0273-2289/99/81/0153/$12.00

# Properties of the *Macrophomina phaseolina* Endoglucanase (EGL 1) Gene Product in Bacterial and Yeast Expression Systems

**Haiyin Wang[1] and Richard W. Jones*[,2]**

*[1]Plant and Microbial Biology, Koshland Hall, University of California, Berkeley, CA 94720; and [2]USDA-ARS, Vegetable Laboratory, Plant Science Institute, BARC West, Beltsville, MD 20705, E-mail: rwjones@asrr.arsusda.gov*

**Received December 10, 1998; Revised August 17, 1999; Accepted August 24, 1999**

## Abstract

Functional expression of a β-D-1,4 glucanase–encoding gene (*egl1*) from a filamentous fungus was achieved in both *Escherichia coli* and *Saccharomyces cerevisiae* using a modified version of pRS413. Optimal activity of the *E. coli*–expressed enzyme was found at incubation temperatures of 60°C, whereas the enzyme activity was optimal at 40°C when expressed by *S. cerevisiae*. Enzyme activity at different pH levels was similar for both bacteria and yeast, being highest at 5.0. Yeast expression resulted in a highly glycosylated protein of approx 60 kDa, compared to bacterial expression, which resulted in a protein of 30 kDa. The hyperglycosylated protein had reduced enzyme activity, indicating that *E. coli* is a preferred vehicle for production scale-up.

**Index Entries:** Cellulase; heterologous expression; *Escherichia coli*; *Saccharomyces cerevisiae*.

## Introduction

Cellulose, a linear chain of β-D-1,4-linked glucose molecules, represents the most abundant biopolymer in nature. Recycling of this fixed carbon requires a series of enzymes capable of hydrolyzing the recalcitrant β-D-1,4 linkages. The cellulolytic enzymes required, endo β-1,4-glucanase (EC 3.2.1.4) and exo-β-1,4-cellobiohydrolase (EC 3.2.1.91), are produced by various bacteria and fungi *(1)*. These proteins are also produced by plants, which utilize cellulose hydrolytic enzymes to modify their cell walls during growth and development *(2)*.

*Author to whom all correspondence and reprint requests should be addressed.

Analysis of β-D-1,4 glucanase has advanced rapidly during the last decade owing to the ability to clone individual β-D-1,4 glucanase–encoding genes *(1)*. Individual genes can be expressed in heterologous hosts, allowing identification of specific enzyme activities in the absence of a complex background mixture of cellulolytic enzymes *(3,4)*. Heterologous expression of β-D-1,4 glucanase–encoding genes also allows production of enzymes used in an array of industrial applications, ranging from the production of wood pulp fiber to laundry detergents *(5)*.

Heterologous expression of enzymatically functional β-D-1,4 glucanases has been largely restricted to related hosts. For example, functional expression of β-D-1,4 glucanase–encoding genes from the filamentous fungus *Trichoderma reesei* can be achieved in other filamentous fungi such as *Aspergillus awamori (6)*. Expression can also be achieved in the unicellular yeast *Saccharomyces cerevisiae*; however, the protein product may be hyperglycosylated *(4,6–8)*.

Filamentous fungal β-D-1,4 glucanase–encoding genes have not been effectively expressed as functional enzymes in bacteria such as *Escherichia coli*. Therefore, no information exists on the differences in the enzyme expressed in a bacterial system relative to a yeast system. We have recently cloned and characterized two β-D-1,4 glucanase–encoding genes (*egl1* and *egl2*) from the filamentous fungal phytopathogen *Macrophomina phaseolina*, which can be expressed as functional enzymes in *E. coli (2,9)*. Although these β-D-1,4 glucanase–encoding genes can be expressed as functional enzymes in a bacterial system, we did not know whether they could be functionally expressed in a yeast system. Initial research showed that *egl1* could be expressed; thus, we pursued a comparative analysis of the EGL 1 enzyme produced in two disparate expression systems. This article reports on the production of EGL 1 in the unicellular yeast *S. cerevisiae* and the bacterium *E. coli*, using a modified version of the shuttle vector pRS413.

