

CONSTRUCTION OF AN ENDOGLUCANOLYTIC BREWING YEAST STRAIN

BY QI XIE AND ANTÓNIO JIMÉNEZ

(Centro de Biología Molecular, CSIC-UAM, Universidad Autónoma de Madrid, Canto Blanco, 28049 Madrid, Spain.)

AND DANIEL RAMÓN AND JOSÉ A. PÉREZ-GONZÁLEZ

(Departamento de Biotecnología de los Alimentos, Instituto de Agroquímica y Tecnología de los Alimentos, CSIC, Jaime Roig 11, 46010 Valencia, Spain)

Received 25 March 1995

An endo- β (1,4)glucanase encoding gene from fungal origin has been expressed in a brewing yeast strain. The yeast transformation was carried out using a previously reported system based in the acquisition of cycloheximide resistance. The plasmid transferred to brewing yeast showed some changes in restriction pattern of the 2 μ portion after transformation while cycloheximide resistance marker and endoglucanase gene were not affected. The drug resistance phenotype showed by the recombinant yeast was highly stable in non-selective conditions. The endoglucanase enzyme was detected in cell-free culture medium and showed high activities in liquid and solid media.

Key words: Brewing yeast, transformation, cycloheximide resistance, fungal endoglucanase expression

INTRODUCTION

β -Glucanase preparations of microbial origin are used in beer production. These enzymes degrade the barley β -glucans, present in wort, which can produce filtering problems and the presence of hazes and precipitates in beer. The use of highly purified enzyme preparations would increase the costs of the production process hence crude preparations are usually added. These mixtures may contain some other undesirable activities and therefore the construction of recombinant yeasts able to secrete β -glucanase directly into the wort has been the objective of several groups. An endo- β (1,3)-(1,4)glucanase gene from *Bacillus subtilis*^{1,6} and an endo- β (1,4)glucanase gene from *Trichoderma reesei*^{10,14} have been introduced into brewing yeast. The use of recombinant yeast strains secreting the fungal enzyme in pilot-scale brewings improves the filterability of the wort and the quality of the finished beer is good^{3,14}.

Industrial yeast strains used in beer production are polyploid and the transfer of genes to them requires the use of a dominant selectable marker. Brewing yeasts are very sensitive to the antibiotic cycloheximide and in this respect the *cyh2* gene, which confers cycloheximide resistance, has been successfully used in transformation of *Saccharomyces cerevisiae* CC45, a strain used in lager beer production^{2,9}. Recently, this selection system has been used for the transformation of a wine yeast strain with a *Trichoderma longibrachiatum* endoglucanase gene (*egl1*)¹¹. We report here the transformation of *S. cerevisiae* CC45 with the *egl1* gene and its expression in this strain using the cycloheximide selection system.

MATERIALS AND METHODS

Strains, plasmids and media

S. cerevisiae CC45 is used by the brewery, La Cruz del Campo S. A. (Sevilla, Spain), for the production of lager beer. This strain grows well at 24°C but is not viable at 37°C, hence growth and incubation of this strain was carried out at 24°C. This strain has a natural resistance to cycloheximide up to 0.15 μ g/ml drug concentration in minimal MM (0.7% yeast nitrogen base w/o amino acids, 2% glucose, 3% agar) or YEPD (1% yeast extract, 2% peptone, 2% glucose, 2% agar) media.

Escherichia coli DH5 α was grown on LB medium¹² and, when required, was supplemented with 100 μ g/ml ampicillin.

Plasmids YEpCR21⁹ which contain the cycloheximide resistance gene *cyh2* and pTLEGY3¹¹ which contain the endo- β (1,4)-glucanase encoding *egl1* gene from *Trichoderma longibrachiatum* inserted in YEpCR21 under the control of a yeast actin gene promoter, were used in *S. cerevisiae* CC45 transformations.

Transformation protocols

E. coli was transformed following the method described by Hanahan⁵ and transformants selected in LB medium plus ampicillin at 100 μ g/ml. Transformation of *S. cerevisiae* CC45 was performed by lithium acetate treatment of intact cells as previously described¹³ except that the temperature of all incubations was 24°C. After heat shock, cells were collected in 5 ml YEPD medium and incubated for 2 h at 24°C before spreading on selection plates. The selective media used were YEPD or MM, each containing 0.20 μ g/ml cycloheximide.

Endoglucanase determinations

Carboxymethyl cellulose was used as substrate in endoglucanase activity plate assays following a previously described procedure⁴ Figure 1. Endoglucanase activity was determined in culture supernatants in vitro. The reaction mixtures (500 μ l) contained 250 μ l of a 10 mg/ml azobarley β -glucan solution (Biocon), 100 μ l of culture supernatant and 150 μ l of 50 mM sodium acetate buffer pH 4.5 and were incubated for 20 min at 50°C. One unit of endoglucanase activity was defined as the amount of enzyme that gave one unit of absorbance at 590 nm in one hour. Western blot detection of the *T. longibrachiatum* EGL1 protein was achieved using a monoclonal antibody raised against the homologous *Trichoderma reesei* EGL1 protein⁸ following published procedures⁷.

