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Construction and characterization of *Pichia pastoris* strains for labeling aromatic amino acids in recombinant proteins

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Abstract

Strains of the methylotrophic yeast *Pichia pastoris* auxotrophic for the aromatic amino acids (tyrosine, phenylalanine, and tryptophan) have been constructed by targeted gene disruption for protein labeling applications. Three strains, with defects in *ARO1* (coding for a homolog of the arom pentafunctional enzyme), *ARO7* (coding for chorismate mutase), and *TYR1* (coding for prephenate dehydrogenase), have been engineered in a *P. pastoris ura*3Δ1 parent strain using standard methods. The nutritional requirements of these auxotrophic strains have been characterized and their utility as expression hosts for labeling recombinant proteins has been demonstrated. All three strains show a surprising sensitivity to rich culture medium and must be grown in supplemented minimal medium. The *tyr1*::*URA3* strain in particular is strongly inhibited by tryptophan, and to a lesser extent by phenylalanine, leucine, and isoleucine. Highly efficient incorporation of exogenously supplied amino acids by these three auxotroph strains has been demonstrated using recombinant galactose oxidase. Stereochemically pure L-amino acids and racemic D,L-mixtures serve nearly equally well to support protein expression and labeling. These strains allow efficient labeling of aromatic amino acids in recombinant proteins, supporting NMR structural biology and a wide range of other biophysical studies.

Keywords: *Pichia pastoris*; Aromatic amino acids; Auxotroph; Strain construction; Galactose oxidase

The methylotrophic yeast *Pichia pastoris* has emerged as one of the most important expression hosts for high-level production of recombinant proteins in structural genomics research and biotechnology [1–3]. These applications take advantage of a number of features that make this expression host unique, including the availability of the tightly regulated yet highly inducible *AOX1* promoter system [1], the adaptability of *Pichia* cultures to high density fermentation [4], the simplicity of methanol induction, the suitability of *Pichia* yeast for expression of proteins requiring eukaryotic post-translational processing (including glycosylation) [1], and the robust secretory system supporting expression levels that in some cases can reach gram per liter of recombinant protein in the culture medium [1–3].

Most of these applications have utilized the wild type *P. pastoris* strain that was originally isolated for biomass production from methanolic fermentation [1,5]. A limited number of auxotrophic strains of *P. pastoris* (including strains with defects in *HIS4* [6], *ARG4* [7], *ADE1* [7], *URA3* [7], *URA5* [8], *CYS4* [9], and *FLD1* [10]) are currently available for selection and genetic complementation experiments, far less than are available in alternative expression hosts, such as *Saccharomyces cerevisiae* (for which genome-wide gene knock-out coverage is now available [11] and designer deletions may be routinely prepared [12]) and *Escherichia coli*. The sequencing of the complete genome of *P. pastoris* will

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ultimately permit a wide range of strains to be engineered for specific applications by gene replacement techniques. In particular, strains that are defective in amino acid biosynthesis will be useful not only for selection/complementation studies in yeast genetics but will have additional important applications in labeling recombinant proteins with amino acid analogs (e.g., fluoro derivatives) or isotopically enriched amino acids for NMR structural biology [13], supporting the emerging role of NMR spectroscopy in structural genomics [14]. Uniform labeling of recombinant protein with ^{15}N and ^{13}C has already been achieved with wild type *P. pastoris* grown on isotopically enriched nitrogen and carbon sources [15,16]. However, such labeling is unselective, and specific amino acids cannot be labeled efficiently with the wild type strain because dilution by endogenously formed amino acids limits the incorporation of label into the protein. Techniques such as NMR spectroscopy that will be used to define the structure of protein gene-products in the proteomic analysis of emerging genomes (including the human genome) will benefit from expanding the capabilities of this proven expression host through auxotrophic strain construction.

To use the *Pichia* expression system to specifically label tyrosine residues in recombinant proteins for spectroscopic studies, we have engineered *P. pastoris* strains that are auxotrophic aromatic amino acids (tyrosine, phenylalanine, and tryptophan) using gene disruption techniques (Fig. 1). The nutritional requirements of these strains have been determined and their application in isotopic labeling of protein has been demonstrated for recombinant galactose oxidase [17]. Galactose oxidase is a metalloenzyme containing a unique tyrosyl-cysteine free radical cofactor in its catalytic active site [18], allowing the extent of incorporation of exogenous deuterium-labeled amino acid to be quantitatively determined through analysis of the free radical EPR spectrum.

