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Proteinase yscE, the yeast proteasome/multicatalytic-multifunctional proteinase: mutants unravel its function in stress induced proteolysis and uncover its necessity for cell survival

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Proteinase yscE is the yeast equivalent of the proteasome, a multicatalytic-multifunctional proteinase found in higher eukaryotic cells. We have isolated three mutants affecting the proteolytic activity of proteinase yscE. The mutants show a specific reduction in the activity of the complex against peptide substrates with hydrophobic amino acids at the cleavage site and define two complementation groups, *PRE1* and *PRE2*. The *PRE1* gene was cloned and shown to be essential. The deduced amino acid sequence encoded by the *PRE1* gene reveals weak, but significant similarities to proteasome subunits of other organisms. Two-dimensional gel electrophoresis identified the yeast proteasome to be composed of 14 different subunits. Comparison of these 14 subunits with the translation product obtained from *PRE1* mRNA synthesized *in vitro* demonstrated that *PRE1* encodes the 22.6 kd subunit (numbered 11) of the yeast proteasome. Diploids homozygous for *pre1-1* are defective in sporulation. Strains carrying the *pre1-1* mutation show enhanced sensitivity to stresses such as incorporation of the amino acid analogue canavanine into proteins or a combination of poor growth medium and elevated temperature. Under these stress conditions *pre1-1* mutant cells exhibit decreased protein degradation and accumulate ubiquitin-protein conjugates.

Key words: multicatalytic-multifunctional proteinase/proteasome/proteinase yscE/stress response/ubiquitin

Introduction

Proteolysis has been found to be of vital importance for cellular life. A major degradative pathway in the eukaryotic cell has been traced in the lysosome (Glaumann and Barrett, 1987). Selective, non-lysosomal proteolysis has been attributed to a pathway mediated by post-translational modification of proteins by ubiquitin, an abundant 76-residue protein (for reviews see Hershko, 1983, 1988; Finley and Varshavsky, 1985; Rechsteiner, 1987; Ciechanover and Schwartz, 1989; Jentsch *et al.*, 1990).

As a highly developed experimental system, the yeast *Saccharomyces cerevisiae* has become a pacemaker in studies on the functions of proteinases in cellular physiology (for reviews see Wolf, 1982; Jones, 1984; Achstetter and Wolf, 1985; Fuller *et al.*, 1988; Suarez Rendueles and Wolf, 1988; Hirsch *et al.*, 1989). We had studied extensively the proteolytic pathway in the vacuole, the yeast counterpart of the lysosome (Suarez Rendueles and Wolf, 1988; Hirsch *et al.*, 1989; Teichert *et al.*, 1989).

Using mutants defective in the major vacuolar proteinases of yeast, we were able to detect a multitude of new proteolytic enzymes (Achstetter *et al.*, 1984a), many of which were of non-vacuolar origin (Emter and Wolf, 1984). One of the prominent activities—called proteinase yscE—was purified and characterized as a high molecular weight enzyme of ~600 kd. It consists of a variety of different subunits with molecular weights between 20 and 38 kd (Achstetter *et al.*, 1984b). We identified proteinase yscE to be the homologue of the 20S cylinder particles found in *Xenopus laevis* (Kleinschmidt *et al.*, 1988). This highly conserved protein complex contains between 10 and 14 subunits of different size and is present in archaebacteria and in all eukaryotes from yeast to man. Commonly used names for this complex are: cylinder particle, prosome, proteasome, multicatalytic proteinase complex, multifunctional protease (Wilk and Orlowski, 1980; Kleinschmidt *et al.*, 1983, 1988; Schmid *et al.*, 1984; Kloetzel, 1987; Arrigo *et al.*, 1988; Falkenburg *et al.*, 1988; Tanaka *et al.*, 1988; Dahlmann *et al.*, 1989; Scherrer, 1990). The high evolutionary conservation of the proteasome from yeast to man implies central functions for this protein complex in the physiology of all eukaryotic cells. So far, the *in vivo* function of this complex is unclear. Based upon two biochemically distinct characteristics of the protein complex found *in vitro*, namely its association with RNA (for review see Scherrer, 1990) and its existence as a proteinase complex carrying multiple proteolytic activities (for review see Rivett, 1989a,b) many different models about the *in vivo* functions of the protein complex have been proposed. Speculative functions for the particle range from controlling transport, distribution and activity of specific mRNAs in the cell (Scherrer, 1990) to specific processing and post-translational modification of newly synthesized proteins or involvement in non-lysosomal protein degradation (for review see Rivett, 1989b).

Changes in the subunit pattern of the protein complex have been observed (Haass and Kloetzel, 1989; Scherrer, 1990). Recently, Eytan *et al.* (1989) as well as Driscoll and Goldberg (1990) showed that the proteasome of rabbit reticulocytes can be part of a 26S (1500 kd) proteolytic complex (Hough *et al.*, 1986, 1987), which is able to degrade ubiquitinated proteins *in vitro* in an ATP-dependent reaction.

As assumed for the proteasome of higher eukaryotic cells (Wilk and Orlowski, 1980, 1983; Tanaka *et al.*, 1988; for review see Rivett, 1989b) the yeast proteasome has three distinct proteolytic activities. The enzyme complex can cleave bonds on the carboxyl side of neutral/hydrophobic, basic and acidic amino acids (Tanaka *et al.*, 1988; C.Escher and D.H.Wolf, unpublished results). These activities have been called 'chymotrypsin-like', 'trypsin-like' and

'peptidylglutamyl-peptide hydrolysing' activity, respectively (Wilk and Orlowski, 1983).

We started a combined genetic and biochemical approach to uncover the functions of these activities in yeast. Here, we report the isolation and characterization of mutants defective in the 'chymotrypsin-like' activity of proteinase yscE. The mutants define two complementation groups (*PRE1* and *PRE2*). Analysis of the *PRE1* gene revealed that it is essential for life and encodes the 22.6 kd subunit (number 11) of the proteinase yscE complex. Furthermore, data presented indicate that one function of the complex rests in the degradation of ubiquitinated proteins.

