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Expression and Secretion of a *Cellulomonas fimi* Exoglucanase in *Saccharomyces cerevisiae*

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We used the yeast *MEL1* gene for secreted α-galactosidase to construct cartridges for the regulated expression of foreign proteins from *Saccharomyces cerevisiae*. The gene for a *Cellulomonas fimi* β-1,4-exoglucanase was inserted into one cartridge to create a fusion of the α-galactosidase signal peptide to the exoglucanase. Yeast transformed with plasmids containing this construction produced active extracellular exoglucanase when grown under conditions appropriate to *MEL1* promoter function. The cells also produced active intracellular enzyme. The secreted exoglucanase was N-glycosylated and was produced continuously during culture growth. It hydrolyzed xylan, carboxymethyl cellulose, 4-methylumbelliferyl-β-D-cellobiose, and *p*-nitrophenyl-β-D-cellobiose. A comparison of the recombinant *S. cerevisiae* enzyme with the native *C. fimi* enzyme showed the yeast version to have an identical K_m and pH optimum but to be more thermostable.

Cellulose is a linear polymer of 100 to 10,000 glucose residues linked by β-1,4-glucosidic bonds. Its enzymatic hydrolysis involves at least three types of cellulase, the endo-1,4-β-D-glucanases (EC 3.2.1.4.), the exo-1,4-β-D-glucanases (EC 3.2.1.91), and the β-1,4-glucosidases (EC 3.2.1.21) (for a review, see reference 20). A more detailed understanding of the mode of action of these enzymes, whether they act individually or in concert, is hindered by the difficulties associated with their preparation from the typically complex enzyme mixtures produced by cellulolytic microorganisms. Gene cloning followed by expression in noncellulolytic cells provides one solution. Heterologous expression also holds out the promise of higher cellulase yields. We undertook the expression of cellulase genes cloned from the bacterium *Cellulomonas fimi* in the yeast *Saccharomyces cerevisiae*, both as part of a program to examine the effects of heterologous expression on cellulase function and in an effort to engineer yeast strains that use cellulose as a carbon source for ethanol production.

Two *C. fimi* cellulase genes, *cex* and *cenA*, have been analyzed in detail (25, 47). The *cex* gene encodes a secreted exoglucanase (Exg) of 48.5 kilodaltons (kDa), and the *cenA* gene encodes a secreted endoglucanase (Eng) of 50.0 kDa. In each case the coding sequences exhibit an extreme codon bias, with over 98% of codons containing C or G at the third position. We have shown previously (34) that fusion on an expression plasmid of a modified version of the leader region of the yeast preprotoxin-coding sequence to a truncated form of the *cenA* gene allows for the expression of active, secreted, Eng from transformed yeast cells. In this report we describe the expression and secretion in *S. cerevisiae* of a second *C. fimi* cellulase, the Exg encoded by the *cex* gene. This was done by using one member of a set of expression cartridges that we constructed from the yeast *MEL1* gene for secreted α-galactosidase (α-Gal). The construction and use of one of these cartridges, pMV1, has been described previously (33).

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MATERIALS AND METHODS

Strains, media, and growth conditions. *S. cerevisiae* 284 (*MATα leu2-3 leu2-112 ura3-52 adel MEL1*^{0}) and 324 (*MATa adel ura3-52 gal80-disruption MEL1*$^+$) were provided by J. E. Hopper. The construction of strain 324 has been described previously (strain 21R-804-1 in reference 40). *S. cerevisiae* strains were grown at 30°C in YEPD medium (10 g of yeast extract, 20 g of peptone, and 20 g of glucose, per liter). Yeast transformed to uracil independence by plasmids carrying *URA3* were grown at 30°C in a complete minimal medium lacking uracil (ura-minimal medium; 6.7 g of yeast nitrogen base without amino acids, 20 mg of adenine, arginine, histidine, isoleucine, leucine, methionine, tryptophan, and tyrosine; 30 mg of lysine and threonine; 150 mg of valine, per liter) and buffered at pH 7.0 (6.0 g of Na_2HPO_4, 3.0 g of KH_2PO, per liter). The carbon sources added to ura-minimal medium were noninducing (20 g of sodium lactate, 30 g of glycerol, per liter), inducing (20 g of galactose per liter), or repressing (20 g of glucose per liter) for the *MEL1* promoter (28). *Escherichia coli* RR1 (19) was grown at 37°C in 2YT medium (16 g of tryptone, 10 g of yeast extract, 5 g of NaCl, per liter), which was supplemented with 100 μg of ampicillin per ml when appropriate.

Transformation. *S. cerevisiae* strains were transformed following treatment with LiCl, as described previously (11). Plasmid-treated cells were then plated onto ura-minimal medium containing 2% glucose. Bacterial cells were made competent and transformed to ampicillin resistance as described previously (18).

Plasmids. *E. coli* RR1 was used for the propagation of plasmids. The alkaline-sodium dodecyl sulfate (SDS) method described by Birnboim and Doly (1) was used for both small- and large-scale plasmid preparations. General procedures for the preparation of restriction fragments and DNA ligations were as described previously (19). Synthetic oligonucleotides were made with an instrument from Applied Biosystems. Mutagenesis with nonphosphorylated oligonucleotides was done as described previously (13). DNA sequencing was done by the chain-termination method (30). Expression plasmids pMV1, pMV2, and pMV3 were constructed as follows (Fig. 1). A 4.1-kilobase-pair (kbp) *Hin*dIII-*Bam*HI fragment from pMP550 (28), containing the

