

Characterization of a Nucleus-Encoded Chitinase from the Yeast *Kluyveromyces lactis*

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Endogenous proteins secreted from *Kluyveromyces lactis* were screened for their ability to bind to or to hydrolyze chitin. This analysis resulted in identification of a nucleus-encoded extracellular chitinase (KICts1p) with a chitinolytic activity distinct from that of the plasmid-encoded killer toxin α -subunit. Sequence analysis of cloned *KICTS1* indicated that it encodes a 551-amino-acid chitinase having a secretion signal peptide, an amino-terminal family 18 chitinase catalytic domain, a serine-threonine-rich domain, and a carboxy-terminal type 2 chitin-binding domain. The association of purified KICts1p with chitin is stable in the presence of high salt concentrations and pH 3 to 10 buffers; however, complete dissociation and release of fully active KICts1p occur in 20 mM NaOH. Similarly, secreted human serum albumin harboring a carboxy-terminal fusion with the chitin-binding domain derived from KICts1p also dissociates from chitin in 20 mM NaOH, demonstrating the domain's potential utility as an affinity tag for reversible chitin immobilization or purification of alkali-philic or alkali-tolerant recombinant fusion proteins. Finally, haploid *K. lactis* cells harboring a *cts1* null mutation are viable but exhibit a cell separation defect, suggesting that KICts1p is required for normal cytokinesis, probably by facilitating the degradation of septum-localized chitin.

Chitin, a β -1,4-linked unbranched polymer of *N*-acetylglucosamine (GlcNAc), is the second most abundant polymer on earth after cellulose. It is a major component of insect exoskeletons (21), the shells of invertebrate crustaceans, and fungal cell walls (23). Chitinases are enzymes that hydrolyze the β -1,4-glycosidic bond of chitin and are encoded in the genomes of many prokaryotic, eukaryotic, and viral organisms. In the yeast *Saccharomyces cerevisiae*, chitinase plays a morphological role in promoting efficient cell separation (19). Additionally, plants express chitinases as a defense against chitin-containing pathogens. In fact, the heterologous expression of chitinase genes in transgenic plants has been shown to increase their resistance to certain plant pathogens (7, 16, 18).

Chitinases belong to either family 18 or family 19 of the glycosylhydrolases based on their amino acid sequence similarities (13). Familial differences in chitinase catalytic domain sequences reflect the different mechanisms of chitin hydrolysis that result in either retention (family 18) or inversion (family 19) of the anomeric configuration of the product (24). Most chitinases have a modular domain organization with distinct catalytic and noncatalytic domains that function independent of each other. An O-glycosylated Ser-Thr-rich region often separates the two domains and may serve to prevent proteolysis or aid in secretion of the chitinase (1). Noncatalytic chitin-binding domains (ChBDs) belong to one of three structural classes (type 1, 2, or 3) based on protein sequence similarities (12). Depending on the individual chitinase, the presence of a ChBD can either enhance (19) or inhibit (11) chitin hydrolysis by the catalytic domain. The ability of a ChBD to function independent of the catalytic domain and to selectively bind

chitin has led to the use of ChBDs as affinity tags to facilitate attachment of recombinant proteins to chitin-coated surfaces (3, 9).

Kluyveromyces lactis is an industrially important yeast due to its ability to grow to a very high cell density and abundantly secrete heterologous proteins (10, 25, 30, 31). We are interested in using *K. lactis* to secrete commercially important recombinant proteins carrying ChBD affinity tags to facilitate their capture on chitin beads. Thus, in the present study, we conducted a survey of endogenous secreted *K. lactis* proteins having chitinolytic or chitin-binding properties that would compromise such an application.

To date, the only described *K. lactis* protein having chitin-binding or chitinolytic activity is the secreted killer toxin (28), a heterotrimeric glycoprotein composed of α , β , and γ subunits encoded by the killer plasmid k1. Killer toxin is thought to aid *K. lactis* by inhibiting the growth of competitive yeasts (17, 26). Toxicity is associated with the γ subunit but is dependent upon the exochitinase activity of the α subunit because chitin deficiency (29) and the chitinase inhibitor allosamidin (6) render competitive yeasts resistant to killer toxin.

In this paper, we report identification and characterization of a second chitinase (KICts1p) secreted from *K. lactis* cells. We demonstrate that KICts1p is a nucleus-encoded endochitinase containing a family 18 catalytic domain and a type 2 ChBD. Genetic disruption of *K. lactis* *CTS1* (*KICTS1*) indicated that KICts1p is required for efficient cell separation, although it is not required for cell viability. Additionally, *K. lactis* Δ *cts1* cells produce no detectable chitinase activity, secrete no chitin-binding proteins, and are still able to grow to a high density, making this strain background suitable for production of recombinant ChBD-tagged proteins. Finally, we demonstrate the use of the ChBD derived from KICts1p as an

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TABLE 1. Yeast strains used in this study

Organism	Strain	Genotype	Source
<i>K. lactis</i>	GG799	<i>MAT</i> α [pGKI1 ⁺]	This study
<i>K. lactis</i>	PCK11	<i>MAT</i> α <i>cts1</i> ::Kan ^R [pGKI1 ⁺]	This study
<i>K. lactis</i>	PCK12	<i>MAT</i> α <i>LAC4</i> :: <i>P</i> _{LAC4} <i>HSA-BcChBD</i> [pGKI1 ⁺]	This study
<i>K. lactis</i>	PCK13	<i>MAT</i> α <i>LAC4</i> :: <i>P</i> _{LAC4} <i>HSA-KIChBD</i> [pGKI1 ⁺]	This study
<i>K. lactis</i>	CBS 2359	<i>MAT</i> α [pGKI1 ⁺ pGKI2 ⁺]	ATCC 8585
<i>K. lactis</i>	CBS 683	<i>MAT</i> α [pGKI1 ⁺ pGKI2 ⁺]	ATCC 56498
<i>S. cerevisiae</i>	BY4741	<i>MAT</i> α <i>his3</i> Δ 1 <i>leu2</i> Δ 0 <i>met15</i> Δ 0 <i>ura3</i> Δ 0	Research Genetics
<i>S. cerevisiae</i>	RG6947	<i>MAT</i> α <i>his3</i> Δ 1 <i>leu2</i> Δ 0 <i>met15</i> Δ 0 <i>ura3</i> Δ 0 <i>Octs1</i> :: <i>kanMX4</i>	Research Genetics
<i>S. cerevisiae</i>	PCSc1	<i>MAT</i> α <i>his3</i> Δ 1 <i>leu2</i> Δ 0 <i>met15</i> Δ 0 <i>ura3</i> Δ 0 <i>Octs1</i> :: <i>kanMX4</i> [pMW20- <i>KICTS1</i>]	This study

affinity tag for purification of recombinant proteins expressed in *K. lactis*.

