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Evaluation of a New Episomal Vector Based on the GAP Promoter for Structural Genomics in *Pichia pastoris*

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Abstract A new constitutive episomal expression vector, pGAPZ-E, was constructed and used for initial screening of eukaryotic target gene expression in *Pichia pastoris*. Two reporter genes such as *beta*-galactosidase gene and *GFPuv* gene were overexpressed in *P. pastoris*. The expression level of the episomal pGAPZ-E strain was higher than that of the integrated form when the *beta*-galactosidase gene was used as the reporter gene in *P. pastoris* X33. The avoiding of both the integration procedure and an induction step simplified the overall screening process for eukaryotic target gene expression in *P. pastoris*. Nine human protein targets from the Core 50, family of Northeast Structural Genomics Consortium (http://www.nesg.org), which were intractable when expressed in *E. coli*, were subjected to rapid screening for soluble expression in *P. pastoris*. HR547, HR919, and HR1697 human proteins, which had previously been found to express poorly or to be insoluble in *E. coli*, expressed in soluble form in *P. pastoris*. Therefore, the new episomal *GAP* promoter vector provides a convenient and alternative system for high-throughput screening of eukaryotic protein expression in *P. pastoris*.

Key words: *Pichia pastoris*, episomal vector, *GAP* promoter, heterologous expression system, eukaryotic structural genomics

There is increasing interest in the structural genomics field in tackling target proteins chosen from complex metazoans such as humans [15]. Although "workhorse" *E. coli* expression systems will be able to produce usable samples for many of these targets (particularly when they are parsed into individual domains that are expressed separately), there will be a growing demand for robust eukaryotic expression systems that can produce soluble and correctly folded proteins for cases where *E. coli* systems cannot [1, 14, 18, 24, 31].

Most of the current choices for candidate eukaryotic expression systems for structural genomics fall into three main categories: simple eukaryotes such as yeast; insect or animal cell tissue culture systems; and *in vitro* wheat germ-based systems [14, 27, 31]. Yeast expression systems, particularly those based on the methylotrophic yeast *Pichia pastoris*, are especially attractive [4, 6, 8, 10, 18, 26]. *P. pastoris* is well established as a host for high-level recombinant protein biosynthesis, including production of isotope-enriched samples for 3D structure determination by NMR spectroscopy. Moreover, because it is a simple microbe, high-throughput laboratory protocols involving *P. pastoris* can take advantage of the installed base of robotics and other automation equipment in existing *E. coli*-based protein production facilities [3, 12, 14].

However, *P. pastoris* is not yet ideal for structural genomics applications [3, 31]. Its main disadvantage resides in the fact that primary tranformants of expression constructs often exhibit low and heterogeneous expression levels of the gene of interest. This typically necessitates the screening of many colonies to search for the few clones with sufficiently high expression to justify carrying them forward [6]. *P. pastoris* expression systems would be better suited for high-throughput applications like structural genomics if one could readily get reliably good expression, such as one sees with the best modern *E. coli* expression systems [2].

Previously, several *Pichia* expression vectors were designed to facilliate the high-throughput screening of expression using inducible promoters such as AOX and CUP promoters [20, 21]. However, the use of inducible promoters introduces additional steps in protein expression. Another difficulty with using *P. pastoris* as a host to express foreign protein is that most *P. pastoris* expression vectors are designed to integrate into the host chromosome [16].

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As an approach to modify *P. pastoris* host/vector systems to improve this protein production technology for structural genomics applications, the strong constitutive *GAP* (glyceraldehyde-3-phosphate dehydrogenase) promoter was chosen for episomal expression in *P. pastoris* [17, 30]. One of the reasons that the constitutive *GAP* promoter has not been widely used is the belief that constitutive production of foreign proteins in *P. pastoris* may have cytotoxic effects [22]. However, recent studies have found not only that cytotoxic effects are not necessarily observed, but also that production levels of a recombinant exo-levanase (LsdB) using the *GAP* promoter were similar to those using the *AOX1* promoter [23]. In order to proceed systematically, we have chosen as proteins for expression optimization a standard panel of 50 eukaryotic structural genomics targets, many of which are difficult to express in *E. coli*-based systems [1]. Using a large multiprotein panel will also allow us to filter out gene-specific effects and focus on those modifications to vectors and hosts that yield general improvements in the expression of most proteins.

