An oral vaccine against candidiasis generated by a yeast molecular display system

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This paper deals with the development of an oral vaccine against Enolase 1 (Eno1p) of Candida albicans. The approach taken by using cell surface engineering of Saccharomyces cerevisiae is original and a proper example of how modern oral vaccines can be produced.

Keywords
Eno1p; Candida albicans; Saccharomyces cerevisiae; cell surface engineering.

Abstract
Enolase 1 (Eno1p) of Candida albicans is an immunodominant antigen. However, conventional technologies for preparing an injectable vaccine require purification of the antigenic protein and preparation of an adjuvant. To develop a novel type of oral vaccine against candidiasis, we generated Saccharomyces cerevisiae cells that display the Eno1p antigen on their surfaces. Oral delivery of the engineered S. cerevisiae cells prolonged survival rate of mice that were subsequently challenged with C. albicans. Given that a vaccine produced using molecular display technology avoids the need for protein purification, this oral vaccine offers a promising alternative to the use of conventional and injectable vaccines for preventing a range of infectious diseases.

Introduction
Candidiasis is a serious infectious disease caused by the fungus Candida albicans and other Candida species (Nucci & Marr, 2005; Pfaller & Diekema, 2007). Superficial or systemic candidiasis is observed when the host immunity is compromised by AIDS, chemotherapies for cancer treatment or the administration of immunosuppressants. Together, C. albicans and Candida glabrata are responsible for 70–80% of Candida infections in patients with candidiasis or candidemia. In general, options for treating candidiasis remain unsatisfactory because of delayed diagnosis and a lack of reliable tools to detect Candida species inside the body of a patient (Noble & Johnson, 2007).

Pharmacotherapy of candidiasis often involves the administration of caspofungin, micafungin, anidulafungin and amphotericin B. However, mutants of Candida with reduced susceptibility to these drugs have emerged (Rodloff et al., 2011). Although genetic and molecular mechanisms that confer resistance of C. albicans to antifungal drugs have been studied for a long time (Morschhäuser, 2002), at present, there is no general strategy to prevent the emergence of resistance to antifungal drugs. In addition, unwanted side effects of chemical drugs against Candida species as well as other fungi also pose a serious problem (Benko et al., 1999). Therefore, prevention of infection by vaccination against Candida species is thought to be an important complementary strategy to pharmacotherapy in efforts to control candidiasis.

Vaccination with Eno1 protein (Eno1p) of C. albicans, which encodes the glycolytic enzyme enolase 1 (2-phospho-D-glycerate hydrolyase), can protect against
infection with *C. albicans* (Montagnoli et al., 2004; Li et al., 2011). Besides Eno1p, the *C. albicans* proteins, e.g. hyphal wall protein, glyceraldehyde-3-phosphate dehydrogenase and phosphoglycerate kinase can also induce protective immune functions against candidiasis when administered with appropriate adjuvant compounds to enhance the immune response to the antigen (Xin & Cutler, 2011). However, the technologies available to purify antigenic proteins, prepare adjuvant, and deliver both in a convenient and noninvasive manner pose a challenge to vaccine development.

In recent years, we have developed the use of molecular display technology, which uses genetically engineered microorganisms to produce foreign proteins (Shibasaki et al., 2007). In general, the approach involves fusion of a heterologous protein, such as an antigen, to a cell-wall protein, thereby anchoring the hybrid protein to the cell surface and exposing the heterologous protein to the exterior of the cell. The use of molecular display to prepare target proteins is especially well established for use with the yeast *Saccharomyces cerevisiae*, and it is also termed cell surface engineering (Shibasaki et al., 2009). For instance, an antigen from red sea bream iridovirus (RSIV) was displayed on the surface of yeast cells to develop an oral vaccine for use by fisheries (Tamaru et al., 2006). A major component of the yeast cell wall, β-glucan, is expected to serve as an adjuvant (Rodríguez et al., 2009), thus obviating the need to prepare an adjuvant for codelivery. Yeasts are generally recognized as safe (GRAS) organisms, and thus appropriate for the preparation of oral vaccines without amino acids, 1% (w/v) casamino acids and supplemented with appropriate amino acids).