## Materials and Methods

### Microbial Strains

*E. coli* strain DH5α was used as a host in the plasmid construction and gene expression as previously described *(2,9)*. *S. cerevisiae* strain YP500 (α ura3-52 lys2-801 ade2-101 tyr1-D63 his3-D200 leu2-D1) and the *S. cerevisiae / E. coli* shuttle vector pRS413, which replicates autonomously in yeast, were from Stratagene (La Jolla, CA).

### Plasmid Construction

A 1.2-kbp *egl1* cDNA fragment was recovered from pSPORT-*egl1 (9)* by polymerase chain reaction (PCR), using Thermopol DNA polymerase (New England Biolabs, Beverly, MA). An *Sal*I site was introduced during PCR amplification by the addition of the restriction site sequence in the 3' primer. The PCR product was *Sal*I digested, gel purified, and subcloned

into pRS413 at the *Sal*I site. Bacterial transformants containing pRS413-*egl1* were screened for endoglucanase activity using carboxymethylcellulose (CMC)-agar overlay followed by Congo Red staining as previously described *(9)*. Plasmid was purified from one of the endoglucanase-positive colonies and used for yeast transformation as described by Elble *(10)*. Yeast transformants were selected by plating on a selective medium containing 0.67% yeast nitrogen base without amino acids, 2% glucose, and 2% agar. This medium was supplemented with 20 mg/L of adenine sulfate, uracil, and L-tryptophan, plus 30 mg/L each of L-leucine and L-lysine.

### *Cultivation and Enzyme Production*

Yeast transformants expressed endoglucanase activity, as revealed through the use of the Congo Red–stained CMC overlay. A colony was chosen and grown in yeast extract-potato dextrose liquid medium at 30°C for 3 d. A bacterial culture was grown in Luria Broth for 1 d as previously described *(11)*. Supernatant, containing endoglucanase activity, was collected from each culture after centrifugation and pelleting of the cells. Samples from bacterial and yeast culture supernatants were concentrated 10-fold by centrifugation in a Centriprep-10 according to the manufacturer's directions (Amicon, Beverly, MA). Total EGL 1 production, determined by enzyme-linked immunosorbent assay (ELISA) using a peptide-specific antibody as previously described *(11)*, was similar for both *S. cerevisiae* and *E. coli.*

### *Enzyme Assays*

Endoglucanase-containing samples were tested for pH optimum by adding concentrated enzyme preparation to Tris-buffered CMC solution (1%). The buffered CMC was preincubated at 30°C for 2 h, and then 100 µL of concentrated enzyme preparation was added to 10-mL vol of Tris-buffered 1% CMC. Samples were incubated at 30°C for 30 min. Samples were irreversibly inactivated by incubation in a boiling water bath. Each sample was assayed with a Brookfield viscometer (Model DV11, Stroughton, MA). Control samples consisted of pH-adjusted CMC that was incubated at 30°C for 30 min without the addition of enzyme.

Temperature optima was determined after preincubating the CMC substrate (pH 5.0) for 2 h at temperatures ranging from 30 to 80°C. Enzyme preparations were then added and the substrate incubated for 30 min at a given temperature. Control samples consisted of CMC substrate, which was adjusted to previously tested pH values, or incubated at temperatures ranging from 30 to 80°C. Samples were irreversibly inactivated by incubation in a boiling water bath. Each sample was incubated at the test temperature, then assayed by viscometry immediately after 30-min incubations. Values given are reduction in viscosity relative to the control. A comparison of relative activities of bacterial EGL 1 and yeast EGL 1 was made by incubating equal amounts (determined by ELISA) of EGL 1 in 1% CMC (pH 5.0) at 50°C for 30 min. Samples were irreversibly inactivated by incu-

