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Efficient antibody production in the methylotrophic yeast *Ogataea minuta* by overexpression of chaperones

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A production system for a therapeutic monoclonal antibody was developed using the methylotrophic yeast *Ogataea minuta* IFO10746. The genetically engineered *O. minuta* secreted a detectable amount of anti-TRAIL receptor antibody into the culture supernatant, and the secreted antibody was purified by multiple column chromatography steps. In the purification process, both fully and partially assembled antibodies were detected and isolated. The fully assembled antibody from *O. minuta* showed almost the same biological activity as that derived from mammalian cells despite the distinct glycosylation profile, whereas the partially assembled antibody showed no cytotoxic activity. To increase the production of active antibody in *O. minuta*, we overexpressed selected chaperone proteins (included protein disulfide isomerase (OmPDI1), thiol oxidase (OmERO1), and immunoglobulin heavy chain binding protein (OmKAR2)) known to assist in the proper folding (in the endoplasmic reticulum) of proteins destined for secretion. Each of these chaperones enhanced antibody secretion, and together these three factors yielded 16-fold higher antibody accumulation while increasing the ratio of the fully assembled antibody compared to that from the parental strain. Supplementation of a rhodanine-3-acetic acid derivative (R3AD_1c), an inhibitor of *O*-mannosylation, further increased the secretion of the correctly assembled antibody. These results indicated that the co-overexpression of chaperones is an effective way to produce the correctly assembled antibody in *O. minuta*.

[**Key words:** Antibody production; Chaperones; Correct protein assembly; *Ogataea minuta*; Protein folding]

In recent years, multiple antibodies have been developed as therapeutic agents, primarily for treatment of cancer and rheumatoid arthritis. These agents prime the endogenous human immune system by binding to a specific molecular target and inducing antibody-dependent cellular cytotoxicity (ADCC), complement-dependent cytotoxicity, and other useful processes. These biologics exhibit strong therapeutic activities, increased affinities for specific antigens, and decreased side-effects; as a result, the demands for novel monoclonal antibodies (mAbs) as well as biosimilar and bio-better products have increased over time. The mAb therapeutic sector represented around 7% of the market in 2011 and was expected to exceed 10% in 2015 (1).

In most cases, therapeutic antibodies are produced by mammalian cells such as Chinese hamster ovary (CHO) cells (2,3). These systems are adequate for post-translational modification of the antibodies, especially complex-type N-linked sugar chain modification, and for higher levels of production. Productivity of these systems reached around 5 g/L by 2004 as a result of the combination of optimization of culture conditions and development of useful host cells (4). However, the overall cost of antibody production typically is quite high, due to the time-consuming and laborious scheme of establishing production cells, expenses of media and column chromatography resins, and long cultivation time. The high production costs can necessitate high drug prices, which in turn pose economic and industrial problems for expanding clinical applications. Consequently, alternative low-cost production systems are desired.

Yeasts are among the most promising expression systems for the production of antibodies. In these unicellular fungi, proteins can be (i) targeted for secretion into the culture broth, (ii) properly folded by eukaryotic quality control mechanisms, and (iii) glycosylated by post-translational modification; additionally, (iv) the doubling time of yeasts are shorter than those of mammalian cells during cultivation, and (v) less expensive media can be used. Recently, we reported the use of the methylotrophic yeast *Ogataea minuta* as an antibody production system (5,6). In early experiments, the produced antibody was degraded by the yeast's endogenous proteases and was modified via yeast-specific O-glycosylation. We therefore inactivated some proteases by genetic manipulation and supplemented the medium with a derivative of rhodanine-3-acetic acid (R3AD), a compound known to suppress O-glycosylation; these modifications yielded an increased amount of fully assembled antibody composed of two identical heavy chains and two identical light chains (H2L2). However, the productivity and the product

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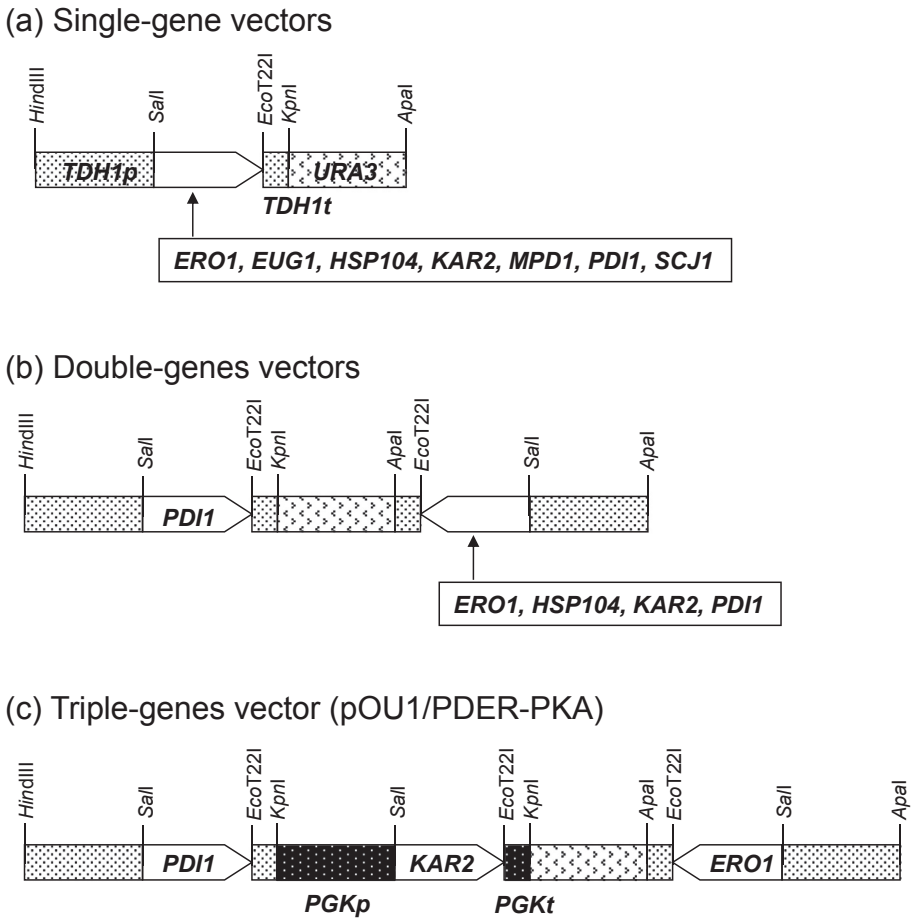

