ORIGINAL ARTICLE

CSU52, a novel regulator functions as a repressor of L-sorbose utilization in *Candida albicans*

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ABSTRACT

Background and Objectives: Monosomy of chromosome 5 associated with utilization of non-canonical sugar L-sorbose is one of the well-studied aneuploidies in *Candida albicans*. Stress-induced ploidy changes are crucial determinants for pathogenicity and genetic diversity in *C. albicans*. The five scattered regulatory regions (A, B, C, 135, and 139) comprising of two functionally redundant pathways (SUR1 and SUR2) were found to be responsible for the growth on L-sorbose. So far, three genes such as *CSU51, CSU53* and *CSU57* have been identified in region A, region 135 and region C, respectively. In this study we have verified the role of region B in this regulatory pathway.

Materials and Methods: We employed a combinatorial gene deletion approach to verify the role of region B followed by co-over expression studies and qRT-PCR to identify the regulatory role of this region.

Results: We confirmed the role of region B in the regulation of *SOU1* gene expression. The qRT-PCR results showed that regulation occurs at transcriptional level along with other two regions in SUR1 pathway. A previously uncharacterized open reading frame in region B has been implicated in this regulation and designated as *CSU52*. Integrating multiple copies of *CSU52* in the genome at tandem, suppresses the growth of recipient strain on L-sorbose, establishing it as a repressor of *SOU1* gene.

Conclusion: This finding completes the identification of regulators in SUR1 pathway. This result paves the way to study the underlying molecular mechanisms of *SOU1* gene regulation that in-turn helps to understand stress induced aneuploidy.

Keywords: *Candida albicans*; L-sorbose; Aneuploidy; Fungal gene expression regulation; Stress; Gene dosage

INTRODUCTION

Candida albicans is a common human fungal commensal that resides in mucosal membranes of the gut and genital-urinary tract of humans (1). It can cause superficial infections and invasive candidiasis, which can be life-threatening in immune-compromised patients, leading to mortality (2). *C. albicans* contains eight pairs of homologous chromosomes (3). Parasexual cycle is thought to be a critical means for generating genetic and phenotypic diversity in absence of meiosis (4). It can undergo mating with the cells of opposite sex forming tetraploid intermediates. These tetraploid cells attain a stable euploid state (disomic) and/or aneuploid cells by concerted

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chromosome loss depending upon the environmental cues (5). Factors contributing to this process include exposure to antifungals, growth on alternative carbon sources, high temperature, and host-pathogen interactions result in aneuploidy (5-7).

C. albicans, naturally disomic, produces spontaneous mutants, thus acquiring the ability to assimilate alternate carbon sources like L-sorbose and D-arabinose. These acquired assimilatory phenotypes have been linked to specific chromosomal alterations. Monosomy of chromosomes 5 (Chr5) and 6 (Chr6) are the chromosomal changes found to be associated with growth on L-sorbose and D-arabinose, respectively (5, 8). The *SOU1* gene present on chromosome 4 (Chr4) encodes a NADPH- dependent sorbose reductase. This enzyme catalyzes the first step in the L-sorbose metabolic pathway by converting L-sorbose to D-sorbitol and subsequently routed to the glycolytic pathway (9). The copy number of chromosome 5 regulates the expression of the *SOU1* gene and the utilization of L-sorbose. *Candida* strain monosomic to Chr5 can grow on L-sorbose (Sou$^+$) otherwise it remains Sou$^-$ (8). Also, overexpression of *SOU1* in a Chr5 disomic strain using a low copy number plasmid makes the strain Sou$^+$ (8, 9). These observations suggest that the Chr5 and *SOU1* gene ratio is the key to growth on L-sorbose (8, 9). Therefore, the loss and gain of Chr5 homolog make the strain Sou$^+$ or Sou$^-$, suggesting that Chr5 harbors repressors of L-sorbose utilization. Systematic truncations established that these negative regulators are located in a region of approximately 341 kb on the right arm of Chr5 (8). Further analysis identified five dispersed regions, such as A, B, C, 135, and 139 which might harbor repressors for L-Sorbose utilization. These five regions were classified into two independent functionally redundant pathways- one involving regions A, B, and 135 and the other involving regions A, C, and 139 where region A is part of both the pathways. The genes *CSU51* and *CSU53* have already been identified from regions A and 135, respectively (8, 10). However, region B has not been investigated and the possible repressor present in this region was not identified. In this study, we have adopted a combinatorial gene deletion approach to implicate region B in the regulation of L-sorbose utilization and to identify the gene present in this region. Using this strategy, we have identified a previously uncharacterized ORF (open reading frame) orf19.1105.3 in region B for L-sorbose utilization and

designated it as *CSU52*. The coding region of *CSU52* is 258 bp long (855689-855432, coordinates on Chr5) (www.candidagenome.org). This gene does not have any homologs in any other sequenced *Candida* species, suggesting its unique function in L-sorbose regulation. This finding completes the identification and implication of all the three genes present in three different regions of the SUR1 pathway (Sorbose Utilization Regulatory) of L-sorbose utilization in *C. albicans.*

MATERIALS AND METHODS

Strains, media, and growth conditions. *Candida albicans* strain CAF4-2 (Δ*ura3::imm434/*Δ*ura3::imm434*) was used for all genetic modifications (11). Integration of additional copies of the *CSU52* gene was done in the strain C89 (ura$^-$leu$^-$), a derivative of CAF4-2 (12). The *Candida* strain C571 (*CSU51*Δ *CSU57*Δ*/CSU51CSU57* trn.2) has been used as a positive control for L-sorbose assay (12). The *Candida* strain C587 was generated by tandem integration (7-13 copies) of *CSU52* using a modified pRC2312 plasmid, where ura$^+$ integrated colonies were selected (12). *Escherichia coli* XL1-Blue was used for regular cloning and plasmid isolation (13). Plasmids pUC19 (14) and pSFU1 (15) were used as vectors to prepare deletion cassettes. Plasmid pRC2312, a low copy number vector, was used for co-overexpression studies (12). Plasmid pKA712, a modified version of pRC2312 in which the *LEU2* gene was inactivated (12), was used for integrating multiple copies of *CSU52*.