Our first step was the construction of a new episomal constitutive expression vector using the *GAP* promoter and the test of a defined set of *P. pastoris* clones. The construction and preliminary expression testing of this set of clones are described here.

MATERIALS AND METHODS

Plasmid DNA and Strains

The *P. pastoris* expression plasmids, pGAPZB, pPIC6lacZ, and expression host strain, X33, were obtained from Invitrogen (U.S.A.). pPICHOLI-1 expression vector was purchased from Mobiotec (Germany). The LacZ control strain from an Invitrogen kit was used for constitutive expression of the *lacZ* gene. *E. coli* XL10-Gold cells (Stratagene) were used as the transformation host for construction of recombinant *Pichia* expression plasmids.

Construction of pGAPZ-E and Recombinant pGAPZ-E Harboring Reporter Genes

PCR primers for a *Pichia*-specific autonomous replication sequence (PARS) was designed according to previously published data [7] and it was used for PCR amplication. Two kinds of ARS, known as PARS1 and PARS2, were subcloned into the pUC18 vector and its DNA sequences were analyzed. PARS1 was subsequently subcloned into the BamHI site of pGAPZB and its inserted orientation was determined by DNA sequencing. The recombinant pGAPZB plasmid, designed as pGAPZ-E, was used for overall episomal expression of human target proteins. In order to test its episomal expression in *P. pastoris*, reporter genes such as *lacZ* and the *GFPuv* gene were tested. The *lacZ* reporter gene from pPIC6lacZ (Invitrogen) was subcloned into the *Bsp*T1041 and NotI sites of pGAPZ-E and resulted in pGAPZ-E/lacZ. The *GFPuv* gene from pGFPuv (Clontech, U.S.A.) was amplified by PCR and subcloned into the EcoRI and NotI sites of pGAPZ-E and resulted in pGAPZ-E/GFPuv. The PCR primers were as follows: GFPuv-F(EcoRI), 5'-CGG GAA TTC CAT ATG AGT AAA GGA GAA GAA C; GFPuv-R(NotI), 5'-GGT AGG CGC GGC GGC CGC TTA TTT GTA GAG CTC ATC.

Subcloning of Core50 Targets into the *Pichia* Expression Vectors

Recombinant pET14 or pET15 series plasmids, constructed for expression of the Core50 target ORFs [1] in *E. coli* were used as a source of template for PCR cloning into pGAPZ-E. All Core50 target ORFs had *N*-terminal His-tags. Taq polymerase (BD Bioscience) was used for PCR cloning experiments. The primers used here were as follows: Core50 (EcoRI)-F, 5'-GGT AGG CGG AAT TCC ACC ATG GGC CAT CAC CAT CAC C-3'; Core50 (NotI)-R, 5'-GGT AGG CGG CGG CCG CCT TTG TTA GCA GCC GGA TCT CGA G-3'.

Pichia Transformation

Recombinant pGAPZ-E plasmids or pPICHOLI-1 harboring Core50 human targets were electroporated into *P. pastoris* X33. *Pichia* transformants were selected for growth on YPD plates supplemented with Zeocin (100 μg/ml). Colony PCR analyses were carried out to verify the insertion of recombinant plasmid DNAs in *P. pastoris*.

Protein Expression and Purification

Pichia transformants of X33 were grown at 30°C for 2 days in 20 ml of YPD broth with Zeocin (100 μg/ml). When their optical density reached about 35 at 600 nm, they were then centrifuged and resuspended in 0.5 ml of Ni-NTA column loading buffer with protease inhibitor cocktail (Sigma Co.). The cell suspension was transferred into a 2-ml Mini-BeadBeater tube and 0.5 g of acid-washed glass beads (425–600 diameter, Sigma) was added to the cell suspension. Cell breakage was done with a Mini-BeadBeater (three 30-sec treatments at 5,000 rpm with 1-min intervals cooling on ice). The lysate was centrifuged for 10 min at 9,000 rpm and then the supernatant was further centrifuged for 15 min at 15,000 rpm. The clear supernatant was subjected to Ni-NTA column chromatography.

Expression Detection of *Pichia* Transformants

Cell lysates and NTA column elutes were analyzed by 4–12% NuPAGE SDS gel (Invitrogen). Total protein was visualized by staining with Coomassie blue and His-tagged proteins were highlighted by staining with InVision (Invitrogen) and visualizing the fluorescent bands on a UV transilluminator.