MATERIALS AND METHODS

Yeast strains and culture conditions. Strains of *K. lactis* and *S. cerevisiae* (Table 1) were routinely cultured in YPD medium (1% yeast extract, 2% peptone, and 2% glucose) or YPGal medium (1% yeast extract, 2% peptone, and 2% galactose) at 30°C. Transformation of *K. lactis* and *S. cerevisiae* was achieved using standard methods. Transformants of *K. lactis* were selected by growth on YPD agar containing 200 μ g G418 ml⁻¹, whereas *S. cerevisiae* transformants were obtained by growth on SD medium (0.67% yeast nitrogen base, 2% glucose) or SGal medium (0.67% yeast nitrogen base, 2% galactose) containing the appropriate supplements needed to complement strain auxotrophies.

Detection and isolation of secreted *K. lactis* chitin-binding proteins. Western blotting was used to detect secreted *K. lactis* proteins that cross-reacted with a polyclonal anti-chitin-binding domain antibody (α -ChBD) raised against the chitin-binding domain derived from *Bacillus circulans* chitinase A1 (New England Biolabs, Beverly, MA). Spent culture medium was isolated from *K. lactis* GG799 cultures following 48 to 96 h of growth by centrifugation at 4,000 \times g for 10 min. Proteins in the spent medium were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) on a 4 to 20% Tris-glycine polyacrylamide gel (Daiichi Pure Chemicals Co., Tokyo, Japan) and were transferred to a Protran nitrocellulose membrane (Schleicher & Schuell, Keene, NH). The membrane was blocked overnight in phosphate-buffered saline containing 0.05% Tween 20 (PBS-T) supplemented with 5% (wt/vol) nonfat milk at 4°C and probed with α -ChBD polyclonal antibodies (1:2,000 in PBS-T containing 5% nonfat milk), followed by a horseradish peroxidase-conjugated anti-rabbit secondary antibody (1:2,000 in PBS-T containing 5% nonfat milk; KPL, Gaithersburg, Md.). Protein-antibody complexes were visualized using LumiGlo detection reagents (Cell Signaling Technology, Beverly, MA).

To isolate secreted proteins that bind chitin, *K. lactis* GG799 cells were grown in 20 ml YPD medium for 96 h. Cells were removed from the culture by centrifugation, and the spent medium was transferred to a fresh tube containing 1 ml of water-washed chitin beads (New England Biolabs) and incubated at room temperature with gentle rotation for 1 h. The chitin beads were harvested by centrifugation, washed with 10 ml of water, and resuspended in 1 ml of water. Approximately 50 μ l of protein-bound chitin beads was removed and boiled in SDS sample buffer (New England Biolabs) for 2 min to elute bound proteins, after which the mixture was microcentrifuged for 2 min to remove the chitin beads. The supernatant, containing eluted proteins, was separated by SDS-PAGE and subjected either to Western analysis with α -ChBD polyclonal antibodies or to amino-terminal protein sequencing.

Analysis of chitin-binding properties of KICTs1p ChBD. KICTs1p from 20 ml of *K. lactis* GG799 spent culture medium was bound to 1 ml of chitin beads as described above. The KICTs1p-containing beads were washed with 10 ml water and resuspended in 1.5 ml of water. Minicolumns were prepared by dispensing 100- μ l aliquots of the KICTs1p-containing beads into individual disposable columns (Bio-Rad, Hercules, CA). One milliliter (~10 bed volumes) of each of the following buffers was passed over separate minicolumns: 50 mM sodium citrate (pH 3.0), 50 mM sodium citrate (pH 5.0), 100 mM glycine-NaOH (pH 10.0), unbuffered 20 mM NaOH (pH 12.3), 5 M NaCl, and 8 M urea. Each column was then washed with 2 ml water, after which the beads were resuspended in 200 μ l of water, transferred to microcentrifuge tubes, and collected by brief centrifugation. The protein that remained bound to the chitin was eluted by boiling the beads in 50 μ l of 3 \times SDS sample buffer (New England Biolabs) for 5 min. Eluted

proteins were separated by SDS-PAGE and detected by Western analysis as described above.

A KICTs1p elution profile with various concentrations of NaOH (0 to 40 mM) was obtained in a similar manner. Aliquots (100 μ l) of chitin-bound KICTs1p were prepared as described above and were distributed into 0.45- μ m nylon Spin-X microcentrifuge spin columns (Corning Life Sciences, Corning, NY). The flowthrough was collected by microcentrifugation at 15,800 \times g for 1 min and discarded. The beads were then resuspended in 100 μ l of NaOH at each desired concentration (0 to 40 mM), and the eluates were collected by centrifugation at 15,800 \times g for 1 min. The chitinase activity in each eluate was measured as described below.

Expression of ChBD-tagged fusion proteins. To create a fusion between the ChBD of KICTs1p and human serum albumin (HSA), primers 5'-GGAAGATC TGACTCTGGGCTGTTACAAGA-3' (BglII site underlined) and 5'-ATAAG AATGCGGCCGCTAGAAAGACGACGTCGGGTTTCAAATA-3' (NotI site underlined) were used to amplify a DNA fragment encoding the C-terminal 81 amino acids of KICTs1p with Deep Vent DNA polymerase (New England Biolabs). The KICTs1p ChBD fragment was cloned into the BglII-NotI sites of the *K. lactis* integrative expression plasmid pGBN2 (New England Biolabs) to produce pGBN2-KIChBD. HSA was amplified with primers 5'-CCGCTCGAGAAAAG AGATGCACACAAGAGTGAGGTTGCT-3' (XhoI site underlined) and 5'-C GCGGATCCTAAGCCTAAGCAGCTTGACTTGC-3' (BamHI site underlined) and cloned into the XhoI-BglII sites of pGBN2-KIChBD. When integrated into the *K. lactis* genome, the resulting expression construct produced a single polypeptide consisting of the *S. cerevisiae* α -mating factor pre-pro secretion leader (present in pGBN2), HSA, and KIChBD.