### Plasmid construction and transformation of microbial cells

The plasmid pQE-eno1, which was used to produce Eno1p in *E. coli*, was constructed by first using PCR to generate the Eno1p-encoding sequence using primers 5′-ATGGATCCTCTTACGCACTAAAAATCCAGC-3′ and 5′-TTAAAGCTTACAAATGAGAAGGCTTTGAAATTTACCTTGACC-3′ and genomic DNA of *C. albicans* strain SC5314 (American Type Culture Collection) (Hube et al., 1997), which was purified as described previously (Aoki et al., 2011). The fragment of Eno1p-encoding sequence was inserted into the pQE30 plasmid (Qiagen, Hilden, Germany) that had been digested with BamH1 and HindIII to construct the recombinant plasmid pQE-eno1.

The plasmid pULD-eno1, which was used to display Eno1p from *C. albicans* on the surface of *S. cerevisiae* cells, was constructed by amplifying the Eno1p-encoding sequence by PCR using primers 5′-ACGCCACTAAAAATCCAGC-3′ and 5′-TGCTCGAGCATTTGAGAAGGCTTTGAAATTTACCTTGACC-3′ and the same genomic DNA of *C. albicans* described above. The fragment of the gene encoding ENO1 was inserted into plasmid pULD1 (Kuroda et al., 2009) that had been digested with NolI and Xhol. Thus, the Eno1p-encoding sequence was fused to the 5′ end of the cell-wall anchoring protein, α-agglutinin-encoding sequence in the plasmid. The constructed plasmid pQE-eno1 was introduced into *E. coli* BL21 as described previously (Hanahan, 1983) for propagation. The constructed plasmid pULD1-eno1 and parent plasmid pULD1 were introduced into *S. cerevisiae* BY4741 using the lithium acetate method (Ito et al., 1983) for surface display of the protein. The nucleotide sequence of all constructed plasmids was confirmed by using an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA).

### Protein production and purification

Expression of the pQE30-eno1 plasmid in *E. coli* BL21 (DE3) produced Eno1p as a fusion protein with an N-terminal His6 tag. The cells were inoculated in 14 mL LB medium, containing 100 μg L⁻¹ ampicillin, and grown in shaking flasks overnight at 37 °C. Fresh LB medium containing 100 μg L⁻¹ ampicillin (120 mL) was inoculated with 6 mL of the overnight cultures and the cells were grown at 37 °C to an OD₆₀₀ of 0.6–1.0. Gene expression was then induced by the addition of isopropyl β-D-thiogalactoside (IPTG; Wako Pure Chemical, Osaka, Japan)

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The strains and media

The *E. coli* strain DH5α [F− endA1 hsdR17 (rK−, mK+) supE44 thi1 recA1 gyrA96 ΔlacU169 Δφ80 lacZ1M15] (Hanahan, 1983) was used as a host for manipulation of recombinant DNA. The *E. coli* strain BL21 [F−ompT hsdSB (rK−, mK+) gal dcm (DE3)] was used to produce antigenic proteins. Both *E. coli* strains were grown in Luria–Bertani (LB) medium [1% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl and 0.1% (w/v) glucose]. The *S. cerevisiae* strain BY4741 (MATα his3-1 leu2 met15 ura3) was used for cell surface display of antigenic proteins. YPD medium [1% (w/v) yeast extract, 2% (w/v) polypeptone and 2% (w/v) glucose] was used for the transformation of yeast cells. Yeast cells that carried a plasmid were grown in SDC medium [2% (w/v) glucose, 0.67% (w/v) yeast nitrogen base without amino acids, 1% (w/v) casamino acids and supplemented with appropriate amino acids].
to a final concentration of 1 mM. After 3–4 h of cultivation at 37 °C, the cell cultures were harvested by centrifugation (4000 g, 20 min, 4 °C). The cell pellets were subsequently resuspended in 10 mL of B-PER bacterial protein extraction reagent (Thermo Fisher Scientific, IL), and shaken gently for 10 min at room temperature (25 °C). Soluble proteins were separated from insoluble proteins by centrifugation at 27 000 g for 20 min. Supernatant containing soluble proteins were purified by recovering the His$_6$-Eno1 fusion protein after passage through nickel-chelated agarose (Thermo Fisher Scientific) columns. The column was equilibrated with 10 mL of B-PER Bacterial Protein Extraction Reagent before application of the supernatant. After washing the column with wash buffer of the B-PER reagent, the bound proteins were released with elution buffer [50 mM Tris, 300 mM NaCl, 200 mM imidazole, 10% (v/v) glycerol]. Endotoxin was removed from the eluate by passage through Detoxi-Gel endotoxin-removing columns (Pierce, Rockford, IL), resulting in levels of < 0.1 endotoxin units mL$^{-1}$, as indicated using Limulus Amebocyte Lysate (LAL) PYROGENT single-test vials (Lonza, Walkersville, MD) as described in the manufacturer’s protocol.

