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Gateway cloning is compatible with protein secretion from *Pichia pastoris*

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Abstract

Secretion of a recombinant protein from the yeast *Pichia pastoris* requires the presence of a signal peptide at the amino terminus. Maintaining the full amino acid sequence of the signal peptide is thought to be important for proper signal processing and protein secretion. We show that at least for one protein, a synthetic human interferon, the presence of a Gateway recombination site within the signal peptide is fully compatible with high levels of protein secretion. The amino termini of the secreted interferon proteins cloned with Gateway and cloned with restriction enzymes and ligase are identical, and the proteins were highly active in biological assays. Compatibility with Gateway cloning simplifies construction of plasmids directing secretion of recombinant proteins from *P. pastoris*.

Keywords: *Pichia pastoris*; Gateway cloning; Secretion; Interferon

Interferons (IFNs) are widely used as human therapeutics [1]. A new class of hybrid IFNs [2] is being investigated for possible human use against cancer and viral pathogens such as the coronavirus that causes severe acute respiratory syndrome (SARS). One of these, a chimeric human IFN-α called Hy3 (GenBank AF085805), was insoluble when expressed in *Escherichia coli* (unpublished data), so the yeast *Pichia pastoris* was tested as an expression host.

Pichia pastoris is a widely used host for expressing recombinant proteins (http://faculty.kgi.edu/cregg/Pichia2004.htm). Because it can attain very high cell densities in chemically defined medium, and because it naturally secretes few proteins of its own, *P. pastoris* can be an efficient producer of secreted proteins. Secretion of the protein of interest is often directed by fusing the gene to be expressed to the *Saccharomyces cerevisiae* α mating factor (AMF) signal sequence [3]. The AMF signal sequence comprises the amino terminal 89 amino acids of the AMF protein. During secretion of the AMF from *S. cerevisiae* the signal peptide is removed by two processing enzymes. The enzyme Kex2 cleaves after a specific lysine-arginine, then Ste13 clips off the dipeptides EA and EA to yield the mature AMF [4] (Fig. 1). Homologous enzymes operate in *P. pastoris*. It is preferable to keep the amino acid sequence of the signal peptide unchanged if possible while fusing it to a gene to be expressed. The standard way to do this is to remove part of the cloning vector from the *Xho*I site 6 amino acids upstream of the processing sites to a point downstream, then to construct the *Xho*I fragment containing the gene of interest with a 5′

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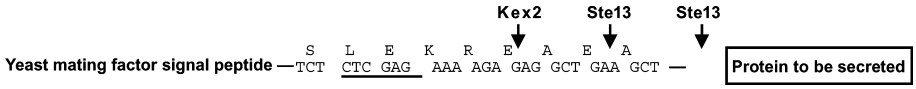

Fig. 1. Locations of cleavage in the *S. cerevisiae* AMF signal peptide for proteases Kex2 and Ste13. Homologous enzymes operate in *Pichia pastoris*. The indicated *Xho*I site is used as a cloning site in either REaL or Gateway construction of secretion plasmids. A *Not*I site and another *Xho*I site are located further downstream.

*Xho*I site and add a sequence encoding the missing AMF amino acids onto the gene during its construction, e.g., by adding a sequence encoding the missing amino acids in frame via PCR (Fig. 2). These steps reconstitute the complete natural AMF signal peptide fused to the gene of interest, such that the activities of *P. pastoris* homologs of Kex2 and Ste13 should yield secreted protein with the desired amino terminal structure. Even with the full mating factor signal peptide, however, the extent of processing during secretion varies with the particular protein being expressed (for example, see [5,6]).

Executing this construction with restriction enzymes and ligase (REaL) is sometimes complicated by the presence of enzyme sites within the gene to be cloned. Specifically, the native *Xho*I site within the AMF signal peptide is the only useful site for cloning protein genes fused to the signal peptide. If the gene of interest contains an internal *Xho*I site, as is the case with the Hy3 gene, it must be removed before cloning can proceed, or a different cloning strategy (e.g., incorporation of a *Sal*I site on the amino end of the gene of interest) must be used.

