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Differential secretion pathways of proteins fused to the *Escherichia coli* maltose binding protein (MBP) in *Pichia pastoris*

Pachai S. Moua [a], Alfonso Gonzalez [a], Kristin T. Oshiro [a], Vivian Tam [a], Zhiguo Harry Li [b], Jennifer Chang [a], Wilson Leung [a], Amy Yon [a], Der Thor [c], Sri Venkatram [a], Andreas H. Franz [b], Douglas D. Risser [a], Joan Lin-Cereghino [a], Geoff P. Lin-Cereghino [a, *]

[a] Department of Biological Sciences, University of the Pacific, Stockton, CA 95211, United States
[b] Department of Chemistry, University of the Pacific, Stockton, CA 95211, United States
[c] Department of Biomedical Sciences, Arthur A. Dugoni School of Dentistry, University of the Pacific, San Francisco, CA 94103, United States

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ABSTRACT

The *Escherichia coli* maltose binding protein (MBP) is an *N*-terminal fusion partner that was shown to enhance the secretion of some heterologous proteins from the yeast *Pichia pastoris*, a popular host for recombinant protein expression. The amount of increase in secretion was dependent on the identity of the cargo protein, and the fusions were proteolyzed prior to secretion, limiting its use as a purification tag. In order to overcome these obstacles, we used the MBP as C-terminal partner for several cargo peptides. While the Cargo-MBP proteins were no longer proteolyzed in between these two moieties when the MBP was in this relative position, the secretion efficiency of several fusions was lower than when MBP was located at the opposite end of the cargo protein (MBP-Cargo). Furthermore, fluorescence analysis suggested that the MBP-EGFP and EGFP-MBP proteins followed different routes within the cell. The effect of several *Pichia pastoris beta-galactosidase supersecretion* (*bgs*) strains, mutants showing enhanced secretion of select reporters, was also investigated on both MBP-EGFP and EGFP-MBP. While the secretion efficiency, proteolysis and localization of the MBP-EGFP was influenced by the modified function of Bgs13, EGFP-MBP behavior was not affected in the *bgs* strain. Taken together, these results indicate that the location of the MBP in a fusion affects the pathway and *trans*-acting factors regulating secretion in *P. pastoris*.

1. Introduction

Pichia pastoris is a methylotrophic yeast that has been genetically engineered to express heterologous proteins valued for applied and basic research purposes [1,2]. Since its development as a recombinant expression system in the 1980's, over 5000 proteins from bacteria to humans have been produced in this yeast according to pichia.com. The use of *Pichia pastoris* as a foreign host offers many advantages, as compared to other expression systems [3,4]. First, gene targeting by homologous recombination, high frequency DNA transformation, and cloning by functional complementation are all available techniques for genetic manipulation. Second, the yeast can express proteins at high levels, in shake-flask or fermenter, intracellularly or extracellularly, with the methanol-inducible alcohol oxidase I (*AOXI*) promoter. Third, unlike prokaryotes, *P. pastoris* is capable of performing many eukaryotic posttranslational modifications, such as glycosylation and proteolytic processing. Additionally, *P. pastoris* is safe, economical, and many reagents are commercially available. Finally, compared to baker's yeast, *P. pastoris* is able to reach higher cell densities in fermenters because of its preference for respiratory growth and shows a lesser tendency to hyperglycosylate secreted proteins [5].

For the *P. pastoris* expression system, there are several secretion signal peptides that can be attached to the recombinant protein, causing it to be exported out of the cell [6]. The 85 amino acid

Abbreviations: MBP, Maltose binding protein; EGFP, Enhanced Green Fluorescent Protein; *bgs*, *beta-galactosidase supersecretion*; FXa, Factor Xa; tER, Transitional ER.

* Corresponding author. Department of Biological Sciences, University of the Pacific, 3601 Pacific Avenue, Stockton, CA 95211, United States.
E-mail address: glincere@pacific.edu (G.P. Lin-Cereghino).
URL: http://www.pacific.edu/Academics/Schools-and-Colleges/College-of-the-Pacific/Academics/Departments-and-Programs/Biological-Sciences/Our-Faculty/Directory/GLin-Cereghino.html

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Saccharomyces cerevisiae MATα prepro signal peptide has been the most successful in this role and is the one commonly included in commercially available vectors [7,8]. The secreted recombinant protein is the major polypeptide species found in the extracellular growth medium because *P. pastoris* secretes few of its own proteins [3]. This programmed export acts as a valuable step in the purification of the heterologous protein, making it a convenient host in terms of time and resources. However, despite these strengths, there are limitations to the *P. pastoris* system. Foremost, some recombinant proteins, which are engineered to be secreted, are retained inside the yeast cell and are often misfolded and/or degraded, creating a major obstacle [9–13]. They may: 1) accumulate in regions of the ER, Golgi, or endosomal compartments; 2) undergo ER-associated protein degradation (ERAD), where the protein is translocated from the ER to the cytosol and degraded by the proteasome; or, 3) be degraded in the vacuole. Retention and degradation are common problems in *Escherichia coli* and other bacteria as well, but in these prokaryotic systems the production of several proteins has been improved by an in frame translational fusion with a partner protein [14,15]. Fusion escorts or partners, such as the small ubiquitin-like modifier (SUMO), the *Trichoderma harzianum* cellulose binding domain (CBD), and the *E. coli* maltose binding protein (MBP), are believed to assist in the proper folding of the desired protein so that the hybrid can arrive at its extracellular destination [16–19]. Not only do these partner proteins improve secretion efficiency, but, these escort peptides usually aid in purification by affinity chromatography. After the escort domain binds to a matrix and the hybrid protein is purified, the partner is removed by protease treatment, liberating the desired cargo protein [20].