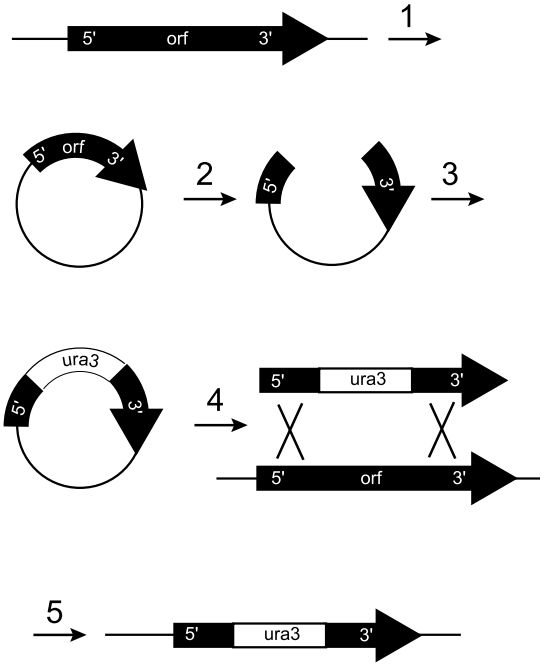

Fig. 1. General procedure for targeted gene disruption. (1) PCR amplification and cloning of target gene; (2) linearization and dephosphorylation; (3) ligation with *URA3* marker; (4) PCR amplification of the disruption cassette and transformation into host; and (5) homologous recombination with target locus generates gene disruption strain.

Materials and methods

Culture media

Pichia pastoris X33 was grown in YPD. *P. pastoris ura3*Δ1 was grown in YPD supplemented with 50 mg/L uridine. *Pichia pastoris ura3*Δ1 *aro1*::*URA3* and *aro7*::*URA3* were grown in YNB-AS-AA medium (Difco) containing ammonium sulfate and 2% glucose, supplemented with 150 mg/L each of tyrosine, tryptophan, and phenylalanine. *P. pastoris ura3*Δ1 *tyr1*::*URA3* was grown in YNB-AS-AA medium (Difco) containing ammonium sulfate and 2% glucose, supplemented with 100 mg/L tyrosine.

Strain construction

Pichia pastoris X33 wild type strain was from Invitrogen (Carlsbad, CA). *Pichia pastoris* X33 genomic DNA was isolated from a 5 mL overnight YPD culture using Qiagen Genomic-tip 100/G column purification. The *P. pastoris URA3* locus was amplified from genomic template using 5′-phosphorylated primers 5′-P-CTCG ATCCTGAGGGCTTGAGGAACTTTTCTGCAC-3′ and 5′-P-AGACCTAGGCAGTTGGTGAGCTTACA TGAG-3′, based on the published sequence (Genbank Accession No. AF321098) [7]. The 1364 bp PCR product was cloned into pPCRScript (Stratagene) and a plasmid containing the insert was isolated and the sequence was verified by automated DNA sequencing (Molecular Biology Core Facility, OHSU). Phosphorylated internal deletion primers having opposite orientations (5′-P-TGTCTAGGTATCTATCCCTTTGATCAGGT TG-3′ and 5′-P-TGGGAAGCTTACCAAAATATTCT GAGG-3′) were used to generate blunt-ended vector arms terminating in the 5′-flanking region and 3′-terminus of the insert from the pPCRScript template. The PCR product was treated with *Dpn*I to remove the template and ligated to generate the *ura3*Δ1 deletion vector. Following plasmid selection and characterization, the *ura3*Δ1 deletion product was amplified by PCR using the *URA3* primer set and transformed into electrocompetent *P. pastoris* X33 using standard methods. Transformants were allowed to recover in 1 M sorbitol (1 h, 30 °C) and selected on YNB + uridine + glucose + 0.1% FOA agar. Positive clones were purified by single colony isolation,