Beta-Galactosidase Assay

β-Galactosidase activity of *Pichia* transformants was tested according to Miller's [25] method using ONPG as substrate. Prior to the reaction, all reaction components were equilibrated to 28°C. Samples of 10–20 μl were added to 1 ml Z buffer (60 mM $Na_2HPO_4 \cdot 7H_2O$, 40 mM $NaH_2PO_4 \cdot H_2O$, 10 mM KCl, 1 mM $MgSO_4 \cdot 7H_2O$, 50 mM β-mercaptoethanol, pH 7.0), and then 200 μl ONPG (4 mg/ml) solution was added. After 10 min, the reaction was stopped by addition of 0.5 ml of 1 M sodium carbonate. The absorbance at 420 nm was determined using a UVmini1240 UV-Vis spectrophotometer. (Simaduzu, Japan). The protein concentration was determined by a Bio-Rad (Richmond, U.S.A.) protein assay kit with bovine serum albumin as the standard.

Yeast Plasmid Isolation

P. pastoris strain was cultured at 30°C in 3 ml YPD and YPD plus Zeocin (100 μg/ml) media with shaking at 200 rpm for 24 h. After culture, cells were harvested by centrifugation at 5,000 rpm for 5 min, and washed once with 1 ml DDW. The washed cells were resuspended in 1 ml lysis buffer (100 mM Tris pH 8.0, 50 mM EDTA, 1% SDS). Acid-washed glass beads 0.2 g were added, and then were shaken three times at 5,000 rpm for 30 sec using a Mini-BeadBeater (Biospec Product, U.S.A.). The liquid phase, which contained total DNA, was recovered by centrifugation at 6,000 rpm for 5 min. Then, 550 μl of 7 M ammonium acetate, pH 7.0, and 10 μl of RNase solution (10 mg/ml) were added. The samples were incubated for 5 min at 65°C, and then 5 min on ice. After equal volume of chloroform extraction, total DNA was recovered with isopropanol precipitation. The DNA pellet was dissolved in 60 μl of DDW and 2 μl of DNA solution was analyzed by 0.8% agarose gel electrophoresis.

Results

Construction of an Episomal Expression Vectors Using PARS1

Two *Pichia*-specific autonomous sequences (PARS) were amplified by PCR from X-33 chromosomal DNA. The PARS, known as PARS1 and PARS2, respectively, were subcloned into pUC18 vectors. The corresponding 168 bp of the PARS1 region as well as 330 bp of the PARS2 region were analyzed by DNA sequencing (data not shown). The PCR amplified sequences were identical with previously reported sequences except for two base-pair alterations in PARS2 (data not shown). Recently, PARS1 was used for episomal expression of the *AOX* promoter-containing pPICHOLI-1 dual expression vector (Mobitec, Germany). In an attempt to compare a similar episomal form with the pPICHOLI-1 inducible vector, PARS1 was chosen for construction of episomal plasmids. PARS1 was inserted into the BamHI site of the pGAPZB vector (Invitrogen, U.S.A.) and the resulting recombinant pGAPZ harboring PARS1 was named as pGAPZ-E (Fig. 1). The pGAPZB without PARS1 did not produce any transformant in *Pichia* transformation, whereas the pGAPZ-E yielded 10^2 transformants/μg. The episomal state of pGAPZ-E was confirmed by its retransformation into *E. coli* as well as agarose gel electrophoresis of yeast plasmids in *Pichia* transformants (data not shown). Restriction enzyme analyses of *E. coli* plasmids showed that the back-transformed pGAPZ-E was identical with the original

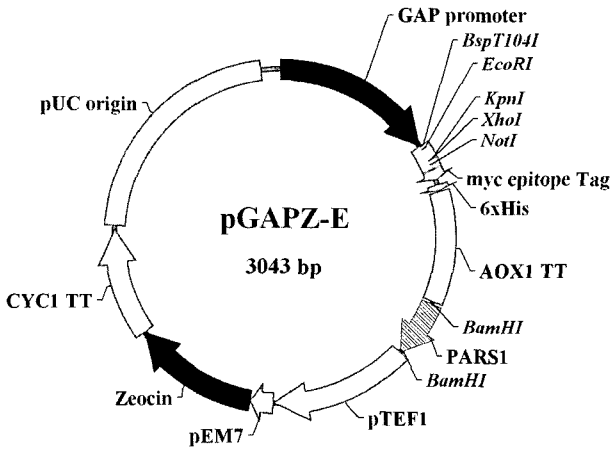

Fig. 1. Constitutive episomal expression vector pGAPZ-E.