YPD (1% yeast extract, 2% peptone, and 2% dextrose) and SD (synthetic dextrose) media were used to grow the *Candida* strains and were prepared as described (16). SD media with D-sorbitol was used for *Candida* transformations. SD media with 2% L-Sorbose (Calbiochem, USA) was used for phenotypic analyses. Uridine (50 μg/mL) was added whenever necessary, and mycophenolic acid (MPA) (8 μg/mL) was added for the selection of transformants carrying *IMH3* marker. Mycophenolic acid (Sigma, USA) stock solution (10 mg/ml) was prepared in dimethyl sulfoxide (DMSO). YT media (0.5% NaCl, 0.5% yeast extract and 1% tryptone) was used for growing *E. coli* strains. Ampicillin (100 μg/mL) was added to YT media for growing *E. coli* strains containing plasmids. 2% Agar was added while preparing solid

media. A stock solution of zymolyase (Sigma, USA) of concentration 1 mg/ml was made in 20% glycerol and stored at -20°C. All the *C. albicans* and bacterial strains were stored in 15% glycerol and maintained at -80°C. *C. albicans* and *E. coli* strains were grown at 30°C and 37°C, respectively.

Molecular biology methods. All basic molecular biology techniques such as PCR amplification, cloning, restriction digestion, and gel elutions were done as described (13). Plasmid isolation was carried out using the alkaline lysis method (13). The calcium chloride method was used for *E. coli* transformation (13). *C. albicans* transformation was done by the spheroplast method as described (17).

Plasmid cassettes for internal deletions and overexpression. Plasmid vectors pUC19 and pSFU1 were used for making deletion cassettes (14, 15). Briefly, 500-1000bp upstream and downstream flanking sequences of the region to be deleted were amplified by PCR from CAF4-2 genomic DNA. The amplified sequences were cloned into pTZ57R/T vector using InsTA PCR cloning kit following the manufacturer's instructions (Thermo Fisher Scientific, Vilnius, Lithuania). Subsequently, PCR products were cloned into the pUC19-based vector at *Bam*HI-*Sac*I and *Pst*I-*Hind*III sites, whereas *Kpn*I-*Xho*I and *Not*I-*Sac*I sites were used for pSFU1. *Candida* strains were transformed with the resulting deletion constructs after digesting with appropriate restriction enzymes.

For co-overexpression studies, *CSU52* and *SOU1* genes were cloned together in the replicative plasmid pRC2312. *CSU52* was amplified as a 649bp PCR product using oligonucleotides KC448/KC449 and cloned into TA cloning pTZ57R/T vector. The *CSU52* gene was released from the TA vector as a *Pst*I fragment and cloned into the plasmid pKA444 (*SOU1* gene in pRC2312) (12) generating a co-overexpression vector pKA648. As a positive control for suppression assay, pKA534 (pRC2312 with *CSU51* and *SOU1*) was used (12). The plasmid pKA712 (12) was used for multiple copy integration of *CSU52* gene. The *CSU52* gene was cloned at the *Pst*I site of pKA712 generating plasmid pKA833, for tandem integration of multiple copies of *CSU52*. Plasmids used in this study are listed in (Table 1).

PCR-based verification of gene deletions. Specific PCR primers were used to confirm accurate gene deletions. Primers were designed from the marker gene and outside the flanking sequences on both 5' and 3' ends of the chromosomal DNA. PCR amplifications were performed for both the 5' and 3' ends to confirm the marker-genomic DNA junctions. The location and orientation of primers used for verification of gene deletions are illustrated (Fig. 1A and 1B). PCR products were run on 0.8% agarose gel along with molecular size markers, stained with ethidium bromide, and visualized using gel documentation system (Bio-Rad Laboratories, USA). Primers used in this study are listed in (Tables 2-4).

L-sorbose phenotypic assay. Spot dilution assay was performed on L-sorbose plates to determine the phenotypes of mutants generated by gene deletions as described (8). Strains were streaked from glycerol stocks onto YPD plates with uridine and were allowed to grow 16-24 h at 30°C. Fresh cells were collected from the plate and washed thrice with distilled water to remove traces of glucose. Cells were then counted using a hemocytometer, and approximately 10^6 cells were taken, serially diluted, and spotted on L-sorbose plate where glucose plate served as a control. Images of glucose plates were taken at 24-36 h of spotting, whereas, L-sorbose plates were monitored and imaged for four days to observe the confluent growth of Sou+ colonies. Uridine was added to both glucose and L-sorbose plates except for maintaining replicative plasmids.

Gene expression studies. Real-time quantitative PCR was used to analyze the changes in *SOU1* gene expression. Total RNA was isolated from the mutant and wild type strains using the acid phenol method (18), and RNA purity was assessed by NanoDrop spectrophotometer (Eppendorf, Germany). RNA was treated with DNaseI (Thermo Fischer Scientific, Vilnius, Lithuania) to remove genomic DNA contamination, followed by PCR amplification to verify complete DNA removal. cDNA was synthesized from total RNA using Revert Aid first-strand cDNA synthesis kit (Thermo Fisher Scientific). cDNA synthesized was either stored at -80°C or proceeded for qRT-PCR.