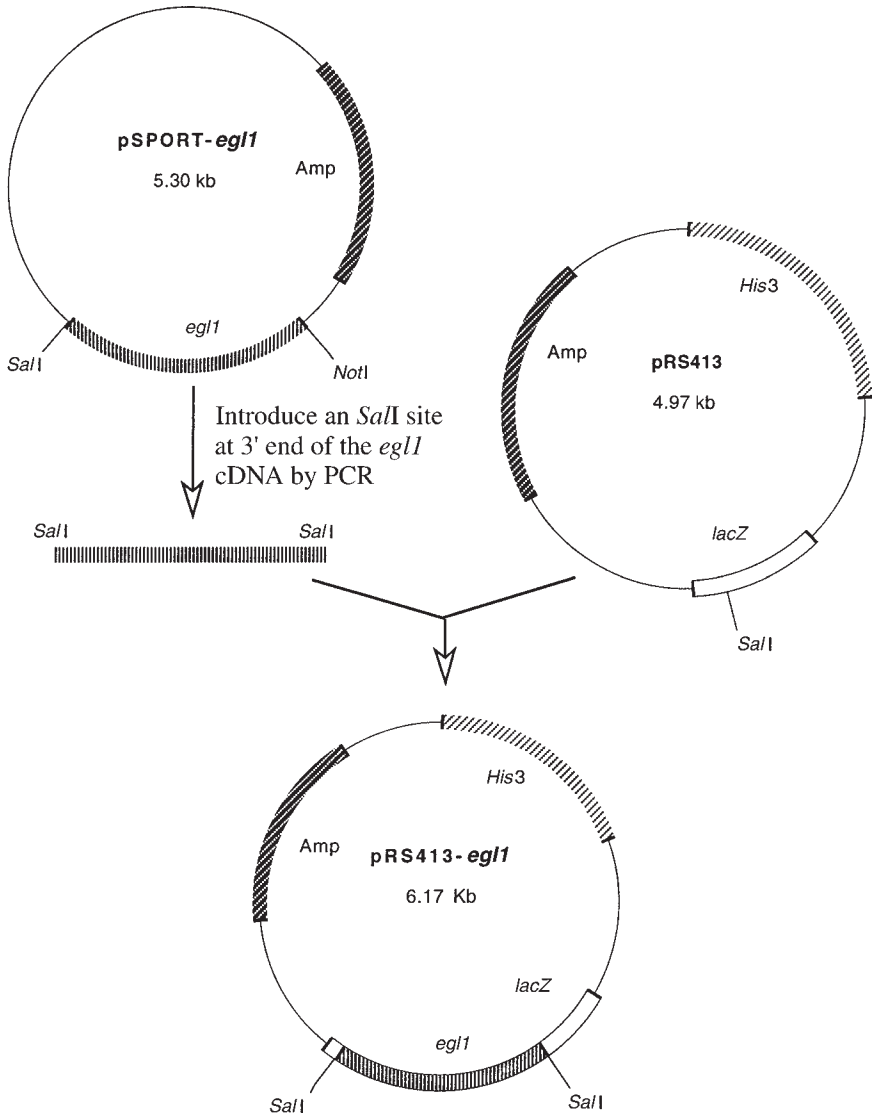


Fig. 1. Construction of the *S. cerevisiae*/*E. coli* shuttle vector (pRS413-*egl1*) for expression of EGL 1.

bation in a boiling water bath. All enzyme assays were repeated twice to verify relative activities.

## Results

Figure 1 summarizes the construction of pRS413-*egl1*. When the plasmid was present, *S. cerevisiae* and *E. coli* produced endoglucanase. This activity was visualized as a clear halo surrounding the colonies, after Congo Red staining of a CMC-agar overlay. A pH optimum of 5.0 was found for EGL 1, whether expressed in *S. cerevisiae* or *E. coli*, as shown in Fig. 2.