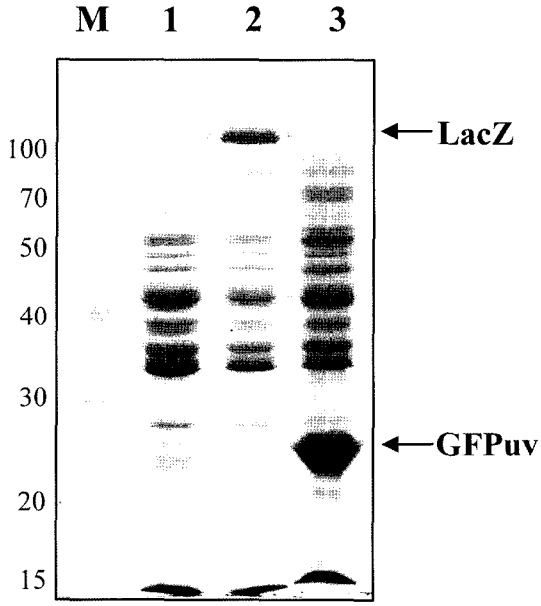

Fig. 2. Expression of recombinant pGAPZ-E harboring reporter genes in *Pichia pastoris*. Cleared lysate samples (3 ul) were analyzed on 12% SDS-PAGE gel.
Lane 1, X33/pGAPZ-E; lane 2, X33/pGAPZ-E/lacZ; lane 3, X33/pGAPZ-E/GFPuv.

pGAPZ-E (data not shown). Reporter genes such as the *lacZ* gene and the *GFPuv* gene were employed for testing foreign genes in constitutive gene expression. The episomal expression of two reporter proteins in *Pichia* strains yielded detectable levels of corresponding proteins (110, 27 kDa, respectively) in protein gel without Ni-NTA column purification (Fig. 2).

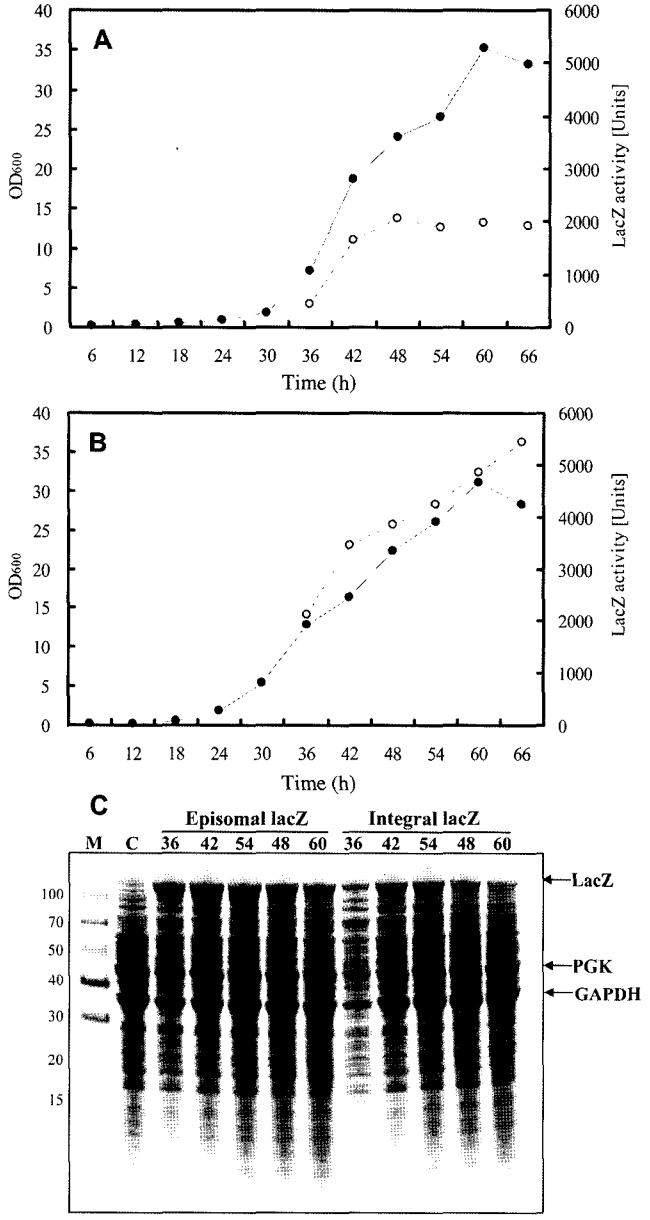

Fig. 3. Comparison of *lacZ* gene expression levels in two different forms of X-33 strain.

