those of Been *et al.* (*3*). However, upon termination of the topo I reactions with SDS, butanol (rather than ethanol) was used to precipitate the reactions. The modifications of the Maxam and Gilbert (*17*) sequencing reactions described by Bencini *et al.* (*18*) were used to isolate markers. The 3′-labeled sequencing markers were dephosphorylated as follows. After butanol precipitation of the piperidine reactions, the samples were dried and resuspended in 47 μl of H_2O, 2.5 μl of 1*M* tris (*p*H 8.0), and 0.5 μl of bacterial alkaline phosphatase (International Biotechnologies Inc., 24 unit/ml). After incubation at 65°C for 60 minutes, 2 volumes of 1 percent SDS and 1 ml of butanol were used to precipitate the dephosphorylated markers. Polyacrylamide-urea sequencing gels (8 percent; 40 cm long and 8.3 mm thick) were run for various times at 1600 V.

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Secretion of a Bacterial Cellulase by Yeast

Abstract. *Gene fusions were constructed between a yeast expression plasmid and a* Cellulomonas fimi *DNA fragment encoding an endo-1,4-β-D-glucanase or carboxymethylcellulase. Yeast transformed with the recombinant plasmids secreted carboxymethylcellulase activity. Secretion of active enzyme was greatly increased when the leader of a secreted yeast protein, the K1 toxin, was inserted immediately upstream of and in frame with the bacterial cellulase sequence. This is the first step in constructing a functional cellulase complex in* Saccharomyces cerevisiae. *It also provides an excellent system for the detailed examination of the determinants of protein secretion because of the ease with which secreted cellulase can be detected.*

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The microbial utilization of crystalline cellulose as a source of glucose for growth requires a group of enzymes, the cellulases. These include an extracellular endo-1,4-β-D-glucanase and exo-1,4-β-D-glucanase and an intracellular 1,4-β-glucosidase (*1*). The glucanases are not produced by the yeast *Saccharomyces cerevisiae*, an organism widely used to ferment glucose to ethanol and carbon dioxide. We introduced a DNA sequence encoding a naturally secreted bacterial cellulase into a yeast expression vector and report that yeast transformed with the vector secreted the bacterial cellulase efficiently if a yeast secretion signal was incorporated into the construction.

The gene for an endo-1,4-β-D-glucanase (carboxymethylcellulase or CM-cellulase) has been cloned on a 5-kilobase (kb) DNA fragment from the cellulolytic bacterium *Cellulomonas fimi* (*2*). The gene is expressed in *Escherichia coli* C600 and the CM-cellulase is largely periplasmic (*2*). A subclone containing a 2.4-kb fragment behaves in a similar manner. This subclone, plasmid pEC2.2, was used for the experiments with yeast. The 2.4-kb fragment may lack the sequence encoding the NH_2-terminal region of the CM-cellulase; it is expressed as a fusion protein when inserted in the tetracycline-resistance gene of plasmid pBR322. Plasmids pOP and pN3 (Fig. 1) were used to examine CM-cellulase expression in yeast. Plasmid pOP contains a modified complementary DNA (cDNA) copy of a yeast double-stranded RNA sequence that encodes preprotoxin (*3*, *4*). The cDNA is contained between the alcohol dehydrogenase 1 promoter and the iso-1–cytochrome c terminator. Expression of the cDNA from the alcohol dehydrogenase 1 promoter in yeast has been described; the cells produce extracellular K1 toxin, and a toxin immunity determinant (Fig. 1) (*4*). Protoxin, the first detectable precursor of K1 toxin in yeast cells (*5*), enters the yeast secretory apparatus. The pre-region of preprotoxin is probably a signal peptide for cotranslational insertion of protoxin into the endoplasmic reticulum and as such might be useful for directing secretion of the CM-cellulase. The Bgl II restriction site in pOP (Fig. 1) provides a cloning site for insertion of the CM-cellulase gene a few codons downstream from this pre-region. Plasmid pN3 was a derivative of pOP in which a 0.17-kb Bal I fragment from the cDNA had been replaced by a synthetic oligonucleotide. This generated an Nco I restriction site, suitable for insertion of the CM-cellulase gene downstream from the $Lys^{232} \cdot Arg^{233}$ codons of preprotoxin (Fig. 1). Since the Tyr^{234} of preprotoxin is the NH_2-terminal residue of the β-polypeptide of secreted toxin (*5*), the $Lys^{232} \cdot Arg^{233}$ residues may specify cleavage of protoxin and so the release of the β-polypeptide or of any polypeptide substituted for β.