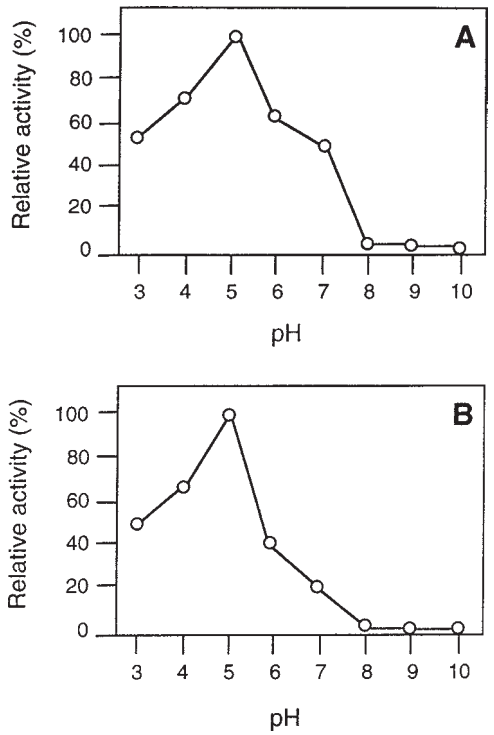


Fig. 2. Hydrolysis of 1% CMC solutions adjusted to different pH levels. Values are relative to the maximum viscosity reduction found after 30 min at 50°C. Activity of EGL 1 expressed by **(A)** *E. coli* and **(B)** *S. cerevisiae*.

Bacterial enzyme preparations retained greater relative activity at pH values of 6.0 and 7.0 than the yeast preparations, whereas both preparations were inactive at pH values of 8.0 or higher. In Fig. 3 it can be seen that activity at different temperatures varied, with the bacterial EGL 1 being most active at 60°C, whereas the yeast EGL 1 was most active at 40°C. Both enzyme preparations were inactive at 70°C and higher. Subsequent incubation of the high-temperature samples at a conducive temperature (50°C) demonstrated that, based on the reduction of CMC viscosity, both bacterial and yeast EGL 1 were reversibly inactivated at 70°C and irreversibly inactivated at 80°C.

Yeast-produced EGL 1 reduced viscosity of a 1% CMC solution considerably less than bacterial-produced EGL 1, when equal amounts of EGL 1 were tested. At the end of a 30-min incubation, bacterial EGL 1 reduced CMC viscosity by 80%, compared to yeast EGL 1, in which viscosity was reduced by 5%. Therefore, production of EGL 1 in *E. coli* provides an enzyme with higher activity than that of *S. cerevisiae.*

Analysis of EGL 1 samples by Western blotting, as previously described *(11)*, revealed that the bacterial EGL 1 has the same molecular weight (30 kDa) as the native enzyme produced in *M. phaseolina*, whereas *S. cerevisiae* EGL 1 was twice the molecular weight (*see* Fig. 4). Hyper-

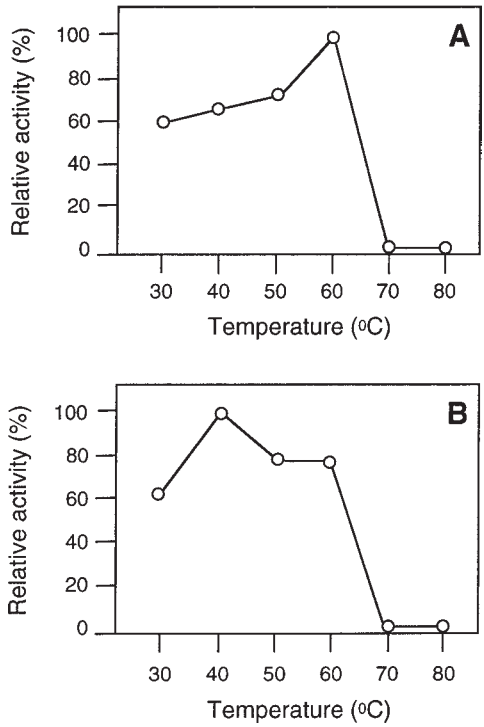


Fig. 3. Hydrolysis of 1% CMC solutions (pH 5.0) after equilibration to different temperatures. Values are relative to the maximum viscosity reduction found after 30 min. Activity of EGL 1 expressed by **(A)** *E. coli* and **(B)** *S. cerevisiae*.