Real-time quantitative PCR was performed using SYBR Green/Rox qPCR Master-mix (Thermo Fischer Scientific, Vilnius, Lithuania) in a Bio-Rad CFX 96 system. Briefly, PCR reaction mixture consists of 10 µl SYBR green master mix, 2 µl each of forward

Table 1. Plasmids used in this study

Sl.No	Plasmid Name	Description	References
1.	pUC19	Vector	(14)
2.	pSFU1	Vector	(15)
3.	pRC2312	Vector	(12)
4.	pKA444	*SOU1* gene cloned in pRC2312 for overexpression	(12)
5.	pKA534	*CSU51* and *SOU1* genes cloned in pRC2312	(12)
6.	pKA712	KpnI inactivated in pRC2312	(12)
7.	pKA34	*CSU51* deletion cassette using *URA3 flipper*	This Study
8.	pKA70	*Region 135 deletion cassette using IMH3 marker*	This Study
9.	pKA140	Region 135 deletion cassette using *URA3* marker	This Study
10.	pKA822	*CSU51* deletion cassette using *URA3* flipper	This Study
11.	pKA139	Deletion cassette for Region A+B using *URA3* marker	This Study
12.	pKA648	*CSU52* and *SOU1* genes cloned in pRC2312	This Study
13.	pKA833	*CSU52* in pKA712, for integrating *CSU52*	This Study
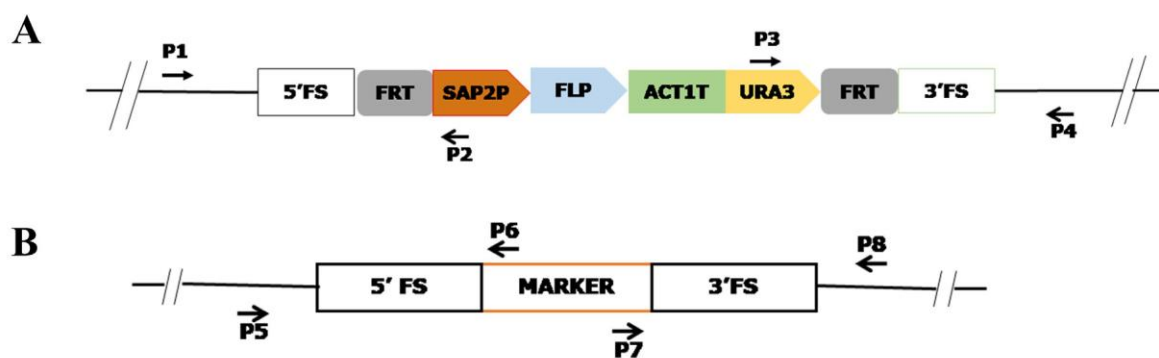
Fig. 1. Schematic representation of the location of primers on deletion cassettes. (A) Vector pSFU1- based deletion cassette: The cassette consists of FRT, FLP under *SAP2* promoter (SAP2P), *ACT1* terminator (ACT1T), and *URA3* as the selection marker. Primers P1 and P2 are designed for 5´end verification, where P1 is present on Chr5, upstream of 5´FS (flanking sequence), and P2 is the marker primer located within the *SAP2* promoter. Primers P3 and P4 are for 3´end verification, where P3 is the marker primer located in *URA3* and P4 is present downstream of 3´FS on the chromosome. (B) Vector pUC19-based deletion cassette: Primers P5 and P6 are for 5´ end verification. Here, P5 is the on chromosome upstream of 5´FS, and P6 is the marker primer. P7 and P8 are for 3´ end verification. P8 is present downstream of 3´FS and P7 is the marker primer. The primers designed on the chromosome vary with the region to be deleted.

and reverse primers (0.3mM final concentration) and 2 μl of cDNA (25 ng/μl), finally made up to 20 μl total volume with nuclease-free DEPC water. *ACT1* and *RDN18* (18S rRNA) genes were used as internal controls. An in-built 2 step amplification protocol (CFX_ 2 step amplification protocol) was used with additional melt curve analysis to verify the presence of a single amplified product. Briefly, initial denaturation at 95ºC for 3 min, followed by 95ºC for 10 sec and 55ºC for 30 sec for 40 cycles. Following the amplification, melt curves were generated at 65ºC to 95ºC for 5 sec with an increment of 0.5ºC. Gene expression changes

were measured as fold change using the ΔΔCt method (19).

Minimum inhibitory concentration (MIC) assay. Minimum inhibitory concentration (MIC) for the mutants generated was performed following the Clinical and Laboratory Standards Institute (CLSI) protocols (20). Briefly, fresh cells were collected from YPD plates with uridine and approximately 5×10^6 cells were mixed with 0.7% molten agar and poured onto Mueller Hinton Agar plates. Ezy MIC strips coated with caspofungin (0.002-32 μg/ml) (Himedia, Mum-

Table 2. Primers used for making deletion cassettes

Primer Name	Sequence (5' to 3')	Gene or Region deletion
KC-01	TCG GAG CTC ACA ACA AAA GCC GAA CAC ACA *Sac*I	*CSU51*
KC-02	TCG GCG GCCGCA TGT GCA CAA CAA TAC ATT ATA AG *Not*I	
KC-03	TCG CTC GAG TGT TGA TTA TAT ATA TGT GTA ATT *Xho*I	
KC-04	TCG GGT ACC ATG AGC AGT TTA GTG GGA CGA A *Kpn*I	
KC-51	TCG GAG CTC CCC GAA ATC CAT TCT TTT GC *Sac*I	Region 135
KC-52	TCG GGA TCCCCAAATGAGAACAAACCGTG *Bam*HI	
KC-53	TCG CTG CAG CAGTAACGTCTTTGGCAAATG *Pst*I	
KC-54	TCG AAG CTTTCCCAATAGGGTGAAATGTTG *Hind*III	
KC-05	TCG CTC GAG TAG ATT CAA TAA TCA AGA TCA *Xho*I	Region B
KC-06	TCG GGT ACC GCA TGT ACG AAG ATG ATG GTG *Kpn*I	
KC-58	GTG GTG GAA CAC AAG AAT ACC AGT	
KC-03	TCG CTC GAG TGT TGA TTA TAT ATA TGT GTA ATT *Xho*I	
KC-78	TCG GGA TCC TAG ATT CAA TAA TCA AGA TCA *Bam*HI	*CSU51* & Region B together
KC-79	TCG GAG CTC GCA TGT ACG AAG ATG ATG GTG *Sac*I	
KC-80	TCG AAG CTT TGGAATTTGATATTGAAATTTGA *Hind*III	
KC-81	TCG CTG CAGCAAAAACTATTTAATATGATCGATCC *Pst*I	

