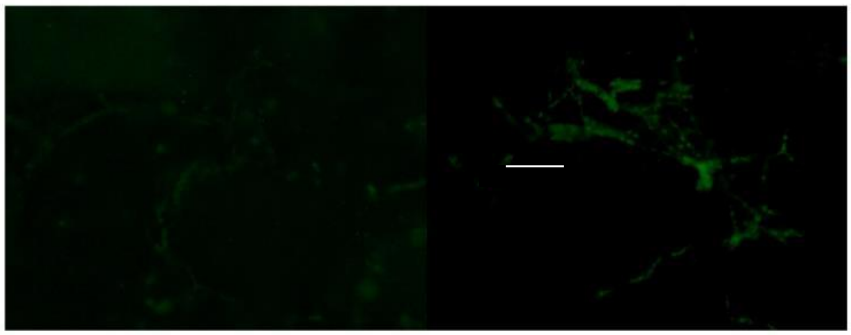


Fig. 1 – Preliminary studies on *E. ashbyii* transformation using the pSV-2 vector constructed using *A. gossypii* genes. The image on the top is the plate on which *E. ashbyii* is transformed with a pSV-2 vector constructed using *A. gossypii* genes, showing the successful transformation. The image below is the fluorescent microphotograph of the *E. asbhyii* filaments expressing *A. gossypii RIB*3p GFP (pSV-2) (Bar, 100 μm) (right panel), thus showing that *A. gossypii* vectors are functional in *E. ashbyii*. The left panel is wild type of *E. ashbyii* with no GFP expression, indicated by the dark background.

Characterisation of Plasmid D

The plasmid D sequencing data was subjected to bioinformatics searches using the KEGG database, Unit Prot and SGD for homology. The nucleotide sequence of both the transcribed and the flanking regions of *SPR*3 revealed a 640-bp open reading frame (Fig. 2) capable of encoding a 210-aa polypeptide chain (Fig. 3). The data showed a significant sequence homology with genes belonging to the septin family.

The gene sequence of Plasmid D harbouring homologue to SPR3 gene

ACGGACTGGCGAGTGGTTCCGTTAGTGAGGGAATGCGATAATGAATGGCCCTGGCGTTGAGGCTGGTTTA
AGTATTCTCCTTATG
ACTGTTCTTAACCTTTAGGAACACTAGGGGCTTACCAGGTTTCGCAAAATGAACCAACCGCAGGTGGGGG
TAGATGGGCAAACA
GCCTCTTAAGATTGAGATAATATGCTTCTGGGAGGGTTCGGTAGGTGATCTTGCTGGAATCCCCAGTATG
GCACGAGAATTGGGT

AGGATGTGGACTCGAGGCTACCCAATCATCGGGATGAGTTAATGGCCGACTGATTTGATCAGAGATGATT
TATCAACCAATTAAC
AAACTGATTGTTGTTGGGAAGAGGGGGGTAGGGAGTAAAGGGCGCTCTTAGGAGCGAAGGAATTGCTAT
TTAATTCCATGGGGA
TGCAAGGGCTATTTCTGTGGTGTGGGCGGAGCATAAAATAAGATCCAAGTGTGGGTTTGCGGCGCAGTGC
CAGTTGCGCTTGGGT
GTTGGGAAGGGGCAGCAGTCCGATATCCGCTGTGAGGAGTGGGCGGGGAGGAGGTAGATGCCAGAATA
GCGGCTGTTGGAGAAG GGAAATAAGGACTTAGAGAAGCGGAGAGCCCGTGGAGACAAATCT

Amino acid sequence analyses in the ProSite database showed the presence of the highly conserved P-loop region (Fig. 2). The presence of the P-loop (ATP/GTP binding site motif) is a characteristic feature of many septin family proteins. The analysis also revealed the presence of two leucine zipper domains in the *Ag SPR*3p (Fig. 3). The leucine zipper pattern is present in many gene regulatory proteins such as C/EBP, CREB, C-myc, etc. As *Ag SPR*3p belongs to the septin family, which has protein regulatory functions, it is important to probe if *Ag SPR*3p is a nuclear DNA-binding protein. If it turns out to be a DNA-binding protein, *Ag SPR*3p's regulatory role may not be limited to the protein level alone.

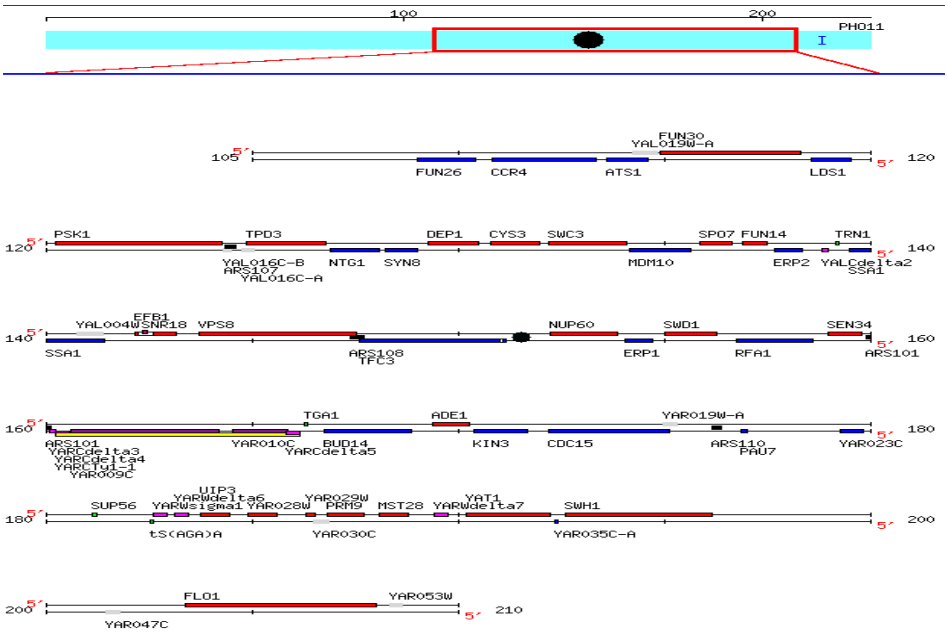


Fig. 2 – KEGG Database**:** http**://**www**.**genome**.**jp/tmp/blast/150311182730YL62W/result_blast**.**html

Comparison of the predicted amino acid sequence using the UniProtKB

The database revealed a significant sequence homology with genes belonging to the septin family (Fig. 4). The *AgSPR*3-like protein has a maximum percentage of sequence identity (100% similarity with 1st score bit and 49% with 2nd score bit) with the *Kluyveromyces lactis* protein KLLA0B08129g, which belongs to the septin family of proteins (Figs. 5 & 6), a high degree (almost 98%) of homology to *A. gossypii* (strain ATCC 10895) as shown in Fig. 4. In contrast to our point of interest, 78% homology has been seen with septin-type G protein of *Eremeothecium cymbalariae* (Fig. 7) and 51% homology with sporulation-regulated protein 3 of *S. ludwigii* (Fig. 8) *and least with S. cerevisiae SPR3p of 47%*, (Fig. 9). The highest match of 73% with *Kluyveromyces marxianus* (strain DMKU3-1042/BCC 29191/NBRC 104275) protein has been found which is known as cell division protein 3 (Fig. 10) and the least match of 38% with *CDC*12p of *Neurospora crassa*, a zinc finger protein, which is involved in cell division (Fig. 11). This indicates that the *Ag SPR*3 gene could have a potential role in the process of septation and can be probed further for such a role as well as a regulatory role on account of its homology to zinc finger proteins.

MSENQGTTSGGLHEDDSQNYTNSETISMSADQRAVSQAQTLGSQSETAASHSGQQATLDPEEHHTSLGYY
EVERKADDGEQDASSNNNMDQLLEGVYFQHDDTETNNAHDKTRKLVEKKPISDRYRVGIECLPLQREFVT
AKKGGHFTVMVVGQTGLGKTTFVNTLFRTSLLPSVWDTLEGNKPNVQFKKTTRIIRHQALIEEKNIKLKLT
VIDTPGFGDNANNSFAWSPIISYIDEQFRSYIFQEEQPDRRRLSDNRIHCCLYFLNPSNKGISPLDIEAMQEISK
RVNLIPVIAKADSLGTQSIAAFKEDVRRIINAQGIRICAFLDESDSECQSVIRDSPYALVCCDSYVQKPNGEK
VRGRKYKWGIAEVENPKHSDFCQLRDILMSKNMVDLVVSSEKYYETCRSHMLMTRINQAKDGLAAETSE
DNLILKNMNYEDPDANGMLNYKCYQIYNKQYMHELIIEWSPEFIHKQWEAKKRLNEIAHSEETKFTWKRA
LMFKHTN(LDSEIED(LHNRVKNL)$_1$QIDCEEL)$_2$ESILLQTGGLGSMSSKRMSKHDLLQ

ATP/GTP-binding site motif A (P-loop) 153 – 160: GqtglGKT

LEUCINE ZIPPER Pattern 500 - 521: LmfkhtnLdseiedLhnrvknL

Fig. 3 – Predicted polypeptide sequence of *Ag SPR*3p**.** The P-loop region is highlighted in blue, and the two leucine zipper patterns are highlighted in red.