Results

Isolation and characterization of proteinase yscE mutants deficient in the 'chymotrypsin-like' activity

To isolate mutants deficient in the 'chymotrypsin-like' activity of proteinase yscE we used strain cl3-ABYS-86, deficient in the four major vacuolar peptidases proteinase yscA, proteinase yscB, carboxypeptidase yscY and carboxypeptidase yscS. After mutagenesis, three out of 2×10^4 colonies screened stained weakly with Cbz-Gly-Gly-Leu-*p*-Nan (Cbz, benyloxycarbonyl; *p*-Nan, *p*-nitroanilide) as a substrate in a proteolysis test (see Materials and methods). Crude extracts of these mutants (E81, E95 and E119) were retested against Cbz-Gly-Gly-Leu-*p*-Nan and two other substrates carrying neutral/hydrophobic amino acids at the cleavage site, Suc-Phe-Leu-Phe-β-NA (Suc, succinyl; β-NA, β-naphthylamide) and Suc-Leu-Leu-Val-Tyr-AMC (AMC, 7-amido-4-methylcoumarine). Only between 5 and 20% of wild type activity against these substrates could be found. The residual activity was not heat sensitive in any of the three mutants. Activities against substrates carrying acidic (Cbz-Leu-Leu-Glu-β-NA) or basic (Cbz-Ala-Arg-Arg-4MeO-β-NA; 4 MeO, 4-methoxy) amino acids at the cleavage site were not affected in the three different mutants.

Table I. Proteinase yscE activity of spore tetrads originating from crosses of wild type and mutant strains E95, E81 and E119

Strain	Specific activity against Cbz-Gly-Gly-Leu-*p*-Nan (mU/mg)
E95-2-6A	0.015
E95-2-6B	0.35
E95-2-6C	0.35
E95-2-6D	0.02
E81-1-9A	0.43
E81-1-9B	0.36
E81-1-9C	0.054
E81-1-9D	0.071
E119-1-1A	0.40
E119-1-1B	0.018
E119-1-1C	0.35
E119-1-1D	0.033

For all three mutants crosses with strains wild type for proteinase yscE showed that the mutations were recessive. Diploids from the three different crosses were sporulated and asci were dissected. From each spore clone a crude extract was analysed for proteinase yscE activity against Cbz-Gly-Gly-Leu-*p*-Nan. A minimum of 10 tetrads were analysed for each of the three crosses. The ascospores tested showed $2^+:2^-$ segregation of proteinase yscE activity against Cbz-Gly-Gly-Leu-*p*-Nan (Table I). This indicates, that the mutants analysed bear a single chromosomal mutation.

Complementation tests between mutants E81, E95 and E119 revealed two complementation groups. Diploids constructed from mutant progeny of strains E81 and E119 did not show considerable proteinase yscE activity against substrate Cbz-Gly-Gly-Leu-*p*-Nan whereas heterozygous diploid combinations of mutant progeny of strains E95 and E81 as well as strains E95 and E119 resumed proteinase yscE activity against this substrate (not shown). The wild type allele of the mutation in strain E95 was called *PRE1* (*p*roteinase yscE or *p*roteasome), the wild type allele of mutations in strains E81 and E119 was called *PRE2*. Obviously, mutations in two different genes lead to the same phenotype, namely a defective activity of proteinase yscE against substrates carrying neutral/hydrophobic amino acids at the cleavage site.

We purified proteinase yscE from mutant strains E95 (*pre1-1*) and E81 (*pre2-1*) and, for comparison, from wild type (Achstetter *et al.*, 1984b). Table II shows that the 'trypsin-like' (cleavage after Arg) and the 'peptidylglutamyl-peptide hydrolysing' activities remained unchanged in the purified enzymes of the mutant strains. In contrast the 'chymotrypsin-like' activity, resulting in hydrolysis of peptides harbouring a neutral or hydrophobic amino acid at the cleavage site (Leu, Phe, Tyr), is dramatically disturbed in the purified enzyme of mutant strain E81 or nearly absent in the enzyme of mutant strain E95.

Analysis of purified proteinase yscE from mutants E95 and E81 by SDS−PAGE showed the same subunit pattern (mol. wts between 20 and 38 kd) as published for wild type proteinase yscE (Kleinschmidt *et al.*, 1988). Separation of wild type proteinase yscE subunits in two dimensions (first dimension: isoelectric focussing, second dimension: SDS−PAGE) resulted in 14 clearly distinguishable protein spots of different molecular weight and charge (Figure 1). Identical treatment of purified proteinase yscE from mutants E95 and E81 did not result in any significantly altered subunit

Table II. Activity of purified wild type and mutant proteinase yscE against chromogenic and fluorogenic substrates

Substrate	Wild type	E95(pre1-1)		E81 (pre2-1)	
	sp. act. mU/mg	sp. act. mU/mg	% of wild type	sp. act. mU/mg	% of wild type
Cbz-Gly-Gly-Leu-*p*-Nan	168	1.6	1	13	8
Suc-Phe-Leu-Phe-*p*-Nan	81	3.8	5	18	22
Suc-Leu-Leu-Val-Tyr-AMC	360	17	5	61	17
Cbz-Leu-Leu-Glu-β-NA	1.8	2.1	117	2.3	128
Cbz-Ala-Arg-Arg-4MeO-β-NA	1	0.8	80	1.2	120

pattern as compared with wild type (not shown). Thus, subunit composition and subunit charge are not significantly altered in the mutant proteins.

When the purified enzymes of mutant strains E95 and E81 were analysed by electron microscopy, the typical ring-shaped, cylindrical particles observed for wild type proteinase yscE (Kleinschmidt *et al.*, 1988) were found. This indicates that no gross alteration of the proteasome structure had occurred in the mutants.

Thus, loss of proteolytic activity in the mutants is neither due to drastic alterations in size or charge of the subunits nor in the quarternary structure of the enzyme complex. Nevertheless the dramatic reduction of the 'chymotrypsin-like' activity is a feature inherent to the mutant proteasomes.

Cloning and sequence of the PRE1 gene. PRE1 is essential

To clone the *PRE1* gene strain E95-1-16A was transformed with a yeast genomic library available in the centromere shuttle vector pCS19 (Sengstag and Hinnen, 1987). Transformants were screened for restoration of the 'chymotrypsin-like' activity of proteinase yscE against Cbz-Gly-Gly-Leu-*p*-Nan. Two different complementing plasmids with overlapping inserts were identified. The DNA sequence responsible for complementation was traced down to a 1.15 kb *Sca*I–*Eco*RI fragment present in both plasmids (Figure 2). Sequence analysis of this region revealed an open reading frame of 594 nucleotides capable of coding for a protein of 198 amino acids with a predicted molecular weight of 22.6 kb (Figure 3).