A. LacZ activity of integrated form of X-33 strain. **B.** That of X-33/pGAPZ-E/LacZ as episomal form. Solid line with closed circle means the cell growth at 600 nm; dotted line with open circle means the *beta*-galactosisase activity. **C.** SDS-PAGE gel analysis of LacZ expression. Lane C denotes control strain X-33 harboring pGAPZ-E at 60 h. The numbers correspond to culture hour.

Comparison of Episomal *vs.* Integral Expression of *lacZ* Gene

Previous study demonstrated that increasing the copy number of a β-galatosidase gene (*lacZ*), under the control of the FLD1 promoter, increased the relative expression of the enzyme proportionately [28]. It was previously known that the copy number of a PARS1-containing plasmid (pYJ30) was 13 copies per host cell [7]. Based on the assumption that the increased copy numbers in an episomal expression vector might contribute to enhanced gene expression, we tested whether the episomal expression level of pGAPZ-E/lacZ in *P. pastoris* was equal to that of a chromosomally integrated construct (Figs. 3A and 3B). The integrated pGAPZ/*lacZ* control strain provided in the manufacturer's kit (Invitrogen, U.S.A.) was used for direct comparison of *lacZ* gene expression. Even though the two strains differed slightly in cell growth, the *lacZ* gene expression level from the episomal form was two-fold higher than the level from the integrated form. The SDS-PAGE gel analysis as well as enzymatic assay of LacZ from both *Pichia* strains further demonstrated the higher expression level of the episomal form of pGAPZ-E/*lacZ* in *Pichia*. Two overexpressed intracellular proteins, GAPDH (glyceraldehyde phosphate dehydrogenase) and PGK (phosphoglycerate kinase), were used for the intracellular expression control in *P. pastoris* [9, 30]. The two overexpressed proteins marked as arrows in the SDS-protein gel (Fig. 3C) were further analyzed by MALDI-TOF MS analyses (data not shown). Therefore, these proteins can be used for intracellular protein expression references. There were no significant differences in levels of protein expression references between integral and episomal LacZ *Pichia* strains. These data suggest that the higher expression levels of episomal *lacZ* gene are vector-specific and not

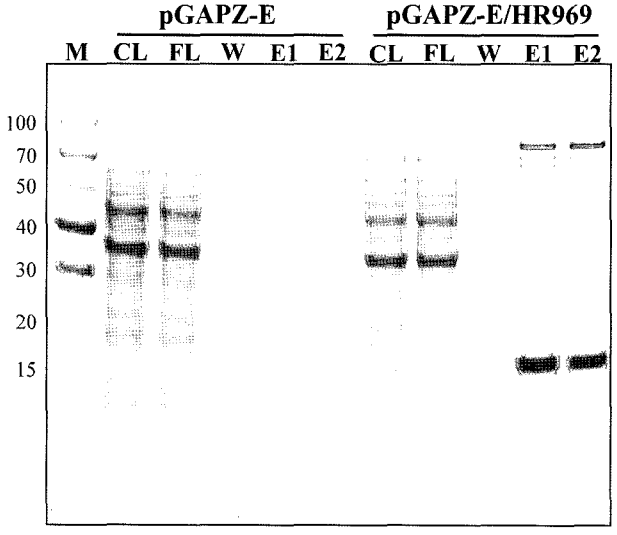

Fig. 4. His-tag column purification of HR969 protein. CL, clear lysate; FL, filtrate; E1, E2, elutes.

due to an overall nonspecific enhancement of general intracellular protein expression [11].