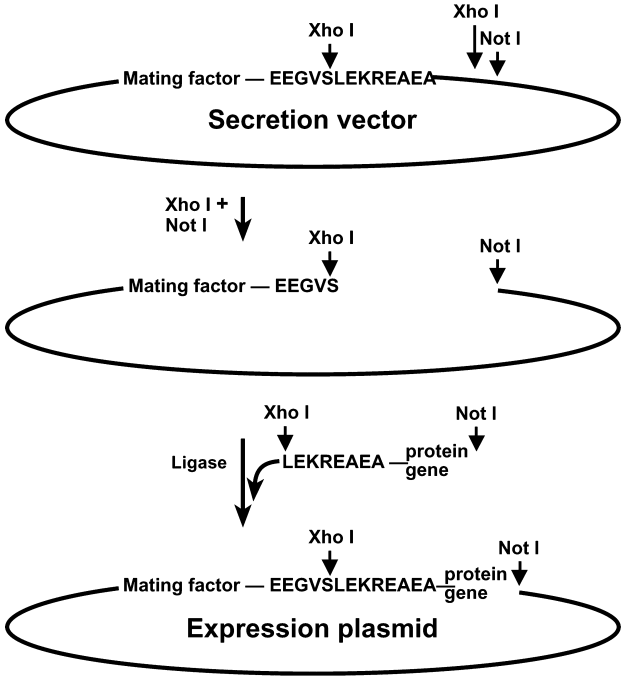

Fig. 2. Reconstitution with REaL of intact mating factor signal peptide following *Xho*I + *Not*I cleavage of vector pPICZα. Bases encoding AMF signal peptide amino acids in the small *Xho*I + *Not*I fragment are added to the PCR primer for the amino side of the protein to enable its secretion.

Gateway technology [7] is widely used for cloning genes for protein expression (http://web.ncifcrf.gov/rtp/pel/pubs.asp). Gateway greatly simplifies the planning and execution of cloning projects, but it requires the presence of Gateway recombination sites in expression vectors. Here we show that Gateway cloning is compatible with secretion of proteins from *P. pastoris*. This improvement simplifies cloning for secretion from this yeast.

Methods

The *P. pastoris* secretion vector pPICZα (Invitrogen) was converted into a Gateway destination vector by cutting with *Xho*I, filling in the ends with Klenow polymerase, and ligating in the Gateway reading frame B cassette (Invitrogen). The new destination vector was named pDest-910.

An internal *Xho*I site in the Hy3 gene was removed by site-directed mutagenesis (QuickChange, Strategene). The product of this mutagenesis was used as the template for PCR amplification. The Hy3 coding sequence was amplified with primers in two stages. For five cycles the reaction contained the amino primer GGCCTCGAGAAAAGAGAGGCTGAAGCTTGTGATCTGCCTCAGACCC (*Xho*I site underlined) and the carboxy primer GGGGACAACTTTGTACAAGAAAGTTGCGGCCGCTTATTCCTTCCTCCTTAATCTTTCTTG (*Not*I site underlined). After 5 cycles an adapter primer for the 5′ side of the gene was added (GGGGACAACTTTGTACAAAAAAGTTGGCCTCGAGAAAAGAGAGGCTG) and cycling was continued for 15 more cycles (Platinum Taq HiFidelity Supermix (Invitrogen), primers at 200 μM). This PCR product contained modified attB sites, attB1.1, CAACTTT GTACAAAAAA GTTG, and attB2.1 CAACTTTGTACAAGAAAG TTG. These sites give about four times as many colonies in a Gateway BP reaction as the original attB1 and attB2 sites [8]. The PCR product was purified with QiaQuick PCR purification columns (Qiagen). An aliquot of PCR product was digested with *Xho*I and *Not*I and ligated into pPICZα cut with the same enzymes (REaL expression clone). A second aliquot was cloned into pDonr223 (spectinomycin-resistant version of pDonr221, Invitrogen) by the Gateway BP reaction according to manufacturer's instructions (Invitrogen). The inserts in both REaL and Gateway clones were fully sequenced. Then the insert in the Gateway entry clone was transferred by recombina-

tion into pDest-910 to make the Gateway expression plasmid. The Gateway and REaL expression plasmids were electroporated into *P. pastoris* strain X33 and selected on Zeocin (100 μg/ml) plates (http://www.teknova.com) at 30 °C (Invitrogen "EasySelect" manual, http://www.invitrogen.com/content/sfs/manuals/easyselect_man.pdf). Colonies were picked into YPDS medium, grown at 30 °C, and glycerol stocks were made according to the EasySelect manual.