Table 3. Primers used for overexpression of genes/ORF's

Primer Name	Sequence (5'to 3')	Gene/ORF
KC-105	TCG GGT ACCTCC AGT AAC AGG TAT TTC TGA TCT CT *Bam*HI	*SOU1*
KC-117	TCG GGA TCC TCT TCA AGA AAA AGA GGA TGA TG *Kpn*I	*CSU51*
KC-60	TCA CGG TAA TAATAA AAT TCT GTA ACA TC	*Orf19.1105.3*
KC-235	GGT ACCCAC AAC ATT CAA ATT AAA ATC CC *Kpn*I	*(CSU52)*
KC-448	CTG CAG CAC ACA CAC CCT TTA CCG TGA ATA *Pst*I	
KC-449	CTG CAG GAA GCC AAG CAA AGC ACA ATA *Pst*I	

Table 4. Primers used for verification of deletions (marker and chromosomal primers)

Primer Name	Sequence (5' to 3')	
KC-16	GTC TTG ATT AAG CAT ACA TAA GGA C	*URA3*
KC-17	TGA AGT TGT TAG CAC TGG AAC TG	
KC-44	ACA CAG AGA GAT ATT ACC AAA CTA	*IMH3*
KC-156	GAT TTT TAG ACG CAC GTG ACA TAC ACA CAG	
KC-102	TCC TCC TAT TCC TTC TCC TTA TGG C	*SAP2*
KC-14	TCG GGT ACC CCA TGT TTT GAT ACC ACT GAT GTA G	Region B
KC-412	AAT AAA GCA GCA CCA ACA GCA G	*CSU51*
KC-146	ATG AGT AGT TTG TTA ATT AAT CAT AGC	
KC-119	TCG AAG CTT CTA GTA CAA TAA AGC AGC ACC AAC	Region B
KC-114	TGG AGA AGC TGT ACG TGT ACC TAA	Region 135
KC-115	AAT TGT GGT GAT ATT CAT GTA AAC C	
KC-116	TGC TTT GGA CTT GAG TGA AGC CTA C	

bai, India), was placed in the middle of the plate and incubated at 30°C. The minimum inhibitory concentration was recorded after 24 hrs of incubation; also the plate photographs were taken using Quantity One Software (Bio-Rad Laboratories, USA). Minimum inhibitory concentrations were determined in µg/ml against the caspofungin.

RESULTS

Deletion and phenotypic analyses of genes/regions in the SUR1 pathway. The SUR1 pathway contains three regions A, B, and 135, responsible for negatively regulating the expression of *SOU1*, and subsequently, the utilization of L-sorbose as a sole carbon source. The genes present in regions A and 135 have been identified as *CSU51* and *CSU53*, respectively. However, the gene present in region B remains uncharacterized. Schematic representation of regions in the SUR1 pathway has been shown in (Fig. 2). To identify the gene/ORF in region B responsible for the regulation of L-sorbose utilization, we performed a series of systematic deletions of the genes/regions.

Single-copy deletion of the genes/regions and phenotypic analyses. To confirm the role of region B in the regulation of L-sorbose utilization, we have first deleted a single copy of the genes/regions individually and assessed the Sou phenotype. The *CSU51* gene was deleted as described (12). The region 135 was deleted by transforming *Candida* strain with the plasmid pKA140. The 5' and 3' junctions of deletion
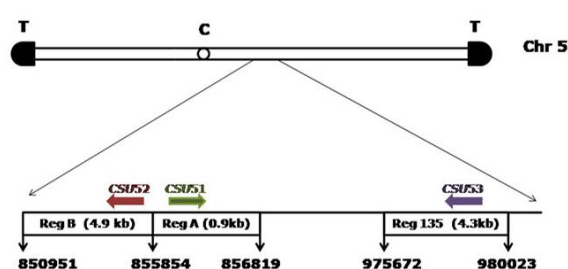
Fig. 2. Schematic representation of the regions in SUR1 pathway on chromosome 5. The genes, *CSU51, CSU52* and *CSU53* are shown along with the regions in which they are present. The centromere (C) and telomeres (T) are also shown on Chr5.

of region 135 were confirmed by PCR using oligonucleotides KC16/KC114 and KC17/KC116, respectively (Fig. 3A; lanes 8 and 10). Similarly, region B was deleted using the plasmid pKA822, and the transformants were verified using oligonucleotides KC102/KC14 and KC17/KC119, respectively (Fig. 3A; lanes 3 and 5). Therefore, single copies of all the three regions/genes were deleted one at a time in *Candida* strain, keeping the second copy intact. The strains harboring these individual deletions were spotted on L-sorbose plates, and the growth was monitored. It has been observed that the *Candida* strains carrying these individual deletions produced Sou- phenotype (Fig. 3B). This finding strongly suggests the possibility of combinatorial effects of the genes located in these regions for the regulation of L-sorbose utilization.