**Immunofluorescence staining**

Yeast cells were collected by centrifugation at 6000 g for 5 min, washed with phosphate-buffered saline (PBS; 50 mM phosphate, 150 mM sodium chloride, pH 7.4) and adjusted to $3.2 \times 10^8$ cells mL$^{-1}$ with PBS. Thereafter, 200 µL of this cell suspension was centrifuged at 6000 g for 5 min. Collected cells were incubated with 200 µL of a 1 : 250 dilution of rabbit IgG in PBS containing 1% (w/v) BSA at room temperature for 1 h (Shibasaki et al., 2007).

Surface-blocked cells were incubated with 3 µg mL$^{-1}$ of mouse monoclonal antibody against the FLAG tag (Sigma-Aldrich, St Louis, MO) in PBS for 1.5 h at room temperature. After washing with PBS, the cells were incubated with 3 µg mL$^{-1}$ of AlexaFluor488-conjugated goat antimouse IgG antibody (Invitrogen, CA) in PBS for 1.5 h at room temperature, and then washed again. Fluorescence of the yeast cell surface was observed using an Olympus BX51 microscope (Olympus, Tokyo, Japan). Fluorescence units were measured using the SpectraMax M2 Microplate Reader (Molecular Devices, CA) with

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**Fig. 1** Subcutaneous administration of the recombinant antigens with IFA (incomplete Freund’s adjuvant). (a) Antibody response ($P < 0.01$). (b) Survival test. Closed circles, administration of Enol1p; closed triangles, administration of PBS.

**Fig. 2** Intranasal administration of the recombinant antigens with CT (cholera toxin). (a) Antibody response ($P < 0.05$). (b) Survival test. Closed circles, administration of Enol1p; closed triangles, administration of PBS.
excitation and emission wavelengths of 495 and 519 nm, respectively.

**Animals**

Female C57BL/6 mice were obtained from Japan SLC, Inc. (Shizuoka, Japan). Mice were maintained in a specific-pathogen-free manner and allowed to drink and eat ad libitum.

**Immunization by intranasal or subcutaneous administration using recombinant proteins**

Seven-week-old female C57BL/6 mice (eight per group) were immunized with 30 µg of *E. coli*-expressed recombinant Eno1 protein by intranasal delivery (i.n.) or subcutaneous injection (s.c.), using it in 20-µL volumes containing 1 µg of cholera toxin (Sigma-Aldrich) (i.n.) or 100-µL volumes containing Freund’s incomplete adjuvant (DIFCO Laboratories, Detroit, MI) (s.c.). Eight female C57BL/6 mice of the same age, which received adjuvant alone mixed with PBS, served as a control group. Mice were immunized at weeks 0, 2 and 4. Blood samples were collected at week 6 from the tail vein for determination of the titer of serum IgG that binds to Eno1p.

**Immunization by oral administration using yeast cells that display Eno1p on their surfaces**

Seven-week-old female C57BL/6 mice were used for immunization experiments. A solution of *S. cerevisiae* cells that display Eno1p on their surfaces (1.6 × 10^9 cells/400 µL) were administered to 10 mice per dose at weeks 0, 1 and 3 (priming) and at week 7 (booster). Wild-type *S. cerevisiae* was used as a control. All inoculums were suspended in PBS (400 µL per animal) and administered via an intragastric tube after 2 h of fasting, once per day for 5 days per week. Blood samples were collected at week 9 from the tail vein to determine the titer of serum IgG.

