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The available *SRL3* deletion strain of *Saccharomyces cerevisiae* contains a truncation of DNA damage tolerance protein Mms2: Implications for Srl3 and Mms2 functions

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Abstract

A screen of the commercially available collection of haploid deletion mutants of *Saccharomyces cerevisiae* for spontaneous mutator mutants newly identified a deletion of *SRL3*. This gene had been previously isolated as a suppressor of lethality of checkpoint kinase deletions if overexpressed. We found DNA damage sensitivity and extended checkpoint arrests to be associated with this strain. However, when crossed to wild-type, a mutant gene conferring these phenotypes was found to segregate from the *SRL3* deletion. The mutation was identified as a C-terminal truncation of Mms2, an E2 ubiquitin conjugating enzyme involved in error-free replicative bypass of lesions. This confirmed an earlier report that Mms2 may be required to restrain error-prone polymerase ζ activity and underscored that residues of the C-terminus are necessary for Mms2 function. Srl3, on the other hand, does not appear to influence DNA damage sensitivity or spontaneous mutability if deleted. However, the absence of these phenotypes does not contradict its likely role as a positive regulator of dNTP levels.

Keywords

Yeast; DNA Repair; DNA Damage Tolerance; Mutagenesis; dNTP Pools; Checkpoints

Introduction

A largely conserved network of proteins is in place to prevent lethal and mutagenic consequences of DNA damage and replication stress in eukaryotes (Friedberg et al., 2006). In this context, the regulation of dNTP levels has attracted considerable attention and many details surrounding the regulation of the heterotetrameric ribonucleotide reductase (RNR) are known in *Saccharomyces cerevisiae*. RNR activity and level is increased in S-phase and in response to DNA damage or replicational stress (e.g. by hydroxyurea [HU]) through

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various mechanisms: by transcriptional regulation (inactivation of the Crt1 co-repressor), by subunit localization, by inhibitor interaction and degradation, and by altered feedback inhibition (Chabes et al., 2003; Elledge et al., 1993; Huang et al., 1998; Lee and Elledge, 2006; Wu and Huang, 2008; Yao et al., 2003; Zhao et al., 2001).

In budding yeast, an elevated dNTP level can prevent the lethality of checkpoint kinase (Mec1 or Rad53) deletions. These checkpoint kinases are necessary for cell cycle arrest and facilitation of DNA repair (Friedberg et al., 2006; Nyberg et al., 2002). Elevated dNTP levels can be achieved by deletion of negative regulators such as Sml1, Ctr1 or Dif1 or by overexpression of RNR subunit Rnr1 (Desany et al., 1998; Lee et al., 2008; Zhao et al., 1998).

Additional genes of unknown function have been identified through a selection for genes that, if overexpressed, suppress the lethality of a *RAD53* and/or *MEC1* deletion (*srl*, for suppressor of *R*ad53 *l*ethality) (Desany et al., 1998). Some of these genes have remained uncharacterized. The present study concerns *SRL3* whose deletion in a commercially available strain was identified by us in a screen for spontaneous mutator mutants. However, its high spontaneous mutability and DNA damage sensitivity phenotype were found to be associated with an independent mutation which was identified as a truncation of *MMS2*. *MMS2* is involved in a replicative DNA damage tolerance pathway (Broomfield et al., 1998; Broomfield et al., 2001; Friedberg et al., 2006; Gangavarapu et al., 2006; Hofmann and Pickart, 1999; Torres-Ramos et al., 2002; VanDemark et al., 2001; Xiao et al., 1998). While we found no direct connection between *SRL3* and *MMS2*, important information pertaining to both gene products was uncovered.

Materials and Methods

Yeast strains and strain construction

Most haploid strains were derived from BY4741 (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*). The collection of deletions of non-essential genes made in this strain was purchased from OpenBiosystems. Additional deletions were introduced by replacing a specific gene with a PCR product containing micro-homology upstream and downstream of the gene and a selectable marker such as G418 resistance gene *kanMX4* (Rothstein, 1989). Yeast transformation was performed by the lithium acetate method (Gietz and Schiestl, 1995). Standard methods of yeast genetics were applied for mating, sporulation and tetrad dissection (Amberg et al., 2005).