Combinational deletions of the regions and phenotypic verification. As the strains carrying dele-
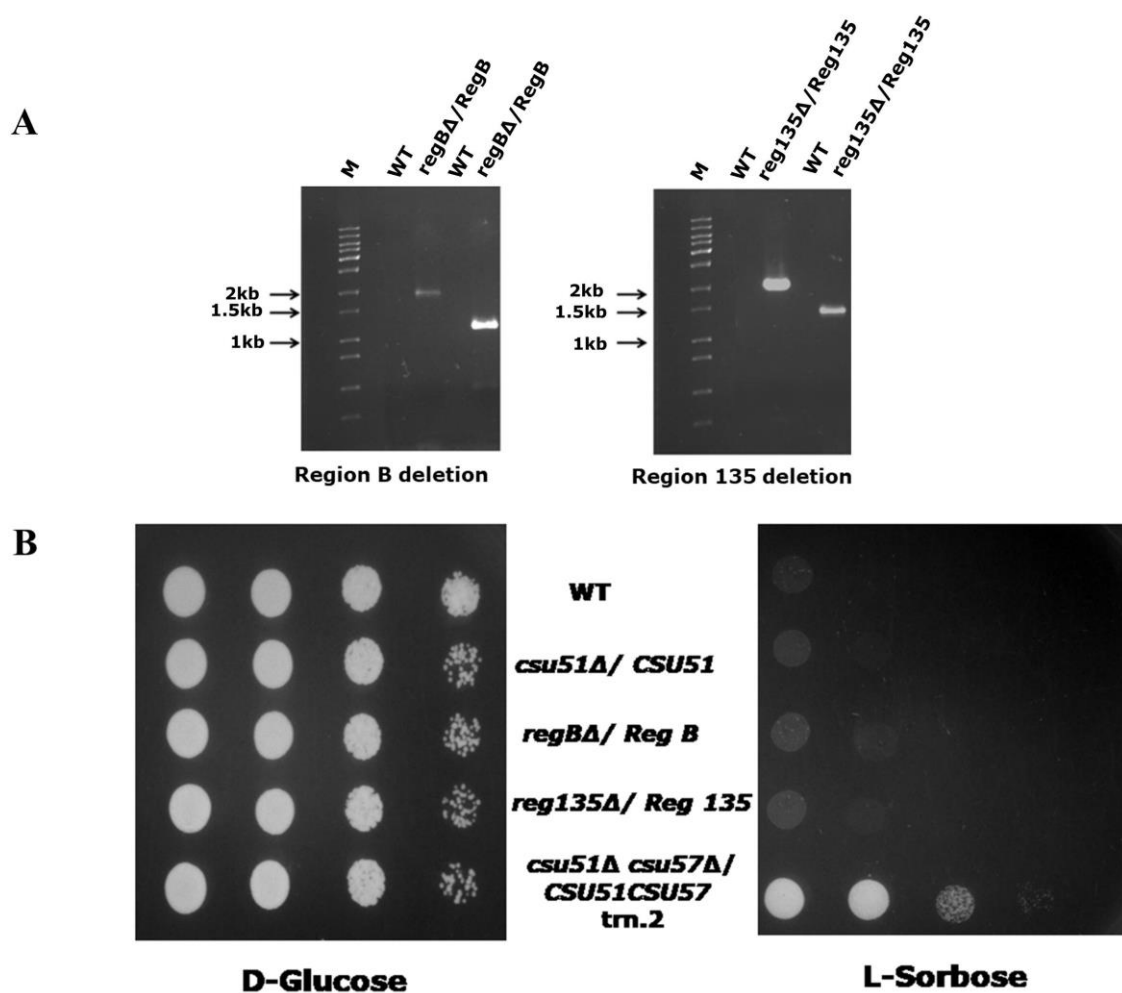
Fig. 3. (A) PCR verification of independent single-copy deletions of region B and region 135. Lanes 3 and 5 are 5' and 3' junctions' verification of region B deletion, using primers KC102/KC14 (5ʹ junction) and KC17/KC119 (3ʹ junction). Lane WT is CAF4-2 (negative control), Lane M, 1 kb ladder. Both the junctions gave expected amplification of 1.7 kb (5ʹ) and 1.2 kb (3ʹ), no amplification is seen in WT. Lane 8 and 10 are 5ʹ and 3' junctions' verification of region 135 deletion using primers KC16/KC114 (5ʹ junction) and KC17/KC115 (3ʹ junction). Lane WT is CAF4-2 (negative control), Lane M, 1 kb ladder. Both the junctions gave expected amplification of 1.65 kb (5ʹ) and 1.3 kb (3ʹ), no amplification is seen in WT. (B) L-Sorbose spot assay for independent deletions of three regions: CAF4-2, wild type; *CSU51Δ/CSU51*, single copy deletion of *CSU51;* regBΔ/RegB, single copy deletion of region B; reg135Δ/Reg135, single copy deletion of region 135; C-571(*csu51Δ* c*su57Δ/ CSU51CSU57* trn.2) strain used as Sou[+] control

tions of single copies failed to produce Sou[+] phenotype, it becomes evident that more than one region/gene is to be deleted for utilization of L-sorbose as a carbon source. Therefore, we have designed the experiments to delete two regions/genes simultaneously in the same strain to assess L-sorbose utilization. As there are three regions/genes, three possible combinations of double deletions such as *CSU51* plus region B, *CSU51* plus region 135, and region B plus region 135 have been carried out. For the deletion of

CSU51 and region B simultaneously, *Candida* strain was transformed with plasmid pKA139 after digesting with appropriate enzymes. The ura[+] transformants were screened by PCR. The oligonucleotides KC16/KC14 and KC17/KC146 were used for verification of 5ʹ and 3'junctions, respectively (see Fig. 4A; lanes 1 and 5). For generating double deletions of (*CSU51* +region135) and regions (B +135), we initially deleted region 135 using a deletion cassette with *IMH3* as the selection marker. The transformants

were selected on SD plates containing mycophenolic acid (MPA) and they were re-streaked on MPA plates for confirmation. The MPA^R (MPA-resistant) transformants were screened by PCR by verifying both 5' and 3' junctions (Fig. 4B; lanes 1 and 5). A single-copy deletant of region 135 has been designated as C85. Subsequently, both *CSU51* and region B were independently deleted in the *Candida* strain C85 using deletion cassettes pKA34 and pKA822, respectively, generating the stains carrying deletions (*CSU51*+region 135) and regions (B+135).