A control construct that produced HSA containing a carboxy-terminal type 3 ChBD derived from *B. circulans* chitinase A1 (BcChBD) was assembled in a similar manner. Primers 5'-GGAAGATCTACGACAAATCCTGGTGTATCC GCT-3' (BglII site underlined) and 5'-ATAAGATGCGGCCGCTTATTGAA GCTGCCACAAGGCAGGAAC-3' (NotI site underlined) were used to PCR amplify BcChBD from pTYB1 (New England Biolabs). The amplified product was cloned into the BglII-NotI sites of pGBN2 to create pGBN2-BcChBD. HSA was amplified and cloned into the XhoI-BglII sites of pGBN2-BcChBD as described above.

Disruption of *KICTS1*. A PCR-based method was used to construct a linear DNA disruption fragment consisting of an *ADH2* promoter-G418 resistance gene cassette having 80 to 82 bp of *KICTS1* DNA on either end. When integrated at the *KICTS1* locus, this fragment caused replacement of DNA encoding the first 168 amino acids of KICTs1p with the G418 resistance cassette. Primers containing DNA that hybridized to the *ADH2*-G418 sequence (not underlined) and having tails consisting of the *KICTS1* DNA sequence (underlined), 5'-CCA GTAATGCAACTATCAATCATTGTGTTAAACTGGTCCACCAGAAATAC AAGATATCAAAAATTACTAATACTACCATAAGCCATCATCATATCG AAG-3' and 5'-CCAACTAGCGTATCCGGTTGGATTATTGTTTTTCGATA TCGAAATCGAAACCATCGACGACAGCAGTGTGCAATGGTCTTTCCC CGGGGTGGGCGAAGAACTCC-3', were used to amplify the disrupting DNA fragment from the *ADH2*-G418-containing vector pGBN2 using *Taq* DNA polymerase. The amplified product was used to transform *K. lactis* GG799 cells, and colonies were selected on YPD agar containing 200 μ g G418 ml⁻¹. Whole-cell PCR using a *KICTS1*-specific forward primer (5'-GGGCACAACAATGGCAG G-3'; designed upstream of the integration site) and a G418-specific reverse primer (5'-GCCTCTCCACCCAAGCGGC-3') was used to amplify an ~600-bp diagnostic DNA fragment from cells that had correctly integrated the disrupting DNA fragment at the *KICTS1* locus. Of 20 transformants tested in this manner, 2 *cts1* *K. lactis* strains were identified and characterized further.

Heterologous expression of *KICTS1* in *S. cerevisiae*. To express *KICTS1* in *S. cerevisiae*, the gene was PCR amplified with primers 5'-GGCGGATCCGCCACCATGTTTACCCTCGTTTACTT-3' (BamHI site underlined) and 5'-ACATGCATGCCTAGAAGACGACGTCGGGTTTCAA-3' (SphI site underlined) and cloned into the BamHI-SphI sites of pMW20 (32) to place expression of *KICTS1* under the control of the galactose-inducible, glucose-repressible *S. cerevisiae* *GAL10* promoter. This expression construct was introduced into *S. cerevisiae* Δ *cts1* (RG6947) cells to produce strain PCScl. To induce production of KICts1p, 2-ml starter cultures were grown in SD medium containing 20 μ g uracil ml⁻¹ overnight at 30°C, after which 1 ml of each culture was used to inoculate 20-ml YPD medium and 20-ml YPGal medium cultures. Each culture was grown overnight with shaking at 30°C prior to analysis of the spent culture medium for chitinase production and cell morphology by microscopy.

Chitinase activity measurements. The following chitin oligosaccharides containing one to four GlcNAc residues and derivatized with 4-methyl umbelliferone (4-MU) were used as substrates: 4-methylumbelliferyl *N*^l-acetyl- β -D-glucosaminide (4MU-GlcNAc), 4-methylumbelliferyl *N*^l,*N*^{ll}-diacetyl- β -D-chitobioside (4MU-GlcNAc₂), 4-methylumbelliferyl *N*^l,*N*^{ll},*N*^{lll}-triacetyl- β -D-chitotriose (4MU-GlcNAc₃), and 4-methylumbelliferyl *N*^l,*N*^{ll},*N*^{lll},*N*^{lV}-tetraacetyl- β -D-chitotetraoside (4MU-GlcNAc₄) (Sigma, St. Louis, MO, and EMD Biosciences, San Diego, CA). Chitinase activity was determined by measuring the release of 4-MU using a Genios fluorescent microtiter plate reader (Tecan, Maennedorf, Switzerland), a 340-nm excitation filter, and a 465-nm emission filter at 37°C. The 100- μ l reaction mixture in each well of 96-well black microtiter plates contained 50 μ M substrate, 1 \times McIlvaine's buffer (the pH ranged from 3 to 7 in different experiments), and 5 to 10 μ l of sample. The initial rates of release were recorded, and enzyme activity was expressed as pmol of 4-MU released min⁻¹. Standard curves for 4-MU (Sigma) were prepared under conditions used for the reactions for conversion from fluorescent units.

Microscopy. Approximately 1 to 2 optical density units (600 nm) of cells was harvested and fixed in 1 ml of 2.5% (vol/vol) glutaraldehyde on ice for 1 h. Cells were washed twice with water and resuspended in approximately 100 μ l mounting medium (20 mM Tris-HCl, pH 8.0, 0.5% *N*-propylgallate, 80% glycerol). In septum-staining experiments, calcofluor white (fluorescent brightener 28; Sigma) was added to the mounting medium to a final concentration of 100 μ g ml⁻¹. Cells were viewed with a Zeiss Axiovert 200 M microscope using light-phase Normaski imaging or fluorescent 4',6'-diamidino-2-phenylindole (DAPI) filter settings.

Cellular chitin measurements. Cells were extracted with KOH, and the chitin in the alkali-insoluble material was hydrolyzed to GlcNAc with chitinase for quantification by a micro Morgan-Elson assay as previously described (5).