To study the phenotype of a *pre1* null mutation we replaced *in vitro* the open reading frame of the *PRE1* gene by the *URA3* gene. The construction left the 5′ and 3′ non-coding regions of *PRE1* essentially intact (Figure 2A). A linear fragment of the construct was transformed into a *PRE1/PRE1* wild type diploid (strain YS18/18) homozygous for a *ura3* deletion. A diploid transformant harbouring the *URA3*-marked null mutation (*pre1Δ::URA3*) and a wild type copy of *PRE1* was sporulated and the resulting asci were dissected. In each tetrad only two spores were viable. All viable spores were auxotrophic for uracil and exhibited wild type proteinase yscE activity against Cbz-Gly-Gly-Leu-*p*-Nan. These experiments clearly show that the *PRE1* gene is essential for life.

To prove that the *pre1-1* mutation is in fact a lesion in the *PRE1* gene cloned we constructed the heterozygous diploid YWH6 carrying the *pre1-1* mutation and the *pre1Δ::URA3* deletion. As found for *pre1-1* haploids, this strain is defective in the 'chymotrypsin-like' activity (cleavage of Cbz-Gly-Gly-Leu-*p*-Nan), in contrast to the diploid control strain YWH7 heterozygous only for the *pre1-1* allele (YWH6, genotype *pre1-1/pre1Δ::URA3*: 0.028 mU/mg; control, YWH7, genotype *PRE1/pre1-1*: 0.225 mU/mg).

PRE1 codes for subunit 11 of proteinase yscE

The deduced amino acid sequence of the PRE1 protein shows weak, but significant similarity to sequences of proteasome subunits of other organisms (Fujiwara *et al.*, 1989; Tanaka *et al.*, 1990; Tamura *et al.*, 1990; Kumatori *et al.*, 1990; Haass *et al.*, 1989, 1990). The similarities are most pronounced in boxes II and III defined by Haass *et al.* (1990). As documented for the proteasome subunits of other organisms, an accumulation of charged amino acid is also found in the carboxy-terminal part of the PRE1 protein. Interestingly, the PRE1 protein also contains a potential nuclear targeting sequence (Garcia-Bustos *et al.*, 1991) at amino acid positions 109–113 (Figure 3).

To determine which of the proteinase yscE subunits was encoded by the *PRE1* gene its coding region was inserted into the transcription vector pSP65 under the control of the SP6 RNA polymerase promoter. After transcription of the linearized plasmid DNA, the resulting mRNA was translated in a wheat germ extract in the presence of [^{35}S]methionine. We added an aliquot of the translation assay to purified pro-

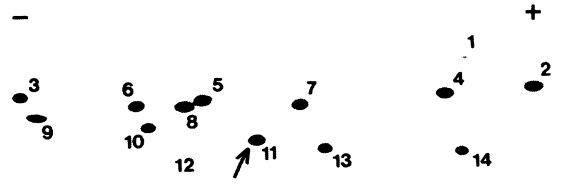

Fig. 1. Two-dimensional electrophoresis of purified proteinase yscE. Horizontal axis: isoelectric focussing, electrodes are marked '+' and '–'; vertical axis: SDS–PAGE. 50 μg of protein were applied. Arrow marks subunit spot comigrating with the protein product of the *in vitro* transcribed and translated *PRE1* gene.

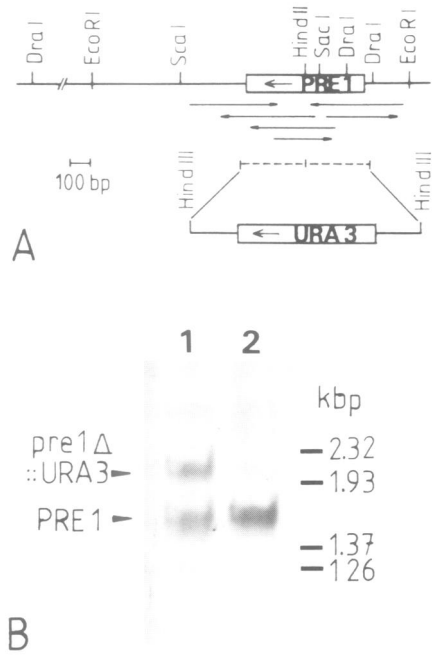

Fig. 2. (**A**) Physical map of the *PRE1* locus and outline of the *PRE1* gene replacement. Open boxes represent coding regions with the direction of transcription indicated by arrows. Arrows outside of boxes represent sequenced stretches of both DNA strands. The broken line marks the extent of the *PRE1* gene deletion brought about by *Bal*31 exonuclease digestion. (**B**) Southern analysis of diploid wild type strain YS18/18 (lane 2) and diploid heterozygous *PRE1/pre1Δ::URA3* mutant strain YS18/18ΔE1 (lane 1). *Eco*RI digested chromosomal DNA was probed with a digoxigenin labelled 2.1 kbp *Dra*I gene fragment (A) as outlined in Materials and methods. Lengths of marker fragments are indicated.

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      5' TCATCTACATCTAGGCCGGGCATCTTTACGGTGGCAAAAAATAAAGAAAAGTG
      AATATTGAACACCAATTACAAGAAAAGGGAGCACCTGTCAATCGAATAAAAAAGAACAA
      ACTTCGGGCACGTCTTTTAAATAGGTTCATATACAATACTAAGAAAGATATATTAAGAA

      ATG GAT ATT ATT CTG GGC ATC CGT GTA CAG GAT TCT GTC ATT CTA
    1 Met Asp Ile Ile Leu Gly Ile Arg Val Gln Asp Ser Val Ile Leu

      GCG TCT TCT AAG GCA GTC ACA AGA GGT ATT TCT GTT TTA AAA GAT
   16 Ala Ser Ser Lys Ala Val Thr Arg Gly Ile Ser Val Leu Lys Asp

      TCT GAT GAT AAA ACG AGA CAA TTA TCG CCA CAT ACA TTG ATG AGT
   31 Ser Asp Asp Lys Thr Arg Gln Leu Ser Pro His Thr Leu Met Ser

      TTT GCC GGT GAA GCT GGT GAC ACC GTT CAA TTC GCC GAG TAC ATT
   46 Phe Ala Gly Glu Ala Gly Asp Thr Val Gln Phe Ala Glu Tyr Ile

      CAA GCC AAT ATC CAA TTA TAC TCC ATT AGA GAA GAT TAT GAG CTC
   61 Gln Ala Asn Ile Gln Leu Tyr Ser Ile Arg Glu Asp Tyr Glu Leu