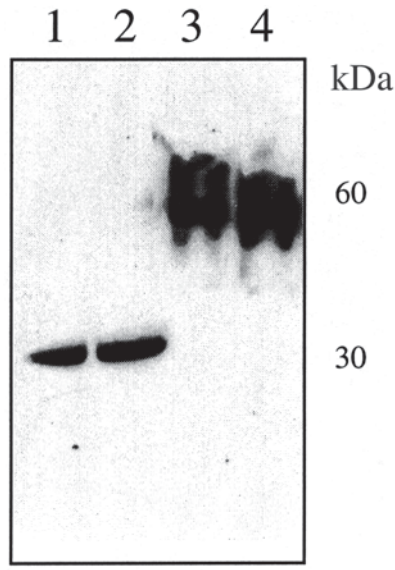


Fig. 4. Western blot analysis of EGL 1 expressed by *M. phaseolina* (lane 1), *E. coli* (lane 2), *S. cerevisiae* (lane 3), and *S. cerevisiae* (lane 4) after Endo H treatment.

glycosylation of the protein resulted in a heterogeneous banding pattern. Treatment with Endo H did not cause a visible reduction in the size of EGL 1 produced by the yeast. Therefore, either the glycosylation is not the high mannose N-linked type, or it is resistant to Endo H cleavage.

## Discussion

The ability to express β-D-1,4 glucanase–encoding genes from filamentous fungi as functional enzymes in *E. coli* was unprecedented before discovery of the endoglucanase genes from *M. phaseolina (2,9)*. We have expanded our knowledge of *egl1* expression by showing that it is readily expressed from pRS413, in which bacterial production had not been previously demonstrated, and that expression results in functional enzyme whether in *S. cerevisiae* or *E. coli*.

Certain β-D-1,4 glucanase–encoding genes have been cloned from filamentous fungi and functionally expressed in yeast; however, the enzymes were heavily glycosylated by the yeast. We found that expression of *egl1* in yeast also led to hyperglycosylation. The yeast enzyme showed altered properties, relative to the enzyme produced in bacteria. The overall activity was lower for yeast EGL 1, and the temperature optimum was shifted down about 20°C.

The only other fungal β-D-1,4 glucanase–encoding genes, which have been expressed as functional enzymes in a bacterial system, are those cloned from the certain lower Mastigomycotina fungi. These fungi produce rhizoids and are found in the rumens of sheep and bovines. In one case it was suggested that the Mastigomycotina β-D-1,4 glucanase–encoding genes actually originated from a prokaryote *(12)*, thus facilitating their functional expression in *E. coli*. In another singular study, a β-D-1,4 glucanase–encoding gene (CMCase 1) from the unicellular yeast *Cryptococcus flavus* was functionally expressed in *E. coli (13)*. Neither of the aforementioned studies involved true filamentous fungi.

There is considerable amino acid homology between three fungal endoglucanases, which can be functionally expressed in *E. coli*—CMCase from *C. flavus* and EGL 1 and EGL 2 from *M. phaseolina*. These endoglucanases show a high degree of amino acid homology to *Pseudomonas solanacearum* EGL 1, a bacterial endoglucanase. All the aforementioned endoglucanases share extensive amino acid homology to EGL 3 from the filamentous fungus *T. reesei*, which has not been functionally expressed in *E. coli*. It will be interesting to begin a mutational analysis of *T. reesei* EGL 3, in an effort to determine the regions of the protein that may limit functional expression in bacterial production systems. The only obvious regions in which *T. reesei* EGL 3 differs from *M. phaseolina* EGL 1 are the signal peptide and the cellulose-binding domain, both located at the N-terminus of EGL 3. However, fusing these regions to the N-terminal region of *M. phaseolina* EGL does not limit functional expression in *E. coli* (Wang, H. and Jones, R. W., unpublished data). Regions affecting differential functional expression in

*E. coli* thus exist beyond the N-terminal region of *T. reesei* EGL 3. Eventually, functional expression of β-D-1,4 glucanase–encoding genes from filamentous fungi such as *T. reesei*, in a bacterial system instead of a yeast system, may lead to improved enzyme production.

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