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Acta Fac. Pharm. Univ. Comen. LXII, 2015 (2): 43-50.
ISSN 1338-6786 (online) and ISSN 0301-2298 (print version),
DOI: 10.1515/afpuc-2015-0031

ACTA FACULTATIS PHARMACEUTICAE UNIVERSITATIS COMENIANAE

Increased expression and secretion of recombinant *hIFNγ* through amino acid starvation-induced selective pressure on the adjacent *HIS4* gene in *Pichia pastoris*

Original research article/Review

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Received August 10, 2015, accepted October 31, 2015

Abstract Transcriptional co-regulation of adjacent genes has been observed for prokaryotic and eukaryotic organisms, alike. High levels of gene adjacency were also found in a wide variety of yeast species with a high frequency of co-regulated gene sets. The aim of this research was to study how selective pressure on the Histidinol dehydrogenase gene (*HIS4*), using amino acid starvation, affects the level of expression and secretion of the adjacent human interferon gamma gene (*hIFNγ*) in the recombinant *Pichia pastoris* GS115 strain, a histidine-deficient mutant. *hIFNγ* was cloned into the *pPIC9* vector adjacent to the *HIS4* gene, a gene essential for histidine biosynthesis, which was then transformed into *P. pastoris*. The transformed *P. pastoris* was cultured under continuous amino acid starvation in amino acid-free minimal medium for ten days, with five inoculations into unspent medium every second day. Under these conditions, only successfully transformed cells (*hIFNγ –HIS4*$^{+}$) are able to synthesise histidine and therefore thrive. As shown by ELISA, amino acid starvation-induced selective pressure on *HIS4* improved expression and secretion of the adjacent *hIFNγ* by 55% compared to unchallenged cells. RT-qPCR showed that there was also a positive correlation between duration of amino acid starvation and increased levels of the *hIFNγ* RNA transcripts. According to these results, it is suggested that these adjacent genes (*hIFNγ* and *HIS4*) in the transformed *P. pastoris* are transcriptionally co-regulated and their expression is synchronised. To the best of the knowledge of the authors; this is the first study demonstrating that amino acid starvation-induced selective pressure on *HIS4* can alter the regulation pattern of adjacent genes in *P. pastoris*.

Keywords *Human interferon gamma – Histidinol dehydrogenase – Gcn4p – Serial passage – Transcriptional co-regulation*

1. INTRODUCTION

There is increasing evidence that eukaryotic genes are co-regulated based on their location within the genome. Adjacent genes are subjected to tighter transcriptional co-regulation compared to distantly placed genes. This type of co-regulation appears to be an evolutionary conserved and a vital regulatory mechanism in eukaryotes including yeasts and has a functional significance for maintaining coordinated levels of gene expression (Arnone et al., 2012). For example, adjacent genes in *Saccharomyces cerevisiae* display similar patterns of expression (Kruglyak & Tang, 2000), which is substantiated by genome-wide expression studies in a number of organisms, such as *Drosophila* (Boutanaev et al., 2002), nematodes (Lercher et al., 2003), mice (Purmann et al., 2007), humans (Purmann et al., 2007), and *Arabidopsis* (Arnone et al., 2012; Wei et al., 2011; Williams & Bowles, 2004).

Another remarkable example are the genes encoding ribosomal proteins and the rRNA biosynthesis pathway exhibiting a high percentage of adjacent gene pairs (Wade et al., 2006). This phenomenon is wide-spread in a variety of yeast species with approximately 24% of the ribosome and rRNA biosynthesis genes being positioned as adjacent gene pairs in *Candida albicans* (Arnone & McAlear, 2011). These genes remain tightly co-regulated even under changing cellular growth status (Arnone et al., 2012; Dai & Lu, 2008; Grewal et al., 2005). In addition, elevated levels of gene expression, and silencing/repression of expression have also reported for adjacent genes (Grunstein, 1997). This correlation between the expression levels of genes and their relative location to each other can be explained by multiple biochemical, evolutionary, genetic, and technological factors

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(Bozinovic et al., 2013; Fraser, 2013; Gilad et al., 2006; Hurst et al., 2004; Michalak, 2008; Sproul et al., 2005). For example, it has been theorised that co-expression of adjacent genes can be defined by chromatin domains (Hurst et al., 2004), i.e. unzipping chromatin during gene expression can concurrently facilitate expression of genes from neighbouring opened region (Sproul et al., 2005).