RESULTS

Identification of KICts1p. A polyclonal antibody raised against the *B. circulans* Chi1A chitin-binding domain was used in a Western blotting analysis of *K. lactis* GG799 spent culture medium in an effort to identify native secreted *K. lactis* proteins that contain a cross-reacting chitin-binding domain. Lane 1 in Fig. 1 shows that three secreted proteins with molecular masses of approximately >200, 85, and 50 kDa cross-reacted with the α -ChBD antibody. To test if any of these proteins was able to bind chitin, spent culture medium was mixed with chitin beads for 1 h at room temperature. Proteins that bound to the chitin beads were eluted by boiling the beads in SDS-PAGE sample buffer. Western blotting indicated that only the 85-kDa α -ChBD cross-reacting protein was able to bind chitin (Fig. 1, lane 2). Protein purified directly from chitin beads in this manner was subjected to amino-terminal protein sequencing, which resulted in identification of the first 20 amino acids of the mature protein (FDINAKDNVAVYWGQASAAT). A tBLASTn search using this amino acid sequence as the query to probe sequence databases identified a partially sequenced *K. lactis* gene having a translation sequence that exactly matched the query sequence and that had significant homology to the *S. cerevisiae* extracellular chitinase Cts1p (ScCts1p).

Cloning *KICTS1* and sequence analysis. At the onset of this work, the complete sequence of the *K. lactis* genome had not

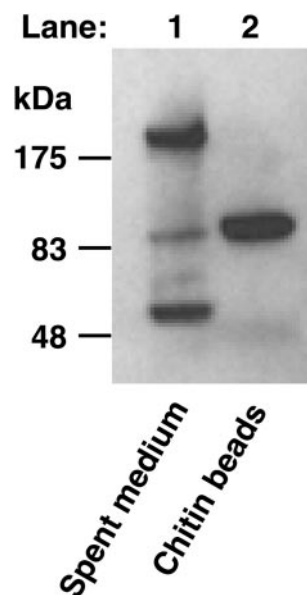


FIG. 1. Identification of a secreted 85-kDa *K. lactis* chitin-binding protein. Secreted proteins in 10 μ l of *K. lactis* GG799 spent culture medium (after 96 h of growth) were separated by SDS-PAGE and screened for the presence of a chitin-binding domain by Western blotting with α -ChBD antibodies (lane 1). Secreted proteins were incubated with chitin beads, after which chitin-bound proteins were eluted by boiling, separated by SDS-PAGE, and detected by α -ChBD Western blotting (lane 2).

been reported yet. Therefore, we used a combination of Southern hybridization and anchored PCR (data not shown) to clone the remainder of a partial *KICTS1* sequence originally identified by database searches with the tBLASTn algorithm (see above). Our KICts1p sequence is identical to the translated product of *K. lactis* open reading frame KLLA0C04730g in the recently reported *K. lactis* genome sequence (8).

KICTS1 encodes a protein that is 53% identical and 82% similar to the *S. cerevisiae* Cts1p chitinase and has a similar modular domain organization consisting of a signal peptide, a catalytic domain, a Ser-Thr-rich domain, and a chitin-binding domain (Fig. 2). Signal P software (22) predicted the presence of a signal peptide that is cleaved after A¹⁹, in agreement with the amino-terminal protein sequence determined from purified secreted KICts1p that begins at F²⁰ (Fig. 2). The KICts1p catalytic domain showed significant homology to family 18 chitinases (12, 13), including hevamine from the rubber tree and *S. cerevisiae* Cts1p (Fig. 2). Additionally, SMART domain prediction software (20, 27) indicated the presence of a C-terminal type 2 chitin-binding domain containing six conserved cysteine residues, as indicated in Fig. 2.

KICTS1 encodes a 551-amino-acid protein having a predicted molecular mass of 56 kDa, yet its apparent molecular mass after separation by SDS-PAGE is 85 kDa. In *S. cerevisiae*, the discrepancy between the predicted (60 kDa) and actual (130 kDa) molecular masses of ScCts1p has been attributed to extensive O-mannosylation of a Ser-Thr-rich domain (19). Because KICts1p contains a similar Ser-Thr-rich domain, we speculate that O-mannosylation may be present in KICts1p, but this was not tested experimentally. Additionally, the KICts1p