Optimization of *Pichia* Expression Using pGAPZ-E Vector

It was previously known that HR969, one of the NESG Core50 targets, yielded soluble protein in *Pichia* expression using the pPIC3.5 vector (data not shown). Therefore, HR969 protein was initially used to test the effectiveness of an alternative *Pichia* expression vector. The results of protein expression revealed that two days of cell culture are enough to test the expressions of eukaryotic target genes (Fig. 4). In contrast to some previous *Pichia* expression vectors that required additional affinity tags such as the streptavidin tag [12, 13], purification *via* a simple *N*-terminal histidine tag system yielded almost pure target protein. Furthermore, the levels of gene expression in *Pichia pastoris* were detectable by simple Coomassie-blue gel staining rather than Western methods.

Episomal Expression of Structural Genomic Target Proteins

A total of nine human target proteins from the Core50 list were tested for episomal expression using pGAPZ-E. All target proteins except HR969 (which served as a positive control) either did not express or were insoluble in previous *E. coli* expression attempts [1]. The His-tagged human proteins were constitutively expressed, purified by Ni-NTA column chromatography, and detected by Invitrogen's Invision gel staining kit (Fig. 5). Among nine human targets, three target proteins including HR547, HR969, and HR919 were expressed at easily detectable levels, whereas HR1697 was expressed at a very low level. The success of expressing human proteins in a soluble form validates this *Pichia* system as an alternative eukaryotic expression system. The overall expression results for nine human target proteins are summarized in Table 1.

Table 1. Protein expression properties of the expressed clones.

No.	Target (Core50)	Predicted size (MW)	*E. coli*	*P. pastoris*
1	HR547	26,371	NE/NA	++/S
2	HR894	30,358	++/NS	NE/NA
3	HR919	23,651	++/NS	++/S
4	HR945	17,776	++/NS	NE/NA
5	HR969	15,588	++/NS	++/S
6	HR1686	14,956	++/NS	NE/NA
7	HR1697	5,967	NE/NA	+/S
8	HR1889	16,377	NE/NA	NE/NA
9	HR1913	16,809	++/NS	NE/NA

NE, Not expressed; NS, Not soluble; NA, Not applicable (proteins that were not expressed have unknown solubility); S, Soluble; ++, Highly expressed; +, Expressed.

Discussion

Yeasts such as *P. pastoris* could potentially be ideal hosts for structural genomics projects, but current approaches to optimizing expression often proceed empirically *via* the testing of various host strains, vectors, and amplification procedures coupled with a screening step to find over-expressing clones. The multiple steps involved are cumbersome and impractical to employ in a high-throughput protein production environment. Furthermore, lack of a general understanding of the molecular factors limiting expression has led to a case-by-case optimization approach, whereby specific high-yield strains may be selected that over-express a particular recombinant protein, but the fruits of the effort are usually non-transferable to the next protein, and the optimization process often has to be repeated again for each new target.

We are attempting to streamline *Pichia pastoris*-based expression systems so that they can be used routinely and with minimal screening efforts to produce ample quantities

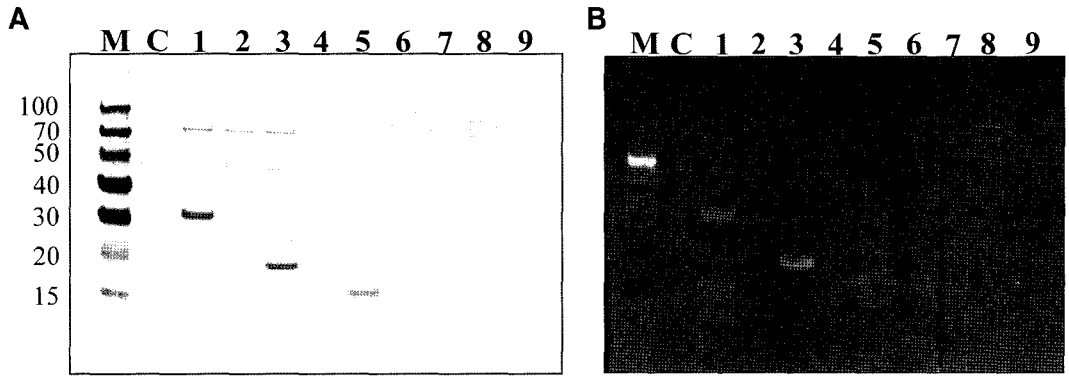

Fig. 5. Analysis of Human proteins expression in *Pichia*.
A. Coomassie-blue staining of His-tag column elutes on Nu-PAGE (4–12%). **B.** His-tag gel staining. Lane C, control pGAPZ-E; lane 1, HR547; lane 2, HR894; lane 3, HR919; lane 4, HR945; lane 5, HR969; lane 6, HR1697; lane 7, HR1686; lane 8, HR1889; lane 9, HR1913.