Determination of spontaneous mutation rates

Parallel cultures were inoculated with 2,000 cells in 4 ml YPD liquid medium (1% yeast extract, 2% peptone, 2% dextrose). Cultures were incubated at 30°C (75 rpm) until the titer of each culture reached at least 1×10^8 cells/ml. From each culture, 2×10^7 cells were plated on canavanine plates (containing 1.4% agar, 2% dextrose, 6.7 g/l yeast nitrogen base w/o amino acid and w/o ammonium sulfate, complete supplement mixture minus arginine [CSM-Arg; Sunrise Science] and 60 mg/l canavanine sulfate) to determine the frequency of canavanine-resistant mutants. Titer of all colony-forming cells was determined on medium

of the same composition without canavanine. Plates were incubated at 30°C until formation of colonies (three to four days). The median mutant colony number was used to determine relative spontaneous mutation frequencies according to the method of Lea and Coulson, as described (Lea and Coulson, 1949; von Borstel, 1978).

Survival assays

For UV irradiation, appropriate dilutions of cell suspensions were plated on YPD plates and irradiated with 254 nm UV (germicidal lamp). For methyl methane sulfonate (MMS) and streptonigrin (both from Sigma Chemicals), liquid cultures were treated for 30 minutes at 30°C with various concentrations of the chemicals. Cells were washed, then appropriate dilutions of untreated and treated cells were plated on YPD plates. Plates were incubated at 30°C until surviving cells formed colonies (about 3 to 4 days). Colonies on each plate were counted and the surviving fractions were calculated. Surviving fraction is defined as titer of colony forming cells with treatment divided by the titer of colony forming cells without treatment.

Checkpoint assays following UV irradiation

To measure exit from G1 phase arrest, logarithmic-phase phase cultures of a titer of approximately 1×10^7 cells/ml were first synchronized in G1 by exposure to alpha-factor (1 mg/ml stock solution, US Biological) at 5 μg/ml in fresh YPD. After cells were incubated at 30°C for 1 hour and 15 minutes, another aliquot of alpha-factor was added up to a final concentration of 10 μg/ml and cells were incubated for another 30-45 minutes. After microscopic confirmation of G1 arrest, 8×10^7 cells were spun down, washed, briefly sonicated and resuspended with 8 ml of sterile water. 4 ml of this cell suspension was transferred to a sterile petridish (6 cm diameter) and irradiated with 254 nm UV (80 J/m^2) under constant stirring. 4 ml of irradiated and 4 ml of non-irradiated cells were centrifuged and each sample was resuspended with 4 ml of YPD. During incubation at 30°C, the percentage of small-budded cells was microscopically determined every 10 to 15 minutes.

To measure exit from S phase arrest, logarithmic-phase cultures were synchronized with HU (at 0.2 M, US Biological) for 2 hour and 30 minute at 30°C. S phase arrested cells were washed, sonicated, streaked on YPD plates and UV-irradiated or not. During incubation at 30°C, the percentage of microcolonies (defined as [No. of colonies composed of more than two cells/No. of total colonies counted] × 100) was microscopically determined every hour.

To measure exit from M phase arrest, logarithmic-phase cultures were synchronized with nocodazole (at 10 μg/ml, US Biological) for 3 hours at 30°C, washed, briefly sonicated, streaked on YPD and observed as described above. Since the microscopic determination of small buds is difficult on solid media, this determination of UV-induced S and M phase arrest may also include the arrest in the subsequent G1 phase.

Results

The commercially available collection of deletion mutants of non-essential genes of a haploid yeast strain (BY4741) was screened for strains with higher spontaneous mutation frequencies, using canavanine resistance as a forward mutation marker (Gong and Siede,

2009). While this screen was similar to the one described earlier by others (Huang et al., 2003), two novel genes were identified: *SRL3* and *TOP3*.