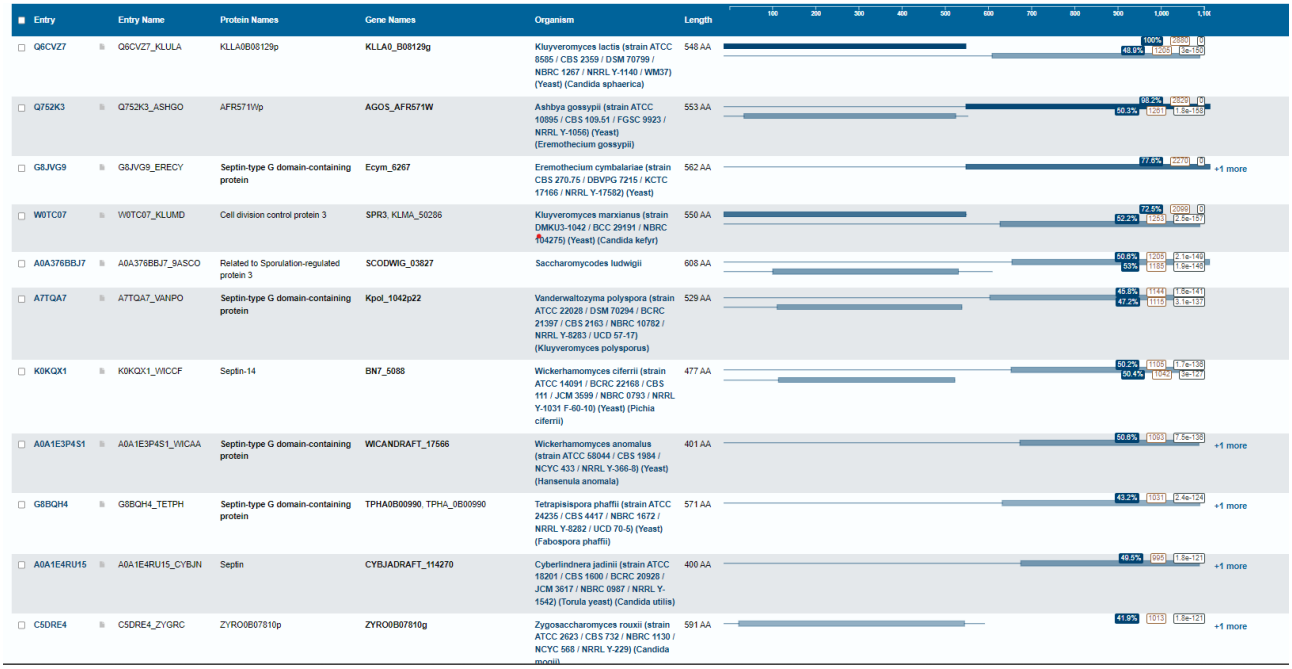
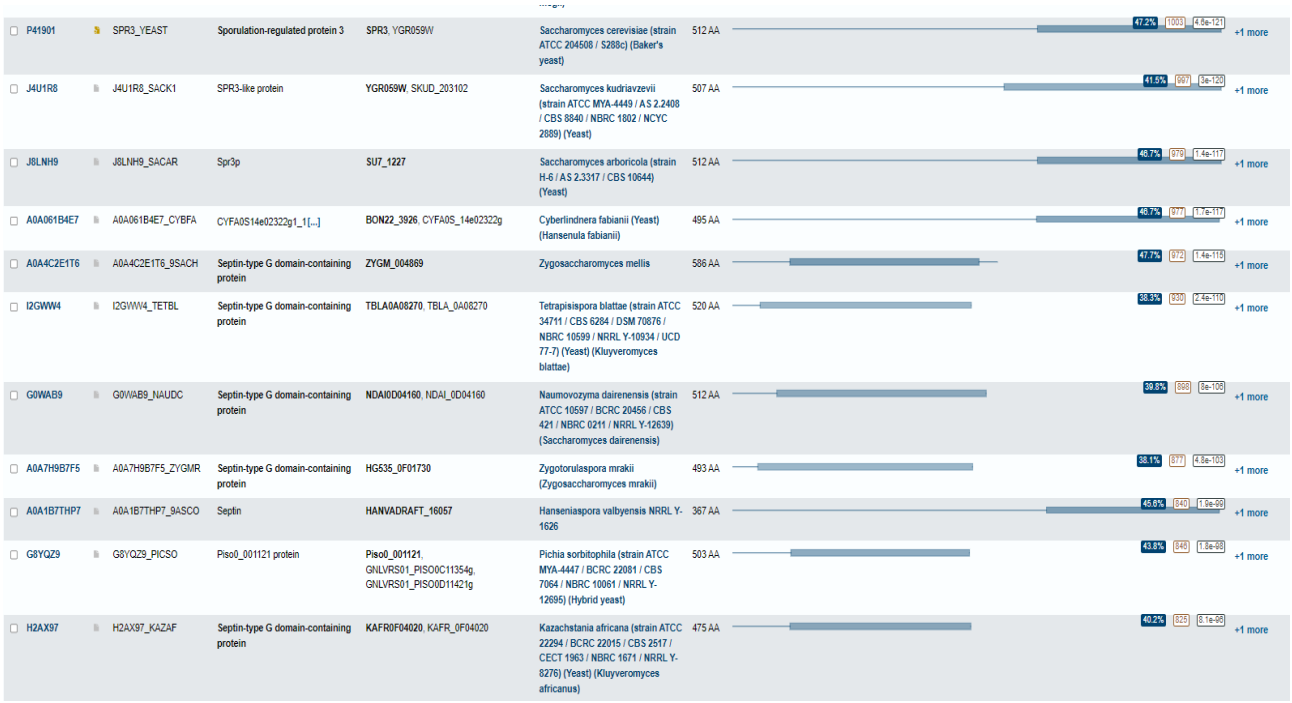


Fig. 4 – Comparison of predicted amino acid sequences for homology studies with the septin family.



P41901	SPR3_YEAST	Sporulation-regulated protein 3	SPR3, YGR059W	Saccharomyces cerevisiae (strain ATCC 204508 / S288c) (Baker's yeast)	512 AA	47.2% 1003 4.6e-121 +1 more
J4U1R8	J4U1R8_SACK1	SPR3-like protein	YGR059W, SKUD_203102	Saccharomyces kudriavzevii (strain ATCC MYA-4449 / AS 2.2408 / CBS 8840 / NBRC 1802 / NCYC 2889) (Yeast)	507 AA	41.5% 997 3e-120 +1 more
J8LNH9	J8LNH9_SACAR	Spr3p	SU7_1227	Saccharomyces arboricola (strain H-6 / AS 2.3317 / CBS 10644) (Yeast)	512 AA	46.7% 979 1.4e-117 +1 more
A0A061B4E7	A0A061B4E7_CYBFA	CYFA0S14e02322g1_1[...]	BON22_3926, CYFA0S_14e02322g	Cyberlindnera fabianii (Yeast) (Hansenula fabianii)	495 AA	46.7% 977 1.7e-117 +1 more
A0A4C2E1T6	A0A4C2E1T6_9SACH	Septin-type G domain-containing protein	ZYGM_004869	Zygosaccharomyces mellis	586 AA	47.7% 972 1.4e-115 +1 more
I2GWW4	I2GWW4_TETBL	Septin-type G domain-containing protein	TBLA0A08270, TBLA_0A08270	Tetrapisispora blattae (strain ATCC 34711 / CBS 6284 / DSM 70876 / NBRC 10599 / NRRL Y-10934 / UCD 77-7) (Yeast) (Kluyveromyces blattae)	520 AA	38.9% 930 2.4e-110 +1 more
G0WAB9	G0WAB9_NAUDC	Septin-type G domain-containing protein	NDAI0D04160, NDAI_0D04160	Naumovozyma dairenensis (strain ATCC 10597 / BCRC 20456 / CBS 421 / NBRC 0211 / NRRL Y-12639) (Saccharomyces dairenensis)	512 AA	39.8% 898 8e-108 +1 more
A0A7H9B7F5	A0A7H9B7F5_ZYGMR	Septin-type G domain-containing protein	HG535_0F01730	Zygotorulaspora mrakii (Zygosaccharomyces mrakii)	493 AA	38.1% 877 4.8e-103 +1 more
A0A1B7THP7	A0A1B7THP7_9ASCO	Septin	HANVADRAFT_16057	Hanseniaspora valbyensis NRRL Y-1626	367 AA	45.6% 840 1.9e-99 +1 more
G8YQZ9	G8YQZ9_PICSO	Piso0_001121 protein	Piso0_001121, GNLVRS01_PISO0C11354g, GNLVRS01_PISO0D11421g	Pichia sorbitophila (strain ATCC MYA-4447 / BCRC 22081 / CBS 7064 / NBRC 10061 / NRRL Y-12695) (Hybrid yeast)	503 AA	43.8% 848 1.8e-98 +1 more
H2AX97	H2AX97_KAZAF	Septin-type G domain-containing protein	KAFR0F04020, KAFR_0F04020	Kazachstania africana (strain ATCC 22294 / BCRC 22015 / CBS 2517 / CECT 1963 / NBRC 1671 / NRRL Y-8276) (Yeast) (Kluyveromyces africanus)	475 AA	40.2% 825 8.1e-96 +1 more

Fig. 4 – Continued.