FIG. 1. Schematics of the vectors used for the overexpression of *O. minuta* chaperones. (a) Single-gene vectors. The genes encoding chaperones were inserted between the *TDH1* promoter (TDH1p) and the *TDH1* terminator (TDH1t). (b) Double-gene vectors. *OmPDI1* and another gene were cloned in convergent orientations. (c) Triple-gene vector. *OmKAR2* was inserted between the *PGK* promoter (PGKp) and the *PGK* terminator (PGKt), such that *OmPDI1* and *OmKAR2* were oriented in the same direction.

quality were still insufficient to permit use as an alternative antibody expression system.

One technique for increasing the yield and quality of heterologous protein production is reinforcement of the function of endoplasmic reticulum (ER) -resident chaperones. For example, BiP/KAR2 and protein disulfide isomerase (PDI), which play a central role in the unfolded protein response (UPR) (7), have shown desirable effects in the production of single-chain antibody fragments by the yeasts *Saccharomyces cerevisiae* and *Pichia pastoris* (8–11). In the context of antibody production in mammalian cells, several reports have demonstrated that overexpression of PDIs contributes to correct folding, resulting in increased efficiency of antibody secretion (12,13).

In the present study, we tried to increase the production of an anti-TRAIL receptor antibody (14) by an *O. minuta* expression system. Genes corresponding to *O. minuta* ER-resident chaperones were cloned and tested for their effects on the titer and cytotoxicity of anti-TRAIL receptor antibody production by an *O. minuta* host. Among the chaperones tested in this study, the proteins encoded by the *O. minuta* homologues of *S. cerevisiae PDI1*, *ERO1*, and *KAR2* each showed strong positive effects on antibody production. Furthermore, the combination of these three chaperones dramatically enhanced active anti-TRAIL receptor antibody production, indicating that the increased production of ER-resident chaperones is an effective way to improve the productivity and activity of heterologous antibody synthesis.

MATERIALS AND METHODS

Yeast strain and media *O. minuta* YK6 (*Δoch1Δyps1Δura3Δade1*), which has been described previously (5), was used as a host strain for antibody production. SD−CA agar (0.67% yeast nitrogen base without amino acids, 0.05% casamino acids, 2% glucose, 0.002% tryptophan) was used for regeneration of cells after transformation. YPDA (1% Difco yeast extract, 2% Bacto peptone, 2% glucose, 0.004% adenine) was used for seed cultivation, and 2xYP-P6-GG medium (2% Difco yeast extract, 4% Bacto peptone, 0.5% glucose, 2% glycerol, 0.1 M phosphate buffer, pH 6.0) was used for antibody production. Stocks of representative clones were stored as suspensions in 17% glycerol at −80°C.

Chemicals and general recombinant DNA techniques *Escherichia coli* JM109, restriction enzymes, and DNA modifying enzymes were purchased from Takara Bio, Inc. (Shiga, Japan). Media, growth conditions, and general recombinant DNA techniques used in this study were as described by Sambrook and co-workers (15). Zeocin was purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, USA). All oligonucleotides in this study were synthesized by FASMAC Co., Ltd. (Kanagawa, Japan). PCR amplification was performed using a GeneAmp PCR System 9700 (Thermo Fisher Scientific, Inc.). Automated DNA sequencing was performed on an ABI PRISM 3700 DNA Analyzer (Thermo Fisher Scientific, Inc.). Rhodanine-3-acetic acid derivative 1c (R3AD_1c) was chemically synthesized according to the method described by Orchard et al. (16).

Transformation of *O. minuta* Electro-transformation of *O. minuta* was performed using an ECM 600 (BTX-Harvard Apparatus, Inc., Holliston, MA) as described previously (17); the transformed cells were regenerated on SD−CA agar under an appropriate selection pressure at 30°C for approximately 3 days. The transformants were transferred to the same selection media to confirm the auxotrophy or antibiotic resistance. *O. minuta* genomic DNA was isolated using Y-DER Yeast DNA Extraction Reagent (Thermo Fisher Scientific, Inc.). AccuPrime *Pfx* DNA Polymerase (Thermo Fisher Scientific Inc.) and Takara LA Taq with GC Buffer (Takara Bio, Inc.) were used for PCR cloning and colony PCR, respectively.

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