      TCT CCA CAA GCA GTA TCT AGT TTT GTT AGG CAA GAA TTA GCC AAG
   76 Ser Pro Gln Ala Val Ser Ser Phe Val Arg Gln Glu Leu Ala Lys

      TCA ATT AGG TCG AGA AGA CCA TAC CAA GTC AAC GTA TTG ATT GGA
   91 Ser Ile Arg Ser Arg Arg Pro Tyr Gln Val Asn Val Leu Ile Gly

      GGC TAT GAC AAA AAG AAG AAC AAA CCG GAA CTA TAT CAA ATT GAC
  106 Gly Tyr Asp Lys Lys Lys Asn Lys Pro Glu Leu Tyr Gln Ile Asp

      TAC TTG GGT ACT AAA GTC GAA TTA CCC TAT GGT GCT CAT GGT TAC
  121 Tyr Leu Gly Thr Lys Val Glu Leu Pro Tyr Gly Ala His Gly Tyr

      TCG GGG TTT TAC ACA TTC TCT TTA CTA GAT CAT CAT TAT AGA CCT
  136 Ser Gly Phe Tyr Thr Phe Ser Leu Leu Asp His His Tyr Arg Pro

      GAT ATG ACT ACT GAG GAG GGT TTA GAT TTA TTA AAA CTA TGT GTA
  151 Asp Met Thr Thr Glu Glu Gly Leu Asp Leu Leu Lys Leu Cys Val

      CAA GAG CTT GAA AAA AGA ATG CCA ATG GAC TTC AAG GGC GTC ATT
  166 Gln Glu Leu Glu Lys Arg Met Pro Met Asp Phe Lys Gly Val Ile

      GTT AAA ACT GTG GAT AAA GAT CGG ATA AGA CAA GTA GAT GAC TTC
  181 Val Lys Thr Val Asp Lys Asp Arg Ile Arg Gln Val Asp Asp Phe

      CAG GCA CAG TGAAAGATATTCAGTGTAATCACACGGAAAAGGTGATTGCTAATGAT
  196 Gln Ala Gln
      ATACTAAAGTAATTATCTTCCTCGTTCATAAAATTAAATAAAGTTATTATAGTTTTTTC
      ATCCGTTATAATAGCAAATGTATCAGAAATAAGACAATTTCTCATTTTTTGTTGGCAGA
      GTGAGATATCATCTGATCTATTGCAAATGAATCCCAATCAATGCTGATACCGCTTCGTT
      ATAACCAATTCGAAAAGTTGTAATTATGTTACCCGCATGAGGAT 3'
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Fig. 3. Nucleotide sequence of the *PRE1* gene and predicted amino acid sequence of the encoded protein. Dotted lines indicate parts of the protein identified by amino acid sequencing of tryptic peptides. Solid line indicates a potential nuclear targeting sequence.

teinase yscE and separated the mixture by two-dimensional gel electrophoresis. Autoradiography showed the *PRE1* translation product comigrating with subunit 11 of the enzyme complex (not shown; see arrow in Figure 1).

Trypsin digestion of the HPLC-purified 22.6 kd subunit 11 from wild type proteinase yscE and separation of the peptide fragments allowed us to obtain protein sequences of four peptides (N.Tröndle, unpublished results). The peptide sequences match perfectly with parts of the protein sequence deduced from the *PRE1* gene (see Figure 3). These data show unambiguously that *PRE1* indeed encodes the 22.6 kd subunit of proteinase yscE.

The pre1-1 mutation leads to canavanine and temperature sensitivity as well as sporulation inability of cells

As the chromosomal *PRE1* deletion leads to lethality, we started a comparative study on the physiology of wild type and *pre1-1* mutant cells. We used diploids—mutant strain 31 (*pre1-1/pre1-1*) and wild type strain 33 (*PRE1/PRE1*)—to minimize the effects of background mutations potentially present in non-isogenic haploid strains.

No sporulation of diploid *pre1-1/pre1-1* mutant cells was detectable (not shown).

In YPD (not shown) or mineral media at 30°C, no considerable differences in growth or viability were found between the mutant and wild type diploids (Figure 4). At 38°C, however, when grown in mineral medium, *pre1-1/pre1-1* mutant cells ceased to grow after ~7.5 h and

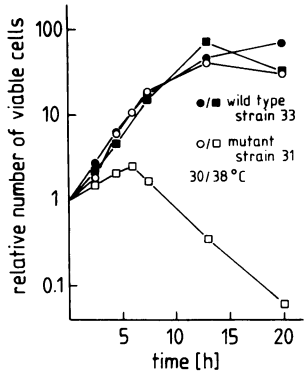

Fig. 4. Surviving cells from wild type strain 33 (*PRE1/PRE1*) and mutant strain 31 (*pre1-1/pre1-1*) during incubation in liquid MV medium at 30°C and 38°C. Absolute numbers of surviving cells were determined by plating appropriate cell dilutions onto YPD agar plates and incubating at 30°C. Relative numbers are given as the ratio of cells present at time points indicated in the figure and time point zero (A_{578} = 0.05).

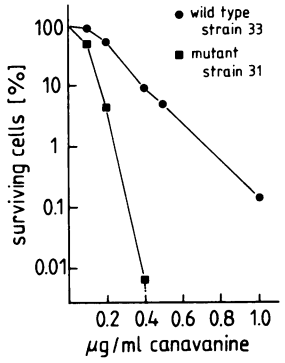

Fig. 5. Survival of wild type strain 33 (*PRE1/PRE1*) and mutant strain 31 (*pre1-1/pre1-1*) on canavanine-containing MV agar medium. Number of colonies growing up without canavanine is given as 100%.

lost viability in a dramatic fashion. Wild type diploids remained unaffected by this treatment (Figure 4).

Experiments with canavanine, an arginine analogue, showed a drastically enhanced sensitivity of the mutant cells towards this compound (Figure 5).

Introduction of the *PRE1* gene into mutant strain 31 restored wild type behaviour with respect to heat and canavanine sensitivity (not shown).