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>TR:Q6CVZ7 Q6CVZ7_KLULA KLLA0B08129p OS=Kluyveromyces lactis (strain ATCC
8585 / CBS 2359 / DSM 70799 / NBRC 1267 / NRRL Y-1140 / WM37)
OX=284590 GN=KLLA0_B08129g PE=3 SV=1
Length=548

 Score = 1113 bits (2880),  Expect = 0.0
 Identities = 548/548 (100%), Positives = 548/548 (100%), Gaps = 0/548 (0%)

Query  1    MNSTKNGWGSSMDHYIEATGVEYDSISYGPAKYGSNADNLEVINEQPEEECTEVKPQSQE  60
            MNSTKNGWGSSMDHYIEATGVEYDSISYGPAKYGSNADNLEVINEQPEEECTEVKPQSQE
Sbjct  1    MNSTKNGWGSSMDHYIEATGVEYDSISYGPAKYGSNADNLEVINEQPEEECTEVKPQSQE  60

Query  61   KKEVLDSVRRLFEADSTESNSALKSLDVDINVQGDFYTLNSVMNSSTANTTQENCSKFIT  120
            KKEVLDSVRRLFEADSTESNSALKSLDVDINVQGDFYTLNSVMNSSTANTTQENCSKFIT
Sbjct  61   KKEVLDSVRRLFEADSTESNSALKSLDVDINVQGDFYTLNSVMNSSTANTTQENCSKFIT  120

Query  121  PSVIDEHYHIGIDSIPLQKETFIEKNGVQFTMMVVGQSGLGKTTFINTLFGTSLLPTVWE  180
            PSVIDEHYHIGIDSIPLQKETFIEKNGVQFTMMVVGQSGLGKTTFINTLFGTSLLPTVWE
Sbjct  121  PSVIDEHYHIGIDSIPLQKETFIEKNGVQFTMMVVGQSGLGKTTFINTLFGTSLLPTVWE  180

Query  181  SDMTERGVTKTTKIVRHESELVENGFTLRYTVIDTPGFGDLANNNFSWSPIVNYIDEQYR  240
            SDMTERGVTKTTKIVRHESELVENGFTLRYTVIDTPGFGDLANNNFSWSPIVNYIDEQYR
Sbjct  181  SDMTERGVTKTTKIVRHESELVENGFTLRYTVIDTPGFGDLANNNFSWSPIVNYIDEQYR  240

Query  241  SYIFQEEQPLRASLKDNRIHCCLYFINLTRNGLSALDIAAMEEISKRVNLIPVIAKIDGL  300
            SYIFQEEQPLRASLKDNRIHCCLYFINLTRNGLSALDIAAMEEISKRVNLIPVIAKIDGL
Sbjct  241  SYIFQEEQPLRASLKDNRIHCCLYFINLTRNGLSALDIAAMEEISKRVNLIPVIAKIDGL  300

Query  301  TSADLEMYKRKIRETLQKQEIKVCSFLDQNHPNCQTIFDTYPFGIVCSDEMVTNNEGKLV  360
            TSADLEMYKRKIRETLQKQEIKVCSFLDQNHPNCQTIFDTYPFGIVCSDEMVTNNEGKLV
Sbjct  301  TSADLEMYKRKIRETLQKQEIKVCSFLDQNHPNCQTIFDTYPFGIVCSDEMVTNNEGKLV  360

Query  361  RGRKYKWGNVEVENPLHSEFTALRTVLMSKNLVDFAVGCENYYEKCRSHILLSRIQQAKT  420
            RGRKYKWGNVEVENPLHSEFTALRTVLMSKNLVDFAVGCENYYEKCRSHILLSRIQQAKT
Sbjct  361  RGRKYKWGNVEVENPLHSEFTALRTVLMSKNLVDFAVGCENYYEKCRSHILLSRIQQAKT  420
```

Fig. 5 – Sequence identity of *Ag SPR*3p with *K. lactis* KLLA0B08129g using UniProtKB: Identical matches are shown in the figure.

```
Query  421  NCPDHLDLTNLDLDNPDQNGLENYKFYEAFNKKFMDDLIIEWSPEFIHKQWEAKKRLSEI  480
            NCPDHLDLTNLDLDNPDQNGLENYKFYEAFNKKFMDDLIIEWSPEFIHKQWEAKKRLSEI
Sbjct  421  NCPDHLDLTNLDLDNPDQNGLENYKFYEAFNKKFMDDLIIEWSPEFIHKQWEAKKRLSEI  480

Query  481  VSMEEKRFKDWKQDLLNKQNLFNHEIEDLHTIVQQIRSECNELETRVNKQRPRRFSKLGL  540
            VSMEEKRFKDWKQDLLNKQNLFNHEIEDLHTIVQQIRSECNELETRVNKQRPRRFSKLGL
Sbjct  481  VSMEEKRFKDWKQDLLNKQNLFNHEIEDLHTIVQQIRSECNELETRVNKQRPRRFSKLGL  540

Query  541  SSHSELAR  548
            SSHSELAR
Sbjct  541  SSHSELAR  548


 Score = 468 bits (1205),  Expect = 3e-150
 Identities = 238/487 (49%), Positives = 321/487 (66%), Gaps = 25/487 (5%)

Query  608   PEEHHTSLGYYEVERK-----------ADDGEQDASSNN-NMDQLLEGVYFQ----HDDT  651
             PEE  T +     E+K           AD  E +++  + ++D  ++G ++      + +
Sbjct  47    PEEECTEVKPQSQEKKEVLDSVRRLFEADSTESNSALKSLDVDINVQGDFYTLNSVMNSS  106

Query  652   ETNNAHDKTRKLVEKKPISDRYRVGIECLPLQREFVTAKKGGHFTVMVVGQTGLGKTTFV  711
               N   +   K +    I + Y +GI+ +PLQ+E    K G  FT+MVVGQ+GLGKTTF+
Sbjct  107   TANTTQENCSKFITPSVIDEHYHIGIDSIPLQKETFIEKNGVQFTMMVVGQSGLGKTTFI  166

Query  712   NTLFRTSLLPSVWDTLEGNKPNVQFKKTTRIIRHQALIEEKNIKLKLTVIDTPGFGDNAN  771
             NTLF TSLLP+VW   E +       KTT+I+RH++ + E    L+ TVIDTPGFGD AN
Sbjct  167   NTLFGTSLLPTVW---ESDMTERGVTKTTKIVRHESELVENGFTLRYTVIDTPGFGDLAN  223

Query  772   NSFAWSPIISYIDEQFRSYIFQEEQPDRRRLSDNRIHCCLYFLNPSNKGISPLDIEAMQE  831
             N+F+WSPI++YIDEQ+RSYIFQEEQP R  L DNRIHCCLYF+N +  G+S LDI AM+E
Sbjct  224   NNFSWSPIVNYIDEQYRSYIFQEEQPLRASLKDNRIHCCLYFINLTRNGLSALDIAAMEE  283

Query  832   ISKRVNLIPVIAKADSLGTQSIAAFKEDVRRIINAQGIRICAFLDESDSECQSVIRDSPY  891
             ISKRVNLIPVIAK D L +  +  +K  +R  +  Q I++C+FLD++   CQ++    P+
Sbjct  284   ISKRVNLIPVIAKIDGLTSADLEMYKRKIRETLQKQEIKVCSFLDQNHPNCQTIFDTYPF  343

Query  892   ALVCCDSYVQKPNGEKVRGRKYKWGIAEVENPKHSDFCQLRDILMSKNMVDLVVSSEKYY  951
              +VC D  V    G+ VRGRKYKWG  EVENP HS+F  LR +LMSKN+VD  V  E YY
Sbjct  344   GIVCSDEMVTNNEGKLVRGRKYKWGNVEVENPLHSEFTALRTVLMSKNLVDFAVGCENYY  403

Query  952   ETCRSHMLMTRINQAKDGLAAETSEDNLILKNMNYEDPDANGMLNYKCYQIYNKQYMHEL  1011
             E CRSH+L++RI QAK         D+L L N++ ++PD NG+ NYK Y+ +NK++M +L
Sbjct  404   EKCRSHILLSRIQQAK-----TNCPDHLDLTNLDLDNPDQNGLENYKFYEAFNKKFMDDL  458

Query  1012  IIEWSPEFIHKQWEAKKRLNEIAHSEETKF-TWKRALMFKHTNLDSEIEDLHNRVKNLQI  1070
             IIEWSPEFIHKQWEAKKRL+EI    EE +F  WK+ L+ K     + EIEDLH  V+ ++
Sbjct  459   IIEWSPEFIHKQWEAKKRLSEIVSMEEKRFKDWKQDLLNKQNLFNHEIEDLHTIVQQIRS  518