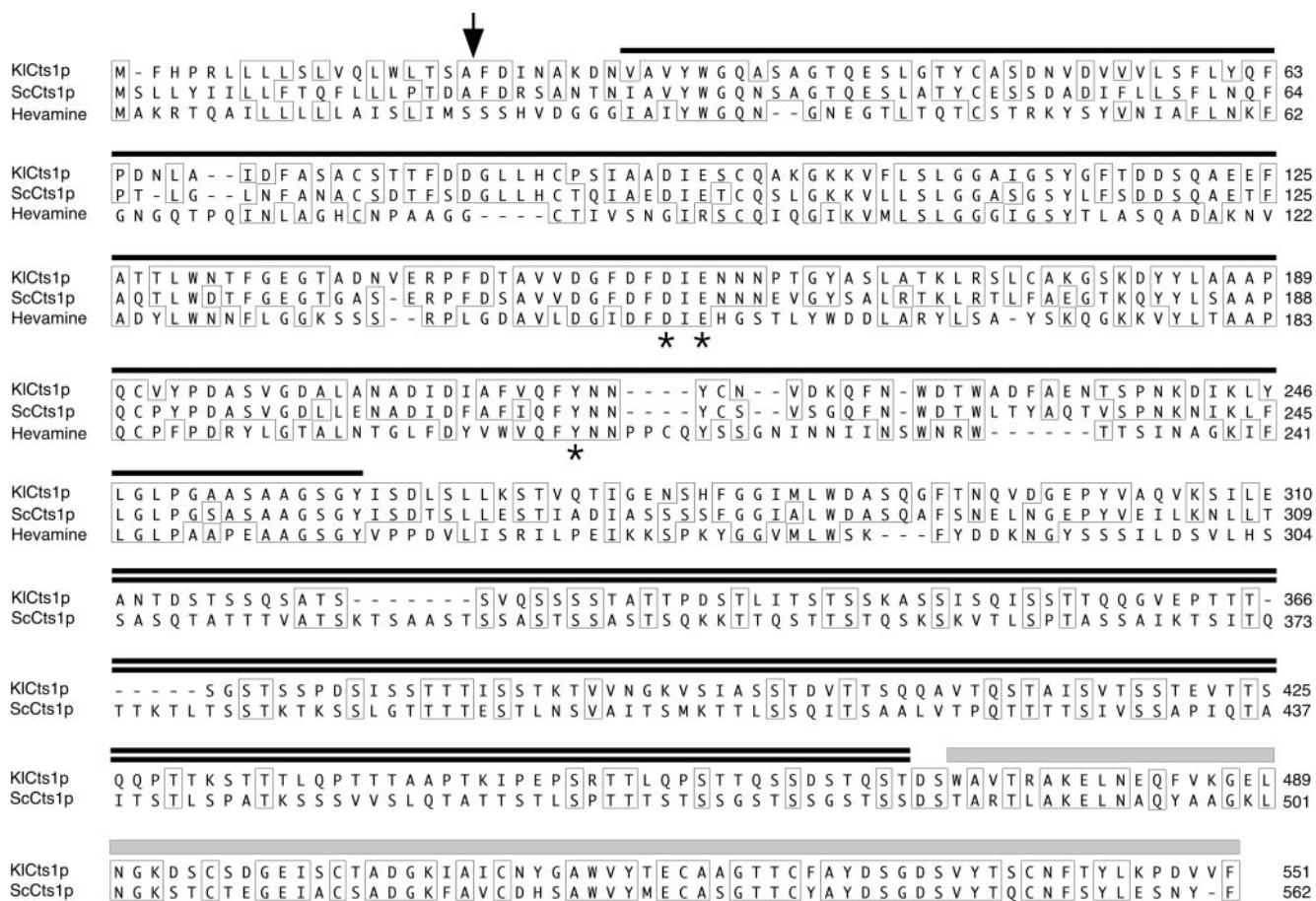


FIG. 2. Domain organization and alignment of KICts1p with family 18 chitinases. The KICts1p sequence was aligned with family 18 chitinases from *S. cerevisiae* (Cts1p; GenBank accession no. A41035) and the rubber tree, *Hevea brasiliensis* (hevamine; GenBank accession no. AJ007701). The amino acids enclosed in boxes are identical in at least two of the three sequences. KICts1p consists of a signal peptide that is cleaved after A¹⁹ (arrow), a family 18 chitinase catalytic domain (single line), a serine-threonine-rich domain (double line), and a type 2 chitin-binding domain (gray box). Conserved catalytic amino acids predicted from the hevamine crystal structure (4) are indicated by asterisks. Six cysteines enclosed in type 2 ChBDs are indicated by solid dots. Hevamine (311 amino acids) does not contain a Ser-Thr-rich domain or a ChBD. Therefore, seven amino acids were truncated from its carboxy terminus to allow alignment of the Ser-Thr-rich domains of KICts1p and ScCts1p.

protein sequence contains one predicted Asn-linked glycosylation site; however, peptide *N*-glycosidase F (PNGase F) treatment did not alter the mobility of KICts1p on an SDS-PAGE gel, indicating that it likely does not contain an Asn-linked glycan (data not shown).

Biochemical characterization of KICts1p. To define the types of chitinolytic activities produced by *K. lactis* cells, we used four 4-methylumbelliferyl-containing substrates that contained from one to four GlcNAc residues (4MU-GlcNAc₁₋₄) in enzyme assays of spent culture medium. The hydrolysis of each substrate was measured as a real-time increase in fluorescence upon enzymatic release of 4-MU. These substrates allowed us to distinguish the activity of KICts1p from the secreted exochitinase activity of the *K. lactis* secreted killer toxin and to determine if *K. lactis* cells produce a β -*N*-acetylhexosaminidase activity.

Vegetatively growing *S. cerevisiae* cells express and secrete only one chitinolytic activity (chitinase ScCts1p) and do not produce a β -*N*-acetylhexosaminidase activity. Therefore, we compared the extracellular chitinolytic activity of *S. cerevisiae*

strain BY4741 with the activities of *K. lactis* strains GG799, CBS2359, and CBS683. No activity was detected using 4MU-GlcNAc₁ as the substrate with *K. lactis* samples of either spent medium or cell homogenates at pH 4.5 or 6.0 (data not shown), indicating that *K. lactis* cells do not produce a detectable β -*N*-acetylhexosaminidase activity. Additionally, no killer toxin exochitinase activity was detectable using 4MU-GlcNAc₂ at pH 6.0 for any of the three *K. lactis* strains. While 4MU-GlcNAc₂ is the only substrate utilized by the killer toxin exochitinase, as reported by Butler et al. (6), the assays of these workers were performed with microgram amounts of purified killer toxin. Therefore, the *K. lactis* strains that we examined likely secrete insufficient amounts of killer toxin to be assayed directly from unconcentrated medium. Conversely, chitinolytic activity was readily detectable in all three *K. lactis* strains at pH 6.0 using both 4MU-GlcNAc₃ and 4MU-GlcNAc₄ as substrates. Furthermore, this activity was completely absent from the spent culture medium of *K. lactis* GG799 cells harboring a Δ cts1 null mutation (strain PCK11), indicating that it was entirely attributable to the KICts1p chitinase.

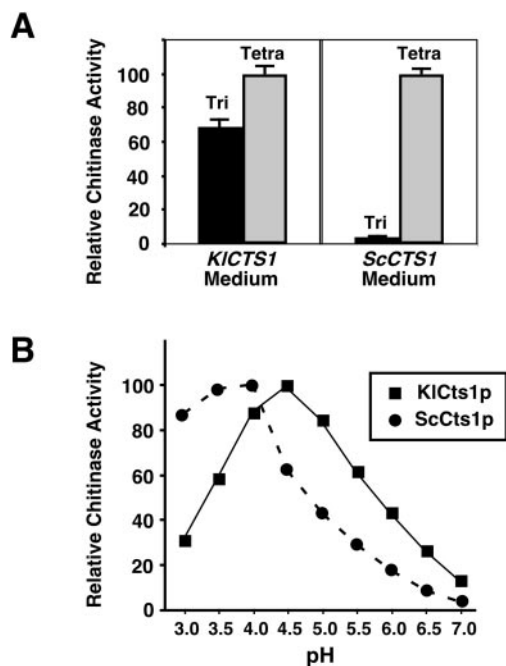


FIG. 3. Enzymatic properties of secreted KICts1p. (A) Substrate preferences of KICts1p and ScCts1p. Spent culture media from cultures of *K. lactis* GG799 and *S. cerevisiae* BY4741 cells were incubated with 50 μ M 4MU-GlcNAc₃ (Tri) (black bars) and with 50 μ M 4MU-GlcNAc₄ (Tetra) (gray bars) at pH 4.5 and 37°C. The relative rates of 4-MU release for each reaction were measured. (B) pH optima of KICts1p and ScCts1p. The relative rates of 4-MU release were measured at 37°C for KICts1p using 50 μ M 4MU-GlcNAc₃ and for ScCts1p using 50 μ M 4MU-GlcNAc₄ in McIlvaine's buffers with pHs ranging from 3.0 to 7.0.