We recently reported that we used the *E. coli* maltose binding protein (MBP) as an *N*-terminal partner protein in *P. pastoris* [21]. We initially hypothesized that the secretion of some foreign proteins by *P. pastoris* would be improved by fusing them in frame to the MBP, the product of the *E. coli malE* gene. We created constructs containing the MATα prepro sequence and a MBP fused to a variety of C-terminal cargo proteins (MBP-Cargo). Inserted between *N*-terminal MBP and the C-terminal cargo protein was a recognition site for a commercially available mammalian protease (Fig. 1A). We wished to determine whether MBP could enhance the export of the proteins, which by themselves displayed different levels of secretion efficiency. However two major problems were encountered. First, the effect on secretion efficiency of the *N*-terminal MBP was variable; the secretion of some cargo proteins increased while others decreased. Second, when MBP was fused as an *N*-terminal partner to several C-terminal cargo proteins expressed in this yeast, proteolysis occurred between the two peptides, and MBP reached the extracellular region unattached to its cargo. Extensive mutagenesis of the spacer region/protease site between MBP and its C-terminal cargo protein could not inhibit the cleavage although it did cause changes in the protease target sites in the fusion proteins, as determined by mass spectrometry. Taken together, these results suggested that the three dimensional structure of MBP triggered attack by an uncharacterized *P. pastoris* protease at a nonspecific region C-terminal of the MBP domain [21].

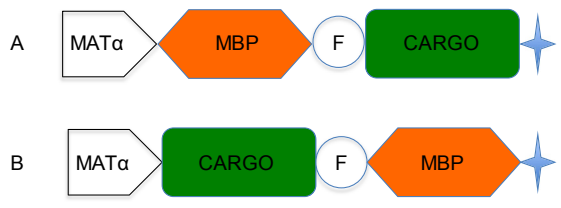

Fig. 1. Diagram of Representative Constructs. Constructs were made with combinations of five elements: the MATα prepro peptide (MATα), *E. coli* maltose binding protein (MBP), a protease site for Factor Xa (F), a cargo protein (CARGO), and a c-myc tag (✦) for antibody detection.

In a related work, our lab isolated a set of mutant strains of *P. pastoris* that secreted increased levels of a beta-galactosidase reporter but differed in their ability to export other reporters including horseradish peroxidase and *Candida antarctica* lipase B [22]. Bioinformatic analysis of these twelve *Beta-Galactosidase Supersecretion (BGS)* genes suggested that they may have functions in intracellular signaling or vesicle transport. Several of these strains also appeared to contain a more permeable cell wall. However, the mechanisms by which the modified function of the *BGS* gene products led to increased secretion need to be elucidated.

In the present work, building upon these two previous endeavors [21,22], we have explored whether the MBP domain could function as a secretion enhancer when it was fused as a C-terminal partner to an *N*-terminal cargo protein (Cargo-MBP) in both wild type and *bgs* strains. Interestingly we received different results with the Cargo-MBP constructs compared to our original MBP-Cargo fusions. Furthermore, the processing of both fusions showed differences in wild type compared to certain *bgs* strains. Overall, our results suggest that not only is the order of partner proteins important for determining secretion efficiency, but different *trans*-acting factors in the *P. pastoris* cell are involved, depending on the location of the MBP domain in the fusions.

2. Material and methods

2.1. Strains and growth conditions

E. coli One Shot® TOP10 chemically competent cells (Life Technologies, Carlsbad, CA) were used for transformation and plasmid DNA amplification unless stated otherwise. The TOP10 transformants were grown in Lennox Broth (LB) media with the addition of 100 μg/mL of Ampicillin or 25 μg/mL of Zeocin™ at 37 °C in the New Brunswick Scientific C25 Incubating Shaker (Edison, NJ) at 225 rpm. The *P. pastoris* strains, yJC100 (wt), yGS115 (*his4*) and yGS200 (*his4 arg4*), were derived from the original strain, NRRL Y11430 (North Regional Research Laboratories, US Department of Agriculture, Peoria, IL). yDT39 (*his4 met2*) [23] and the *bgs* mutants [22] derived from it were generated in our lab. These yeast strains were cultured in YPD (1% yeast extract, 2% peptone, and 2% glucose) supplemented with 100 μg/mL Zeocin or 0.5 mg/mL G418 for antibiotic resistance selection, YND (0.17% yeast nitrogen base (YNB) with 0.5% ammonium sulfate, 0.4% glucose), BMGY (1% glycerol, 2% peptone, 2% glucose, 1.34% YNB, 4×10^{-5}% biotin, 100 mM potassium phosphate with pH 6.0), or BMMY (0.5% methanol, 2% peptone, 1% yeast extract, 1.34% YNB, 4×10^{-5}% biotin, 100 mM potassium phosphate, pH 6.0) [24]. *P. pastoris* cells were grown in liquid culture at 29 °C in a VWR 1585 Shaking Incubator (Batavia, IL) set to 325 rpm, and cells that were grown on agar plates were incubated at 30 °C in a Fisher Scientific Isotemp Incubator. Cell optical densities were measured at 600 nm using a Spectronic Genesys 2 spectrophotometer (Spectronic Instruments Inc., Rochester, NY). DNA concentrations were determined using a NanoDrop 2000c (Thermo Scientific, Wilmington, DE) set to 260 nm.

Recombinant DNA methods, including bacterial transformation, were performed essentially as described [25]. Plasmid DNA was purified from *E. coli* cultures using a QIAprep Spin Miniprep Kit (Qiagen, Chatsworth, CA). PCR products were purified with the QIAprep PCR Cleanup Kit (Qiagen, Chatsworth, CA) prior to restriction digestion. Restriction enzymes were purchased from MBI Fermentas (Hannover, MD). DNA digested with restriction enzymes