Query  1071  DCEELES  1077
             +C ELE+
Sbjct  519   ECNELET  525
```

Fig. 5 – Continued.

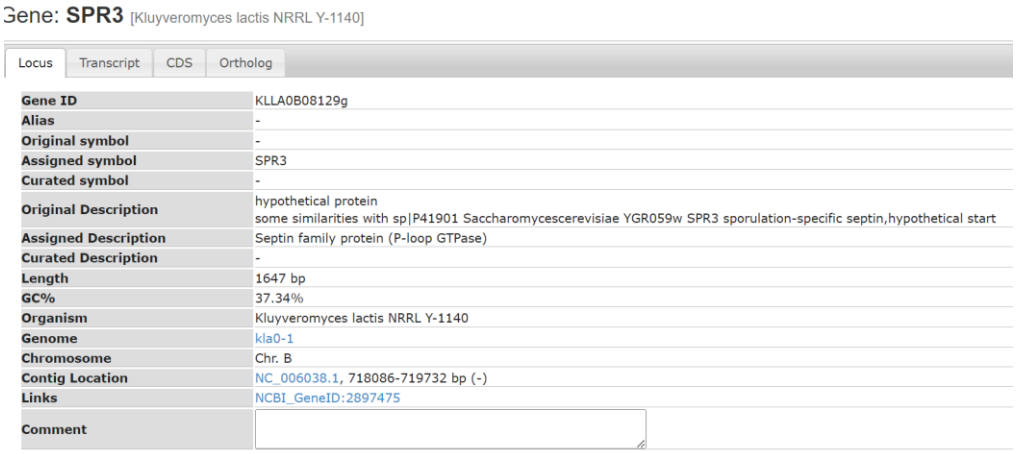
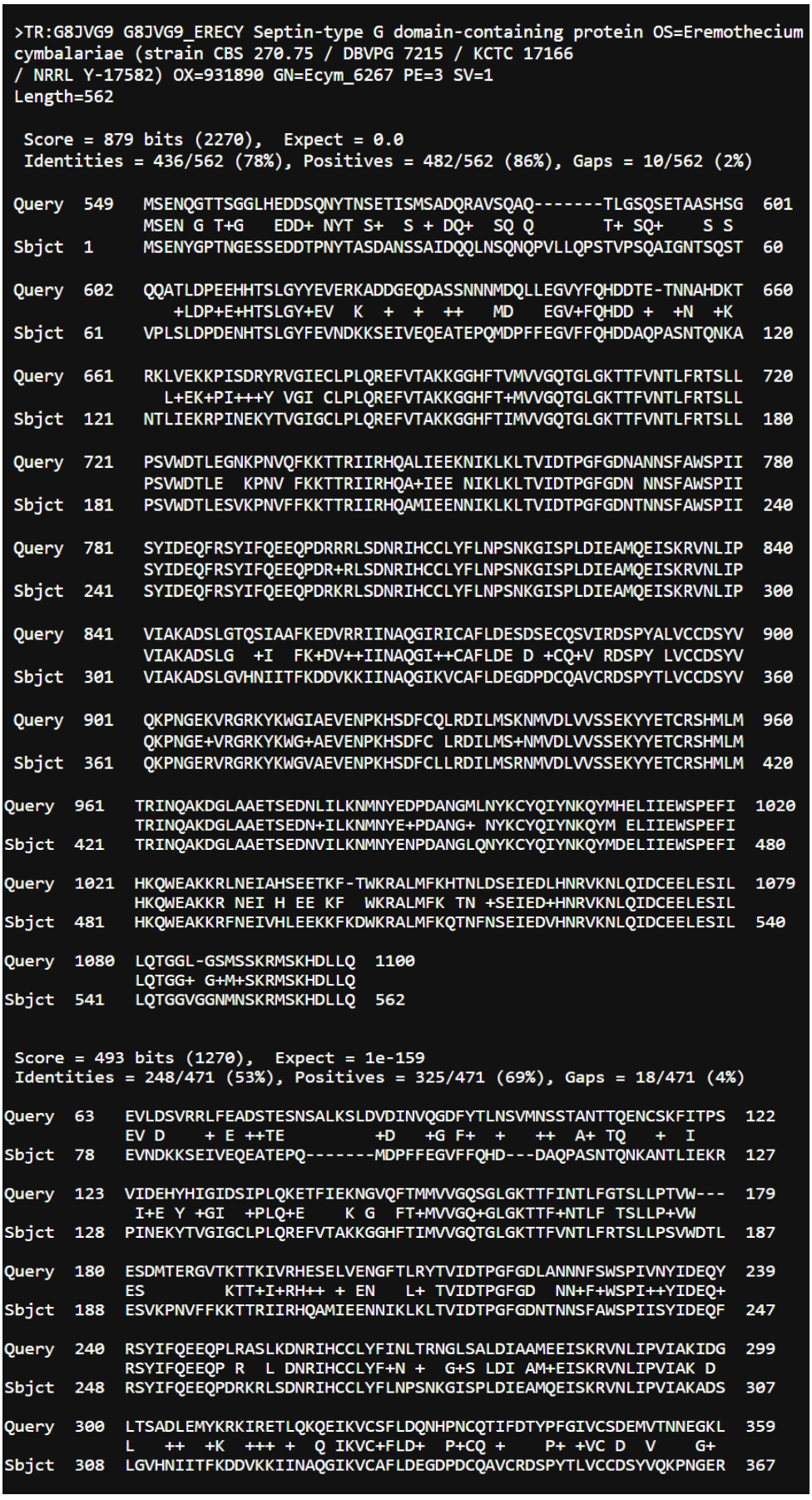


Fig. 6 – CAoGD database showing KLLA0B08129g having similarities with *S. cerevisiae* SPR3 and belongs to septin family protein with P-loop GTPase.



```
>TR:G8JVG9 G8JVG9_ERECY Septin-type G domain-containing protein OS=Eremothecium
cymbalariae (strain CBS 270.75 / DBVPG 7215 / KCTC 17166
/ NRRL Y-17582) OX=931890 GN=Ecym_6267 PE=3 SV=1
Length=562

 Score = 879 bits (2270),  Expect = 0.0
 Identities = 436/562 (78%), Positives = 482/562 (86%), Gaps = 10/562 (2%)

Query  549   MSENQGTTSGGLHEDDSQNYTNSETISMSADQRAVSQAQ-------TLGSQSETAASHSG  601
             MSEN G T+G   EDD+ NYT S+  S + DQ+  SQ Q       T+ SQ+    S S
Sbjct  1     MSENYGPTNGESSEDDTPNYTASDANSSAIDQQLNSQNQPVLLQPSTVPSQAIGNTSQST  60

Query  602   QQATLDPEEHHTSLGYYEVERKADDGEQDASSNNNMDQLLEGVYFQHDDTE-TNNAHDKT  660
                +LDP+E+HTSLGY+EV  K  +  +  ++   MD   EGV+FQHDD +  +N  +K
Sbjct  61    VPLSLDPDENHTSLGYFEVNDKKSEIVEQEATEPQMDPFFEGVFFQHDDAQPASNTQNKA  120

Query  661   RKLVEKKPISDRYRVGIECLPLQREFVTAKKGGHFTVMVVGQTGLGKTTFVNTLFRTSLL  720
               L+EK+PI+++Y VGI CLPLQREFVTAKKGGHFT+MVVGQTGLGKTTFVNTLFRTSLL
Sbjct  121   NTLIEKRPINEKYTVGIGCLPLQREFVTAKKGGHFTIMVVGQTGLGKTTFVNTLFRTSLL  180

Query  721   PSVWDTLEGNKPNVQFKKTTRIIRHQALIEEKNIKLKLTVIDTPGFGDNANNSFAWSPII  780
             PSVWDTLE  KPNV FKKTTRIIRHQA+IEE NIKLKLTVIDTPGFGDN NNSFAWSPII
Sbjct  181   PSVWDTLESVKPNVFFKKTTRIIRHQAMIEENNIKLKLTVIDTPGFGDNTNNSFAWSPII  240

Query  781   SYIDEQFRSYIFQEEQPDRRRLSDNRIHCCLYFLNPSNKGISPLDIEAMQEISKRVNLIP  840
             SYIDEQFRSYIFQEEQPDR+RLSDNRIHCCLYFLNPSNKGISPLDIEAMQEISKRVNLIP
Sbjct  241   SYIDEQFRSYIFQEEQPDRKRLSDNRIHCCLYFLNPSNKGISPLDIEAMQEISKRVNLIP  300

Query  841   VIAKADSLGTQSIAAFKEDVRRIINAQGIRICAFLDESDSECQSVIRDSPYALVCCDSYV  900
             VIAKADSLG  +I  FK+DV++IINAQGI++CAFLDE D +CQ+V RDSPY LVCCDSYV
Sbjct  301   VIAKADSLGVHNIITFKDDVKKIINAQGIKVCAFLDEGDPDCQAVCRDSPYTLVCCDSYV  360

Query  901   QKPNGEKVRGRKYKWGIAEVENPKHSDFCQLRDILMSKNMVDLVVSSEKYYETCRSHMLM  960
             QKPNGE+VRGRKYKWG+AEVENPKHSDFC LRDILMS+NMVDLVVSSEKYYETCRSHMLM
Sbjct  361   QKPNGERVRGRKYKWGVAEVENPKHSDFCLLRDILMSRNMVDLVVSSEKYYETCRSHMLM  420

Query  961   TRINQAKDGLAAETSEDNLILKNMNYEDPDANGMLNYKCYQIYNKQYMHELIIEWSPEFI  1020
             TRINQAKDGLAAETSEDN+ILKNMNYE+PDANG+ NYKCYQIYNKQYM ELIIEWSPEFI
Sbjct  421   TRINQAKDGLAAETSEDNVILKNMNYENPDANGLQNYKCYQIYNKQYMDELIIEWSPEFI  480