MEL1 gene, was cloned into pUC8 (44) to give plasmid pA. This plasmid was linearized by restriction with *Hin*dIII, and the ends were made flush with the Klenow fragment of DNA polymerase 1 and then recircularized in the presence of a synthetic duplex oligonucleotide containing a *Bam*HI site (linker A) to produce plasmid pB. Plasmid pB was linearized with *Sph*I to cut within the sequence encoding the signal peptide of pre-α-Gal and then recircularized in the presence of one of the three duplex oligonucleotides (linkers 1, 2, and 3). This was done to restore the native signal peptide coding sequence and to introduce an adjacent *Bgl*II site for cloning. Recombinant plasmids containing a *Bgl*II site were identified and then screened by DNA sequencing to identify those that contained linker 1, 2, or 3 in the appropriate orientation (plasmids p1, p2, and p3, respectively). These were then each restricted with *Bam*HI, and the 4.1-kbp *Bam*HI fragments (expression cartridges MC1, MC2, and MC3) were each cloned into the shuttle vector YEp24 (2) at the *Bam*HI site. This produced plasmids pMV1, pMV2, and pMV3, respectively. The reading frame at the *Bgl*II site in each plasmid is indicated in Fig. 1. Construction of plasmid pMV3-*cex*, which contained a gene fusion of the pre-α-Gal signal peptide encoded in pMV3 to the Exg sequence, is illustrated in Fig. 2. The Exg gene was a filled in, 1.8-kbp *Eco*RI-*Hin*dIII fragment from p510.507, which is pUC12 (44) containing nucleotides 851 to 2585 of the cloned *C. fimi cex* gene and its flanks (Fig. 1 in reference 25); this is flanked by the sequences 5′-AATTCCCGGG and CTGCAGCCC AAGCT-3′. Plasmid YEp24 *MEL1*, which was constructed as a control for α-Gal expression from the *MEL1* promoter in plasmid YEp24, was made by restricting plasmid pB (Fig. 1) with *Bam*HI, isolating the 4.1-kbp fragment containing *MEL1*, and inserting this into YEp24 at its unique *Bam*HI site.

Culture fractionation. Cultures of 100 ml grown to 1×10^7 to 2×10^7 cells per ml in ura-minimal medium containing galactose were centrifuged to separate the cells from the secreted proteins (extracellular fraction). The cells were washed once with 10 ml of 25 mM Tris hydrochloride (pH 8.0) containing 1 M sorbitol and 50 mM dithiothreitol and once with 1 M sorbitol, and then they were converted to spheroplasts by incubation for 4 min at 37°C in 100 mM sodium citrate (pH 8.0)–1 M sorbitol–10 mM EDTA–1 mg of zymolyase 100T per ml. Spheroplasts were separated from the periplasmic fluids (periplasm fraction) by centrifugation (4,000 rpm for 4 min in a Sorvall SS-34 rotor) and then were suspended in 9 ml of 50 mM Tris hydrochloride (pH 7.5)–5 mM EDTA–0.5% octylglucoside and vortexed for 2 to 3 min. A total of 3 ml of the lysed spheroplasts was retained (total intracellular fraction); the remainder was centrifuged (12,000 rpm for 15 min in a Sorvall SS-34 rotor), and the pellet was kept (membrane fraction). The supernatant was then centrifuged (35,000 rpm in a Ti50 rotor [Beckman Instruments, Inc., Fullerton, Calif.]) to pellet the vesicles, and the supernatant was kept (cytoplasmic intracellular fraction). The vesicles were suspended in 50 mM Tris hydrochloride (pH 7.5)–5 mM EDTA–1.0% octylglucoside and sonicated for 2 min (vesicle fraction). All fractions were then dialyzed against 50 mM Tris hydrochloride (pH 7.5)–5 mM EDTA–1 mM phenylmethylsulfonyl fluoride.

Quantitative enzyme assays. Secreted enzymes were measured routinely by centrifuging culture samples for 10 s in a microcentrifuge and then by assaying the supernatant fluids. For α-Gal, the hydrolysis of *p*-nitrophenyl-α-D-galactopyranoside to *p*-nitrophenol (pNP) was measured spectrophotometrically, as described previously (35); 1 U produced 1 μmol of pNP per min. For Exg, several assays were used. The routine assay (*p*-nitrophenyl-β-D-cellobiase [pNPCase]) measured the release of pNP from *p*-nitrophenyl-β-D-cellobiose (pNPC); 1 U of pNPCase produced 1 μmol of pNP per min at 37°C and pH 6.8 to 7.0. Carboxymethyl cellulase activity was measured by production of reducing sugars from low-viscosity carboxymethyl cellulose (CMC) (21, 22), as described previously (34). Xylanase activity was measured by the production of reducing sugars from 0.66% xylan at pH 7 and 37°C by using the 3,5-dinitrosalicylic acid reagent (21) and D-glucose as a standard. One unit produced 1 μmol of reducing sugar per min. β-Glucosidase was assayed by measuring the production of pNP from *p*-nitrophenyl-β-D-glucopyranoside (pNPG), as described above for the pNPCase assay.

Qualitative enzyme assays. Exg production by yeast colonies was scored by growing cells at 30°C on agar plates containing ura-minimal medium, 2% glucose, and 100 μM 4-methyl umbelliferyl-β-D-cellobiose (MUC). The appearance of fluorescence under UV light indicates the removal of the cellobiose with production of 4-methylumbelliferone (43). Carboxymethyl cellulase activity of the type typically displayed by endo-1,4-β-D-glucanases was scored by growing cells on agar plates supplemented with high-viscosity CMC and then staining the plates with Congo red (38, 48), as described previously (34).

Determination of K_m. Hydrolysis of CMC (low-viscosity grade; nominal degree of polymerization, 400; nominal degree of substitution, 0.7; Sigma Chemical Co., St. Louis, Mo.) at 30°C in 50 mM sodium citrate (pH 6.8) was determined by using *p*-hydroxybenzoic acid hydrazide (15) with reference to a glucose standard curve. Hydrolysis of pNPC at 30°C in 50 mM potassium phosphate (pH 7.0) was measured spectrophotometrically by determining the A_{400} of pNP. Reaction rates were measured at 8 to 10 substrate concentrations in the range of 0.2 to 10 K_m. Data were fitted to the normal form of the Michaelis-Menten equation by the method described by Wilkinson (46).