of $^{13}C/^{15}N$- or selenomethionine-enriched proteins for structural studies. Our first step has been to create a *basis set* of clones, based on a reference panel of eukaryotic structural genomics targets [1]. The aim is to have the recombinant gene for each reference clone to have defined genetic characteristics: constitutive expression and episomal at a known gene copy number in the *P. pastoris*.

Lang and her colleagues have reported on the use of *Pichia pastoris* as an expression host for structural genomics using pPIC3.5 vector [3, 26]. Boettner *et al.* [3] observed a low but detectable expression level for several protein targets by Western blotting analyses and reported that independent clones of the same gene gave consistent expression levels of the gene product, as would be expected for same-context single-copy integrants. These results were not inconsistent with our preliminary expression characterization of the *P. pastoris* clone using the pPIC3.5 vector (data not shown).

New expression vectors for the rapid screening of soluble eukaryotic proteins are in demand as an alternative to integrated *P. pastoris* pPIC3.5 vectors. Generally, foreign protein expression in *P. pastoris* depends on inducing promoters such as the *AOX* or *CUP1* promoter [17, 22]. The integration steps of each gene are another tedious step. Furthermore, efficient expression usually requires the target-by-target screening for multicopy integrated strain from *Pichia* transformants. Recently, episomal expression using pPICHOLI-1 was employed for structural target expression [20, 21], but this also required an additional inducing step in the culture condition.

The *GAP* promoter was known as a strong constitutive promoter in *P. pastoris* [30]. Previously, constitutive expression driven by the *GAP* promoter was used for analyzing the mutants in a directed evolution project [17]. In this case, the expression was not of intracellular proteins but of proteins destined to be secreted into the medium. *Pichia pastoris* is known as an alternative expression host for eukaryotic protein when conventional *E. coli* expression has failed [3, 6, 8, 10, 31].

The biggest drawback of episomal expression in yeast is its instability in plasmid replication [7]. This was also true in the case of the pGAPZ-E vector. The stability of pGAPZ-E was only 35% after ten generations or 24 h in the absence of zeocin selection. However, the rapid expression phenomenon of the *GAP* promoter might compensate for this instability. The episomal state of pGAPZ-E in *P. pastoris* was confirmed by yeast plasmid isolation and back transformation into *E. coli* and restriction enzyme analyses (data not shown). These results also suggested that pGAPZ-E exists as an episomal state in *P. pastoris*.

In order to test the efficiency of episomal expression, we initially tried to express reporter genes such as beta-galactosidase and *GFP*uv genes [5, 28, 29, 32]. These reporter genes resulted in detectable levels of proteins in SDS-protein gels (Fig. 3C). In addition, comparison of the integral *versus* episomal expression was carried out using the beta-galactosidase gene as a reporter gene. Previously, we found that episomal expression levels were inferior to those from integrated plasmids (unpublished). However, with the pGAPZ-E vector expressing beta-galactosidase, SDS-PAGE gels and enzyme activity measurements showed that the episomal lacZ expression level was at least equal to or better than that for integral expression. Based on these results, we introduced recombinant pGAPZ-E constructs carrying the human target genes into *P. pastoris*. HR547, HR919, and HR1697 human targets, for which *E. coli* expression had previously given unsatisfactory results (Table 1), yielded soluble proteins when expressed in *P. pastoris* using pGAPZ-E. The detection of soluble expression was based on the normal Coomassie-blue gel staining rather than Western detection. This is because the *GAP* promoter is strong enough to allow detect of the foreign proteins with a regular protein staining method.

Although yeast, in particular *Pichia pastoris*, offers an attractive option for eukaryotic protein expression, it has lagged somewhat behind other systems in its adoption by many laboratories because of its technical and time-consuming requirements. The simple and easy detection procedure for foreign eukaryotic proteins would make this new *Pichia* expression vector suitable for high-throughput screening of structural genomics target proteins. Therefore, the new *Pichia* expression system will in the future contribute to providing a powerful alternative to other eukaryotic systems such as baculovirus or mammalian expression.

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