Figure 2 shows the structure of plasmids constructed to determine if transformed yeast cells can express CM-cellulase. From previous experiments, it was known that the translational reading frame in these constructions would put the open reading frame of the CM-cellulase in phase with the initiator ATG of preprotoxin. The CM-cellulase sequence was fused to either the first three (pL5.19), the first 52 (pK2.4), or the first 233 (pK1.3) codons of preprotoxin. In this way we could determine whether the bacterial enzyme could be secreted in the absence of a yeast leader peptide, in the presence of the preprotoxin leader peptide or pre-region, or in the presence of all the preprotoxin except for the COOH-terminal region that is the β-polypeptide (Fig. 1) of extracellular K1 toxin. In pL5.19 and pK1.3 there were four and three additional codons, respectively, between the yeast and bacterial sequences. This was due to the cloning manipulations.

Each plasmid was introduced into the tryptophan-dependent *S. cerevisiae* strains 2OB12 (*6*) and SX34-4D (*7*), by LiCl transformation (*8*). Tryptophan-independent cells were then streaked out on a selective medium containing carboxymethyl cellulose, and the colonies were assayed for extracellular CM-cellulase activity by the Congo red procedure (*9*). Each plasmid directed the synthesis of extracellular CM-cellulase (Fig. 3), although pL5.19 and pK1.3 gave much lower activity than pK2.4. Cells contain-

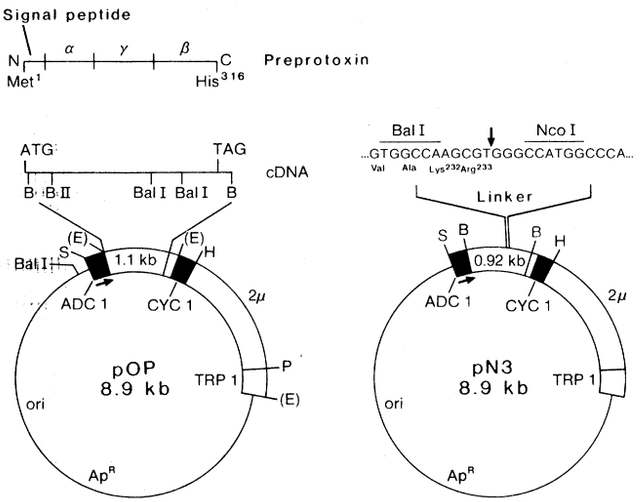


Fig. 1. Construction of *S. cerevisiae* plasmids. (a) pOP is pYCDE-2 (obtained from the Washington Research Foundation) containing a cDNA sequence that encodes the yeast preprotoxin. The 1.1-kb cDNA was obtained from plasmid pADH10A (*4*) in a 1.4-kb Hind III–Hpa I fragment. It was blunt-end ligated into the Eco RI site of pYCDE-2 so as to be flanked by the alcohol dehydrogenase 1 (ADC1) promoter (*16*) and the iso-1-cytochrome c (CYC 1) terminator (*17*). The structure of preprotoxin is indicated. It is cleaved proteolytically during maturation, giving the α and β polypeptides of extracellular K1 toxin (*5*), and the γ polypeptide, which is probably the toxin-immunity determinant (*4, 5*). TRP 1 encodes *N*-(5′-phosphoribosyl)-anthranilate isomerase (*18*). 2μ is a fragment from the yeast 2-μm plasmid (*19*). The remainder of the plasmid is from pBR322 (*20*) and encodes β-lactamase (Ap^R) and an origin of replication (ori). The arrow shows the direction of transcription from the ADC1 promoter. (b) Plasmid pN3 was obtained by digesting pOP with restriction endonuclease Bal I (which cleaved only the two sites within the preprotoxin sequence) and ligating the 8.7-kb fragment to the synthetic linker 5′-CCAAGCGTGGGCCATGGC-3′, as described (*21*). Plasmids containing the linker were identified by digestion with restriction endonuclease Nco I (5′-CCATGG-3′). Plasmid pN3 contains the linker in the orientation shown; this was confirmed by sequencing. As indicated, the linker restores the left-hand Bal I site and inserts an Nco I cloning site, which is separated by three nucleotides from the $Lys^{232} \cdot Arg^{233}$ codons of preprotoxin. The position of cleavage of the β polypeptide of toxin from the precursor (*5*) is shown by the arrow. Standard methods (*22*) were used for preparation and manipulation of plasmids and restriction fragments. The synthetic oligonucleotide linker was made using a Biosearch Sam One instrument and phosphotriester chemistry. DNA sequencing was done by either the chemical cleavage (*23*) or dideoxy chain-termination (*24*) methods. Abbreviations: B, Bam HI; BII, Bgl II; E, Eco RI sites removed by constructions; H, Hind III; P, Pst I; and S, Sph I.