Given the similarity in protein sequence between KICts1p and ScCts1p, we compared the catalytic properties of the two proteins. Because *S. cerevisiae* Cts1p has an acidic pH optimum (19), pH 4.5 was initially chosen for defining the substrate preferences of KICts1p. As shown in Fig. 3A, chitinases from both yeasts hydrolyzed both 4MU-GlcNAc₃ and 4MU-GlcNAc₄. However, the *K. lactis* chitinase differed from ScCts1p in the extent to which it preferred 4MU-GlcNAc₄ to 4MU-GlcNAc₃. Results identical to those shown for *K. lactis* strain GG799 were observed for chitinase secreted from strains CBS2359 and CBS683 (data not shown). Additionally, KICts1p showed maximum activity at pH 4.5, which was approximately 0.5 pH unit more alkaline than the pH for maximum ScCts1p activity (Fig. 3B).

KICts1p ChBD-chitin affinity analysis. The association of KICts1p and chitin beads was examined under a range of conditions. Figure 4A shows that the chitin association of KICts1p was stable in 5 M NaCl and in buffers at pH 3, pH 5, and pH 10. In 8 M urea, which are conditions that normally denature proteins, only about 60% of chitin-bound KICts1p dissociated from the chitin beads. However, complete dissociation was observed in 20 mM NaOH at pH 12.3. An elution profile of chitin-bound KICts1p revealed that an equal amount of the protein eluted in the first two fractions (including the void volume), suggesting that destabilization of KICts1p-chitin association occurs immediately in 20 mM NaOH (Fig. 4B). Sur-

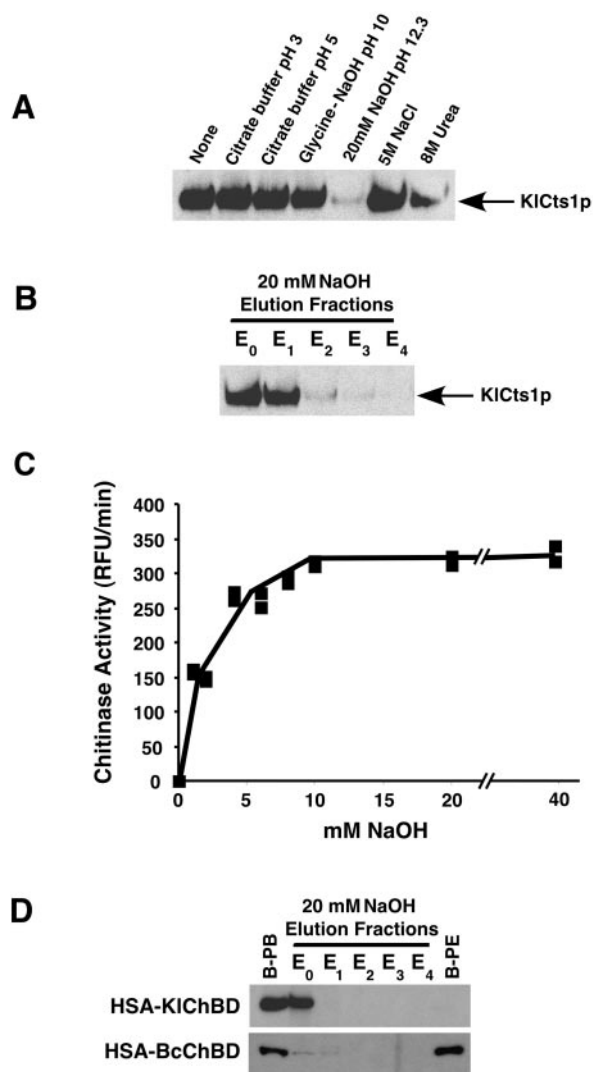


FIG. 4. KICts1p dissociates from chitin at high pH. (A) KICts1p was bound to chitin beads in minicolumns as described in Materials and Methods. The solutions were passed over separate minicolumns, after which KICts1p that was still chitin bound was eluted by boiling, separated by SDS-PAGE, and detected by α -ChBD Western blotting. (B) KICts1p elution from chitin with 20 mM NaOH. Chitin-bound KICts1p was eluted in five successive 1-ml fractions of 20 mM NaOH (E₀ to E₄, where E₀ represents the column void volume), separated by SDS-PAGE, and detected by α -ChBD Western blotting. (C) Base-eluted KICts1p retains chitinolytic activity. Chitin-bound KICts1p was eluted from chitin with various concentrations of NaOH, and the eluates were assayed for chitinase activity with 4MU-GlcNAc₃ at pH 4.5 and 37°C. RFU, relative fluorescence units. (D) KICts1p-ChBD can function as an elutable affinity tag. HSA fusions to ChBDs derived from KICts1p or *B. circulans* ChiA1 were secreted from *K. lactis*, bound to chitin beads, and eluted with five 1-ml 20 mM NaOH fractions (E₀ to E₄). Proteins that were bound to chitin beads prior to elution (B-PB) or that were still bound after elution with 20 mM NaOH (B-PE) were eluted by boiling. All samples were separated by SDS-PAGE and examined by α -ChBD Western blotting.

prisingly, KICts1p eluted in this manner retained chitinolytic activity (Fig. 4C). In fact, measurement of chitinase activity before chitin binding and after elution with 20 mM NaOH showed that nearly 100% of the chitinase activity was recovered (data not shown).