We focused on *srl3*Δ and confirmed quantitatively that spontaneous mutation rates were approximately 10 fold higher in this strain than in the isogenic wild-type (Table 1). (In order to avoid confusion, we have designated the genotype of this strain as *srl3*Δ *uvs* since subsequent analysis indicated the existence of a second mutation.) *SLR3* has been described as a suppressor of lethality of a Rad53 deletion if overexpressed and in general, higher dNTP pools have been identified as a mechanism for this effect (Desany et al., 1998). Therefore, using the *CAN1* system, we determined spontaneous mutation rates of mutants that have presumably lower or higher dNTP levels due to deletion of RNR subunits (*rnr1*Δ, *rnr3*Δ, *rnr4*Δ) or of a negative regulator of RNR (*sml1*Δ) (Zhao et al., 1998), respectively. In no instance, we found an influence on spontaneous mutation rates that is quantitatively comparable to that of *srl3*Δ *uvs* (Table 1). At the most, an approximately 3 fold enhanced rate was found for *RNR1* deletion compared to wild type.

Also, determination of UV sensitivity in *srl3*Δ *uvs* indicated a phenotype different from other mutants with possibly lower dNTP levels. The *srl3*Δ *uvs* strain showed notably increased UV sensitivity while none of the RNR mutants did (Fig. 1 A). Using cells synchronized in G1, S or M, UV sensitivity of *srl3*Δ *uvs* was demonstrated to be cell cycle stage independent (data not shown). Sensitivity was also shown towards the alkylating agent MMS and the double-strand break-inducing agent streptonigrin (Fig. 1 B, C).

UV sensitivity was not due to a checkpoint defect. When cells were synchronized in G1 with alpha-factor, in S with HU and in M with nocodazole before treatment with UV, a more extended arrest was found in all tested cell cycle stages (Fig. 2 A, B, C).

Prompted by the fact that the second commercially available *srl3*Δ strain of opposite mating type did not exhibit DNA damage sensitivity (not shown), we hypothesized that there might be an unlinked mutation present in the used strain. Therefore, we crossed the strain against wild type, sporulated the diploid strain and isolated individual spores by micromanipulation. Indeed, the mutagen sensitivity did not co-segregate with the *SRL3* deletion which can be identified through the *kanMX4* marker gene conferring G418 resistance (Fig. 3 A). The unknown mutation was termed *uvs*. Additionally, elevated spontaneous mutation rates were caused by the *uvs* mutation but not the *SRL3* deletion (Table 1).

In order to identify possible candidate genes whose mutation may result in the observed phenotypes, we performed epistasis analyses with well-characterized single gene deletions conferring similar phenotypes. Briefly, in epistasis analysis, an uncharacterized mutant gene affecting DNA damage sensitivity is assigned to the same DNA repair or tolerance pathway as a characterized mutant gene if the double mutant's phenotype is not more severe than that of the most sensitive single mutant (Brendel and Haynes, 1973). Given the sensitivity of the *uvs* mutant to both UV radiation and the alkylating agent MMS, a role in either homologous recombination (Rad51 pathway) or replicative DNA tolerance (Rad6 pathway) is likely (Friedberg et al., 2006). If combined with a *RAD52* deletion (representing the Rad51 pathway), non-epistatic interaction was found for both UV and MMS (not shown). If

combined with a *RAD5* deletion, representing an error-free sub-pathway of the Rad6 pathway, epistatic interaction was found for both agents (Fig. 3 B, C). We also investigated interaction with *RAD50*, encoding a protein of the MRN complex, involved in several pathways depending on double-strand break recognition and processing (D'Amours and Jackson, 2002; Friedberg et al., 2006). We found an epistatic interaction with *uvs* for MMS and for low doses of UV which became additive at higher doses (Fig. 3 B, C).

Next, we identified candidate genes of the error-free subpathway of the Rad6 epistasis group or the MRN complex whose mutation may account for the *uvs* phenotype. A haploid *uvs* strain was mated with deletion mutants of *RAD50, MRE11, SRL3, RAD5, UBC13* and *MMS2* of opposite mating type and the generated diploid strains were tested for complementation of UV and MMS sensitivity. Absence of complementation as indicated by increased sensitivity was found for *mms2*Δ (Fig. 3 D).