Finally, we generated a strain where all the three regions/genes, *CSU51*, B, and 135, were deleted together in the same strain. For this purpose, both *CSU51* and region B were knocked out simultaneously in *Candida* strain C85 using deletion cassette pKA139 as described above. The transformants, which were both ura$^+$ and MPA^R, were verified using PCR. The oligonucleotides KC16/KC14 and KC17/KC146 were used for verification of 5' and 3' junctions, respectively (Fig. 4A; lanes 1 and 5).
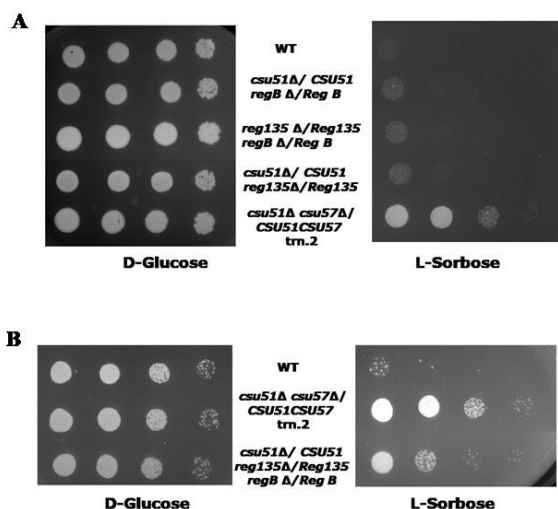
A minimum of three PCR verified positive colonies were taken and checked for Sou phenotype. One representative colony for each deletion was taken and spotted on the L-sorbose plate along with appropriate positive and negative controls. It has been found that even double knockouts did not produce Sou$^+$ phenotype (Fig. 5A). However, upon deleting all the three regions together, the strain acquired Sou$^+$ phenotype (Fig. 5B). Therefore, it clearly established that all three regions/genes act in a concerted manner in the *SUR1* pathway. This result also proves that region B plays a significant role in L-sorbose regulation.

SOU1 gene expression analysis. It was previously reported that *Candida* strain could be Sou$^+$ either by loss of one homolog of chromosome 5 (monosomy) or by incorporation of extra copies of the *SOU1* gene through replicative plasmid (8). Herein we have analyzed the expression of structural gene *SOU1* to comprehend whether the regulation works at the transcriptional level or not. Quantitative real-time PCR was done for the strains such as CAF4-2, double deletion of regions/genes (*CSU51* plus 135), triple
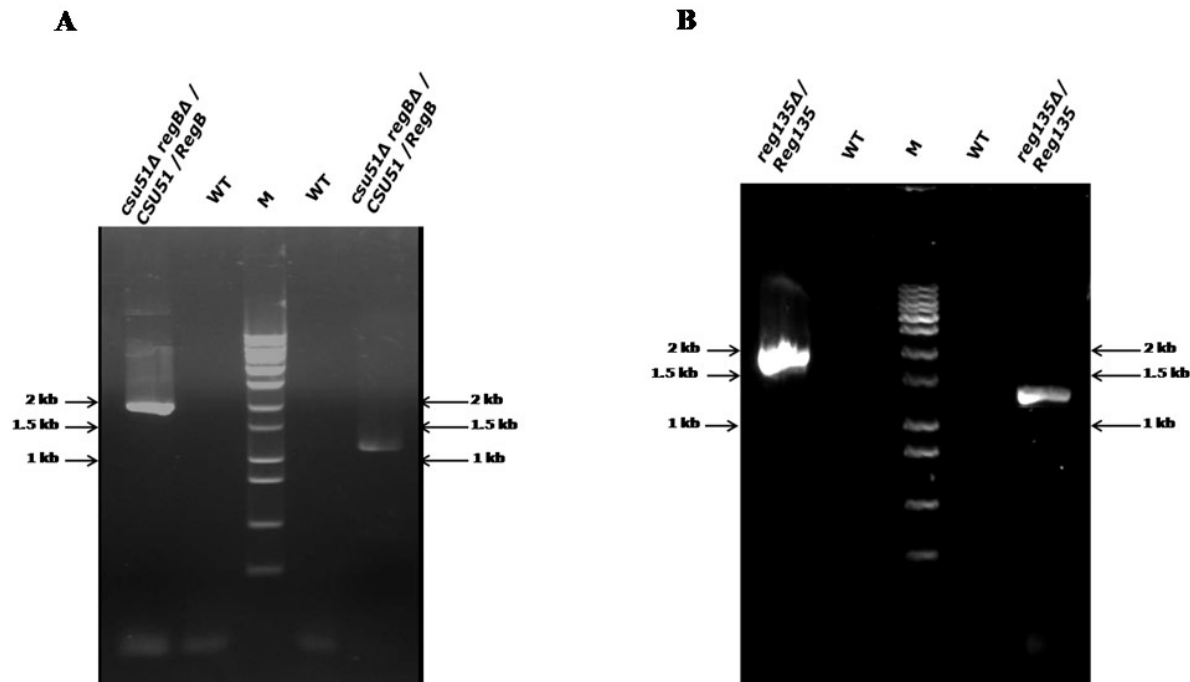
Fig. 4. (A) PCR verification of *CSU51*+ Reg B deletion. Lanes 1 and 5 are 5' and 3' junctions' verification using primers KC102/KC14 (5' junction) and KC17/KC146 (3' junction). Lane WT is CAF4-2 (negative control), Lane M, 1 kb ladder. Both the junctions gave expected amplification of 1.7 kb (5') and 1.3kb (3'); no amplification is seen in WT.(B). PCR verification of region 135 deletion using IMH3 marker. Lane 1 and 5, 5' and 3' junctions' verification using primers KC44/KC114 (5' junction) and KC156/KC115 (3' junction). Lane WT is CAF4-2 (negative control), Lane M, 1 kb ladder. Both the junctions gave expected amplification of 1.7 kb (5') and 1.35 kb (3'); no amplification is seen in WT.
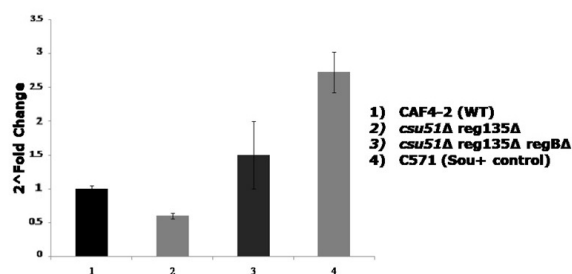
Fig. 6. *SOU1* gene expression analysis using qRT-PCR. WT (CAF4-2), CAF4-2 with both *CSU51* and Reg135 deletion (*csu51Δ reg135Δ /CSU51* Reg135), CAF4-2 with one copy of all three regions deleted (*csu51Δ reg135Δ regBΔ /CSU51* Reg135 RegB), C571 positive control (*csu51Δ csu57Δ/ CSU51CSU57* trn.2). Here CAF4-2 and CAF4-2 with both *CSU51* and Reg135 deletion showed basal *SOU1* gene expression, positive control C571 showed 2.7 times more than expression than CAF4-2, and the strain with all three regions deleted has shown 1.5 times more than CAF4-2.