RESULTS

Transformation of brewer's yeast with a plasmid carrying an endoglucanase encoding gene from *T. longibrachiatum*

Plasmid pTLEGY3¹¹ contains the *T. longibrachiatum* *egl1* gene (encodes the endoglucanase EGL1) under the control of the *S. cerevisiae* actin gene promoter. The vector used for this construction, YEpCR21⁹, carries the cycloheximide resistance

TABLE I. Phenotype Stability in a *S. cerevisiae* CC45 (pTLEGY3) Transformant (see text).

Generations	% of <i>cyh</i> ⁺ clones
12	91
36	70

TABLE II. Endoglucanase Activity in Culture Supernatants of Several *S. cerevisiae* CC45 derived strains. The Results are the Average of Two Independent Experiments.

Strain	Endoglucanase activity (U/ml)
CC45	0.48
CC45 (YEPC21)	0.45
CC45 (pTLEGY3)	10.93

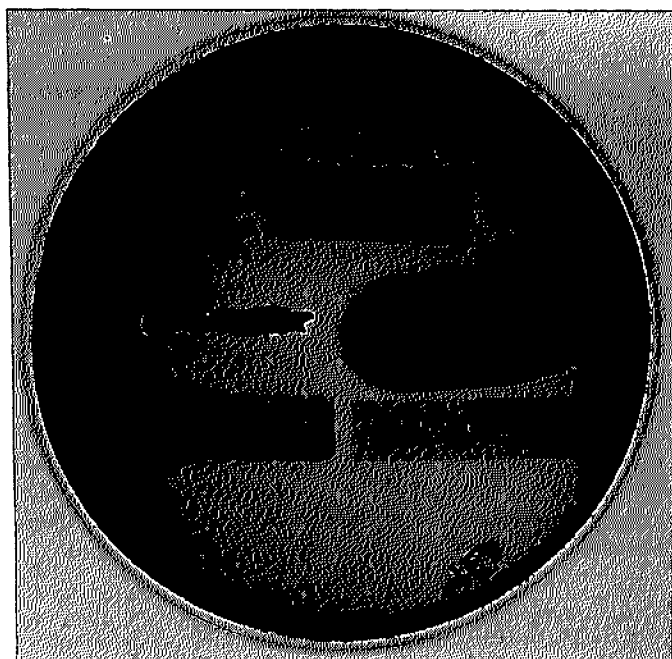


FIG. 1. Plate assay for the detection of endoglucanase activity. The assay was performed as described in Materials and Methods.

gene *cyh2* as a dominant selection marker². The pTLEGY3 plasmid was used to transform the industrial strain *S. cerevisiae* CC45 and transformants were selected for cycloheximide resistance.

The presence of transforming plasmid in some cycloheximide resistant clones was analyzed by retransformation of *E. coli* with DNA from those clones and selection for ampicillin resistance. Plasmids isolated from *E. coli* clones were analyzed with restriction enzymes. Some plasmids showed changes in the restriction pattern with respect to the original pTLEGY3 plasmid. Most of the variations were found within that fragment of the plasmid pTLEGY3 derived from the 2 µ plasmid (data not shown).

Phenotype stability analyses were also carried out using a representative *S. cerevisiae* CC45 (pTLEGY3) transformant with respect to cycloheximide resistance. The transformant was grown in non-selective conditions and after several generations culture dilutions were plated on selective and non-selective solid media and the percentage of cycloheximide resistant colonies was determined. The results obtained are shown in Table I.

Expression of the *T. longibrachiatum* endoglucanase enzyme in *S. cerevisiae* CC45 transformants

Expression of endoglucanase activity in some *S. cerevisiae* CC45 transformants was analyzed using plate activity assays. As can be seen in Fig. 1, transformant CC45 (pTLEGY3) produced a carboxymethylcellulose degradation halo around



FIG. 2. Western blot of culture media inoculated with the strains *S. cerevisiae* CC45 (lane A), *S. cerevisiae* CC45 (YEPC21) (lane B), and transformant *S. cerevisiae* CC45 (pTLEGY3) (lane C).

the colony whereas *S. cerevisiae* CC45 or the same strain transformed with YEPC21 did not. Endoglucanase activity was also tested *in vitro* using culture supernatants. Table II summarizes the results obtained. Transformant CC45 (pTLEGY3) showed high endoglucanase activity in relation to the controls namely the host strain CC45 and the same strain transformed with the YEPC21 plasmid.

Culture supernatants were also analyzed by sodium dodecylsulfate-polyacrylamide gel electrophoresis and western blotting. As can be seen in Fig. 2 the EGL1 protein was only detected in the *S. cerevisiae* CC45 (pTLEGY3) transformant.