The pre1-1 mutation affects degradation of ubiquitinated proteins

We investigated the effect of defective 'chymotrypsin-like' activity of proteinase yscE on the protein degradation in cells exposed to canavanine or heat stress. Following a 5 min pulse-labelling of proteins, the release of radioactivity from cells during the chase period was measured. At 30°C, a temperature where no phenotype is expressed in *pre1-1/pre1-1* mutant cells, no difference in protein degradation between mutant and wild type cells is visible (Figure 6B). However, when cells are grown in mineral medium at 38°C, conditions which lead to a strong phenotype, a considerable reduction (27% within 4 h) in protein degradation is observed in the mutant cells (Figure 6B). Synthesis of canavanyl-peptides results in an even more dramatic decrease (43% within 4 h) of protein turnover in mutant cells as compared with wild type (Figure 6A).

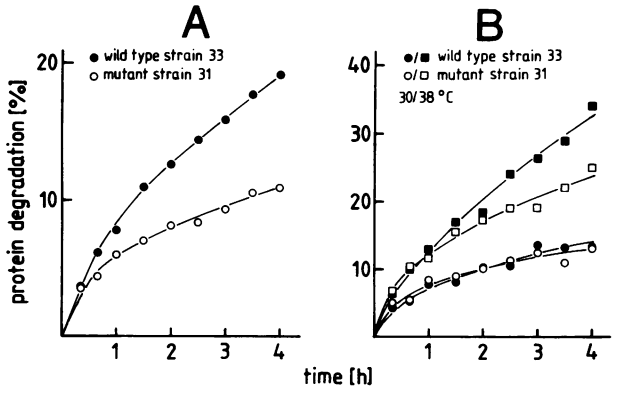

Fig. 6. Degradation of short-lived proteins in wild type strain 33 (*PRE1/PRE1*) and mutant strain 31 (*pre1-1/pre1-1*). (**A**) Protein degradation after canavanine treatment; (**B**) protein degradation at 30°C (○,●) and at 38°C (□,■). For details see Materials and methods.

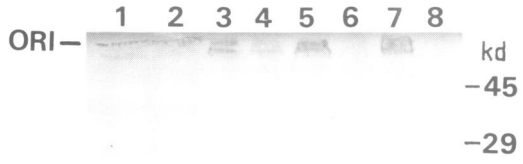

Fig. 7. Western blot analysis of wild type (*PRE1/PRE1*) and mutant (*pre1-1/pre1-1*) crude extracts of cells treated with canavanine using anti-ubiquitin–protein conjugate serum. SDS gel (18%) was loaded with wild type (lanes 2, 4, 6 and 8) and mutant (lanes 1, 3, 5 and 7) cell extracts (50 μg protein). Extracts were prepared before (lanes 1 and 2), at the end of (lanes 3 and 4), 1 h after (lanes 5 and 6) and 2 h after (lanes 7 and 8) a 90 min canavanine treatment. Gel was immunoblotted as described in Materials and methods. Molecular weights of marker proteins and the origin of the gel are indicated.

Since ubiquitin conjugation has been shown to be one prerequisite for the degradation of short-lived and falsely synthesized proteins we analysed the pattern of ubiquitin–protein conjugates in wild type and mutant cells. Proteins were prepared from cells either treated with canavanine or incubated in mineral medium at 38°C, separated by SDS–PAGE and probed by Western blot analysis with anti-ubiquitin protein conjugate serum. This serum recognized a heterogeneous population of high molecular weight proteins (Figure 7). No antigenic reaction was observed in control experiments with pre-immune serum (data not shown). While in wild type cells treated with canavanine the immunoreactive high molecular weight material decreased rapidly with time (Figure 7; lanes 2, 4, 6 and 8), the immunoreactive protein in equally treated *pre1-1/pre1-1* mutant cells accumulated (Figure 7; lanes 1, 3, 5 and 7). Identical results were obtained when wild type and mutant cells were subjected to 38°C heat stress on mineral medium (not shown). These results provide direct evidence that proteinase yscE is linked to the ubiquitin mediated proteolytic pathway.

Discussion

We have isolated three mutants defective in the 'chymotrypsin-like' activity of proteinase yscE, the proteasome of yeast. The mutations fell into two complementation groups, *PRE1* and *PRE2*. We have cloned and sequenced the *PRE1* gene and found it to code for the 22.6 kd subunit (number 11) of proteinase yscE. *Pre1-1* mutants grow like wild type in complete and poor media at 30°C but are sensitive to treatment at 38°C in poor medium as well as to canavanine application. Under these conditions of phenotypic alterations mutant cells exhibit considerably reduced degradation rates of short-lived proteins, and ubiquitinated proteins accumulate. Chromosomal deletion of *PRE1* is lethal.

As found for the proteasome of higher eukaryotic cells (Rivett, 1989a), proteinase yscE carries three proteolytic activities, a 'chymotrypsin-like', a 'trypsin-like' and a 'peptidyl-glutamylpeptide hydrolysing' activity (C.Escher, unpublished; Table II). The *pre1-1* mutation only affects the 'chymotrypsin-like' activity of the multicatalytic protein complex leaving the other two activities intact (Table II). This points to distinct active sites present in the enzyme. The deduced amino acid sequence of the 22.6 kd subunit 11 of proteinase yscE encoded by the *PRE1* gene does not exhibit any homology to the chymotrypsin enzyme family or to any other proteinase family known so far.

Mutations in two genes, *PRE1* and *PRE2* lead to the same phenotype, reduction of the 'chymotrypsin-like' activity of proteinase yscE (Tables I and II). Explanations for this fact could be; (i) *PRE1* or *PRE2* code for the active site subunit responsible for the 'chymotryptic' activity, whereby the gene not coding for the active site subunit encodes a neighbouring subunit whose structural integrity is essential for the integrity of the peptide chain containing the active site; (ii) the gene not coding for the active site subunit encodes a protein designed to modify the active site subunit to render it functional; (iii) the PRE1 and PRE2 proteins each contain part of the active site carrying the 'chymotryptic' activity, which is functional only when the two subunit proteins are properly assembled.

Pre1-1 mutants are viable under conditions which do not impose stress on cells, whereas a chromosomal deletion of *PRE1* is lethal. These phenotypic differences might be due to the small residual 'chymotrypsin-like' activity of proteinase yscE remaining in *pre1-1* mutant cells, enabling these mutants to survive. However, lethality of the chromosomal *PRE1* deletion might not be caused solely by the complete absence of 'chymotrypsin-like' activity of the proteasome expected for the deletion mutant. One can also imagine a complete lack of assembly or a disintegration of the proteinase particle as a result of the absence of the PRE1 protein. This would lead to an additional knock out of the 'trypsin-like' and the 'peptidyl-glutamylpeptide hydrolysing' activities of the enzyme complex and all its *in vivo* functions. This view might be supported by the finding that the proteasome is highly sensitive to structural alterations. Dissociation of the rat liver multicatalytic proteinase using urea or low pH leads to loss of all proteolytic activity, which cannot be recovered by dialysis at neutral pH under a variety of conditions (Rivett, 1989a).