ing plasmid pOP did not produce CM-cellulase (Fig. 3). Cell-free medium from cultures of each strain was assayed for its ability to produce reducing sugars from carboxymethyl cellulose (*10*), as an independent test for endoglucanase activity; only that from cells containing plasmid pK2.4 has activity sufficient to be detected by this procedure (Fig. 4). For each strain, broken spheroplasts and the periplasmic material released during spheroplast formation were devoid of CM-cellulase activity when assayed by the reducing-sugar procedure, establishing that the activity seen in the cultures (Figs. 3 and 4) was not due to cell lysis but to secretion. Antibody specific to CM-cellulase will be used to establish whether the low level of activity produced by cells containing plasmids pL5.19 or pK1.3 (Fig. 3) is due to inappropriate processing or a low level of translation. While the weak expression of plasmid pL5.19 may be attributable to the absence of an appropriate leader peptide (Fig. 1), it is not obvious to us why plasmid pK1.3, containing the same leader as pK2.4, is expressed poorly. It seems possible, however, that substitution of the CM-cellulase polypeptide for

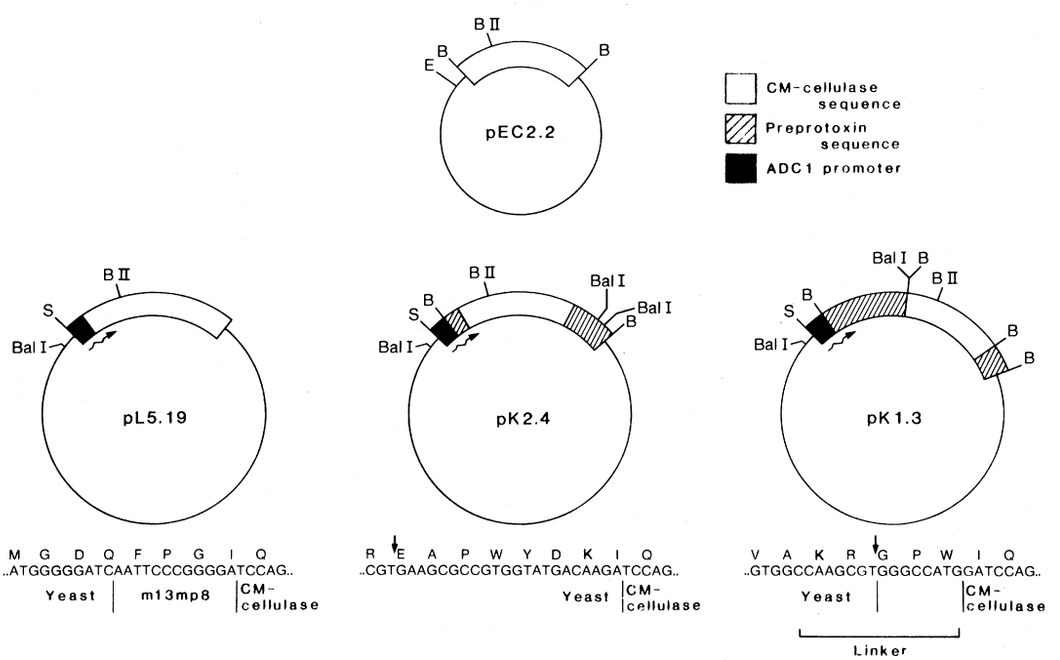
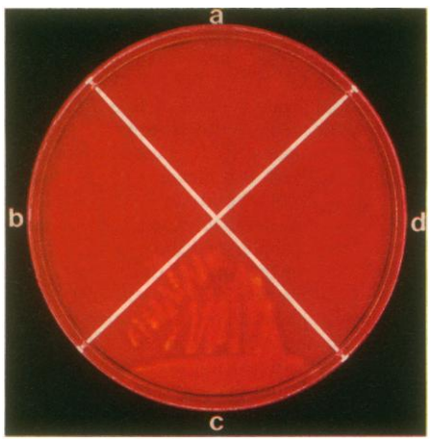