Query  1021  HKQWEAKKRLNEIAHSEETKF-TWKRALMFKHTNLDSEIEDLHNRVKNLQIDCEELESIL  1079
             HKQWEAKKR NEI H EE KF  WKRALMFK TN +SEIED+HNRVKNLQIDCEELESIL
Sbjct  481   HKQWEAKKRFNEIVHLEEKKFKDWKRALMFKQTNFNSEIEDVHNRVKNLQIDCEELESIL  540

Query  1080  LQTGGL-GSMSSKRMSKHDLLQ  1100
             LQTGG+ G+M+SKRMSKHDLLQ
Sbjct  541   LQTGGVGGNMNSKRMSKHDLLQ  562


 Score = 493 bits (1270),  Expect = 1e-159
 Identities = 248/471 (53%), Positives = 325/471 (69%), Gaps = 18/471 (4%)

Query  63    EVLDSVRRLFEADSTESNSALKSLDVDINVQGDFYTLNSVMNSSTANTTQENCSKFITPS  122
             EV D    + E ++TE         +D   +G F+  +   ++  A+ TQ   +  I
Sbjct  78    EVNDKKSEIVEQEATEPQ-------MDPFFEGVFFQHD---DAQPASNTQNKANTLIEKR  127

Query  123   VIDEHYHIGIDSIPLQKETFIEKNGVQFTMMVVGQSGLGKTTFINTLFGTSLLPTVW---  179
              I+E Y +GI  +PLQ+E    K G  FT+MVVGQ+GLGKTTF+NTLF TSLLP+VW
Sbjct  128   PINEKYTVGIGCLPLQREFVTAKKGGHFTIMVVGQTGLGKTTFVNTLFRTSLLPSVWDTL  187

Query  180   ESDMTERGVTKTTKIVRHESELVENGFTLRYTVIDTPGFGDLANNNFSWSPIVNYIDEQY  239
             ES        KTT+I+RH++ + EN   L+ TVIDTPGFGD  NN+F+WSPI++YIDEQ+
Sbjct  188   ESVKPNVFFKKTTRIIRHQAMIEENNIKLKLTVIDTPGFGDNTNNSFAWSPIISYIDEQF  247

Query  240   RSYIFQEEQPLRASLKDNRIHCCLYFINLTRNGLSALDIAAMEEISKRVNLIPVIAKIDG  299
             RSYIFQEEQP R  L DNRIHCCLYF+N +  G+S LDI AM+EISKRVNLIPVIAK D
Sbjct  248   RSYIFQEEQPDRKRLSDNRIHCCLYFLNPSNKGISPLDIEAMQEISKRVNLIPVIAKADS  307

Query  300   LTSADLEMYKRKIRETLQKQEIKVCSFLDQNHPNCQTIFDTYPFGIVCSDEMVTNNEGKL  359
             L   ++  +K  +++ +  Q IKVC+FLD+  P+CQ +    P+ +VC D  V    G+
Sbjct  308   LGVHNIITFKDDVKKIINAQGIKVCAFLDEGDPDCQAVCRDSPYTLVCCDSYVQKPNGER  367
```

Fig. 7 – Sequence identity of *Ag SPR*3p with *Eremeothecium cymbalariae* showing 78**%** homology with Septin-type G domain-containing protein.

```
Query  360  VRGRKYKWGNVEVENPLHSEFTALRTVLMSKNLVDFAVGCENYYEKCRSHILLSRIQQAK  419
            VRGRKYKWG  EVENP HS+F  LR +LMS+N+VD  V  E YYE CRSH+L++RI QAK
Sbjct  368  VRGRKYKWGVAEVENPKHSDFCLLRDILMSRNMVDLVVSSEKYYETCRSHMLMTRINQAK  427

Query  420  -----TNCPDHLDLTNLDLDNPDQNGLENYKFYEAFNKKFMDDLIIEWSPEFIHKQWEAK  474
                      D++ L N++ +NPD NGL+NYK Y+ +NK++MD+LIIEWSPEFIHKQWEAK
Sbjct  428  DGLAAETSEDNVILKNMNYENPDANGLQNYKCYQIYNKQYMDELIIEWSPEFIHKQWEAK  487

Query  475  KRLSEIVSMEEKRFKDWKQDLLNKQNLFNHEIEDLHTIVQQIRSECNELET  525
            KR +EIV +EEK+FKDWK+ L+ KQ  FN EIED+H  V+ ++ +C ELE+
Sbjct  488  KRFNEIVHLEEKKFKDWKRALMFKQTNFNSEIEDVHNRVKNLQIDCEELES  538
```

Fig. 7 – Continued.

```
>TR:A0A376BBJ7 A0A376BBJ7_9ASCO Related to Sporulation-regulated protein 3 OS=Sacchar
ludwigii OX=36035 GN=SCODWIG_03827 PE=3 SV=1
Length=608

 Score = 468 bits (1205),  Expect = 2e-149
 Identities = 238/470 (51%), Positives = 323/470 (69%), Gaps = 23/470 (5%)

Query  653   TNNAHDKTRKLVEKK-------PISDRYRVGIECLPLQREFVTAKKGGHFTVMVVGQTGL  705
              T N+  K   + E K       P+     VGI  LPLQ+  +TA+ G +F +MVVGQ+GL
Sbjct  128   TENSLPKNEDVQESKNVFVVAEPLDSDRLVGIGELPLQKMKLTARNGAYFNLMVVGQSGL  187

Query  706   GKTTFVNTLFRTSLLPSVWDTLEG--NKPNVQFKKTTRIIRHQALIEEKNIKLKLTVIDT  763
              GKTTF+NTLF TS+LP++W+ +E     PNV F KTT I+RH +++EEK+I LK TVIDT
Sbjct  188   GKTTFINTLFGTSILPNIWNQIESLMKTPNVTFNKTTSIVRHTSILEEKDISLKFTVIDT  247

Query  764   PGFGDNANNSFAWSPIISYIDEQFRSYIFQEEQPDRRRLSDNRIHCCLYFLNPSNKGISP  823
              PGFGD +NNSF+W PI +YIDEQFRSY+FQEEQPDR  + D R+HCCLYF+ PSNKG+S
Sbjct  248   PGFGDCSNNSFSWEPITNYIDEQFRSYMFQEEQPDRSPIEDRRVHCCLYFIQPSNKGLST  307

Query  824   LDIEAMQEISKRVNLIPVIAKADSLGTQSIAAFKEDVRRIINAQGIRICAFLD-ESDSEC  882
              LDIE+M+EISKRVNLIP+IAK D L  + +  FK+ +R I+ AQ I+IC F+  E D  C
Sbjct  308   LDIESMKEISKRVNLIPIIAKGDGLLPKDLQIFKKTIRSILQAQNIKICEFIQKEKDPGC  367

Query  883   QSVIRDSPYALVCCDSYVQKPNGEKVRGRKYKWGIAEVENPKHSDFCQLRDILMSKNMVD  942
                + +D PY+++  +S       + V GRKYKWG++EVEN KH DF +LR++LM +N+VD
Sbjct  368   SLIFKDFPYSIIGSESKTLNEEHKLVYGRKYKWGVSEVENEKHCDFVKLRNVLMKENLVD  427

Query  943   LVVSSEKYYETCRSHMLMTRINQAKDGL----AAETSEDNLI--------LKNMNYEDPD  990
              LV+S+E YYE CR+ +L TRI QAKD L      +  ++ +I        L  +++++ +
Sbjct  428   LVLSTEAYYEKCRTKLLETRILQAKDSLINVSGVKKKDEEVINIGITKKGLSELDFDNLN  487

Query  991   ANGMLNYKCYQIYNKQYMHELIIEWSPEFIHKQWEAKKRLNEIAHSEETKF-TWKRALMF  1049
              +N + NYKCY I++K YM EL+IEWSP FIHKQWEAK++ NEI   EE KF  WK+AL
Sbjct  488   SNKLDNYKCYYIFDKLYMDELVIEWSPIFIHKQWEAKQKFNEIVLLEEKKFKDWKKALFN  547

Query  1050  KHTNLDSEIEDLHNRVKNLQIDCEELESILLQTGGLGSMSSKRMSKHDLL  1099
              + T  + EIE+LH +VK LQ +C+ELE  +  +  L +   K   K+ +L
Sbjct  548   RQTGFNIEIENLHKKVKLLQKNCQELEKNINMSRELFNHPHKLAMKNPIL  597
```

Fig. 8 – Sequence identity of *Ag SPR*3p with *Saccharomycodes ludwigii* showing 51% homology with its sporulation-regulated protein3 (*SPR3*).