Fig. 5. (A) L- sorbose spot assay for simultaneous knockout of two gene/regions strains: CAF4-2, wild type; *csu51Δreg-BΔ /CSU51* RegB, a single-copy of both *CSU51* and region B; *csu51Δreg135Δ /CSU51* Reg135, single copy deletion of both *CSU51* and region 135; *regBΔreg135Δ /RegB* Reg135, single copy deletions of both regions B and 135; C-571 (*csu51Δ csu57Δ/CSU51CSU57* trn.2) strain used as Sou+ control. (B) L-sorbose spot assay for deletion of all the three regions together: CAF4-2, wild type; C-571 (*csu51Δ csu57Δ/CSU51CSU57* trn.2) strain used as Sou+ control; *csu51Δ reg135Δ regBΔ /CSU51* Reg135 RegB, combinatorial deletion of all the three regions. All the strains were spotted on L-sorbose plates and glucose plates (control).

deletion of regions/genes (*CSU51,* B and 135), and strain C571 (positive control) to measure the expression of *SOU1* gene. This analysis revealed basal *SOU1* expression in CAF4-2 and double deletions of regions/genes (*CSU51* plus 135) where C571 (positive control) showed 2.7 times more expression of *SOU1* compared to CAF4-2. However, the strain with all the three regions deleted showed 1.5 times more expression than the negative control (Fig. 6). This result suggests the involvement of region B *(CSU52)* in regulating *SOU1* gene expression at the transcriptional level.

Identification of *CSU52* in region B. The size of region B is 4.8 kb spanning from 850951 bp to 855840 bp on the right of chromosome 5 and is located upstream of *CSU51* (region A) (11; www.candidagenome.org). The region B contains only one uncharacterized ORF (orf19.1105.3) of 258 bp in size and located ~900 bp upstream of *CSU51* (Fig. 2).

Co-overexpression analysis of *CSU52* with *SOU1.* It has been shown earlier that overexpressing the *SOU1* gene in wild-type *Candida* strain using a replicative plasmid pRC2312 produced confluent growth on the L-sorbose plate. However, the strain containing *CSU51* and *SOU1* together remained Sou- due to the repressing effect of *CSU51* (8). Herein, we have applied the same approach to verify the repressing ability of *CSU52*. For this purpose, *Candida* strain CAF4-2 was transformed with the plasmids, pKA648 (*CSU52+SOU1* in pRC2312), pKA534 (*CSU51+SOU1* in pRC2312), pKA444 (*SOU1* in pRC2312) and pRC2312 (empty vector). The ura+ transformants were spotted on L-sorbose taking glucose plates as control. CAF4-2 transformed with empty vector pRC2312 (negative control) failed to grow, while the strain with additional copies of *SOU1* exhibited confluent growth. The positive control (*CSU51+SOU1*) for repression did not grow on L-sorbose plate as reported earlier (8). However, transformants containing the plasmid pKA648 (*CSU52+SOU1*) could not suppress the growth on the L-sorbose plate. This indicates that *CSU52* may encode a weak repressor compared to *CSU51* (Fig. 7A). This observation is expected, as *CSU51* is essential for both the regulatory pathways required for negative regulation of L-sorbose utilization and acts as the master molecule. As the copy number of

repressors and *SOU1* is the key in this regulatory system, we suspected that the expression of *CSU52* is insufficient to repress *SOU1*. To test this, we integrated multiple copies of *CSU52* by exploiting the property of the plasmid pRC2312, which can generate 7-13 copies of tandem integration into *Candida* genome and produce much larger colonies compared to strain with autonomously replicating plasmids (12). For tandem integration of multiple copies of *CSU52*, *Candida* strain C89 was transformed with pKA833 (*CSU52* in pKA712), and ura$^+$ integrated colonies were selected, and newly generated strain has been designated as C587. The strain C587 was subsequently transformed with pKA444 (*SOU1* in pRC2312) and pRC2312 (vector control). Simultaneously, the strain C89 was transformed with pKA444 and pRC2312. The ura$^+$ transformants were spotted on L-sorbose plate, taking glucose plate as a control. As expected, the negative controls (vector only) were Sou- while C89 transformed with pKA444 (*SOU1* in pRC2312) grew well on the L-sorbose plate. However, the strain C587 transformed with pKA444 (*SOU1* in pRC2312) showed very little growth on L-sorbose plate. This elegant experiment clearly showed that *CSU52* can suppress the Sou$^+$ phenotype (Fig. 7B). This result strongly suggests that the *CSU52* gene encodes a potential repressor for the regulation of L-sorbose utilization. Further, it also establishes that the copy number of repressors is indeed the key to the regulation of the *SOU1* gene.

MIC values for the mutant strains. It was reported, that L sorbose resembles with echinocandin class of antifungals in repressing cell wall β 1-3 glucan synthesis (21). Here, we have verified the MIC values for caspofungin on the mutants generated. The minimum inhibitory concentrations for the mutant strains varied from 0.094 μg/ml - 0.25 μg/ml (Fig. 8). The observed MIC values suggested that the mutants were not resistant against caspofungin compared to wild type strain.