Protein analysis. Samples were dialyzed thoroughly before protein analysis. Protein concentrations were determined by the method described by Bradford (3) by using a kit supplied by Bio-Rad Laboratories, Richmond, Calif. For deglycosylation with endoglycosidase H (37), samples were incubated at 37°C for 2 h in 0.1 M 2-mercaptoethanol–10 mM Tris hydrochloride (pH 8) and then boiled for 10 min, adjusted to contain 10 mM citrate-phosphate buffer (pH 5.1), and incubated overnight at 37°C with 1.25 mU of enzyme per 2.5 mg of protein. Samples for electrophoresis were boiled in 0.1 M dithiothreitol–0.11 M SDS–0.04 M Tris hydrochloride (pH 6.8)–5% glycerol and then separated by SDS-polyacrylamide gel electrophoresis by the method described by Dreyfuss et al. (5) by using 4% acrylamide (pH 6.8) in the stacking gel and 10% acrylamide (pH 9.1) in the resolving gel. To visualize the proteins, gels were stained with Coomassie blue. To visualize Exg activity, gels that were run with samples that were not treated with dithiothreitol or boiled were equilibrated with 16 mM phosphate buffer (pH 6.8) and then incubated for 10 min at room temperature in 21 mM pNPC. Gels were photographed promptly to record the yellow color caused by pNP production. For the display of antigens, proteins were transferred from SDS-polyacrylamide gels to nitrocellulose by electroblotting, and then they were visualized by immunoassay (ProtoBlot Immunoassay System; Promega Biotech). The first antibody used was rabbit anti-Exg, which was raised against enzyme that was expressed in *E. coli*. The second antibody was the anti-rabbit

immunoglobulin G-alkaline phosphatase conjugate that was supplied with the assay kit.

RNA analysis. Yeast cells grown in ura-minimal medium were harvested, suspended in a 0.1 volume of water, and extracted for 30 min at 65°C in 20 mM Tris hydrochloride (pH 7.5) containing 50% phenol, 10 mM EDTA, and 1% SDS (29). The aqueous phase was extracted twice with phenol-chloroform-isoamyl alcohol (24:24:1) and twice with phenol-chloroform (24:1), and then the RNA was precipitated with ethanol. Samples were electrophoresed in formaldehyde-agarose gels as described previously (19), blotted onto nylon membranes (GeneScreen), and then analyzed for specific sequences by using nick-translated probes. Commercial RNA size markers (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) were used to measure RNA lengths. The DNA used to prepare the probe for Exg mRNA was the 1.8-kbp *Eco*RI-*Hin*dIII piece from plasmid 510.507 (see above).

Enzymes and chemicals. Congo red, *p*-nitrophenyl-α-D-galactopyranoside, pNPC, pNPG, xylan (oat spelts, practical grade), CMC, and MUC were from Sigma. The xylan was suspended in water, centrifuged, and then dialyzed exhaustively against water before use. Restriction enzymes, T4 DNA ligase, and DNA polymerase (the Klenow fragment) were from Pharmacia Fine Chemicals, Piscataway, N.J., or Boehringer Mannheim Biochemicals, Indianapolis, Ind. Low-melting-point agarose was from International Biotechnologies Inc., New Haven, Conn. Endoglycosidase H was from Boehringer Mannheim. Zymolyase 100T was from Miles Laboratories, Inc., Elkhard, Ind.

RESULTS

Construction of the expression cartridges. *S. cerevisiae MEL1*-based expression vectors pMV1, pMV2, and pMV3 were constructed as described above (Fig. 1). They each contained a 4.2-kbp *Bam*HI fragment, which was the expression cartridge, which was derived from a cloned DNA fragment containing the complete *MEL1* gene for yeast pre-α-Gal and its flanking sequences. As such, they each should have contained all of the *cis*-located signals that are necessary for the normal, regulated expression of α-Gal, including 5′ promoter sequences, the upstream activation sequence UAS_M (16, 35), and 3′ transcription termination and polyadenylation signals. In each case two deliberate sequence modifications were made during cartridge construction. First, an additional flanking *Bam*HI site was created, to enable facile cartridge transfer between alternative yeast vectors that contained a cloning site that was compatible with *Bam*HI. Second, a *Bgl*II site was inserted next to the sequence that encoded the amino-terminal signal peptide or the presequence of pre-α-Gal (Fig. 1). Appropriate gene fusions at this *Bgl*II site would then create translational fusions of the yeast signal peptide to the heterologous polypeptide, facilitating secretion of the polypeptide. By leaving all of the *MEL1* sequence 5′ to the Gly_{18} codon unaltered in the cartridges, we hoped to minimize disturbance to the DNA and RNA structures that are important for normal α-Gal expression. After the *Bgl*II cloning site, each cartridge contained a repeat of the codons for the C-terminal region of the α-Gal signal peptide, the structural gene for mature α-Gal, and then the 3′-untranslated region of *MEL1* containing the putative transcription termination and polyadenylation sites. Plasmid pMV1 has been successfully used in constructions involving the *C. fimi cenA* gene for secreted Eng (33). Plasmid pMV3 has been used to express Exg, as described below.

Construction of the α-Gal-Exg fusion. A DNA fragment containing the *cex* gene for *C. fimi* Exg was cloned into pMV3 at the *Bgl*II site of the cartridge to generate plasmid pMV3-*cex* (Fig. 2). The *MEL1.cex* gene fusion in this plasmid encodes a hybrid amino-terminal leader peptide, followed by the mature Exg protein, its translational termination codon, and then the 3′-untranslated sequences from *cex*. The hybrid leader consists of the 18-residue α-Gal signal peptide, an artifactual 6-residue sequence generated by the cloning procedures, and the 17 carboxyl-terminal residues of the 41-residue *C. fimi* pre-Exg signal peptide (Fig. 2).

Exg expression in *S. cerevisiae*. Plasmids pMV3-*cex* and pMV3 were used to transform *S. cerevisiae* 324 to uracil independence. Transformants were assayed qualitatively for production of secreted Exg by growth on agar plates containing the fluorogenic substrate MUC. Cells containing pMV3-*cex* produced fluorescence, while cells containing pMV3 did not (data not shown). Cells containing pMV3-*cex* were then grown in liquid culture and assayed at intervals for secreted Exg activity by using a quantitative assay (pNPC hydrolysis). As a useful positive control for enzyme secretion, the culture was assayed for the α-Gal encoded from the chromosomal *MEL1* gene in strain 324. Both Exg and α-Gal were produced continuously as secreted activities during culture growth (Fig. 3). There was no evidence of cell damage in these cultures; both enzymes were produced from intact cells.