```
 Score = 461 bits (1185),  Expect = 2e-146
 Identities = 235/443 (53%), Positives = 298/443 (67%), Gaps = 23/443 (5%)

Query  112  QENCSKFITPSVIDEHYHIGIDSIPLQKETFIEKNGVQFTMMVVGQSGLGKTTFINTLFG  171
            QE+ + F+    +D    +GI  +PLQK     +NG  F +MVVGQSGLGKTTFINTLFG
Sbjct  139  QESKNVFVVAEPLDSDRLVGIGELPLQKMKLTARNGAYFNLMVVGQSGLGKTTFINTLFG  198

Query  172  TSLLPTVW---ESDMTERGVT--KTTKIVRHESELVENGFTLRYTVIDTPGFGDLANNNF  226
            TS+LP +W   ES M    VT  KTT IVRH S L E   +L++TVIDTPGFGD +NN+F
Sbjct  199  TSILPNIWNQIESLMKTPNVTFNKTTSIVRHTSILEEKDISLKFTVIDTPGFGDCSNNSF  258

Query  227  SWSPIVNYIDEQYRSYIFQEEQPLRASLKDNRIHCCLYFINLTRNGLSALDIAAMEEISK  286
            SW PI NYIDEQ+RSY+FQEEQP R+ ++D R+HCCLYFI  +  GLS LDI +M+EISK
Sbjct  259  SWEPITNYIDEQFRSYMFQEEQPDRSPIEDRRVHCCLYFIQPSNKGLSTLDIESMKEISK  318

Query  287  RVNLIPVIAKIDGLTSADLEMYKRKIRETLQKQEIKVCSFLD-QNHPNCQTIFDTYPFGI  345
            RVNLIP+IAK DGL   DL+++K+ IR  LQ Q IK+C F+  +  P C  IF  +P+ I
Sbjct  319  RVNLIPIIAKGDGLLPKDLQIFKKTIRSILQAQNIKICEFIQKEKDPGCSLIFKDFPYSI  378

Query  346  VCSDEMVTNNEGKLVRGRKYKWGNVEVENPLHSEFTALRTVLMSKNLVDFAVGCENYYEK  405
            + S+    N E KLV GRKYKWG  EVEN  H +F  LR VLM +NLVD  +  E YYEK
Sbjct  379  IGSESKTLNEEHKLVYGRKYKWGVSEVENEKHCDFVKLRNVLMKENLVDLVLSTEAYYEK  438

Query  406  CRSHILLSRIQQAKTNCPD----------------HLDLTNLDLDNPDQNGLENYKFYE  448
            CR+ +L +RI QAK +  +                   L+ LD DN + N L+NYK Y
Sbjct  439  CRTKLLETRILQAKDSLINVSGVKKKDEEVINIGITKKGLSELDFDNLNSNKLDNYKCYY  498

Query  449  AFNKKFMDDLIIEWSPEFIHKQWEAKKRLSEIVSMEEKRFKDWKQDLLNKQNLFNHEIED  508
             F+K +MD+L+IEWSP FIHKQWEAK++ +EIV +EEK+FKDWK+ L N+Q  FN EIE+
Sbjct  499  IFDKLYMDELVIEWSPIFIHKQWEAKQKFNEIVLLEEKKFKDWKKALFNRQTGFNIEIEN  558

Query  509  LHTIVQQIRSECNELETRVNKQR  531
            LH  V+ ++  C ELE  +N  R
Sbjct  559  LHKKVKLLQKNCQELEKNINMSR  581
```

Fig. 8 – Continued.

.

```
>SP:P41901 SPR3_YEAST Sporulation-regulated protein 3 OS=Saccharomyces cerevisiae
(strain ATCC 204508 / S288c) OX=559292 GN=SPR3 PE=1
SV=1
Length=512

 Score = 390 bits (1003),  Expect = 5e-121
 Identities = 195/413 (47%), Positives = 280/413 (68%), Gaps = 19/413 (5%)

Query  675   VGIECLPLQREFVTAKKGGHFTVMVVGQTGLGKTTFVNTLFRTSLLPSVWDTLEGNKPNV  734
             +GI+ LP QRE + AK G  FT+MV GQ+GLGKTTF+N+LF TSL+    D ++ NKP
Sbjct  90    IGIKNLPRQRELLNAKNGIDFTLMVAGQSGLGKTTFINSLFSTSLID---DDIKENKP--  144

Query  735   QFKKTTRIIRHQALIEEKNIKLKLTVIDTPGFGDNANNSFAWSPIISYIDEQFRSYIFQE  794
                    IIR+++++E     L   VIDTPGFG+N +N+F W  +++YIDE+ RSYIFQE
Sbjct  145   -------IIRYKSIVEGDGTHLNFNVIDTPGFGNNMDNAFTWRTMVNYIDEEIRSYIFQE  197

Query  795   EQPDRRRLSDNRIHCCLYFLNPSNKGISPLDIEAMQEISKRVNLIPVIAKADSLGTQSIA  854
             EQPDR ++ DNR+HCCLYFL PSNKGI  LD+  M++++KRVNLIPVIAK+D L  + +
Sbjct  198   EQPDRTKMVDNRVHCCLYFLRPSNKGIDTLDVVTMKKLAKRVNLIPVIAKSDLLTKEELK  257

Query  855   AFKEDVRRIINAQGIRICAFL-DESDSECQSVIRDSPYALVCCDSYVQKPNGEKVRGRKY  913
              FK  VR II  Q I +C F  DE  +  Q + +  P++++  + Y+    GEKV+GR+Y
Sbjct  258   NFKTQVREIIRVQDIPVCFFFGDEVLNATQDIFQKYPFSIIASNEYIFNEKGEKVKGRQY  317

Query  914   KWGIAEVENPKHSDFCQLRDILMSKNMVDLVVSSEKYYETCRSHMLMTRINQAKDGLAAE  973
             KWG  ++EN K+ DF  L+  +   N++DLV S+E YYE CRS ML TR+ +A+D L  +
Sbjct  318   KWGAVDIENEKYCDFKILQKTIFDWNLIDLVESTEDYYEKCRSEMLRTRLLKARDCLTTK  377

Query  974   ----TSEDNLIL-KNMNYEDPDANGMLNYKCYQIYNKQYMHELIIEWSPEFIHKQWEAKK  1028
                 T E    L + MN+++ + N + NYKCY+I NK  M ++  EW PEFI +Q EAKK
Sbjct  378   SVDITEEQRKFLEEEMNFDEIEENKLKNYKCYEIINKTVMDKVATEWDPEFITRQLEAKK  437

Query  1029  RLNEIAHSEETKF-TWKRALMFKHTNLDSEIEDLHNRVKNLQIDCEELESILL  1080
             + NE+++ E +KF  WK++L  +  N + EIE L+++++NLQ++C++LE  LL
Sbjct  438   KFNELSNREISKFRDWKKSLFMEQENFNQEIEQLNHKLENLQLECQDLEYKLL  490
```

Fig. 9 – Sequence identity of *Ag SPR*3p with *S. cerevisiae SPR*3p using UniProtKB: Identical matches are shown in the figure.

```
 Score = 360 bits (923),  Expect = 2e-109
 Identities = 181/410 (44%), Positives = 266/410 (65%), Gaps = 22/410 (5%)

Query  130  IGIDSIPLQKETFIEKNGVQFTMMVVGQSGLGKTTFINTLFGTSLLPTVWESDMTERGVT  189
            IGI ++P Q+E    KNG+ FT+MV GQSGLGKTTFIN+LF TSL+         +  +
Sbjct  90   IGIKNLPRQRELLNAKNGIDFTLMVAGQSGLGKTTFINSLFSTSLI---------DDDIK  140

Query  190  KTTKIVRHESELVENGFTLRYTVIDTPGFGDLANNNFSWSPIVNYIDEQYRSYIFQEEQP  249
            +   I+R++S +  +G  L + VIDTPGFG+  +N F+W  +VNYIDE+ RSYIFQEEQP
Sbjct  141  ENKPIIRYKSIVEGDGTHLNFNVIDTPGFGNNMDNAFTWRTMVNYIDEEIRSYIFQEEQP  200

Query  250  LRASLKDNRIHCCLYFINLTRNGLSALDIAAMEEISKRVNLIPVIAKIDGLTSADLEMYK  309
             R  + DNR+HCCLYF+  +  G+  LD+  M++++KRVNLIPVIAK D LT  +L+ +K
Sbjct  201  DRTKMVDNRVHCCLYFLRPSNKGIDTLDVVTMKKLAKRVNLIPVIAKSDLLTKEELKNFK  260

Query  310  RKIRETLQKQEIKVCSFLDQNHPNC-QTIFDTYPFGIVCSDEMVTNNEGKLVRGRKYKWG  368
             ++RE ++ Q+I VC F      N  Q IF  YPF I+ S+E + N +G+ V+GR+YKWG
Sbjct  261  TQVREIIRVQDIPVCFFFGDEVLNATQDIFQKYPFSIIASNEYIFNEKGEKVKGRQYKWG  320

Query  369  NVEVENPLHSEFTALRTVLMSKNLVDFAVGCENYYEKCRSHILLSRIQQAKTNC--PDHL  426
             V++EN  + +F  L+  +   NL+D     E+YYEKCRS +L +R+ +A+ +C     +
Sbjct  321  AVDIENEKYCDFKILQKTIFDWNLIDLVESTEDYYEKCRSEMLRTRLLKAR-DCLTTKSV  379

Query  427  DLT---------NLDLDNPDQNGLENYKFYEAFNKKFMDDLIIEWSPEFIHKQWEAKKRL  477
            D+T          ++ D  ++N L+NYK YE  NK  MD +  EW PEFI +Q EAKK+
Sbjct  380  DITEEQRKFLEEEMNFDEIEENKLKNYKCYEIINKTVMDKVATEWDPEFITRQLEAKKKF  439

Query  478  SEIVSMEEKRFKDWKQDLLNKQNLFNHEIEDLHTIVQQIRSECNELETRV  527
            +E+ + E  +F+DWK+ L  +Q  FN EIE L+  ++ ++ EC +LE ++
Sbjct  440  NELSNREISKFRDWKKSLFMEQENFNQEIEQLNHKLENLQLECQDLEYKL  489
```

Fig. 9 – Continued.