Consequently, the *MMS2* gene of the *uvs* strain was isolated by PCR and subjected to sequencing using appropriate primers. Inspection of the DNA sequence revealed a G –> A transition at residue 393, resulting in a change of TAG (encoding tryptophan) to TGA (stop codon) and thus a truncation of the C-terminal 35 amino acids of Mms2 (Fig. 4).

Discussion

In a screen of the commercially available deletion collection in the haploid yeast strain BY4741, deletions of *SRL3* and *TOP3* were among previously unidentified mutants enhancing spontaneous mutability. *SRL3* has been isolated before as a gene that can suppress in high copy number the lethality of a deletion of checkpoint kinase Rad53 and, more poorly, Mec1 (Desany et al., 1998). Since no additional phenotype was known, we proceeded with a more detailed characterization and, in addition to approximately 10 fold higher mutation rates in the *CAN1* system, we found sensitivity to UV radiation, MMS and streptonigrin. Extended checkpoint arrest responses correlated well with the likely DNA repair deficiency in this mutant.

Further analysis, however, revealed that the described phenotype in this particular strain originated from another unlinked mutation that results in truncation of the damage tolerance protein Mms2. An *SRL3* deletion alone does in fact neither influence spontaneous mutability nor mutagen sensitivity. Elevated dNTP levels are the only known mechanism to overcome the lethality of deletions of the checkpoint kinases Mec1 or Rad53, possibly by facilitating replication through chromosomal regions that are difficult to replicate and comprise fragile sites (Cha and Kleckner, 2002). Absence of any large effect on spontaneous mutability or UV sensitivity is in agreement with the phenotype of other yeast mutants affecting dNTP pools, negatively (RNR subunits) or positively (Sml1 mutant). However, it should be noted that RNR mutants very well affect *damage-induced* mutation frequencies when dNTP levels appear to become more limiting than in undamaged cells. Reduced UV-mutation frequencies were found for *rnr1* and *rnr4* deletions (Lis et al., 2008; Strauss et al., 2007)(Gong and Siede, unpublished). Similarly, elevated dNTP levels result in higher induced mutation frequencies in response to certain types of DNA damage (Chabes et al., 2003).

Mms2 functions in a sub-pathway of the DNA-damage tolerance pathway (Rad6 group) that facilitates replication of a damaged template (Andersen et al., 2008). Mms2 is a ubiquitin-conjugating (E2) variant enzyme that forms a heterodimer with the E2 enzyme Ubc13 (VanDemark et al., 2001) and interacts with the ubiquitin-ligase Rad5 (Broomfield et al., 1998; Gangavarapu et al., 2006; Hofmann and Pickart, 1999; Torres-Ramos et al., 2002; Xiao et al., 1998). Their primary target during lesion bypass appears to be PCNA that is subject to polyubiquitination (Hoege et al., 2002). Error-free template switching is postulated as the used mechanism of lesion bypass (Branzei et al., 2008). There is one earlier report of higher spontaneous mutation rates in *mms2*Δ measured in a point mutant reversion system (*trp1-289*) (Broomfield et al., 1998). Our studies confirm the phenotype in a forward mutation system which will detect a much broader range of sequence alterations. It has been speculated that Rad5-Mms2-Ubc13 is required to restrain the action of the error-prone bypass polymerase zeta which is responsible for most spontaneous mutations; the exact mechanism, however, is not known. Our data also attest to the importance of the C-terminus of Mms2. The observed truncation does also delete Trp 393, a residue which is well conserved in various E2 enzymes (Ptak et al., 2001).

Given the role of Mms2 in the replicative bypass of UV lesion, extended checkpoint arrest of its mutant in S-phase can easily be rationalized. This is not the case with the observed extension of G1 and M phase arrest where the assumed bypass mechanism cannot easily contribute to UV resistance. We hypothesize that there may be an unknown role of Rad5-Mms2-Ubc13 during nucleotide excision repair. The observed extension of checkpoint arrest was independent of *srl3*Δ (data not shown).