Fig. 2. Structure of plasmids containing the CM-cellulase sequence. Plasmid pEC2.2 contains a 2.4-kb Bam HI fragment encoding CM-cellulase (see text). Plasmid pL5.19 was obtained by cloning the 2.4-kb Bam HI fragment from pEC2.2 into the Bam HI site of phage m13mp8 RF DNA (*25*). It then was excised as a 2.4-kb Eco RI–Hind III fragment and blunt-end ligated into a 7.8-kb fragment isolated from Bam HI–digested pOP. Plasmid pK2.4 contains the 2.4-kb Bam HI fragment from pEC2.2 cloned by cohesive-end ligation into pOP at the Bgl II site. Plasmid pK1.3 contains the 2.4-kb Bam HI fragment from pEC2.2 cloned by blunt-end ligation into the Nco I site of pN3. This reconstituted the Bam HI sites of the insert. Figure 1 shows the structures of pOP and pN3. The arrows indicate the direction of transcription from the ADC1 promoter. The DNA sequence of the promoter-proximal yeast–CM-cellulase junction is shown for each plasmid. In each case, the CM-cellulase sequence begins [G]GATCC · · · , corresponding to the left-hand Bam HI site in pEC2.2. The corresponding amino acid sequence is given in single-letter code: M, Met; G, Gly; D, Asp; Q, Gln; F, Phe; P, Pro; G, Gly; I, Ile; R, Arg; E, Glu; A, Ala; W, Trp; Y, Tyr; K, Lys; and V, Val. Also shown are the positions in preprotoxin at which the α polypeptide (pK2.4) and the β polypeptide (pK1.3) are cleaved during secretion (*5*). Abbreviations: B, Bam HI; BII, Bgl II; E, Eco RI; and S, Sph I.