**Challenge with *C. albicans***

For survival studies, mice were infected with 1.1 × 10^5 cells of *C. albicans* resuspended in 100 µL PBS by tail-vein injection 2 weeks after the last immunization. Mice were observed daily for 4 weeks after challenge. All animal experimental protocols were approved by the Institutional Animal Care and Use Committee, and animal experiments were conducted according to the institutional ethical guidelines for animal experiments. The outcomes of *C. albicans* challenge were shown using the Kaplan-Meier method. Significant differences between groups were tested using the log-rank method.

**Determination of endpoint titer**

Indirect enzyme-linked immunosorbent assay (ELISA) was conducted for antibody analysis for antisera collected at week 6 (i.n. or s.c.) or week 9 (p.o.). Briefly, 96-well microtiter plates (Nalge Nunc International, Rochester, NY) were coated with 50 µL per well of *E. coli*-expressed recombinant Eno1 (0.01 µg µL^-1). The plates were blocked with 1% bovine serum albumin (BSA) dissolved in PBS containing 0.05% Tween-20. Serially diluted antisera and horseradish peroxi-

![Fig. 3](image) **Fig. 3** Immunofluorescence microscopic observation of displayed Eno1 on *Saccharomyces cerevisiae*. (a,b) BY4741 (control cell). (c,d) BY4741 harboring pULD1-eno1. (a,c) Bright-field images. (b,d) fluorescence microscopic images after staining using the anti-FLAG antibody. Scale bars = 10 µm.
Results and discussion

Extraction of Eno1p from E. coli for s.c. injection and i.n. administration

The use of PCR to amplify the Eno1p-encoding sequence from genomic DNA extracted from C. albicans as a template yielded a fragment of the expected size, which was cloned into the pQE30 vector that was designed to produce His-tagged recombinant proteins. After confirmation that the nucleotide sequence of the insert within pQE-Eno1 was confirmed as correct by comparison with the Candida genome database (http://www.candidagenome.org/), the plasmid was introduced into E. coli BL21, and the recombinant protein was produced in the cytosol, and then recovered, and purified by passage of the extract through a nickel-chelated column. The recombinant protein was further purified by passage through an endotoxin-removing column. The concentration of endotoxin in eluate from the nickel-chelated column ranged from 0.06 to 0.125 EU (endotoxin units) mL$^{-1}$. Analysis of Eno1 proteins using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting indicated that anti-His antibody recognized the purified protein (data not shown).

Vaccination by s.c. and i.n. administration of Eno1p

We used both s.c. injection and i.n. administration to examine the efficacy with which delivery of recombinant Eno1 could induce immunity. The ability of vaccination with Eno1p to protect against candidiasis was determined by testing survival after administration of a lethal dose of C. albicans.

Administration of Eno1p with adjuvant by s.c. administration elevated titers of antibody that bound to Eno1p (Fig. 1a). At 35 days after challenge with a lethal dose of C. albicans, 12.5% of mice vaccinated with Eno1p were alive, whereas none of the control animals infected with the same dose of C. albicans SC5314 (Hube et al., 1997) survived longer than 23 days after infection (Fig. 1b).

As was observed for s.c. administration, i.n. administration of Eno1p with adjuvant increased titers of Eno1p-specific antibody (Fig. 2a). However, approximately twice as many animals (25% compared with 12.5%) survived challenge with C. albicans after i.n. administration of the vaccine compared with s.c. injection (Fig. 2b). Although our finding that vaccination with Eno1p can protect against subsequent exposure to C. albicans is consistent with the previous report that showed the ability of Eno1p to induce immunity to C. albicans in mice (Montagnoli et al., 2004), our findings reveal for the first time that i.n. administration of Eno1p appears to induce protective immunity against C. albicans more effectively than s.c. delivery of the protein.

