



Regulated expression of green fluorescent protein under the control of *Aureobasidium pullulans* xylanase gene *xynA*

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Abstract

A mutant form of the jellyfish cDNA encoding green fluorescent protein (GFP) was fused to the promoter of the *Aureobasidium pullulans* xylanase gene *xynA* and the expression vector pxynEGFP was introduced into *A. pullulans*. In a manner consistent with regulation of the native *xynA* gene, *gfp* activity was induced by xylose and repressed by glucose. The marker may be useful for monitoring populations of *A. pullulans* in situ and for identifying transcriptional control elements of *xynA*. © 1999 Federation of European Microbiological Societies, Published by Elsevier Science B.V. AU rights reserved.

Keywords: *Aureobasidium pullulans*; Green fluorescent protein; Xylanase

1. Introduction

Aureobasidium pullulans (de Bary) Arnaud is a cosmopolitan yeast-like fungus that occurs in diverse habitats [1] including the phyllosphere. It is of ecological importance because the fungus has numerous morphotypes [2], produces extracellular polymers including pullulan [3], and has potential use as a bio-control agent for plant pathogens [4]. Thus, its population biology in nature has been studied [5], and methods are being sought to facilitate monitoring populations in situ.

Leaf surface populations of *A. pullulans* have been detected and identified by direct plating [4,5], by oli-

gonucleotide probes complementary to ribosomal RNA [6,7], and most recently by expression of *Aequorea victoria* green fluorescent protein (GFP) [8]. Intense GFP signals were obtained [8] by fusing a mutant GFP cDNA, EGFP [9], to the promoter of the constitutively expressed gene for the translational elongation factor TEF [10]. Although GFP is widely used as a reporter, high levels of expression may alter host fitness, and inducible promoters are more desirable under some circumstances.

Xylanases have received much recent attention as fiber bleaching agents (for review see [II]), and *A. pullulans* xylanases are among the most intensively studied [12,13]. Xylanase activity in *A. pullulans* is strongly repressed by glucose and induced by xylose and xylan [13-15]. A xylanase-encoding gene, *xynA*, was cloned from *A. pullulans* strain ATCC 62921 [16], and the enzyme was successfully ex-

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pressed in *Saccharomyces cerevisiae* [17]. Regulation of *xynA* occurs at the transcriptional level [16], but regulatory elements have not been sequenced and characterized.

We report the cloning and sequence of regions upstream of *xynA* and demonstrate controlled expression of GFP when fused to these regulatory regions.

2. Materials and methods

To assess a *xynA-gfp* fusion, expression vector pxynEGFP was constructed (Fig. 1). Only 58 bp of 5' untranslated *xynA* sequence were previously reported [16], necessitating upstream 'walking'. Based on the published sequence [16], nested primers (5'-TGGGGCAGCAACAGCTCCCACAACGA-3' and 5'-AGCAGCAATGGTGGCGAAGAACTTC-AT-3') were designed for use with the Universal Genome Walker kit (Clontech Laboratories, Palo Alto, CA).

The upstream sequence was fused to EGFP and the *Aspergillus awamori* glucoamylase terminator [18] by overlap extension [19] using *Pfu* polymerase. *EcoRI* restriction sites were incorporated into 'outside' primers to facilitate cloning into pBluescript II KS- (Stratagene Inc., La Jolla, CA). The sequence junctions within pxynEGFP were confirmed and the vector introduced into *A. pullulans* (ATCC 90393) by cotransformation with the hygromycin resistance (*hyg^R*) vector pDH33 [20] as previously described [21,22].

To assess *gfp* expression, a pxynEGFP transformant was selected and grown for 5 days at room temperature on yeast peptone agar medium amended with various proportions of xylose and glucose. Mixtures were adjusted for relative molarity of each sugar and final carbon concentration was kept constant (55 mM). We imaged colonies using long wave UV lighting with Sylvania Blacklight Blue F15T8/BLB bulbs (Osram Sylvania, Danvers, MA) and a Sony 3CCD model DXC-960MD video camera (Sony Electronics Inc., Park Ridge, NJ) equipped with a Yellow 2 filter (Tiffen Manufacturing Corp., Hauppauge, NY).