DISCUSSION

L-Sorbose metabolism in *C. albicans* was previously shown to be an ideal process to study chromosomal changes induced by stress conditions and the effects of gene copy number alterations. The growth of *Candida* strains is correlated with the loss of one
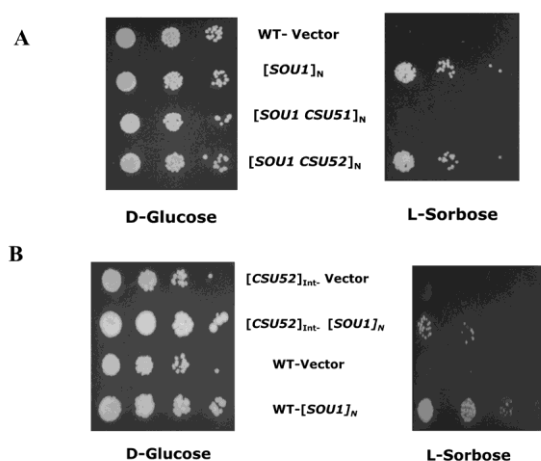
Fig. 7. (A) Co-overexpression and *SOU1* suppression assay: CAF4-2 transformed with pRC2312 (vector control), pRC2312 with *SOU1*, pRC2312 with *CSU51* and *SOU1*, pRC2312 with *CSU52* (orf19.1105.3) and *SOU1*. Transformants were spotted on ura- plate supplemented with 2% L-sorbose, and ura- plate with 2% glucose was taken as the control. *CSU51* and *SOU1* in pRC2312 were taken as the positive suppression control. (B) Multiple Tandem integration of *CSU52* and *SOU1* suppression assay. [*CSU52*]$_{Int}$ transformed with vector pRC2312, [*CSU52*]$_{Int}$ transformed with pKA444 (*SOU1* gene in pRC2312), C89 transformed with vector pRC2312, C89 transformed with pKA444 (*SOU1* gene in pRC2312).

homolog of chromosome 5, suggesting the presence of repressors regulating *SOU1* gene expression required for utilization of L-sorbose as a carbon source (8, 9). A systematic chromosomal truncations approach combined with large internal deletions identified five scattered regions containing putative repressors on the right-hand side of the centromere on Chr5 for the regulation of L-sorbose utilization. These five regions have been categorized into two independent functionally redundant pathways – *SUR1* and *SUR2* (Sorbose Utilization Regulatory pathways 1 and 2). Each pathway involves three regions, with region A being common to both the pathways. The SUR1 pathway consists of three regions (A, B, and 135), whereas the SUR2 pathway contains regions A, C and 139. The regions A and 135 harbor *CSU51* and *CSU53*, respectively which are shown to repress the *SOU1* gene expression and utilization of L-sorbose (8, 10). In this study, we focused on identifying the remaining repressor in region B to understand the different players in the SUR1 pathway.

Neither single-copy independent deletions of all
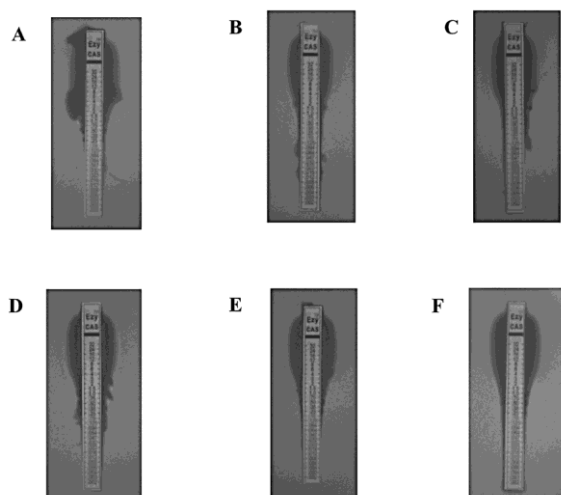
Fig. 8. Minimum Inhibitory Concentration (MIC) for the mutants on Caspofungin: (A) WT (CAF4-2). (B) *regBΔ/ RegB*, single copy deletion of region B (C) *csu51ΔregBΔ /CSU51 RegB*, a single-copy of both *CSU51* and region B. (D) *regBΔreg135Δ /RegB Reg135*, single copy deletions of both regions B and 135. (E) *csu51Δ reg135Δ regBΔ / CSU51 Reg135 RegB*, combinatorial deletion of all the three regions. (F) C-571 (*csu51Δ csu57Δ/CSU51CSU57* trn.2) Sou+ control.

three regions (A, B, and 135) nor combinatorial deletions of two regions together (A+B, A+135, B+135) resulted in Sou+ phenotype. Rather deletion of all the three regions/genes is required to produce Sou+ phenotype. These deletion studies revealed beyond doubt that region B has an essential role in L-sorbose utilization by *C. albicans*. The uncharacterized ORF, orf19.1105.3 located in region B, has been designated as *CSU52*. The *CSU52* gene is unable to suppress the Sou+ phenotype upon co-overexpression with *SOU1* in a replicative plasmid, unlike *CSU51* (8). However, tandem integration of multiple copies of the *CSU52* gene is able to suppress the growth on L-sorbose plate. We obtained similar results for *CSU57,* which is functioning in the SUR2 pathway (12). The qRT-PCR result showed that *CSU52* functions at the transcriptional level of *SOU1* gene expression similar to *CSU57* (12). Except for *CSU51* that appears to be the master molecule, each of the repressors is necessary but insufficient to suppress *SOU1* individually.

Moreover, the copy number of these repressors plays a crucial role in regulating the *SOU1* gene expression. Differential suppression of Sou phenotype by these repressors strongly suggests their function-

al hierarchy where *CSU51* is at the centre, and other repressors such as *CSU52* and *CSU57* might act as additional factors. However, to relieve the repression, at least three repressors must be reduced to half of the copy number of wild-type *Candida* strain. In summary, this study proves that L-sorbose metabolism is regulated by multiple pathways involving chromosomal changes and gene copy numbers. All the regulatory proteins are critical in performing the function and they act in a concerted manner with scope for hierarchy where *CSU51* acts as a master molecule. While *CSU51* was found to be involved in echinocandin drug tolerance (21), so far, none of the other repressors has been reported to perform any function apart from regulating L-sorbose utilization. The MIC values obtained for the generated strains against caspofungin are similar to that of wild type strain and did not show any resistant phenotype. Although, *CSU52* (Orf19.1105.3) expression has been found to be enhanced upon heat shock in *HSP90* depleted cells (22). The interaction between *HSP90* and *CSU52* is still unclear, as *HSP90* involved in plethora of cellular functions.

The underlying molecular mechanisms of how *CSU51* orchestrates *SOU1* suppression employing two independent pathways are potential areas for future studies. As L-sorbose does not occur naturally in the niches inhabited by *C. albicans*, these genes may represent a more general stress response mechanism, the elucidation of which requires further investigation.

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