Regulated expression of secreted Exg. In common with other genes involved in galactose catabolism in *S. cerevisiae*, the expression of *MEL1* is regulated at the level of transcription by the gene products of the regulatory loci *GAL3*, *GAL4*, and *GAL80* (12, 24, 28, 40, 41, 45, 49). The *GAL4* protein is absolutely required for transcription; it acts via an interaction with sequences 5′ to the transcription start site (upstream activation site). This activation is antagonized by the *GAL80* gene product unless galactose or melibiose (the inducer) is available in the culture medium. Glucose overrides *GAL4* and *GAL80* regulation, shutting down expression. The *GAL3* gene regulates the rate of response of the galactose-inducible genes to inducer (24, 41). The strain used for our initial experiments with Exg expression, strain 324, carries a normal chromosomal *MEL1* gene but a disrupted chromosomal *GAL80* gene and, consistent with the current model for *MEL1* regulation (40), expresses α-Gal at the maximal level when grown in nonfermentable carbon sources such as glycerol plus lactate, i.e., in the absence of the normal inducer galactose (Table 1). Similarly, cells of this strain that expressed Exg from the *MEL1* expression cartridge in pMV3-*cex* expressed secreted Exg maximally in the absence of galactose (transformant 165.2; Table 1). For both α-Gal and Exg, the addition of galactose reduced enzyme yield, as has been shown before (40) for α-Gal in *GAL80*-deleted strains. Glucose repression operated normally, although the absence of the functional *GAL80* gene product resulted in the rapid onset of galactose-independent expression from the *MEL1* promoter once glucose in the growth medium was exhausted. In the experiments for which the results are reported in Table 1, in which cultures derived from strain 324 were assayed in the stationary phase, there was appreciable enzyme expression in glucose-grown cultures, presumably due to glucose exhaustion.

To examine regulation in cells with a normal *GAL80* gene, expression was measured in strain 284. This strain is the wild type for *GAL4* and *GAL80* but lacks a chromosomal *MEL1*

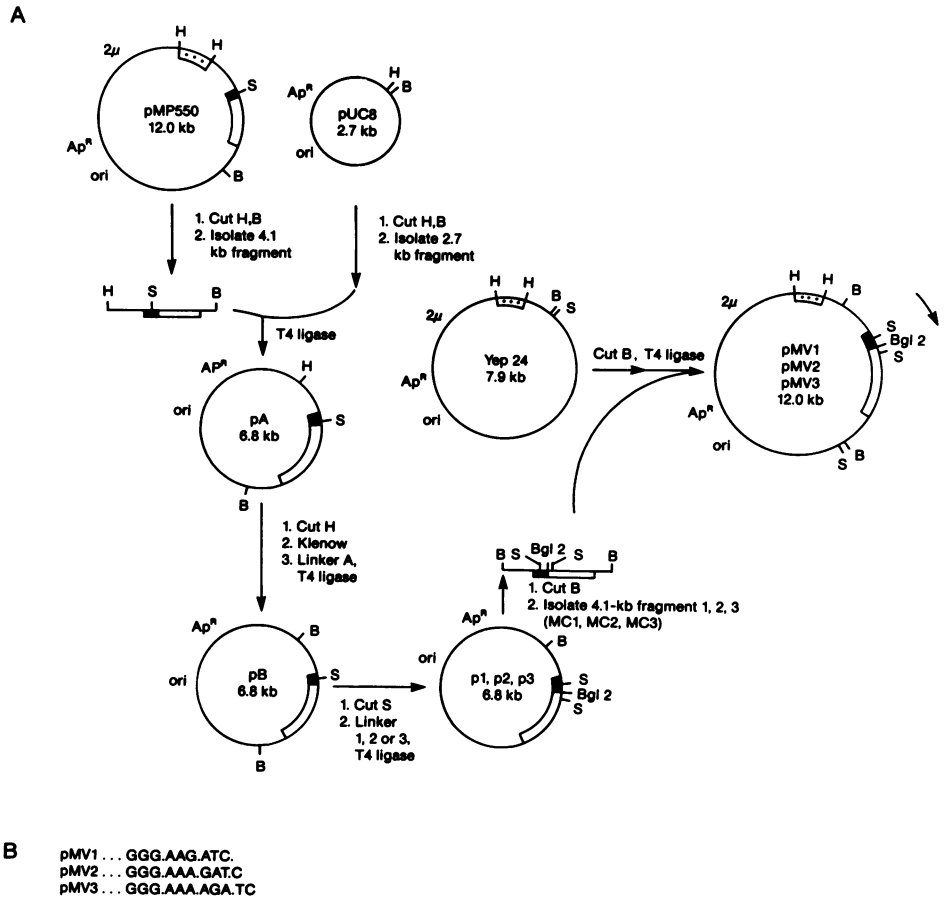

FIG. 1. Construction of expression vectors. (A) Construction. See the text for additional details.

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Linker A: 5'-CCGGATCCGG
          3'-GGCCTAGGCC
Linker 1: 5'-      CATCAGTTTGAAGGGCGTTTTTGGGAAGATCTCTGCATG
          3'-GTACGTAGTCAAACTTCCCGCAAAAACCCTTCTAGAGAC
Linker 2: 5'-      CATCAGTTTGAAGGGCGTTTTTGGGAAAGATCTGATCTCTGCATG
          3'-GTACGTAGTCAAACTTCCCGCAAAAACCCTTTCTAGACTAGAGAC
Linker 3: 5'-      CATCAGTTTGAAGGGCGTTTTTGGGAAAAGATCTGCATG
          3'-GTACGTAGTCAAACTTCCCGCAAAAACCCTTTTCTAGAC
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Key: ■, structural gene for α-Gal signal peptide; □, structural gene for mature α-Gal; →, transcription from *MEL1* promoter; ori, bacterial replication origin; Ap^r, bacterial β-lactamase gene; 2μ, fragment of the yeast 2μm plasmid containing the *S. cerevisiae* replication origin and the *REP3* gene; ▭, yeast *URA3* gene. Restriction sites are abbreviated as follows: B, *Bam*HI; H, *Hin*dIII; S, *Sph*I. (B) Reading frame at the *Bgl*II cloning site (AGATCT) in each vector. The sequence of the filled-in *Bgl*II site is shown in each case. The GGG (Gly) codon is residue 18 of the 18-amino-acid signal peptide of pre-α-Gal.

gene. As a positive control for *MEL1* promoter function in this strain, the *MEL1* gene was introduced on plasmid YEp24 (pYEp24-*MEL1*, transformant 87.2; Table 1). Exg expression in strain 284 was examined in cells that were transformed to uracil independence with plasmid pMV3-*cex* (transformant 164.1; Table 1). Results of an analysis of α-Gal and Exg expression in these cells are shown in Table 1. For both secreted enzymes, expression from the *MEL1* promoter in strain 284 was regulated in the expected way, with a low, basal level in medium containing glycerol plus lactate, a maximal level in medium containing galactose, and a low level in medium containing glucose.