For *P. pastoris* fermentations, seed cultures of each strain (REaL or Gateway) were grown at 28 °C in BMGY medium (EasySelect manual) in shake flasks, and used to inoculate 1 L of basal salts medium containing 4% glycerol with trace mineral (PTM) salts (EasySelect manual) in New Brunswick BioFlow 110 minifermenters. Both fermentations were transitioned to methanol feeding, with a maximum of 12 ml methanol per L per hour. Aliquots were removed and centrifuged, and supernatants were applied to an SDS–PAGE gel, or diluted in PBS and spotted on nitrocellulose, next to aliquots of purified IFN-α2c. The nitrocellulose membrane probed with rabbit anti-human IFN-α2a (1:10,000 dilution in 2% dry milk), followed by HRP-conjugated goat anti-rabbit secondary antibody (Pierce). Chemiluminescence was developed using SuperSignal West Femto reagent (Pierce). Putative interferon proteins were enriched by ion-exchange chromatography, separated by SDS–PAGE, transferred to a PVDF membrane, stained with Coomassie brilliant blue R-250, and excised for N-terminal sequence determination by automated Edman degradation. Antiviral and antiproliferative assays of Hy3 activity were performed according to [9,10], respectively.

Results and discussion

We sought to preserve the proteolytic processing sites of the yeast AMF signal peptide of pPICZα while installing the Gateway cassette just upstream of them. When the Gateway destination vector pDest-910 (Materials and methods) was recombined with a Gateway entry clone of the gene of interest that contained the six amino acids lost during pDest-910 construction, the resulting expression plasmid encoded the complete AMF signal peptide fused to the protein of interest, with 9 extra amino acids encoded by the Gateway attB1 site (Figs. 3 and 4).

The hybrid human IFN-α gene Hy3 was cloned into pPICZα with restriction enzymes *Xho*I and *Not*I and ligase, and into pDest-910 by Gateway recombination. For REaL cloning, it was necessary to remove an *Xho*I site within the Hy3 gene of the PCR template by site-directed mutagenesis. Since both expression vectors had lost 23 bases of the mating factor signal peptide coding sequence as a result of the *Xho*I digestion, the Hy3 gene

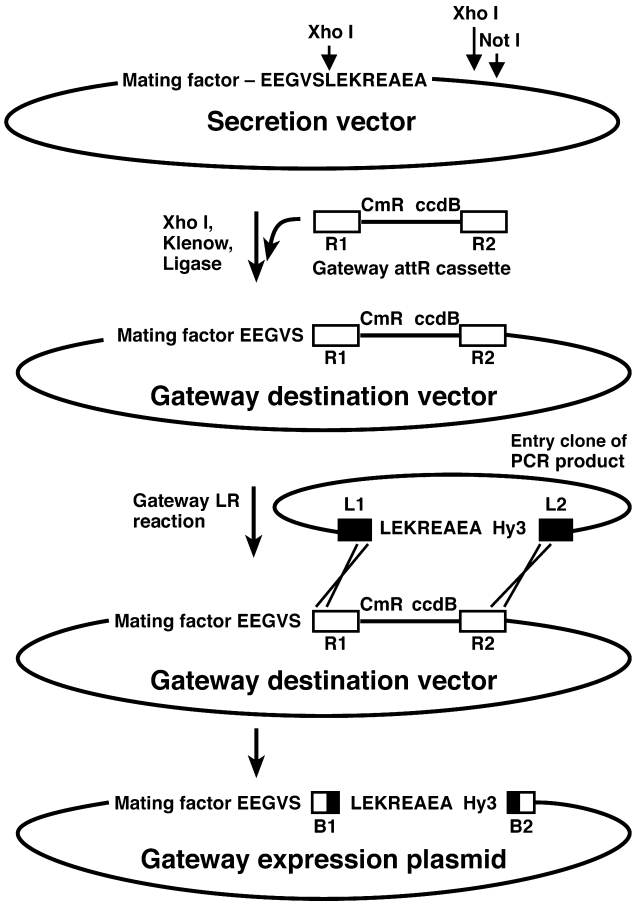

Fig. 3. Reconstitution with Gateway of AMF signal peptide for expressing Hy3. During the Gateway LR reaction, in vitro recombination between the attL sites of the entry clone and the attR sites of the destination vector transfers the Hy3 gene from the entry clone to the destination vector. The presence of the attB1 site results in the insertion of nine new amino acids, two amino acids upstream of the Kex2 processing site. See also Fig. 4.