Preparation of Eno1p-displaying yeast for oral administration

A S. cerevisiae strain that displays Eno1p on its surface was generated by transforming yeast strain BY4741 with the pULD1-eno1 plasmid and confirmation of successful transformation with this plasmid by auxotrophic selection and colony direct PCR. The strain that harbored pULD1-eno1 was named BY4741/eno1. Display of Eno1p on the cell surface of BY4741/eno1 was observed by immunofluorescence microscopy. Whereas no fluorescence was observed on the surfaces of cells of the parent strain BY4741 (Fig. 3a and b), clear fluorescence of AlexaFluor488 was
observed around the surfaces of BY4741/eno1 cells (Fig. 3c and d).

To quantify changes in the relative amounts of Eno1p displayed on the surfaces of yeast cells during cultivation, the fluorescence of yeast cells stained with AlexaFluor488 was analysed using a multiwell plate reader. The amounts of displayed Eno1p increased for the first 24 h of the growth cycle (data not shown).

Vaccination by oral administration of yeast cells that display Eno1p on their surfaces

Cultures used for oral administration of Eno1p-displaying yeast cells to mice were grown for 24 h, given that this was the length of culture when cells displayed the largest number of Eno1p-fusion proteins on their surfaces. Mice were vaccinated by oral administration of the cells four times over the course of a 7-week period before challenge with a lethal dose of *C. albicans*. The average titer of antibody against Eno1p generated after oral administration of yeast cells that display Eno1p was 5.2 x 10^3, although the value varied substantially between animals, 1 x 10^2 to 5.2 x 10^4 (Fig. 4a). Although this dispersion was different from results of conventional administration of the antigenic protein (Figs 1a and 2a), the yeast positively gave enough immunological stimuli to almost all mice. Examination of survival rate after challenge with *C. albicans* for 35 days indicated that 60% of mice that received oral administration of Eno1p-displaying cells survived longer than mice that received oral administration of control cells (Fig. 4b). This survival rate is better than that associated with the more conventional modes of immunogen administration, i.e. s.c. injection and i.n. administration. These results suggest that display of a suitable antigen on the surfaces of orally delivered yeast cells generated by molecular display might provide a convenient and effective type of oral vaccine against various infectious diseases. Moreover, this yeast oral vaccine can be prepared rapidly because unlike proteins produced in *E. coli*, it does not require a complicated purification step (Fig. 5). These advantages are also relevant for emerging pandemics.

The immunological responses shown in Fig. 4a show that administration of control *S. cerevisiae* gave an immune response IgG titer of 2 x 10^2 as well as of Incomplete Freund’s adjuvant and cholera toxin (Figs 1a and 2a). *Saccharomyces cerevisiae* is thought to have a better adjuvant function as a result of β-glucan in its cell wall (Berner *et al.*, 2008; Rodriguez *et al.*, 2009).

Conclusions

After Eno1p was successfully produced by *E. coli* and purified by passage through endotoxin-removing columns, the protein was given to mice before challenge with a lethal dose of *C. albicans* cells. Survival tests confirmed the protective efficacies of this antigen: after more than 1 month, survival rates associated with s.c. injection and i.n. infection were 12.5% and 25%, respectively. Next, Eno1p was produced on the surface of *S. cerevisiae* cells and was administered orally to mice. Oral delivery of yeast cells that displayed Eno1p on their surfaces protected 60% of mice against candidiasis. The present study demonstrates the considerable potential of molecular display of immunogens on microbial cells to generate oral vaccines for...
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convenient protection against infectious diseases. The only requirement is the availability of a DNA sequence that encodes the antigenic protein; no purification step is needed to produce this oral vaccine. However, whether it retains protective effect after attenuation of whole cells as in the previous study using another type of antigen-displaying cell (Adachi et al., 2010) needs to be examined, because S. cerevisiae sometimes causes invasive infection (Enache-Angoulvant & Hennequin, 2005).

For further development of a promising vaccine that protects against candidiasis, we have used proteomic analyses to investigate other candidate antigenic proteins (Aoki et al., 2013). This should lead to novel antigens that have a stronger effect as a vaccine.

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References