This regulation in strain 284 was consistent with the transcriptional control of the *MEL1* promoter in the expression plasmid (Fig. 4). Total RNA from cells grown under noninducing, inducing, or repressing conditions was electrophoresed, blotted onto a membrane, and probed with a radioactive, Exg-specific sequence. The induced sample contained a 3.5-kb RNA species which hybridized to the probe. Since this species corresponded in length to a fusion of the 1.8-kb Exg and 1.7-kb α-Gal sequences contained in pMV3-*cex*, it appears that the mRNA both starts and stops in the positions predicted for normal *MEL1* transcriptional regulation in the expression cartridge. In a derivative of pMV3-*cex*, in which the α-Gal sequence 3′ to the *Bgl*II cloning site was deleted, Northern blot analysis showed that the RNA detected by the Exg probe was 1.8 kb long, which was again consistent with accurate transcription initiation and termination in the expression cartridge (data not shown). In summary, Exg expression from the MV3 cartridge is

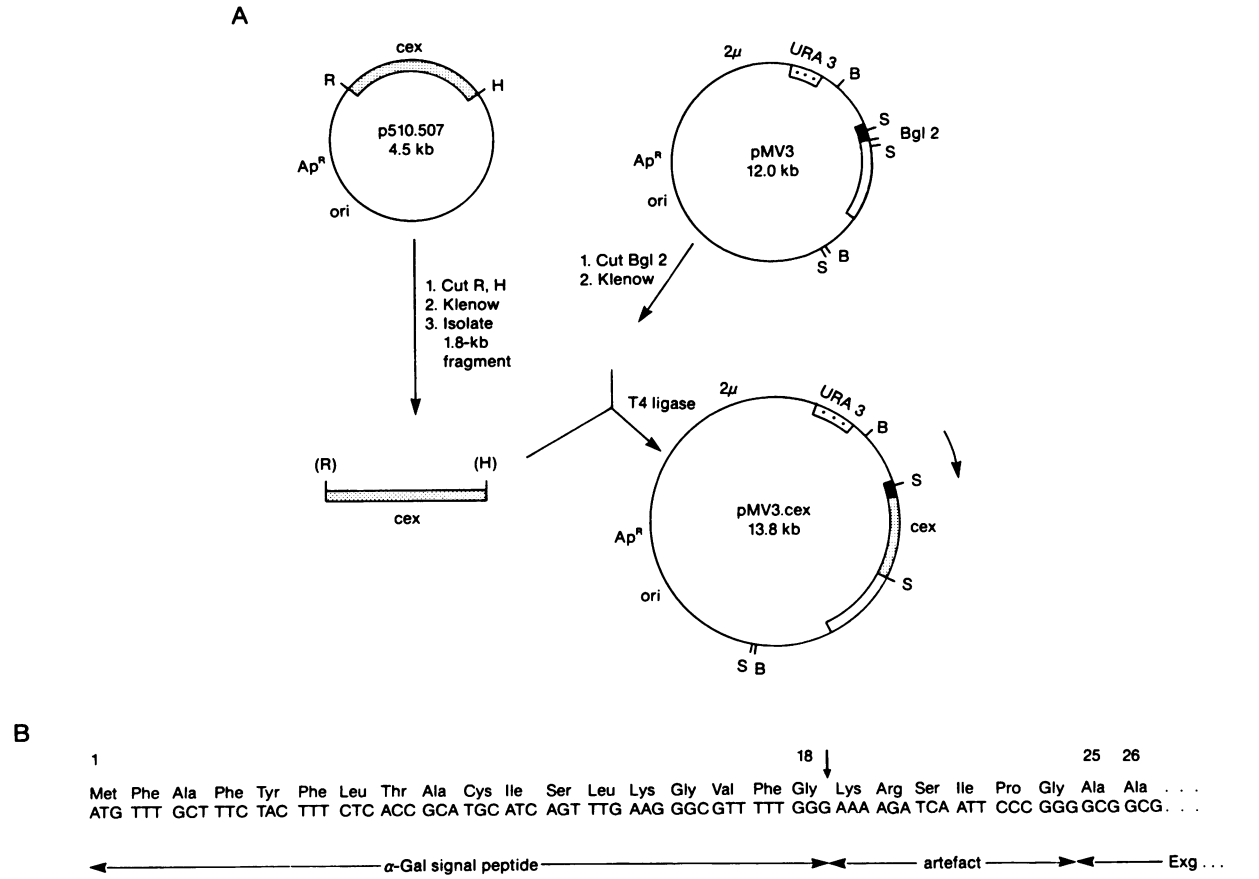

FIG. 2. Construction of the α-Gal-exoglucanase gene fusion. (A) Plasmid construction. Refer to the text for additional details. Key: ■, structural gene for α-Gal signal peptide; ▭, structural gene for mature α-Gal; →, transcription from *MEL1* promoter; ▨, *cex* gene; ▭, *URA3* gene. Other abbreviations are as defined in the legend to Fig. 1. Restriction sites are abbreviated as follows: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; S, *Sph*I. (B) Structure at the α-Gal-Exg gene fusion. The arrow shows the site of cleavage of the signal peptide from mature (secreted) α-Gal. Residues Ala_{25} and Ala_{26} correspond to residues Ala_{26} and Ala_{27}, respectively, of the 41-residue presequence of the pre-Exg protein encoded by the native *cex* gene of *C. fimi* (25).

regulated at the level of transcription, and this regulation appears to be identical to that which is characteristic of normal α-Gal expression from the natural *MEL1* gene.