```
>TR:W0TC07 W0TC07_KLUMD Cell division control protein 3 OS=Kluyveromyces
marxianus (strain DMKU3-1042 / BCC 29191 / NBRC 104275) OX=1003335
GN=SPR3 PE=3 SV=1
Length=550

 Score = 813 bits (2099),  Expect = 0.0
 Identities = 399/550 (73%), Positives = 464/550 (84%), Gaps = 2/550 (0%)

Query  1    MNSTKNGWGSSMDHYIEATGVEYDSISYGPAKYGSNADNLEVINEQPEEECTEVKPQSQE  60
            M S  NGWGS MD  +E++ +   S S    KY +  +++E+I E PEE+  E+   S
Sbjct  1    MTSMNNGWGSYMDRSVESSDIGSSSGSNAQEKYLAPNESMEIIQEHPEEDNLEIASISHG  60

Query  61   KKEVLDSVRRLFEADSTESNSAL-KSLDVDINVQGDFYTLNSVMNSSTANTTQ-ENCSKF  118
            +  VL SV++LF ADS ES+ +L K+ D + N Q D+YT NS +NS   ++   E   +F
Sbjct  61   EDGVLGSVKKLFAADSIESSKSLTKNSDNEQNAQADYYTPNSAINSEVFDSNGGEKNRRF  120

Query  119  ITPSVIDEHYHIGIDSIPLQKETFIEKNGVQFTMMVVGQSGLGKTTFINTLFGTSLLPTV  178
            +   VID +Y+IGID+IP+QK+TFI KNG +FTMMVVGQSGLGKTTFINTLFGTSLLPTV
Sbjct  121  VNAPVIDPNYYIGIDTIPIQKQTFIAKNGGRFTMMVVGQSGLGKTTFINTLFGTSLLPTV  180

Query  179  WESDMTERGVTKTTKIVRHESELVENGFTLRYTVIDTPGFGDLANNNFSWSPIVNYIDEQ  238
            WE D+++R VTKTTKIVRHESELVE  F L++TVIDTPGFGD ANN+FSWSPIVNYIDEQ
Sbjct  181  WEGDLSDREVTKTTKIVRHESELVEGDFALKFTVIDTPGFGDHANNSFSWSPIVNYIDEQ  240

Query  239  YRSYIFQEEQPLRASLKDNRIHCCLYFINLTRNGLSALDIAAMEEISKRVNLIPVIAKID  298
            YRSYIFQEEQPLR SLKDNRIHCCLYFI LTR+GLSALDIAAMEEISKRVNLIP+IAK+D
Sbjct  241  YRSYIFQEEQPLRGSLKDNRIHCCLYFIKLTRHGLSALDIAAMEEISKRVNLIPIIAKVD  300

Query  299  GLTSADLEMYKRKIRETLQKQEIKVCSFLDQNHPNCQTIFDTYPFGIVCSDEMVTNNEGK  358
            GLT  D+ +YK+ IRET+QKQ+IKVC+FLDQN PNCQTIFD YPFGIVCSDEMV N EGK
Sbjct  301  GLTPDDVSIYKKNIRETIQKQQIKVCAFLDQNDPNCQTIFDMYPFGIVCSDEMVPNEEGK  360