For fluorescence-activated cell sorting (FACS), liquid cultures of the transformant were grown in

yeast peptone media with varying amounts of glucose and xylose as the carbon source under constant agitation at 28°C for 60 h. Samples of cells were removed and fixed with 1.5% formaldehyde in 50 mM phosphate buffer (pH 7.4) and kept at 4°C pending our analysis by FACS on a Coulter EPICS ELITE flow cytometer (Coulter Corp., Hialeah, FL), using FITC settings. Five thousand cells were analyzed and data plotted as histograms of log GFP fluorescence vs. number of cells. Mean cell fluorescence intensity was determined with WinMDI v2.7. software (©Joseph Totter, URL <http://facs.scripps.edu/>).

3. Results and discussion

To obtain regulated *gfp* expression in *A. pullulans*, 5' upstream regions of *xynA* were obtained by genome walking (Fig. 1). Approximately 1300 bp of 5' untranslated sequence were amplified from

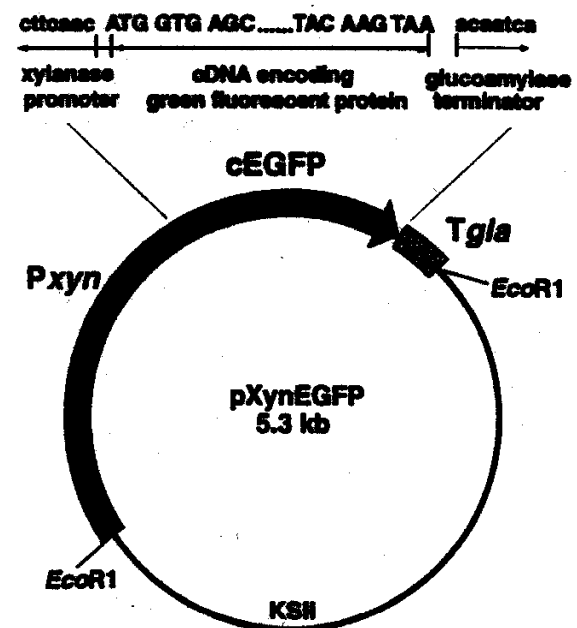


Fig. 1. Expression vector pxynEGFP. The mutant GFP encoding cDNA (cEGFP) was fused to the *A. pullulans xyn* promoter (*Pxyln*) and to the *A. awamori* glucoamylase gene terminator (*Tgla*). The precise nucleotide sequence of cDNA junctions is shown above. To aid subcloning, the expression cassette is flanked by *EcoRI* sites.