Characterisation of *S. cerevisiae* Exg. (i) Enzyme localization. To determine the degree to which the plasmid-encoded Exg was being successfully directed to the culture medium by the α-Gal signal peptide in pMV3-*cex*, cultures of transformant 165.2 were fractionated and assayed for pNPCase activity. In parallel, the fractions were assayed for the α-Gal encoded by the chromosomal MEL1 gene in the host cells. Both activities were found in both the secreted (extracellular plus periplasmic) and nonsecreted fractions (Table 2). The proportion of the total recovered activity for each enzyme present in the extracellular fraction differed markedly for Exg (28%) and α-Gal (72%). The periplasmic fraction and the intracellular fractions contained about the same fraction of activity in each case. We have no ready explanation for the presence of either enzyme in the nonsecreted fractions. Unlike the case of yeast invertase in which the coding sequence allows for signal-plus and signal-minus versions of the protein, which gives rise to secreted (periplasmic) and nonsecreted (cytoplasmic) versions (26, 27), neither the α-Gal nor Exg genes appeared to allow for alternative versions of each enzyme. Presumably, translation of each protein is not strictly coupled to translocation into the endoplasmic reticulum membrane, a fraction of the translocated protein can be lost from the secretory pathway following cotranslational translocation, or the fractionation procedure used artificially created the distribution that was observed. We conclude from results of the fractionation experiments that Exg is less effectively secreted from strain 324 than is α-Gal, perhaps because of suboptimal processing of the hybrid leader peptide. Yeast cells containing plasmids in which the α-Gal signal peptide is joined precisely to the mature Exg protein produce 10 to 15 times more secreted Exg activity than did the cells examined here (R. Wong, C. Curry, D. G. Kilburn, and N. Skipper, manuscript in preparation).

(ii) Glycosylation of the secreted enzyme. Secreted yeast enzymes, whether located primarily in the periplasm (e.g., invertase, repressible acid phosphatase, [7, 17]) or primarily in the truly extracellular fluids (α-Gal [14]), are N-glycosylated during their passage through the secretory apparatus. This occurs at certain Asn residues located in the sequence Asn-X-Ser/Thr (for a review, see reference 32). These N-linked carbohydrate side chains can usually be removed in vitro by using endoglycosidase H, provided that they are accessible under the conditions used. Secreted yeast α-Gal contains eight N-linked carbohydrate side chains, one of which is difficult to remove (35). The *cex* gene sequence

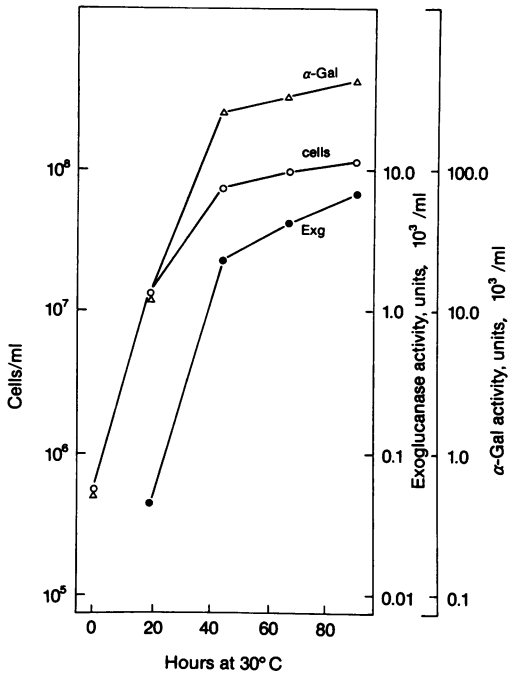

FIG. 3. Exg and α-Gal expression by growing cells. Strain 324 containing plasmid pMV3-*cex* (transformant 165.2) was grown at 30°C in ura-minimal medium containing 2% sodium lactate and 3% glycerol and then sampled at intervals for cell number (○) and centrifuged. The supernatant fluids were then assayed for α-Gal (△) and Exg (pNPCase) (●).

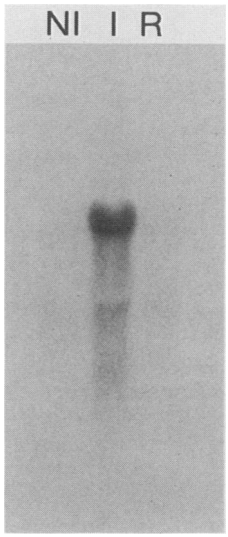

FIG. 4. mRNA analysis. Total RNA was prepared from cells of strain 284 containing plasmid pMV3-*cex* (transformant 164.1; see text and Table 3) which was grown in ura-minimal medium under noninducing (NI), inducing (I), or repressing (R) conditions. An equal amount of each RNA (50 μg) was electrophoresed under denaturing conditions in agarose and then blotted onto a membrane, probed with labeled Exg sequence, and autoradiographed. The Exg mRNA, based on migration of markers, was 3.5 kb in length.

predicts six Asn residues that are potentially suitable for N-linked glycosylation in Exg (25). Electrophoresis of the proteins secreted from yeast cells containing pMV3-*cex*, followed by an in situ activity assay for Exg in the gel, showed active material that covered a wide size range (Fig. 5A). This suggests that the secreted enzyme was extensively glycosylated. Western blots of the Exg antigen confirmed the impression generated by the activity gel (Fig. 5B). Deglycosylation with endoglycosidase H converted the heterogenous Exg proteins into a series of discrete species (Fig. 5B). We do not yet understand the reason for this particular pattern, but it may be due to different accessibility of N-linked carbohydrate side chains in the Exg to the endoglycosidase. O-linked glycosylation of Exg may also contribute to the pattern that was observed; the Exg sequence is rich in Ser and Thr residues. The smallest Exg antigen consistently detected in Western analyses of N-deglycosylated Exg was about 54 kDa, which was larger than the predicted size of nonglycosylated Exg (46.5 kDa [25]).