We have no indication for any functional connection between Srl3 and Mms2 and assume that the appearance of both mutations together is a coincidence. For example, continued propagation of *srl3*Δ did not result in the accumulation of UV-sensitive cells (data not shown). This issue, however, will require more detailed studies in the future.

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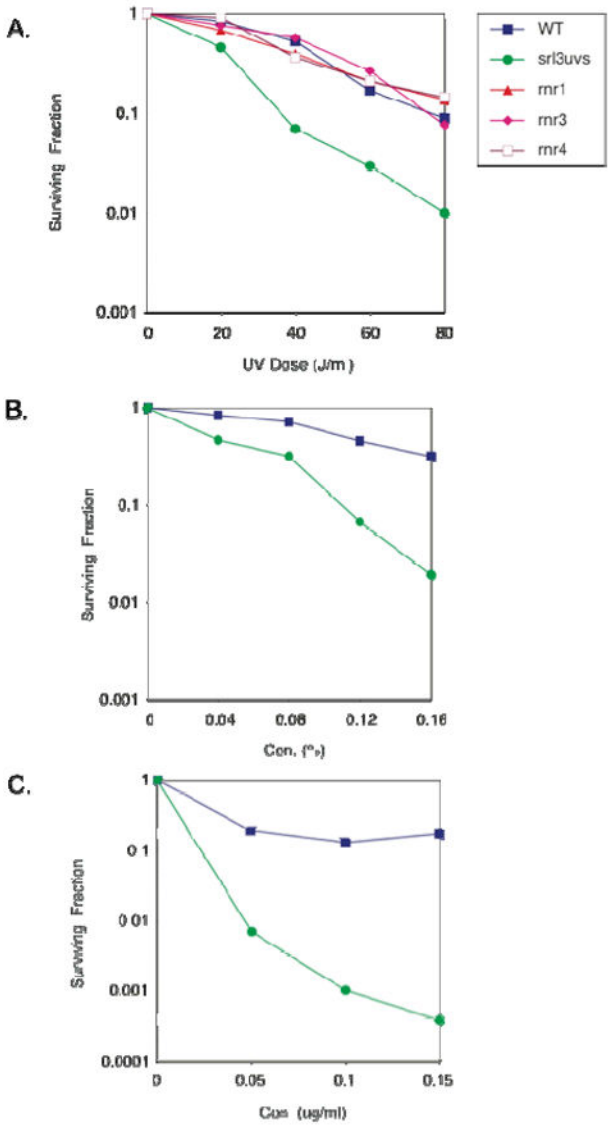

Figure 1.

Survival of BY4741 *srl3*Δ *uvs* following exposure to UV radiation (A), MMS (B) and streptonigrin (C). Additionally, the UV survival of strains BY4741 *rnr1*Δ, *rnr3*Δ and *rnr4*Δ are shown (A). Logarithmic-phase cells were treated, as described in Material and Methods, and the fraction of colony forming cells was plotted as a function of dose.