In spite of the potential importance of variation of gene regulation, so far little is known about the effects of selective pressures acting on regulatory patterns. Correlation between gene expression and selective pressure has been observed in model organisms and primates (Gilad et al., 2006). These findings suggest that statistically significant changes in gene expression contribute to phenotypic changes and large morphological differences (Bozinovic et al., 2013). For example in humans, selective pressure in the form of solar radiation is a probable explanation for observed changes in expression levels of genes involved in the UV radiation response, diabetes-related pathways and immune cell proliferation (Fraser, 2013).

The *HIS4* (histidinol dehydrogenase) gene is essential for histidine biosynthesis and its transcriptional regulation has been studied extensively in *S. cerevisiae*. Transcriptional control of *HIS4* is carried out by either of two mechanisms: "basal control" is driven by transcriptional factors *Bas1* and *Bas2* binding independently to the *HIS4* promoter under amino acid-rich condition while "general control" is driven by the transcriptional factor Gcn4p, which is activated under starvation of even a single amino acid and leads to an induction of 40 genes in 12 pathways required for the biosynthesis of amino acids (Lamas-Maceiras et al., 1999; Zaman et al., 1999). Gcn4p binds as a homodimer protein to the consensus sequence rrTGASTCA(T)n and activates the transcription of genes in either direction at a distance of approximately 600 bp. Five such binding sites have been identified in the *HIS4* promoter *of S. cerevisiae* (Lamas-Maceiras et al., 1999). The *Gcn4p* gene itself is regulated by the fluctuation of amino acid availability (Zaman et al., 1999). It has also been shown that amino acid starvation can increase *HIS4* expression three to four-fold above unstressed levels (Hinnebusch, 2005).

The aim of this study was to assess the effect of amino acid starvation-induced selective pressure on *HIS4* on the level of expression of adjacent genes in the recombinant *Pichia pastoris*. For this purpose; the human interferon gamma (*hIFNγ*) gene, which has a therapeutic value against wide variety of diseases like cancer, hepatitis and tuberculosis (Miller et al., 2009), was cloned into the *pPIC9* vector adjacent to the *HIS4* gene. Then it was transformed into the *Pichia pastoris* GS115 strain, a histidine-deficient mutant. The transformant, containing the *HIS4* gene, was cultured under continuous amino acid starvation in modified Yeast Nitrogen Based medium (YNB) void of amino acids, leading to an expression of the *HIS4* gene. Finally the expression levels of *hIFNγ* were measured to evaluate the possibility of co-regulation of these adjacent genes.

2. MATERIAL AND METHODS

2.1. Cloning and transformation

Cloning: To generate *pPIC9-hIFNγ*, the coding sequence of *hIFNγ*, flanked with *EcoRI* and *NotI*, was synthesised by Life Technologies, GeneArt Strings, and modified based on the codon preference in *P. pastoris*. Subsequently, the fragment was inserted into the *pPIC9* vector between the same restriction sites; adjacent to the *HIS4* gene, which is essential for biosynthesis of histidine. The optimised sequence encoding *hIFNγ* and its resultant amino acid sequences are shown in (Fig.1-A).

Transformation & integration in P. pastoris: The non-linearized plasmid *pPIC9-hIFNγ* was transformed into the GS115 strain of *P. pastoris* by electroporation (Electroporator 2510, Eppendorf) following the protocols for electro-competent cell production and electroporation (Life Technologies). Gene integration occurs at the *AOX* (GS115) locus by a single crossover between the *AOX* locus and any of the three *AOX* regions on the vector: the 5′ *AOX* promoter, the *AOX* transcription termination region (TT) or the 3´ *AOX*. This results in the integration of one or more copies of the vector into the genome with the resultant phenotype of His^+Mut^+ for the transformed *P. pastoris* (GS115) (Fig.1-B).

Screening for Mut^+ transformants: Transformant colonies with $HIS4^+$ phenotype were selected on Minimal Dextrose (MD) (1.34% YNB, 2% dextrose) agar plates based on complementation of histidine auxotrophy. In order to confirm the methanol utilization (*Mut*) phenotype of the strain, colonies with $HIS4^+$ phenotype were transferred to plates with either MD or Minimal Methanol (MM) (1.34% YNB, 0.5% methanol) as the carbon source. This allows to differentiate between Mut^S (slow methanol utilisation) and Mut^+ (can utilise methanol effectively as a carbon source) phenotypes, with the latter growing well on MM agar plates, while the former shows insignificant growth. As expected, only the Mut^+ phenotype was detected based on growth on both agar media after 24h.