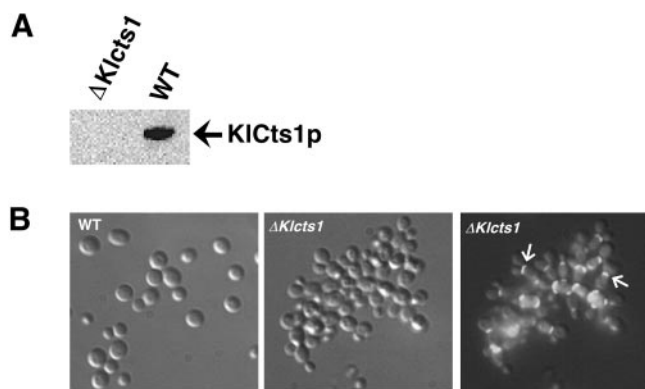


FIG. 5. (A) *K. lactis* $\Delta cts1$ cells do not secrete KICTs1p. Proteins in spent culture medium from wild-type GG799 (WT) and $\Delta cts1$ *K. lactis* cells were incubated with chitin beads. Bound proteins were eluted by boiling and were detected by α -ChBD Western blotting. (B) *KICTS1* is required for efficient cell separation. Wild-type and $\Delta cts1$ *K. lactis* cells were grown in YPD medium and fixed in 2.5% glutaraldehyde. Septum-localized chitin was stained with calcofluor white and detected by fluorescence microscopy using a DAPI filter. The middle and right panels show the same cells visualized by phase-contrast and fluorescence microscopy, respectively. The arrows in the right panel indicate the locations of septa in certain cells.

To determine if the KICTs1p chitin-binding domain could function (i) independent of the KICTs1p catalytic domain and (ii) as an affinity tag on heterologously expressed proteins, HSA containing a C-terminal fusion to the ChBD derived from amino acids 470 to 551 of KICTs1p (KICChBD) was secreted from *K. lactis* strain PCK13. For comparison, HSA was also fused to the *B. circulans* chitinase A1 type 3 ChBD (BcChBD) and secreted from *K. lactis* strain PCK12 in the same manner. ChBD fusion proteins were bound to chitin beads as described in Materials and Methods, and their chitin affinities in the presence of 20 mM NaOH were determined. Figure 4D shows that the HSA-KICChBD fusion protein fully dissociated from chitin beads in 20 mM NaOH, whereas the HSA-BcChBD fusion protein remained bound to chitin even after extensive washing with 20 mM NaOH. These results indicate that KICChBD functions independent of the KICTs1p catalytic domain and that its dissociation from chitin in 20 mM NaOH is an intrinsic property. Furthermore, these data raise the possibility that KICChBD could be used as an elutable affinity tag for purification or reversible chitin immobilization of alkaliphilic or alkali-tolerant proteins.

Disruption of *KICTS1*. To examine the in vivo function of KICTs1p in *K. lactis*, the *KICTS1* allele was disrupted in haploid cells. A PCR-based method was used to assemble a DNA disruption fragment containing a kanamycin-selectable marker cassette as described in Materials and Methods. This fragment was used to transform *K. lactis* cells to G418 resistance. Transformants were screened by whole-cell PCR to obtain transformants that had the disrupting DNA fragment integrated at the *KICTS1* locus. Of 20 colonies tested, 2 had the disrupting DNA fragment correctly integrated (data not shown), indicating that *KICTS1* is not essential for the viability of *K. lactis*. Additionally, *K. lactis* $\Delta cts1$ cells (strain PCK11) did not secrete chitinase, as demonstrated by the absence of KICTs1p (Fig. 5A) and chitinolytic activity in spent culture medium.

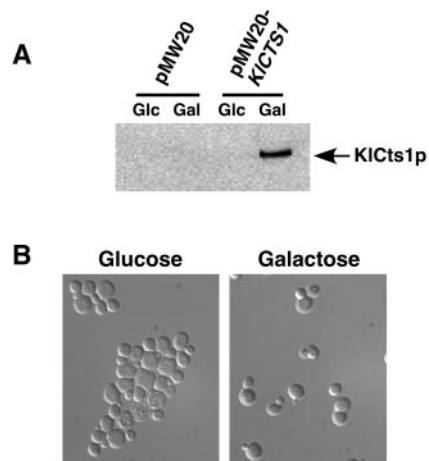


FIG. 6. (A) *S. cerevisiae* $\Delta cts1$ cells expressing *KICTS1* secrete KICTs1p. *S. cerevisiae* $\Delta cts1$ cells harboring either an empty vector (pMW20) or a vector containing *KICTS1* under the control of a galactose-inducible glucose-repressible promoter (pMW20-*KICTS1*) were grown in medium containing glucose or galactose overnight at 30°C. Spent culture medium was tested for the presence of KICTs1p by passage over chitin beads, elution of chitin-bound proteins by boiling, separation by SDS-PAGE, and α -ChBD Western blotting. (B) Expression of *KICTS1* corrects the aggregation phenotype of *S. cerevisiae* $\Delta cts1$ cells. *S. cerevisiae* $\Delta cts1$ cells harboring pMW20-*KICTS1* were grown in medium containing galactose or glucose. Cells were fixed in 2.5% glutaraldehyde and visualized by phase-contrast microscopy. Cells harboring an empty vector maintained an aggregation phenotype regardless of the carbon source (data not shown).

We also examined the growth and cell morphology of *K. lactis* wild-type strain GG799 and $\Delta cts1$ cells. In YPD medium, the $\Delta cts1$ strain grew as small clusters of loosely clumped cells that were easily dispersed into single cells by brief sonication. Fluorescence microscopy of cells stained with the chitin-binding dye calcofluor white showed that $\Delta cts1$ cells were joined via their septa (Fig. 5B, right panel), suggesting that these cells were unable to degrade septal chitin during cytokinesis. A similar phenotype has been observed for *S. cerevisiae* $\Delta cts1$ cells (19). Therefore, we tested the ability of *KICTS1* to restore normal cell separation to *S. cerevisiae* $\Delta cts1$ cells. *KICTS1* was placed under the control of the *GAL10* galactose-inducible promoter in an *S. cerevisiae* expression vector. *S. cerevisiae* $\Delta cts1$ cells expressing *KICTS1* (strain PCSc1) secreted KICTs1p in galactose-containing medium (Fig. 6A) and did not form cell aggregates (Fig. 6B). Considered together, these data suggest that *KICTS1* and *ScCTS1* encode functionally equivalent proteins that participate in cell separation, presumably by facilitating the degradation of septal chitin.

Because KICTs1p is abundantly secreted, we tested the accessibility of secreted KICTs1p to the septum by examining if exogenously added KICTs1p could abolish the cell aggregation phenotype. Purified KICTs1p was added to YPD medium prior to inoculation of *K. lactis* $\Delta cts1$ cells. After 48 h of growth, the cells were examined by microscopy. The aggregation of cells grown in the presence of exogenously added KICTs1p was alleviated but not completely ablated (data not shown). Thus, while exogenously supplied KICTs1p appeared to be able to access and cleave septal chitin, it did so less efficiently than KICTs1p that passed through the cell wall during secretion.