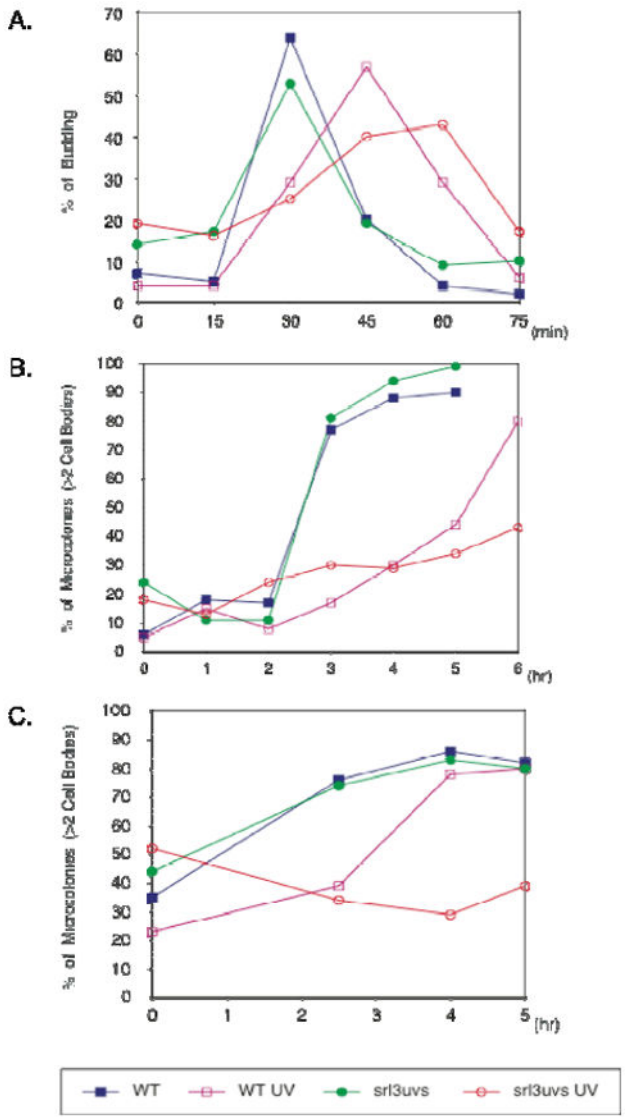

Figure 2.

Checkpoint arrest duration in *srl3*Δ *uvs* in comparison to wild type. Exit from G1 (A), S (B) and M phase (C) following UV radiation treatment (80 J/m^2) was analyzed by determining the fraction of small budded cells in liquid medium (A) or by the emergence of microcolonies (with more than two cells) on plates (B, C).

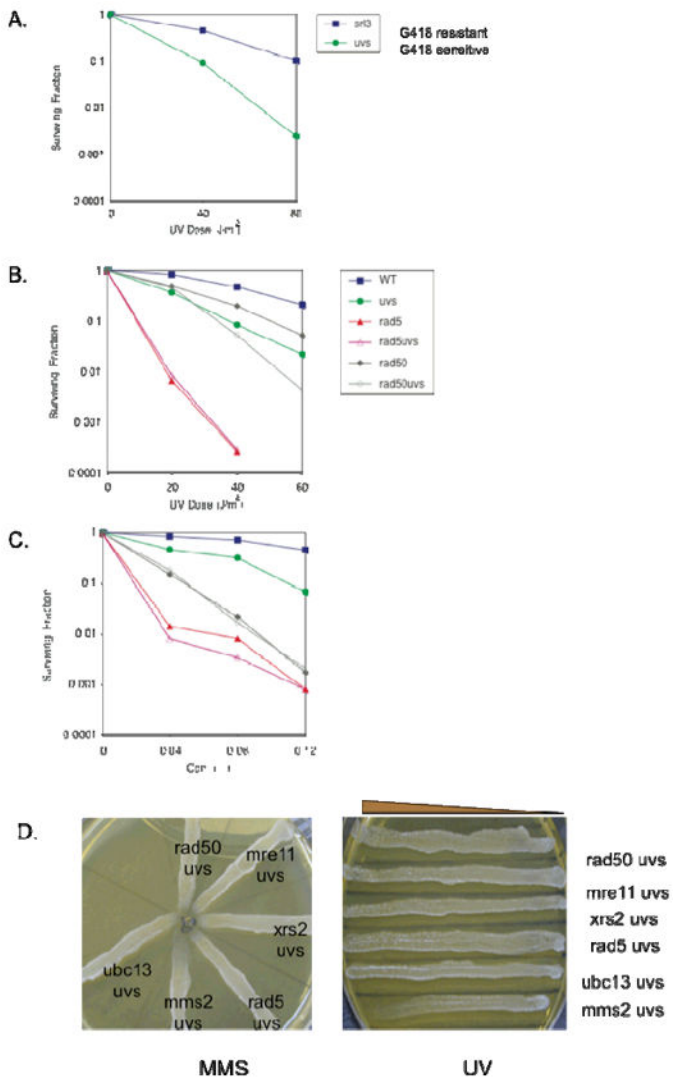

Figure 3.