Query  359  LVRGRKYKWGNVEVENPLHSEFTALRTVLMSKNLVDFAVGCENYYEKCRSHILLSRIQQA  418
            LVRGRKYKWGNVEVENP HSEFTALRTVLMSKNLVD  VGCENYYE+CR+H+LLSRI QA
Sbjct  361  LVRGRKYKWGNVEVENPEHSEFTALRTVLMSKNLVDLVVGCENYYERCRTHMLLSRINQA  420
```

Fig.10 – Sequence identity match of *Ag SPR*3p with cell division control protein 3 of *Kluyveromyces marxianus* using UniProtKB.

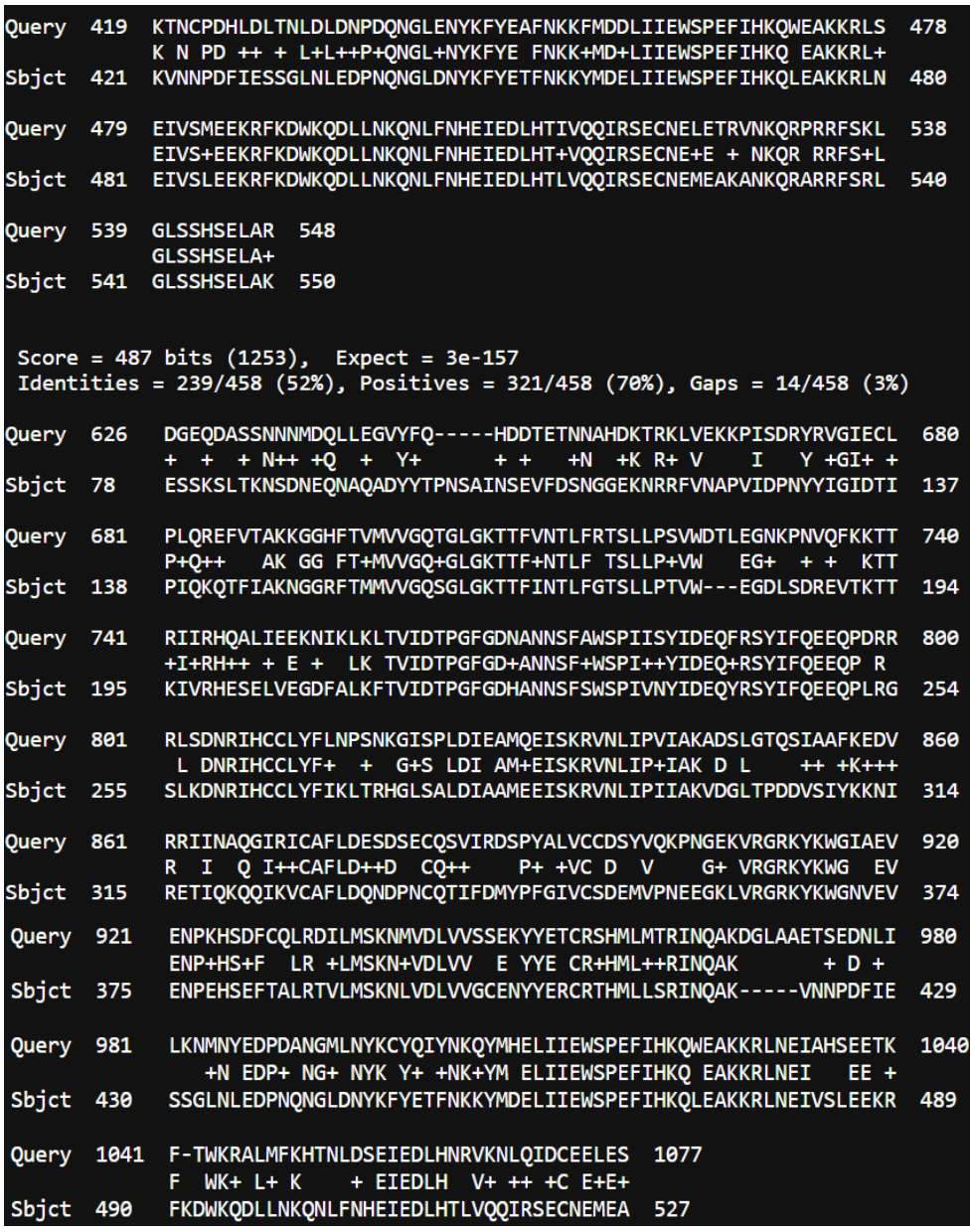


Fig. 10 – Continued.

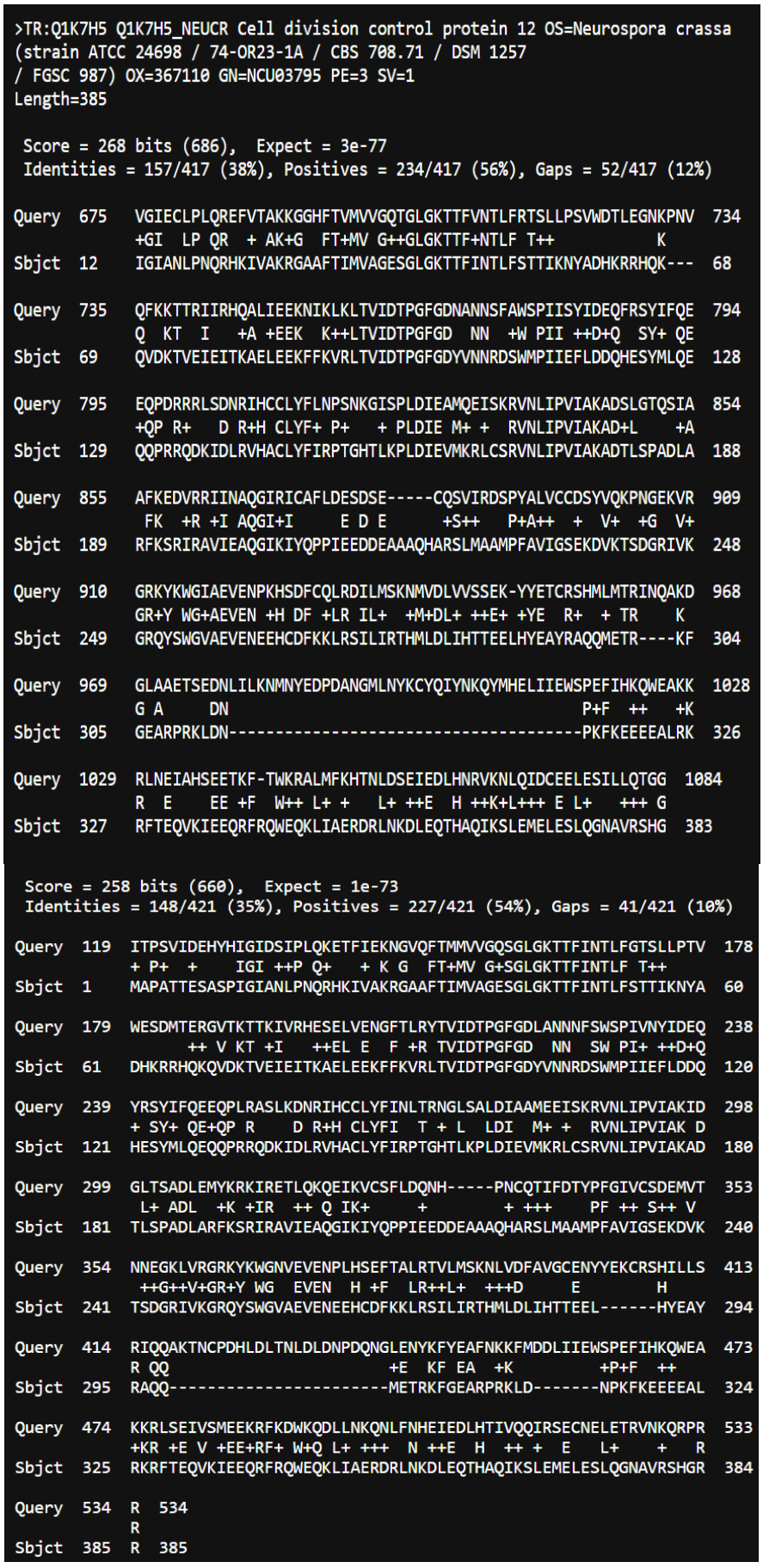


Fig. 11 – Sequence identity of *Ag SPR*3p with *N. crassa CDC*12p using UniProtKB**:** Identical matches are shown in the figure**.**

Characterization of plasmid BB

The plasmid BB is also a derivative of the original integrative plasmid pSV 2. In this plasmid, the *RIB* 3 promoter was replaced by the *A. gossypii RAD* 14 gene/promoter homologue, the Lac Z gene replaced the GFP gene, and the whole fragment was cloned into the *Bgl* II restriction site of plasmid pSV 2.

The plasmid BB was sequenced, and the sequencing data was subjected to bioinformatics searches using the KEGG database, Unit Prot, and SGD for homology. The nucleotide sequence of both the transcribed and the flanking regions of *RAD* 14 revealed an 849-bp open reading frame. The gene sequence showed a high degree of homology (42%) with genes of the *HOG* I pathway of *S. cerevisiae*, which is involved in the stress response. Specifically, it plays a role in combating osmotic stress in *S. cerevisiae* (Nadal & Posas 2022). This pathway may be implicated in combating the hyperosmotic conditions resulting from the accumulation of intracellular riboflavin in *E. ashbyii*, and this could lead to further scrutiny.

Gene sequence homologous to gene for stress response protein RAD 14 like protein involved in hypoxia stress response

CGGGGGGATGCTGGGTTCATTTCTTTGGTGATTATACGATTGTTCCCCGGGGGGTGGTTCGGATGGTTATA
ATTCGCCTGGTGAAT
ATTGTTCGCTTTTATTCCAGCTGAATCGTTAACCACGATGCACCAAATGGAGCAACCCCTGCAAGTGCGG
AAAAAAGAAATATAG
GCCGGAGACTGACCGGATGACAAGTTTGTTCGAGGATACTGAGGCAGTGTTGCTGGGAATTGACTATCGG
ATGGGGGTATCGAGA
TTGAGACTCTTTCCAAGCTTATCATCGGGTGAATTAGTTACGGAGTTTGACTATCCAGGGAGGGTTTATTG
ACCGACGAGAAACCA
ACTGCTGTTCCCCTTAAACGTAGGACGAAGAAATGAGCTCTGAATAGCCTCTCGAGAATATTCGTTATTT
GCATTGACATAGTTGG
CCTACTTCCATATGCGTTGTAACCTACAAAGAACTATAATCAACATAGTATTGGCCGTACCCCTGGGCGG
GCCGTTGCAATTTTTG
GGTGGGCTACCTAGCGCAGGGCTCAAGACACCTTATGGGAGAGGGAAGGCTATCCGACTAATTCCAGTA
CTTATTATCATTGGGG
GAAGAAGCTTTCTTCCGACCACTAGCCCCCAGAAACCGAATCGCCCCACAACTGACCGCGGGCAACCTA
GTAAATCCAACTATTG
CAACAGGGGTATCTGTCCTATTCCCTCTTTACCCAGGTAGCCATCTGAACTGCAACAGACTGTTCCAAGTA
AACACTACGAGAGCG
CCAGTAGCAAGGTGCGAGCTATAGATAGGCCTGTATATAAGTCTTTATCTCCAGAAATTGTTAGTGCCAC
CATGCAAGTGCGCTCA ATATACACCACTCAGAATTACAG

Discussion

Characterisation of plasmid D

This study has shown that the plasmid D, which harbours the *SPR* 3 gene homologue, is probably a regulatory gene since it exhibits a high degree of homology to the zinc finger binding protein and the regulatory *SPR* 3 gene of *S. cerevisiae* and SPR type G gene of *S. ludwigii* and *E. cymbalariae* as well as homology to *the K. marxianus* CDC 3p and *N. crassa* CDC 12 protein. Both of these are regulatory proteins with zinc clusters and zinc finger domains in their structures. Many regulatory proteins in yeast and filamentous fungi employ zinc finger motifs and zinc clusters for mediating transcriptional regulation. Zinc clusters are especially implicated in the regulation of amino acid and vitamin biosynthesis (MacPherson et al. 2006, Schillig & Morschhäuser 2013, Garcia-Estrada et al. 2018, Li & Liu 2020). Since the database search has revealed a significant degree of homology between the *AgSPR* 3 homologue and zinc finger proteins, it is quite possible that the *Ag SPR* 3 homologue encodes a transcription factor which probably regulates cell division and septation by employing the zinc finger motif/zinc cluster to mediate transcriptional regulation of these processes. This aspect of the *AgSPR 3* gene can be probed further.

Characterisation of plasmid BB

This study has shown that the plasmid BB, which harbours the *RAD* 14 gene homologue, is probably involved in the pathway activated in response to osmotic stress. The homology searches in the databases revealed a significant degree of homology to the genes of the *HOG* I pathway of *S. cerevisiae*, which plays a role in combating osmotic stress (Warringer et al. 2010, Nadal & Posas 2022). Since both *A. gossypii* and *E. ashbyii* are overproducers of riboflavin, an intracellular accumulation of the metabolite could lead to a hyperosmotic condition within the hyphae, leading to osmotic stress, which could, in turn, trigger the stress response pathway. Hence, the *RAD* 14 gene homologue could be probed further for its role in combating osmotic stress due to intracellular accumulation of riboflavin, in addition to its possible role in combating radiation stress. The possibility of crosstalk between the osmotic stress response and radiation stress response pathways could be explored as a time course to check temporal gene regulation. Earlier reports implicate the *HOG* pathway's involvement in other stress conditions, such as temperature fluctuations, oxidative stress, and heat stress, where it plays the role of a modulator in fine-tuning the response. (Winkler et al. 2002, Bilsland et al. 2004, Panadero et al. 2006, Gutin et al. 2015). The *RAD* 14 gene in *E. ashbyii* and *A. gossypii* can be studied for such a modulating role.

Conclusions

Knowledge about the biochemistry and genetics of riboflavin overproduction by *E. ashbyii* is of interest both from a fundamental and an applied point of view. A host of cellular processes are involved in the synthesis and secretion of riboflavin. Of these, the process of intracellular compartmentalization and septation is very important. In the present study, a plasmid bearing the *AgSPR* 3 gene (which functions in septation) fused to the *Lac* Z reporter gene was characterized, and it showed homology to the *S. ludwigii* and *E. cymbalariae* septin protein, *S. cerevisiae SPR* 3 gene, and *K. marxianus CDC 3 and N. crassa CDC* 12 genes involved in cell cycle regulation, a possibility that can be explored further. Riboflavin is an essential vitamin, but it causes photo-induced damage to the DNA at higher concentrations. One of the mechanisms of repairing the damaged DNA is by *RAD* proteins. Hence, in this study, the plasmid bearing the *Ag RAD* 14 gene fused to the *Lac* Z reporter gene was characterized, and it showed homology to the *S. cerevisiae HOG* I gene, which could prove to be the focus of future research. Finally, molecular tools for *E. ashbyii* have been characterized in this study and this would aid in carrying out future studies on the biochemistry and genetics of flavinogenesis by *E. ashbyii*. In future, these vectors will be used to transform *E. ashbyii* and pull out the homologous genes to study their respective roles in *E. ashbyii.*

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