The phenotype of *pre1-1* mutant cells, sensitivity against stress factors, such as heat or canavanine, as well as the reduction of the degradation rate of short-lived and canavanyl-proteins resembles the phenotypes of cells defective in components of the ubiquitin mediated pathway of protein degradation (Finley *et al.*, 1987; Seufert and Jentsch, 1990). In this pathway ubiquitin, a protein of 76 amino acids, is covalently attached to proteins via a sequence

of catalytic steps to target those proteins for degradation (Hershko, 1988; Ciechanover and Schwartz, 1989; Jentsch *et al.*, 1990). The accumulation of ubiquitinated proteins in *pre1-1* mutant cells under the stress conditions mentioned above (Figure 7), links proteinase yscE to the ubiquitin mediated protein degradation pathway. Here, the proteasome might be directly involved in the destruction of the ubiquitin–protein conjugates formed. Two other possibilities for a function of the proteasome in relation to the ubiquitin mediated proteolysis pathway could be thought of. (i) The 'chymotrypsin-like' activity of the proteasome is responsible for processing and by this activating an ubiquitin–protein conjugate degrading enzyme. (ii) The proteasome is involved in another proteolytic pathway not dependent on ubiquitin. A block in this pathway could result in the abnormal ubiquitination of accumulated proteins, which then become destined for proteolysis via the ubiquitin dependent pathway. These additional substrates might simply jam the flow through this system or even inhibit the proteinase acting on ubiquitinated proteins.

At the moment we consider possibilities (i) and (ii) less likely, and we favour the hypothesis that the proteasome is an enzyme or part of an enzyme degrading 'physiologically' ubiquitinated proteins. This view is supported by *in vitro* experiments showing that the proteasome of higher eukaryotic cells, even though unable to degrade ubiquitinated proteins by itself, gains this property when assembling to a 26S proteinase complex (Eytan *et al.*, 1989; Driscoll and Goldberg, 1990). As such the 20S proteasome might represent the core unit of the 26S proteinase complex responsible for degradation of ubiquitinated proteins *in vivo*. Formation of this 26S complex from the 20S core and additional units within the cell might be regulated by environmental factors, as are nutrient content of growth media, temperature or amino acid analogues.

Loss solely of the 'chymotrypsin-like' activity of the proteasome leads to accumulation of ubiquitinated proteins in *pre1-1* mutant cells (Figure 7). This phenotype could be explained by the speculation that the site responsible for the 'chymotrypsin-like' activity is the first in a 'channelling' mechanism, which binds and cleaves ubiquitinated proteins after neutral/hydrophobic amino acids and thereafter delivers the peptide fragments in a tightly bound form to the other proteolytically active domains on the proteasome for complete degradation, thus avoiding diffusion of possibly poisonous peptide intermediates into the environment.

Further experiments are needed to unravel the exact mechanism of the proteasome in ubiquitin mediated proteolysis as well as its possible involvement in other cellular processes, such as the control of mRNA activity, which has been hypothesized on the basis of *in vitro* studies (Kuehn *et al.*, 1990; Scherrer, 1990).

Note added

After this work had been submitted to this journal a report of Fujiwara *et al.* (1990) appeared, describing cloning and disruption of two genes different from *PRE1* (named YC1 and YC7-α) coding for subunits of the yeast proteasome.

The *PRE1* gene shows some homology to the YC1 and YC7-α genes which is, however, less pronounced than the homology between the YC1 and YC7-α genes and respective genes of rat and *Drosophila* (Fujiwara *et al.*, 1989; Tanaka *et al.*, 1990; Tamura *et al.*, 1990; Kumatori *et al.*, 1990; Haass *et al.*, 1989, 1990). As found for the chromosomal deletion of *PRE1*, disruptions of the two other yeast proteasomal genes are lethal (Fujiwara *et al.*, 1990).

This again points to the high sensitivity of the essential function(s) of the particle towards lack of a subunit. This phenotypic behaviour makes the search for proteasome function(s) in 'null' mutants impossible. As shown in our work, mutant proteasomal proteins keeping the structural integrity of the enzyme complex mainly intact are powerful tools to uncover its cellular function(s).

Materials and methods

Manipulation of microorganisms

For growth of yeast cells, mating, sporulation, tetrad dissection and plasmid segregation standard protocols were followed (Sherman *et al.*, 1986; Ausubel *et al.*, 1987). Standard media were YPD and mineral (MV) medium (Sherman *et al.*, 1986). Transformation of yeast cells was carried out either by the alkali cation method described by Ito *et al.* (1983) or, especially for screening of plasmid libraries, by the following modification of a procedure published by Klebe *et al.* (1983). Cells from 100 ml culture in YPD medium were harvested in the early logarithmic growth phase (A_{578} = 1), washed with 20 ml of 1 M Sorbitol, 3% ethyleneglycol, 0.01 M Tris–HCl pH 8.35 and resuspended in 3 ml of the same buffer. 180 μl of DMSO were added, the suspension was carefully mixed and after division into 250 μl aliquots, slowly frozen to −70°C. For transformation 0.1–10 μg of DNA dissolved in up to 30 μl TE-buffer (10 mM Tris–HCl pH 7.5, 1 mM EDTA) was pipetted onto the surface of the frozen cells. The mixture was thawed by shaking at 37°C for 5 min and subsequently 1 ml of 40% PEG 1000, 0.2 M Tris–HCl pH 8.35 was added. After incubation at 30°C for 1 h, cells were briefly sedimented, resuspended in an appropriate volume of 0.15 M NaCl, 0.01 M Tris–HCl pH 8.35 and plated onto agar medium selective for growth of transformants.

Mutagenesis was performed with ethylmethanesulphonate as outlined by Fink (1970).

Growth and manipulation of *Escherichia coli* strains HB101 and JM109 were carried out using standard methods described in Sambrook *et al.* (1989) and Ausubel *et al.* (1987).

Yeast strains

The proteinase yscE mutant strains E81, E95 and E119 were obtained after mutagenesis of strain cl3-ABYS-86 (*MATα pra1-1 prb1-1 prc1-1 cps1-3 ura3Δ5 leu2-3, 112 his*$^-$).