(iii) Substrate reactivity. When expressed in *E. coli*, the *C. fimi cex* gene produces an Exg which hydrolyzes CMC, xylan, and pNPC, but not pNPG (8, 9). The reactivity of the secreted yeast version of the enzyme toward these sub-

TABLE 1. Regulated enzyme expression

Host	Plasmid	Transformant	Carbon source[a]	Yield of secreted enzyme activity (mU/10⁸ cells)	
				Exg (pNPCase)	α-Gal
324			NI	0	200
			I	0	159
			R	0	89
324	pMV3-*cex*	165.2	NI	7.6	148
			I	5.0	112
			R	2.7	77
284	pYEp24-*MEL1*	87.2	NI	0	11
			I	0	525
			R	0	0
284	pMV3-*cex*	164.1	NI	0.3	0
			I	1.8	0
			R	0.4	0

[a] Carbon source abbreviations: NI, glycerol plus lactate; I, galactose; R, glucose.

TABLE 2. Localization of Exg and α-Gal[a]

Activity and fraction	% Recovered activity of[b]:	
	Exg (pNPCase)	α-Gal
Total activity		
Extracellular	27.4 ± 4.7	71.9 ± 6.8
Periplasmic	6.3 ± 1.1	7.8 ± 0.4
Intracellular	66.1 ± 5.5	20.3 ± 6.9
Intracellular activity		
Soluble intracellular	68.5 ± 6.8	60.5 ± 10.0
Vesicles	8.0 ± 2.5	6.9 ± 1.1
Membranes	23.5 ± 9.0	32.6 ± 11.8

[a] Cells of strain 324 containing pMV3-*cex* (transformant 165.2) were grown and fractionated as described in the text and then were assayed for β-Gal and Exg activities. The results represent the average of the analysis of three independent cultures.

[b] Activity of 100% was 1.71 ± 0.24 × 10^{-3} U/10⁸ cells for Exg and 218.2 ± 69.2 × 10^{-3} U/10⁸ cells for α-Gal.

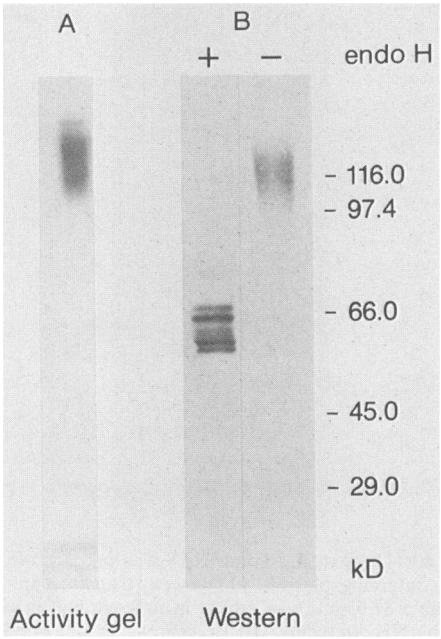

FIG. 5. Analysis of secreted yeast Exg. Strain 324 containing pMV3-*cex* (transformant 165.2) was grown in ura-minimal medium containing glycerol plus lactate. The cells were removed; and the supernatant fluids were dialyzed against water, lyophilized, and then dissolved in a small volume of water. (A) Activity gel. A sample (50 μg of protein) was electrophoresed and then assayed in situ for hydrolysis of pNPC. (B) Western blot. Samples containing equal amounts of protein (2.5 μg) were incubated under deglycosylation conditions (see text), either with or without endoglycosidase H (endo H), and then were electrophoresed, blotted, and analyzed for Exg antigens. Size markers are indicated in kilodaltons.

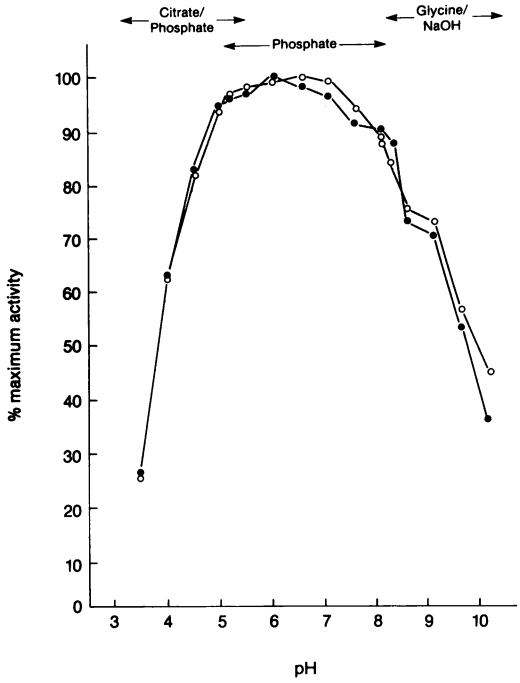

FIG. 6. Determination of pH optimum. Exg (2.2×10^{-3} U/ml) was incubated at 30°C with 12.5 mM pNPC–1 mg of bovine serum albumin per ml in 40 mM sodium citrate-phosphate (pH 3.5 to 5.5), 40 mM potassium phosphate (pH 5.2 to 8.2), or 40 mM glycine-NaOH (pH 8.0 to 10.0). Hydrolysis was stopped by the addition of sodium carbonate to 1.7 M, and pNP production was measured by determining the A_{400}. Symbols: ○, unpurified yeast secreted Exg; ●, purified *C. fimi* Exg.

strates was examined. For comparison, a sample of the *C. fimi* extracellular Eng expressed in *S. cerevisiae* from a plasmid containing the *cenA* gene (plasmid p6 [33]) was assayed in parallel. The yeast Exg, like the version expressed in *E. coli*, hydrolyzed CMC, xylan, and pNPC (Table 3). The yeast recombinant Eng, by contrast, was inactive against xylan or pNPC. Culture supernatants were also assayed for hydrolysis of pNPG, which indicates 1,4-β-glucosidase activity. All cultures, including those of untransformed cells of strain 324, contained *p*-nitrophenyl-β-D-glucopyranosidase activity (Table 3). Despite the endogenous production of this secreted apparent β-glucosidase, none of the cultures could use cellobiose as the sole carbon source. It should be noted that although both the *cex* Exg and the *cenA* Eng produced in yeast hydrolyzed low-viscosity CMC solutions to produce reducing sugars (34) (Table 3), only the *cenA* Eng produced the characteristic clearing on Congo red-stained high-viscosity CMC that is indicative of endocellulolysis.