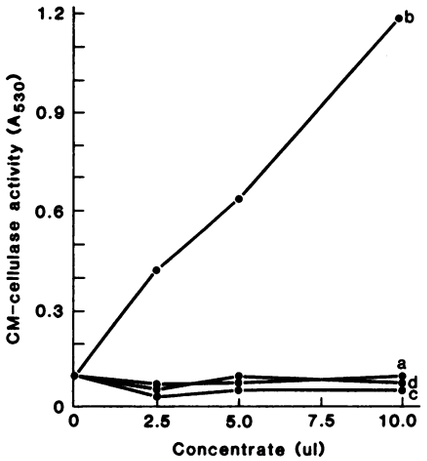


Fig. 3 (left). Secretion of CM-cellulase by yeast. Cells were grown at 30°C on selective medium (*26*), supplemented with 1.2 percent agar, 50 m*M* sodium phosphate, *p*H 6.8, and 0.9 percent carboxymethyl cellulose (Sigma, high viscosity). The colonies were washed off with water. The plate was rinsed with Congo red (2 mg/ml) for a few minutes, then rinsed with 1*M* NaCl. The unstained areas indicate hydrolysis of carboxymethyl cellulose to β(1 → 4)glucans that contain seven or fewer glucose residues (*27*). Cultures were strain 2OB12 containing either plasmid pOP (a), pL5.19 (b), pK2.4 (c), or pK1.3 (d). Transformants of strain SX34-4D were indistinguishable from those shown for strain 2OB12. Fig. 4 (right). Production of reducing sugar by yeast extracellular CM-cellulase. Yeast was grown at 30°C to a density of 1×10^8 to 2×10^8 cell/ml in selective medium (*26*), supplemented with L-tryptophan (20 μg/ml) for untransformed strain 2OB12. The supernatants were concentrated by ultrafiltration, dialyzed against water, and lyophilized. The material was dissolved in water, and samples were incubated for 1 hour at 37°C in 50 m*M* sodium phosphate, *p*H 6.8, containing 2 percent carboxymethyl cellulose (Sigma, low viscosity). One volume of 3,5-dinitrosalicylic acid (DNS) reagent (*10*) was added, the samples were boiled for 10 minutes, and the absorbance at 530 nm (A_{530}) was measured, with water used as a reference. The data were corrected for reactions stopped with DNS reagent at time zero. Cultures: a, untransformed 2OB12; b to d, 2OB12 containing plasmids pK2.4 (b), pK1.3 (c), or pL5.19 (d). Ten microliters of concentrate contained 28, 16, 18, and 26 μg of protein for cultures a to d, respectively. The CM-cellulase activity of culture b, based on reference to a glucose standard curve, is 1.6 units per milliliter of culture supernatant and 340 units per milligram of secreted protein, where 1 unit releases 1 μmol of reducing-sugar equivalents per minute at 37°C.

the β-polypeptide of preprotoxin results in a precursor conformation that interferes with normal processing.

These results demonstrate that *S. cerevisiae* can produce an extracellular CM-cellulase of bacterial origin and suggest that the effectiveness of this depends on the presence of an appropriate yeast leader peptide. *Cellulomonas fimi* grown on 0.1 percent Avicel produces 130 U of CM-cellulase per milliliter (*11*), a membrane-leaky mutant of *E. coli* C600 containing the cloned CM-cellulase gene produces 0.16 U/ml (*12*), and yeast containing plasmid pK2.4 produces 1.6 U/ml (Fig. 4). Since *C. fimi* produces a cellulase complex whose extracellular components act synergistically on cellulose (*13*), the level of CM-cellulase seen in yeast may compare more favorably to that of *C. fimi* than these numbers indicate. Two other examples of expression in *S. cerevisiae* of cloned endoglucanases from Gram-positive bacteria have been reported: an endo-1,3-1,4-β-D-glucanase from *Bacillus subtilis* (*14*), and an endo-1,4-β-D-glucanase or CM-cellulase from *Clostridium thermocellum* (*15*). In neither case was a yeast leader peptide incorporated into the cloned genes and in neither case was the endoglucanase secreted. Our results suggest that further constructions to incorporate genes for an extracellular exo-1,4-β-D-glucanase and 1,4-β-D-glucosidase will allow *S. cerevisiae* to utilize cellulose as a carbon source.

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Neuroendocrine Response to Estrogen and Sexual Orientation

Gladue *et al.* (*1*) report that intravenous injections of 25 mg of Premarin (water-soluble, conjugated estrogens, especially sodium estrone sulfate) exerted a positive feedback action on serum luteinizing hormone (LH) within 48 hours in heterosexual women but not in heterosexual men. Premarin also stimulated a significant increase in serum LH in homosexual men 72 hours after estrogen treatment, and this LH concentration was between that observed in heterosexual males and that observed in heterosexual females. Gladue *et al.* thereby replicated earlier results of Dorner *et al.* (*2*), and concluded that the brain mechanism controlling the pituitary secretion of LH in homosexual men is somehow different from that in heterosexual men. We believe, however, that there may be an alternative explanation for these data.

Gladue *et al.* (*1*) refer to the fact that in females estrogen typically exerts a positive feedback effect on LH secretion during the ovarian cycle, whereas in males an LH response to exogenously administered estrogen is absent, which "presumably reflects 'male' brain differentiation" (*1*). This conclusion derives, however, from studies of nonprimate mammalian species (*3*). In macaques and