Mutant strain E95 was backcrossed twice against proteinase yscE wild type cells. In a first cross strain CPS14-8A (*MATa prc1-1 cps1-3*) was used, and the resulting spore progeny was named E95-1. In the second cross one of these spore clones deficient in proteinase yscE activity, E95-1-16A (*MATa pra1-1 prc1-1 cps1-3 pre1-1 ura3Δ5*), was mated with strain 4545/III-2-2C (*MATα prc1-1*). The haploid descendents were named E95-2. Mutant strains E81 and E119 were crossed once with strain E95-2-1D (*MATa prc1-1*), producing the E81-1 and E119-1 progeny.

Strain 31, homozygously deficient in proteinase yscE activity was constructed by crossing strain E95-2-3C (*MATα pre1-1 prc1-1 ura3Δ5*) with strain E95-2-5D (*MATa pre1-1 prc1-1 ura3Δ5*). Strain 33, homozygously wild type for proteinase yscE resulted from a cross of strain E95-2-2D (*MATa prc1-1 cps1-3*) with strain E95-2-6B (*MATα prc1-1 ura3Δ5*).

The *PRE1* deletion/disruption was done in diploid strain YS18/18 (*MATa/MATα ura3Δ5/ura3Δ5 leu2-3,112/leu2-3,112 his3-11, 15/his3-11, 15*), yielding strain YS18/18ΔE1 (*MATa/MATα PRE1/pre1Δ::URA3 ura3Δ5/ura3Δ5 leu2-3,112/ leu2-3,112 his3-11,15/his3-11,15*).

Allelism of the *pre1-1* mutation with the gene designated *PRE1* was proven with strains YWH6 (*MATa/MATα pre1-1/pre1Δ::URA3 PRA1/pra1-1 PRB1/prb1-1 PRC1/prc1-1 CPS1/cps1-3 ura3Δ5/ura3Δ5 leu2-3,112/leu2-3, 112 HIS3/his3-11,15*) and YWH7 (isogenic to YWH6, except for *pre1-1/PRE1*). For their construction strain YS18/18ΔE1 was transformed with a plasmid carrying the *PRE1* and *LEU2* genes (W.Heinemeyer, in preparation) and sporulated. One spore clone each of α-mating type and prototrophic for leucine and uracil (*pre1Δ::URA3* disruptant) or prototrophic for leucine only (*PRE1* wild type) was crossed with mutant strain E95-1-2A (*MATa pre1-1 pra1-1 prb1-1 prc1-1 cps1-3 ura3Δ5 leu2-3,112*). Diploids

were selected on the basis that they had either lost all their auxotrophies or had retained only the uracil auxotrophy. They were subsequently cured of the *PRE1 LEU2* containing plasmid.

Purification of proteinase yscE

Purification of wild type and mutant proteinase yscE was done after Achstetter *et al.* (1984b).

Proteinase tests

Determination of the 'chymotrypsin-like', 'trypsin-like' and 'peptidyl-glutamyl-peptide hydrolysing' proteinase yscE activities in crude extracts [prepared by vortexing 30% (v/v) cell suspensions in 50 mM Tris−HCl pH 8.2 with equal volumes of washed glass beads for 2 min] or of purified preparations were performed as follows: (i) For substrates with *p*-nitroaniline as leaving group, a 200 μl reaction mixture in 0.05 M Tris−HCl pH 8.2 containing 10 μl of a 10 mM substrate solution in DMSO and alternatively 50 μl crude extract or 2−20 μg of purified enzyme was incubated at 30°C for 1 h. To stop the reaction, 150 μl H_2O, 120 μl 5% $ZnSO_4$ and 30 μl 0.15 M $Ba(OH)_2$ were added, the precipitate was sedimented by centrifugation and the absorbance of the supernatant was measured at 405 nm (modified after Garcia Alvarez *et al.*, 1987). (ii) For substrates with β-naphthylamine as leaving group, reaction mixtures were set up as in (i). Tests were stopped by addition of 190 μl 0.2 M Na-citrate pH 4.4, 4% Tween 20. Thereafter 10 μl of Fast Garnet GBC (Sigma 6504, 10 mM in DMSO) were added and the absorption at 546 nm was determined immediately (modified after Barrett, 1972). (iii) Cleavage of substrates with 7-amino-4-methylcoumarine as leaving group was determined as described previously (Kleinschmidt *et al.*, 1988).

For direct determination of *p*-nitroaniline releasing activity in yeast colonies cells were grown at 30°C to a colony size of ~3 mm on YPD or selective MV agar medium and then replica plated onto fresh agar plates covered with sterile filter discs (GB 002, Schleicher und Schüll). After a 24 h incubation at 30°C colonies were permeabilized by incubating the filters in chloroform for 10 min. Filters were dried and transferred to Petri dishes, overlayed with 10 ml of reagent mixture (1% Agar, 25 mM Tris−HCl pH 8.0, 300 μl of 10 mM Cbz-Gly-Gly-Leu-*p*-Nan or Suc-Phe-Leu-Phe-*p*-Nan in DMSO) and incubated for 3−5 h at 30°C. *p*-Nitroaniline released in colonies was detected by a coupling reaction with 1-naphthyl-ethylenediamine (Ohlsson *et al.*, 1986), resulting in the development of a pink colour.

SDS−PAGE and immunoblotting of extracts of canavanine labelled and heat stressed cells

For canavanine incorporation strains were grown overnight at 30°C in MV medium to early stationary phase. Thereafter the cultures were incubated for 90 min with 20 μg/ml of canavanine, cells were harvested by centrifugation, resuspended in prewarmed canavanine free MV medium and further incubated. Heat stressed cells were generated by growing strains in MV medium at 30°C up to an A_{578} of ~0.5 and subsequently shifting the culture to 38°C. For preparation of cell extracts samples of 20−40 ml culture were taken. The cells were centrifuged, washed with 0.1 M K-phosphate pH 7.4 and resuspended in the same buffer to yield a 50% (w/v) suspension. Cells were broken by vortexing with equal volumes of 95°C hot glass beads for 1 min and immediately denatured by adding two volumes of 4.5% SDS, 2.5 mM EDTA, 2.5 mM EGTA and vortexing again four times for 1 min with intermittent heating. Aliquots from the supernatants (50 μg protein) were separated by SDS−PAGE (18% gels, Laemmli, 1970) and blotted onto nitrocellulose (Towbin *et al.*, 1979). Filters (5 × 7 cm) were blocked by shaking in 0.05% Tween 20, 0.9% NaCl, 40 mM Tris−HCl pH 7.4 (coating buffer) for at least 2 h, then treated overnight with a 1/400 dilution of anti-ubiquitin protein conjugate serum in 12 ml of coating buffer. After washing twice in coating buffer peroxidase-coupled goat anti-rabbit IgG antibody was added and binding was detected by addition of 4-chloro-1-naphthol. Immunoblot controls were done with pre-immune sera.