DISCUSSION

Brewing yeasts are polyploid and the use of dominant selectable markers is very convenient for detection of transformation. The *cyh2* gene, which confers cycloheximide resistance, has been successfully used in the transformation of *S. cerevisiae* CC45, a strain used in lager beer production, and in

other industrial and laboratory yeast strains². Recently, this transformation system has given good results regarding the transformation of the industrial wine yeast strain T73¹¹. An endoglucanase gene from *T. longibrachiatum* was expressed in the T73 yeast strain under the control of the *S. cerevisiae* actin gene promoter. The same construct has been used to transform the brewing strain CC45. A number of cycloheximide resistant transformants were obtained. The presence of free plasmid copies in cells from these transformants was confirmed. Some changes were observed in the physical structure of the plasmids isolated. Most changes were found within the 2 μ fragment. This would result from recombination between the 2 μ fragment carried in the transforming plasmid and the 2 μ from *S. cerevisiae* CC45 itself. Industrial brewing yeasts have been shown to have different forms of the 2 μ plasmid¹⁵. In our case, those changes did not impede the expression of the *T. longibrachiatum* gene in the transformant as endoglucanase activity was detected in plate and in vitro activity assays. In contrast to the T73 wine strain, the CC45 strain does not appear to easily accept foreign DNA sequences, as the pTLEGY3 has been modified in the transformant *S. cerevisiae* CC45 (pTLEGY3). The vector YE_pCR21 itself is also the result of a modification by deletion of the original YE_pCR20 plasmid into the CC45 strain⁹.

Acknowledgements. We are grateful to M. A. Delgado for providing yeast strain CC45, to Mrs A. Martin for expert technical assistance and to C. P. Kubicek who kindly provided us with the *T. reesei* EGI antibody. Thanks are due to A. MacCabe for critical reading of the manuscript. This research was supported by grants AL190-0842; Plan Concertado CDTI-Cruzcampo S. A. (900041) and an institutional grant from Fundación Ramón Areces to the Centro de Biología Molecular.

REFERENCES

1. Cantwell, B., Brazil, G., Hurley, J. & McConnell, D., *European Brewery Convention Proceedings of the 20th Congress, Helsinki*, 1985, 259.
2. Del Pozo, L., Abarca, D., Claros, M. G. & Jiménez, A. *Current Genetics*, 1991, **19**, 353.
3. Enari, T.-M., Knowles, J., Lehtinen, U., Nikkola, M., Penttilä, M., Suihko, M.-L., Home, S. & Vilpola, A. *European Brewery Convention Proceedings of the 21st Congress, Madrid*, 1987, 529.
4. González, R., Ramón, D., & Pérez-González, J. A. *Applied Microbiology and Biotechnology*, 1992, **38**, 370.
5. Hanahan, D. *Journal of Molecular Biology*, 1983, **166**, 557.

6. Hinchliffe, E. & Box, W. G. *European Brewery Convention Proceedings of the 20th Congress, Helsinki*, 1985, 267.
7. Kubicek, C. P., Panda, T., Schrefferl-Kunar, G., Messner, R. & Gruber, F. *Canadian Journal of Microbiology*, 1987, **33**, 689.
8. Luderer, M., Hofer, F., Hagspiel, K., Allmaier, G., Blaus, D. & Kubicek, C. P. *Biochemical and Biophysical Acta*, 1990, **1076**, 427.
9. Navas, L., Esteban, M. & Delgado, M. A. *Journal of the Institute of Brewing*, 1991, **97**, 115.
10. Penttilä, M. E., Suihko, M.-L., Lehtinen, U., Nikkola, M. & Knowles, J. K. C. *Current Genetics*, 1987, **12**, 413.
11. Pérez-González, J. A., González, R., Querol, A., Sendra, J. & Ramón, D. *Applied and Environmental Microbiology*, 1993, **59**, 2801.
12. Sambrook, J., Fritsch, E. F. & Maniatis, T. *Molecular cloning: a Laboratory Manual*, 2nd ed., 1989. Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory Press.
13. Schiesl, R.-H. & Gietz, R.-D. *Current Genetics*, 1989, **16**, 339.
14. Suihko, M.-L., Lehtinen, U., Zurbriggen, B., Vilpola, A., Knowles, J. & Penttilä, M. *Applied Microbiology and Biotechnology*, 1991, **35**, 781.
15. Xiao, W., Pelcher, L.-E. & Rank, G.-H. *Journal of Bacteriology*, 1991, **173**, 1181.

ADDITIONAL REPORT

I have studied the paper "Plant Hormones in Fungi and Bacteria from Malting Barley" and the referee's comments.

Essentially, I agree with the points made concerning the paper. It is a valuable piece of work (or potentially so) and it is such a pity that none of the bacteria involved were identified. With very little extra work it would have been easy to identify them at least to genus level (i.e. *Lactobacillus paediococcus* etc.). Without this information or confirmation that the hormone-producing bacteria were actually proliferating during malting (all the isolates were from dry, stored barley) it is impossible to judge their significance.

The yeasts and fungi were identified but the bacteria not—strange for a laboratory of Biochemistry and Microbiology.

I agree that "non-sparing isolate" would be more exact than "Mycelia Sterilia".

Equating mycelial cell mass with yeast dry mass of known cell number is not precise. I suppose the authors felt that this would give an *approximation*—the best one can do with material like mycelium.

In my view the most important thing to do is *either* establish the identity of the IAA-producing bacteria as ones that are known to proliferate during malting *or* actually show that they do by following cell numbers during malting.

I hope this is helpful.