Finally, we examined the ability of Δ cts1 cells to grow to a high culture density. The aggregation phenotype of *K. lactis* Δ cts1 cells distorted measurements of cell density based on light absorbance at 600 nm so that they were less than 65% of those of wild-type cells. However, cultures of wild-type and Δ cts1 cells grown for 48 h produced nearly identical dry weights of cells (data not shown). Additionally, the amounts of total cellular chitin did not differ significantly for the two strains. Strain GG799 yielded 21.8 ± 1.9 nmol GlcNAc per mg (dry weight) of cells, whereas the Δ cts1 strain yielded 20.8 ± 1.0 nmol GlcNAc per mg (dry weight) of cells upon KOH extraction of cellular chitin and hydrolysis with chitinase. Therefore, despite their cell aggregation phenotype, Δ cts1 cells remained capable of achieving the same cell densities in culture as wild-type cells, suggesting that this strain background would be suitable for commercial production of ChBD-tagged proteins.

DISCUSSION

In this study, we conducted a survey of native secreted *K. lactis* proteins that are capable of binding to chitin or that have chitinolytic activity. We discovered an abundantly expressed and previously undescribed extracellular protein (KICts1p) that cross-reacted with an anti-ChBD polyclonal antibody, bound chitin with high affinity, and exhibited a chitinase activity distinct from that of the secreted killer toxin. We cloned and sequenced *KICTS1* and showed that it encodes a family 18 chitinase with a type 2 chitin-binding domain. We demonstrated that the ChBD derived from KICts1p functions independent of the catalytic domain and readily dissociates from chitin in an alkaline environment. Finally, we showed that *K. lactis* cells require KICts1p for proper cytokinesis, suggesting that cells use this protein to degrade septal chitin.

The small size (~5 to 7 kDa), the substrate binding specificity, and the high avidity of ChBDs for chitin have led to utilization of these domains as affinity tags for immobilization of proteins to chitin surfaces. For example, the *B. circulans* chitinase A1 type 3 ChBD has been used to immobilize fusion proteins on chitin beads (9) and on chitin-coated microtiter dishes (3). However, a drawback of using the *B. circulans* ChBD as an affinity tag is that its binding to chitin is generally irreversible, which limits its utility for many applications, such as protein purification. In this study, we showed that the ChBD derived from KICts1p can (i) bind to chitin in the absence of the catalytic domain, (ii) function as an affinity tag on a heterologously expressed protein in *K. lactis*, and (iii) dissociate from chitin in 20 mM NaOH, whereas BcChBD cannot do this. The latter observation likely reflects intrinsic structural differences between type 2 and type 3 ChBDs. For example, type 3 ChBDs (including BcChBD) contain conserved hydrophobic and aromatic amino acids that likely mediate their interaction with chitin (14), whereas type 2 ChBDs (including KICts1p) contain six conserved cysteine residues that mediate the tertiary structure, most likely through the formation of disulfide bridges (2). This observation also suggests that KICts1p could be used as an affinity tag for reversible immobilization or purification of alkaliphilic or alkali-tolerant proteins. Such proteins are widely used in the laundry detergent industry, where enzymes that function at a high pH (e.g., cellulases and proteases) are utilized to degrade common stains (15). Alterna-

tively, it is possible to generate KICts1p mutants that dissociate from chitin at neutral pH. The plausibility of using mutagenesis to alter the chitin-binding characteristics of a ChBD is supported by a recent study in which a mutant type 3 ChBD that requires 2 M NaCl to bind chitin was created (9).

The ideal yeast strain background for secretion of recombinant ChBD-tagged proteins would (i) produce no abundant chitin-binding proteins or chitinolytic activity that would copurify during downstream applications, (ii) be capable of achieving a high cell density in culture, and (iii) efficiently secrete recombinant proteins. Our data indicate that despite a mild growth phenotype, *K. lactis* Δ cts1 GG799 cells are capable of achieving the same cell density as wild-type cells in culture and produce no proteins with detectable chitin-binding or chitinolytic activities. Additionally, we have recently found that Δ cts1 cells retain the ability to abundantly secrete recombinant maltose-binding protein (B. Taron, P. Colussi, and C. Taron, unpublished data). Thus, the *K. lactis* GG799 Δ cts1 strain is well suited as a host background for production of recombinant ChBD-tagged proteins.

Our data suggest that KICts1p is required for proper cytokinesis of *K. lactis* cells. Disruption of *CTS1* in a haploid *K. lactis* strain produced viable cells that had a separation defect. During growth of this strain, cells remained joined together via their septa, which stained brightly for the presence of chitin. Thus, it is probable that KICts1p is required for removal of septal chitin during cytokinesis. A similar phenotype has been described for *S. cerevisiae* Δ cts1 cells (19). Therefore, we showed that *KICTS1* could restore normal morphogenesis to *S. cerevisiae* Δ cts1 cells, indicating that KICts1p and ScCts1p perform identical functions and that the two organisms likely have similar mechanisms for removing septum-localized chitin during cytokinesis.

Only a small fraction of total KICts1p activity produced by *K. lactis* cells may be required for cytokinesis. Exogenous addition of purified KICts1p to the culture medium prior to growth of *K. lactis* Δ cts1 cells only partially alleviates the cell separation defect. Thus, exogenous KICts1p may have a limited ability to access and cleave chitin in the septum. It is therefore possible that during secretion, some KICts1p is retained in the cell wall, where it may act upon cell wall chitin during cell division. In support of this notion, we found that 99% of KICts1p activity localizes to the culture medium, whereas ~1% remains associated with a cell wall fraction of lysed cells (data not shown). Thus, it is likely that efficient cytokinesis requires only limited hydrolysis of cell wall chitin by KICts1p. However, the abundance of medium-localized KICts1p may reflect a second purpose for the protein. It is possible that secreted KICts1p acts as an antimicrobial agent by digesting exposed chitin on competing fungi. Such a function was suggested by experiments in which transgenic tobacco plants expressing the *S. cerevisiae* Cts1 chitinase could inhibit spore germination and hyphal growth of the fungal pathogen *Botrytis cinerea* (7).

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