Antibodies

Antibodies against ubiquitin−protein conjugates were generated by immunization of rabbits with SDS-denatured ubiquitin-γ-globulin crosslink products according to Hershko *et al.* (1982). The antibodies (a generous gift of A.Simeon) recognize ubiquitin−protein conjugates and also react to some extent with free ubiquitin.

Protein turnover measurements

Cells were grown at 30°C in MV medium up to an A_{578} of ~1. Thereafter cells were concentrated in fresh MV medium yielding an A_{578} of 2.5. 3 ml of the cultures were labelled with [^{35}S]methionine (60 μCi/ml culture) for 5 min. For canavanine incorporation prior to labelling cells were pre-incubated for 90 min with 20 μg canavanine per ml culture medium. For degradation measurements of proteins in heat stressed cells, prior to labelling, cultures were shifted for 4 h to 38°C. After the 5 min labelling period cells were washed twice with pre-warmed MV medium containing 5 mM non-labelled methionine (chase), resuspended in 3 ml of this chase medium and further incubated at 30°C, or, in the case of heat stressed cells, at 38°C. At different time points samples of 100 μl were taken and mixed with 10 μl of 110% TCA. After incubation at 4°C overnight and centrifugation, the radioactivity in the supernatant was determined. Radioactivity at time point zero (TCA-soluble radioactivity of pulsed, washed cells) was subtracted from each value. Percentage of degradation is given as the ratio of radioactivity liberated to the radioactivity incorporated into TCA-insoluble material at time point zero. TCA-insoluble radioactive protein was obtained and treated as described by Betz and Weiser (1976).

Gene cloning and analysis

Complementation of the *pre1-1* mutation was achieved by transforming mutant strain E95-1-16A with a yeast genomic library based on the *CEN15-URA3-ARS1* shuttle vector pCS19 (Sengstag and Hinnen, 1987). Restriction mapping of the genomic inserts found in two complementing plasmids revealed an overlapping region of ~6 kbp in length. Subcloning of common restriction fragments into the *CEN14-URA3-ARS1* shuttle vector pDP83 (D.Pridmore, unpublished) limited the complementing sequences initially to a 1.6 kbp *Eco*RI fragment and later to a 1.15 kbp *Sca*I−*Eco*RI subfragment (plasmid pDP83.E1-L8).

Sequence analysis of both strands of the major part of this fragment (Figure 2A) was carried out by the dideoxy chain termination method (Sanger *et al.*, 1977) using pDP83 based plasmid constructs containing overlapping segments out of this region. The 'universal' primer complementary to a region adjacent to the pUC18/19 derived multiple cloning site in pDP83 or, in one case, a *PRE1* sequence derived oligonucleotide were used for priming the sequencing reactions, which were performed with the Sequenase kit (Pharmacia).

For *in vitro* transcription−translation of the *PRE1* gene the *PRE1* coding region was excised from pDP83.E1-L8 as an *Eco*RI−*Bam*HI fragment and inserted into the vector pSP65, yielding pSP65.E1. For control the fragment was also inserted in opposite orientation into pSP64, yielding pSP64a.E1. Following standard protocols (Sambrook *et al.*, 1989) mRNA capped with dGpppG was synthesized by SP6 polymerase using *Bam*HI linearized pSP65.E1 as template. The control plasmid pSP64a.E1 was linearized with *Eco*RI. Translation of the mRNA into radiolabelled protein was performed in a wheat germ extract (kindly provided by H.O.Krebs, Krebs *et al.*, 1989). For analysis of the translation product in two-dimensional gel electrophoresis standard protocols were followed (O'Farrell, 1975). Separation in the first dimension was done by disc-isoelectric focussing on gel carrier Pharmalytes (Pharmacia), producing a pH gradient of 3−10, in the presence of 9 M urea and run from anode to cathode. Separation in the second dimension was done by SDS−PAGE. Proteins were stained with Coomassie Brilliant Blue.

Chromosomal deletion of the PRE1 gene

The *PRE1* coding region was deleted *in vitro* by exonuclease digestion starting from the unique *Hin*dII site located centrally in the structural gene, and the *URA3* gene was inserted between the remaining 5′ and 3′ flanking regions. For that purpose a second *Hin*dII recognition sequence located in the multiple cloning site of pSP64a.E1 was eliminated by linearizing the plasmid with *Acc*I, filling in the protruding ends and religating the blunt ends formed. The resulting plasmid pSP64b.E1 was then linearized with *Hin*dII, treated with *Bal*31 until ~300 bp of each end had been removed and ligated together with a blunt ended 1.15 kbp *Hin*dIII fragment carrying the *URA3* gene. The extent of the *PRE1* gene deletion in one of the resulting plasmids (pSP64b.ΔE1), which was chosen for further experiments, was shown by restriction analysis to comprise rather exactly the entire coding region, leaving ~200 and 350 bp of the 5′ and 3′ flanking regions intact.

The modified DNA insert was transformed as an *Eco*RI−*Bam*HI fragment into the uracil-auxotrophic diploid wild type strain YS18/18 to achieve recombination with one of the two chromosomal *PRE1* alleles (one step gene disruption, Rothstein, 1983). Ura$^+$ transformants were sporulated, the resulting four haploid spores were dissected and analysed for their ability to germinate.

Heterozygous chromosomal deletion of the *PRE1* gene in one of the transformants producing only two viable spores (YS18/18ΔE1) was verified by Southern transfer to a nitrocellulose filter of *Eco*RI digested total DNA separated on an agarose gel and subsequent hybridization with a labelled 2.1 kbp *Dra*I fragment extending into a large part of the region downstream of the *PRE1* structural gene. As a control, DNA from untransformed strain

YS18/18 was treated the same way. Labelling of the probe and detection of hybridization signals were performed with the non-radioactive digoxygenin kit from Boehringer, following the manufacturer's instructions.

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Note added in proof

The PRE1 sequence will appear in the EMBL, GenBank and DDBJ nucleotide sequence databases under the accession number X56812.