(iv) K_m pH optimum and stability, and thermal stability. The native secreted *C. fimi cex*-encoded Exg (cellulase CB2) is a glycosylated protein; the carbohydrate side chains are entirely composed of mannose. It was of interest to compare some of the properties of the *S. cerevisiae* and *C. fimi* versions of the secreted cellulase. K_m values for the CMC and pNPC substrates for the two versions of the enzyme were very similar (values for the CMC substrate, 3.180 ± 0.214 and 3.156 ± 0.256 for *C. fimi* and *S. cerevisiae*, respectively; values for the pNPC substrate, 0.641 ± 0.027 and 0.706 ± 0.012 for *C. fimi* and *S. cerevisiae*, respectively), suggesting that substrate binding is insensitive to the type or degree of glycosylation. For pNPC hydrolysis the pH

TABLE 3. Substrates hydrolyzed by secreted yeast recombinant cellulases[a]

Yeast transformant	Cellulase gene	Sp act of secreted enzyme (U/mg of protein)				
		α-Gal	pNPCase	pNPGase[b]	CMCase[c]	Xylanase
229.1	None	67	0	1.2	0	0
165.2	*cex*	50	3	0.9	20	37
149.1	*cenA*	77	0	1.6	19	0

[a] Cultures of yeast strain 324 containing plasmid pMV3 (transformant 229.1), pMV3-*cex* (transformant 165.2), or p6 (plasmid pMV1 containing a *MEL1.cenA* gene fusion [33]) (transformant 149.1) were grown to 1×10^8 to 2×10^8 cells per ml in ura-minimal medium containing 2% galactose and centrifuged to remove cells. The supernatant fluids were dialyzed against water, lyophilized, and then dissolved in water and assayed.

[b] pNPGase, *p*-Nitrophenyl-β-D-glucopyranosidase.

[c] Compare CMC hydrolysis by the qualitative Congo red assay (transformant 149.1 ≫ transformant 165.2; transformant 229.1, no reaction).

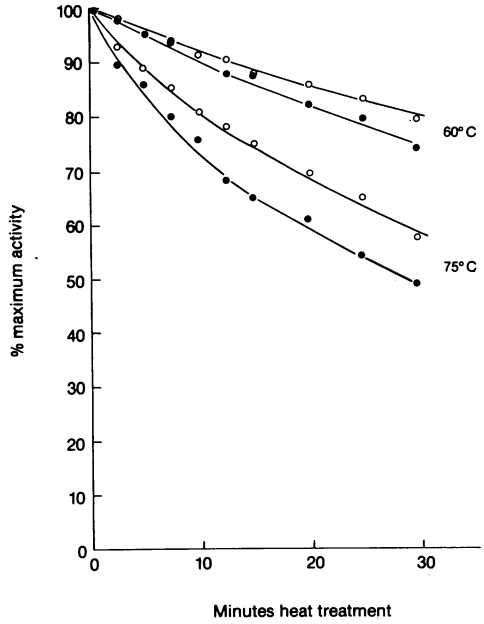

FIG. 7. Determination of thermal stability. Exg (5×10^{-3} U/ml) was incubated at the indicated temperatures in 50 mM potassium phosphate (pH 7.0)–2.5 mg of bovine serum albumin per ml and then assayed at intervals for pNPCase activity at pH 7.0, as described in the legend to Fig. 6. Symbols: ○, unpurified *S. cerevisiae*-secreted Exg; ●, purified *C. fimi*-secreted Exg.

optimum was also insensitive to the production system used (Fig. 6). Measurement of Exg stability at pH extremes showed the yeast enzyme to be more stable at pH 12 but far less stable at pH 2.0 (data not shown). Because we suspected the presence of an acid protease in the unpurified yeast samples, we included the inhibitor pepstatin A in further assays of yeast Exg at pH 2.0. This reduced the acid instability of the enzyme, such that it appeared as stable as the *C. fimi* Exg.

The thermal stability of Exg was also assessed. The yeast enzyme was consistently more stable than the *C. fimi* enzyme at both 60 and 75°C (Fig. 7). Inclusion of 2.5 mg of bovine serum albumin per ml in these experiments, to compensate for the difference in total protein concentration between the yeast and *C. fimi* samples, did not affect the relative temperature stability of the two Exg samples (data not shown). A *Trichoderma reesei* Eng is apparently also more thermostable when expressed as a secreted yeast enzyme than it is when expressed in its native form (42). Together, these results point to a useful role for yeast glycosylation in the production of more stable enzymes. Other properties, however, appear to be insensitive to the type or extent of glycosylation involved.

DISCUSSION

We have described previously (33, 34) the expression and secretion in *S. cerevisiae* of the *C. fimi cenA* Eng. In this report we have demonstrated the expression and secretion of a second member of the *C. fimi* cellulase complex, the *cex* Exg, and have examined some of the properties of the secreted enzyme. We have also shown that production of the enzyme can be controlled by factors that are known to regulate transcription from the *MEL1* promoter used in the expression cartridge. Our understanding of the elements that mediate this regulation, specifically the *GAL80* protein, the *GAL4* protein, and the activation sequences located 5′ to the transcription start site, is advancing rapidly due to intensive investigation focused on regulation of the coregulated *GAL1*, *GAL10*, and *GAL7* genes. This offers several potentially useful routes for improving the utility of *MEL1*-based expression systems. For instance, controlled overproduction of the *GAL4* activator protein may allow for increased transcriptional activity, which may result in increased cellulase yields. The availability of *GAL80*-disrupted host strains provides a means of automatic control in that cultures can be grown in glucose to achieve a high cell density, and then *MEL1* controlled expression can be turned on once the glucose is exhausted. Such a separation of biomass accumulation from product expression may be important to the maintenance of plasmid stability and maximization of product yield.

Our results, and those of others (4, 6, 10, 23, 31, 36, 39, 42), demonstrate that there is no obvious biological barrier to the expression in genetically engineered yeast cells of heterologous secreted enzymes of considerable size. Whether the yield of these secreted enzymes from laboratory yeast strains can be made sufficient for enzyme production per se remains to be established. For processes such as the utilization of cellulosic substrates, however, the ability of engineered yeast to produce active degradative enzymes in a secreted form may be very useful.

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