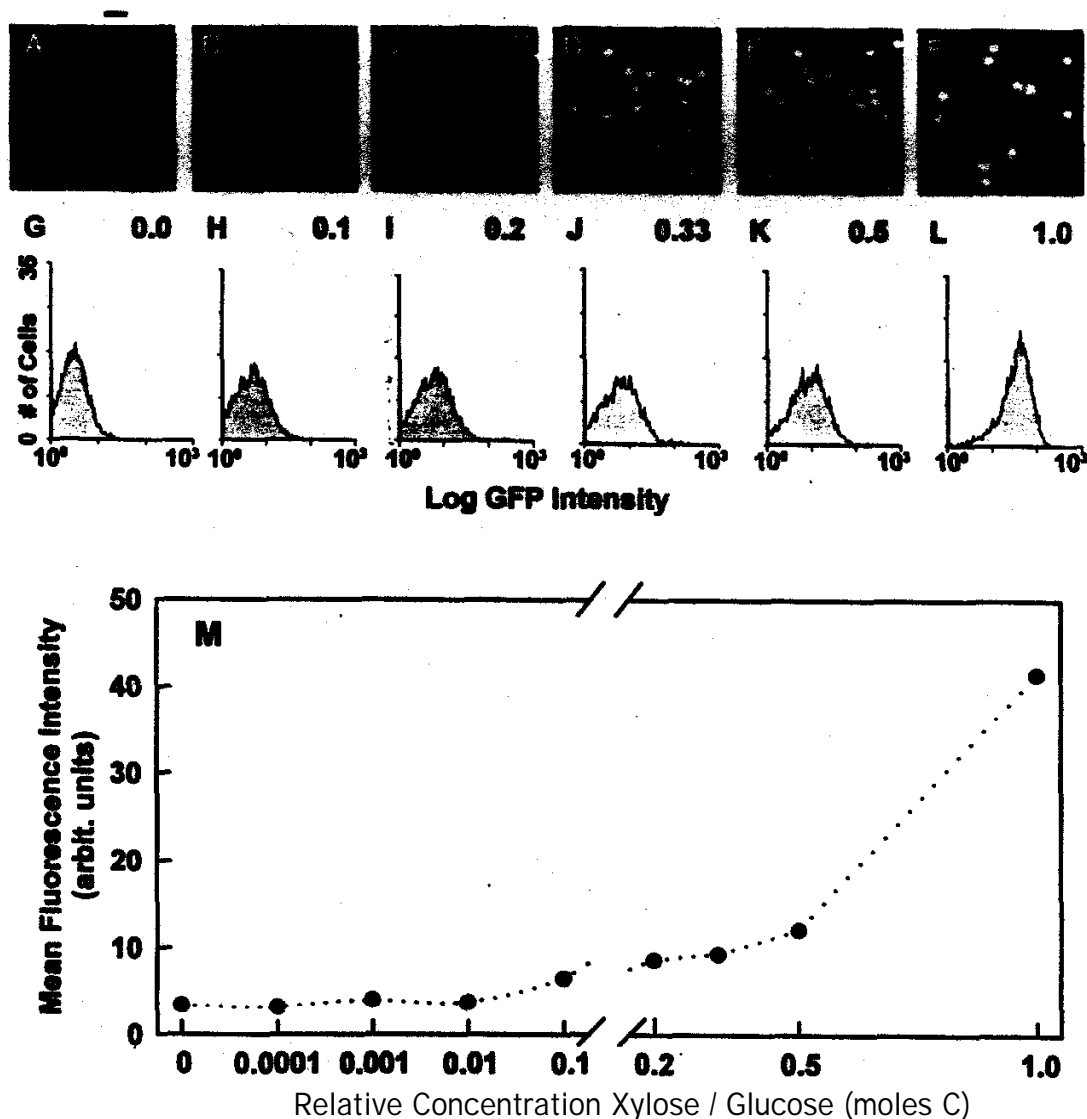


Fig. 2. A-F: Fluorescent images of colonies of pxynEGFP-transformed *A. pullulans* on yeast-peptone agar media with xylose/glucose molar ratios of 0, 0.1, 0.2, 0.33, 0.5, and 1.0, respectively. Magnification bar applies to A-F and equals 5 mm. G-L: FACS histogram from liquid cultures of 5×10^3 cells showing log fluorescence (horizontal axis) vs. number of cells (vertical axis) at indicated xylose/glucose ratios. Note shift in population distribution of fluorescing cells with increasing xylose concentration. M: Mean GFP fluorescence from FACS analysis of 5×10^3 cells at xylose/glucose ratios of 0 (glucose only) to 1.0 (xylose only).

A. pullulans strain ATCC 62921. The 3' and sequence of this fragment diverged slightly from the previously published *xynA* sequence (accession number U10298). Consequently, the entire coding region was amplified with *pfu* polymerase, subcloned, and sequenced. The deduced amino acid sequence of the two xylanase clones differed within the putative se-

cretion signal at residue eleven (A→G) and within the mature peptide at residues 141 (Y→Q), 150 (C → Y), and 206 (A → P). Our identification of these residues agrees with several other fungi, including *Aspergillus niger* (accession number P55329) and *Cryptococcus* sp. (JC4909). The new nucleotide sequence, together with 381 bp 5' untranslated region.

has been assigned GenBank accession number AF169630.

GFP fluorescence intensified with the increasing proportion of xylose in molar relation of carbon to glucose (Fig. 2). GFP could be detected in some cells either by FACS or microscopically at levels of xylose to glucose as low as 0.0001 (data not shown). On solid media, colony fluorescence was not detected below a level of 0.01 xylose. Regulated *gfp* expression is consistent with previous Northern blot analyses of *xynA* expression in *A. pullulans* [16].

The marker may be a useful tool for monitoring released populations and for characterizing specific transcriptional control elements. Deletion analyses with *xyn-hyg^R* fusions have identified upstream regions responsible for glucose repression of *Trichoderma reesei xyn1* [23,24]. In this connection, the sequence reported here contains a putative CreI binding consensus sequence (SYGGRG) 236 bp upstream of the translational start codon. CreI-mediated glucose repression has been implicated in the regulation of a variety of fungal genes including xylanases of *Aspergillus* [23,24] and *Trichoderma* [25,26].

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