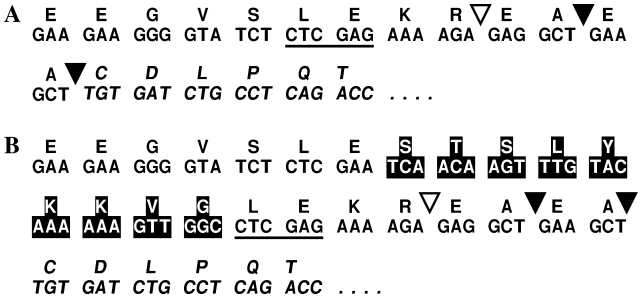

Fig. 4. (A) Sequence of the junction between the AMF signal peptide and the Hy3 gene in the secretion vector constructed with REaL. (B) The same region of the Gateway clone. (Open triangle) Kex2 cleavage site. (Closed triangles) Ste13 cleavage sites. (Underlined) *Xho*I site. Shaded codons in B derive from the attB1 site, and represent amino acids that are added to the signal peptide by conversion of the secretion vector to the Gateway system. The mature Hy3 sequence is italicized, *CDLPQT*...

was PCR amplified with primers that added those 23 bases to the 5′ end of the Hy3 coding sequence. For REaL cloning the PCR primers contained *Xho*I (5′) and

*Not*I (3′) sites. The PCR primers also contained attB sequences for cloning by Gateway recombination into the donor vector pDonr223, i.e., the same PCR product was used for both REaL and Gateway cloning. Inserts and junctions of both types of clones were fully sequenced, then the insert of the Gateway entry clone was transferred into pDest-910 by in vitro recombination. The base and amino acid sequences of the relevant regions of the Gateway and REaL clones are shown in Fig. 4.

The two *P. pastoris* expression plasmids pHy3-REaL and pHy3-Gateway were electroporated into strain X-33 and selected on zeocin plates. Single colonies were picked into broth medium and frozen glycerol stocks were made. Supernatants from shake flask cultures induced with methanol were spotted onto nitrocellulose and probed with anti-IFN-α2a antibody (data not shown), which also detects Hy3; both cultures were positive for secreted Hy3. Both REaL and Gateway strains were then grown in 1 L cultures in minifermenters with pH and dissolved oxygen control. Following initial growth with glycerol as a carbon source, the cultures were transitioned to methanol feeding and grown for 90 h after the start of methanol induction (final cell densities were approximately 0.5 kg wet weight per liter). Culture supernatants were harvested every 24 h and either applied directly to an SDS–PAGE gel that was subsequently stained with Coomassie blue (Fig. 5A) or probed on a dot blot (Fig. 5B).

The human IFN Hy3 produced by both the REaL and Gateway clones migrated identically on an SDS–PAGE gel. Both Hy3 and the IFN-α2c standard migrated as doublets, with the Hy3 doublet migrating more slowly than the α2c doublet. Both the REaL and Gateway Hy3 proteins were partially purified and their N-terminal amino acid sequences were determined. The upper and lower bands of the Gateway and REaL Hy3 proteins (Fig. 5) had the same sequences, NH2–EAEACDL···, showing that while the *Pichia* homolog of Kex2 had cleaved at the expected site (Fig. 4), the Ste13 homolog did not. Inefficient Ste13 processing of the AMF signal peptide in *Pichia* is common (for example, see [5,6]). Migration of IFNs as doublets on SDS–PAGE is presumably due to disulfide bond formation, since extensive heating with reducing agent considerably diminished the doublets (our unpublished observations).