UV sensitive phenotype of *srl3*Δ *uvs* is caused by a mutation in *MMS2* (= *uvs*). A. Segregation of UV sensitivity. Following crossing of *srl3*Δ*uvs* to wild-type, spores were isolated containing *SRL3* deletion as indicated by the transplacement marker *kanMX4*, conferring G418 resistance. As shown, *SRL3* deletion and UV sensitivity did not cosegregate. B, C. Epistasis analysis with uvs. The unknown mutation (*uvs*) was combined with deletions of *RAD5* and *RAD50*. In logarithmic-phase cells, UV (B) and MMS (C) sensitivities of double mutants were determined and compared to single mutant sensitivities. D. A *uvs* strain was mated with strains carrying deletions of *RAD50, MRE11, XRS2, RAD5, UBC13* or *MMS2*. UV and MMS sensitivities of the diploid strains were characterized in gradient assays. Enhanced sensitivity was only found for the *mms2*Δ *uvs* combination.

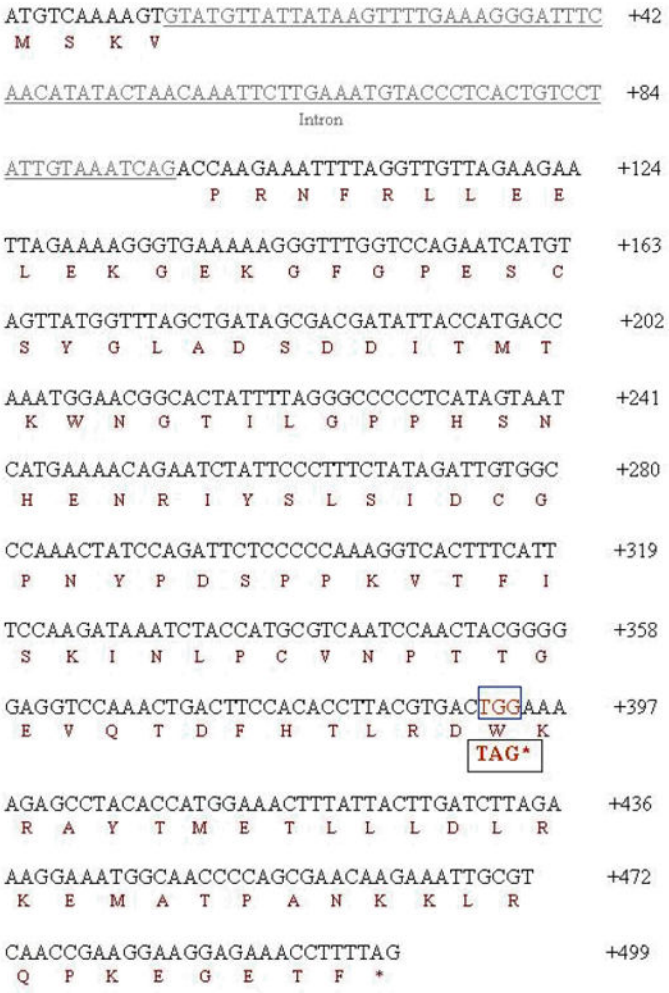

Figure 4.

MMS2 DNA and protein sequence. The mutation identified in *srl3*Δ *uvs* is indicated.

Table 1

Spontaneous rates of canavanine-resistance mutations, expressed as fold difference over wild type (= 1)

Data shown are from representative single experiments, except for strain *srl3*Δ *uvs* which represents the average of three measurements including standard deviation.

Deletion mutant genotype	Spontaneous mutation rate relative to wild type
*srl3*Δ *uvs*	10.3 ± 1.3
*rnr1*Δ	3.0
*rnr3*Δ	2.2
*rnr4*Δ	0.9
*sml1*Δ	0.9
*srl3*Δ	0.6
uvs	8.6