The Hy3 protein is a synthesis of the amino 94 amino acids of IFN-α2c and the carboxy 71 amino acids of IFN-α21a [2]. We used purified α2c protein to estimate the concentrations of Hy3 produced by the Gateway and REaL strains at time points in our fermentation (Fig. 5). The Gateway strain was more productive early in the run, but the REaL strain ended up secreting about as much protein. Comparison to the α2c standard on both the SDS–PAGE gel and the antibody dot blot suggested concentrations of secreted Hy3 protein of 100–150 mg per liter. Since neither of these expression strains was selected for multiple copies of the expressed gene, we assume that each contains one copy per *P. pastoris* genome, the most common number. The Gateway and REaL culture supernatants had similar specific antiviral activities, on the order of 10^7 international units/mg protein (approximately 10% pure). They also had similar antiproliferative activities, approximately 2 ng/ml inhibited cell proliferation by 50%.

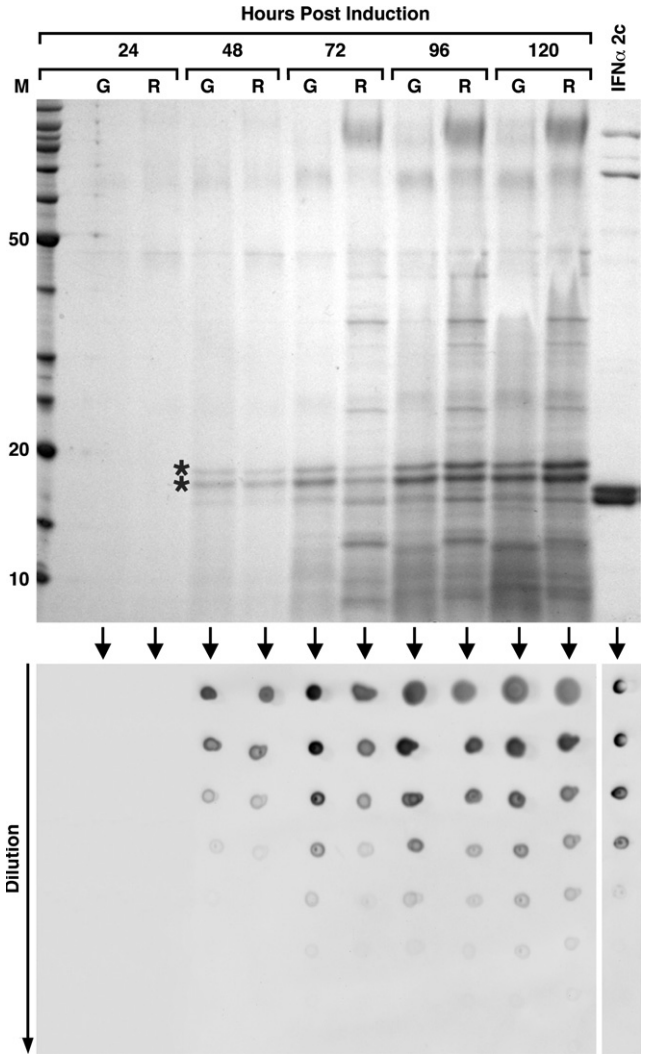

Fig. 5. Secretion of Hy3 from Gateway (G) and REaL (R) strains of *Pichia*. (Upper panel) SDS–PAGE gel of culture supernatants (2 μl) or purified IFN-α2c (1 μg), Coomassie-stained. The stars mark the expected mobility of Hy3. (Lower panel) Western dot blot of 3-fold dilutions of culture supernatants sampled at 24, 48, 72, 96, or 120 h, or dilutions of purified IFN-α2C protein, probed with anti-IFN-α2a. The concentration of the undiluted IFN-2c was 100 μg/ml (100 mg/L).

These results show that the presence of amino acids in the yeast mating factor signal peptide corresponding to the Gateway attB1 site does not significantly affect the secretion of recombinant Hy3 from *P. pastoris* cells during fermenter growth. While other proteins may behave differently, the ease with which genes of any size or

restriction map can be cloned for secretion via Gateway make this method an attractive alternative to standard cloning methods to attempt production of secreted proteins in *P. pastoris*.

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