2.2. Confirmation of integration to genomic DNA by PCR

In order to determine whether *hIFNγ* was integrated into the *P. pastoris* genome, genomic DNA from colonies with $HIS4^+Mut^+$ phenotype were isolated (Wizard® Genomic DNA Purification Kit, Promega). The integration of *hIFNγ* into the genome of *P. pastoris* was confirmed by PCR using the α-Factor sequencing primer as a forward primer 5´-TACTATTGCCAGCATTGCTGC-3´ which hybridises within the 5′ end of the α-factor region paired with the 3´ *AOX1* sequencing primer as a reverse primer 5´-GCAAATGGCATTCTGACATCC-3´ which hybridises with 3´ end of the *AOX1* transcription terminator (TT) region (Fig.1). Genomic DNA of untransformed *P. pastoris* GS115 was used as a negative control. Thirty amplification cycles were

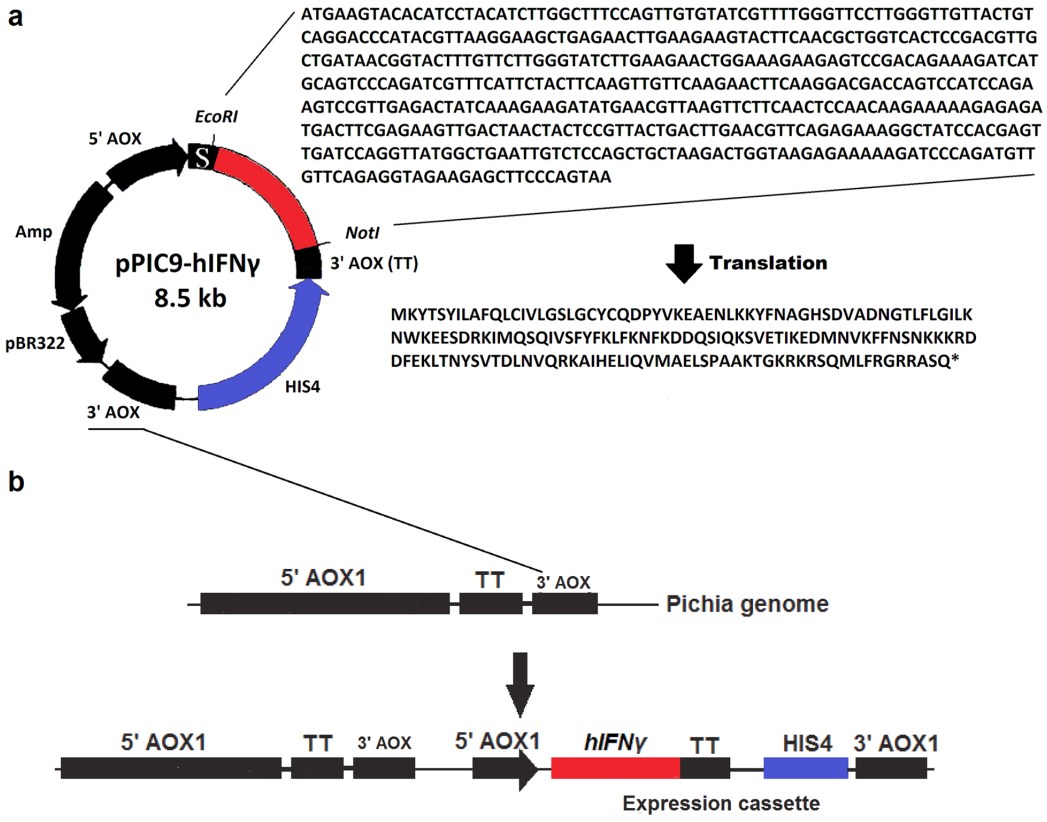

Figure 1. Placement of the two adjacent genes, hIFNγ and HIS4, as part of the pPIC9-hIFNγ vector (a) and result of the integration of the vector between the 3´AOX into the intact AOX1 locus (Mut⁺) and the gain of promoter 5' AOX1, hIFNγ gene , and HIS4 (expression cassette) (b). 5' and AOX1: 5' Alcohol oxidase promotor gene which requires methanol for induction, S: α-factor secretion signal, hIFNγ: optimised human interferon gamma gene for P. pastoris, 3' and AOX (TT): Alcohol oxidase transcription terminator, HIS4: Histidinol dehydrogenase gene which is essential for histidine biosynthesis, pBR322: origins from E. coli, Amp: Ampicillin resistance gene

performed at 94˚C for 30 s, 55˚C for 30 s, and a final extension for 5 min at 72˚C.

Successful integration of the *hIFNγ* into the *P. pastoris* genome was demonstrated by the expected ~700 bp fragment size using agarose (1.5%) gel electrophoresis which was verified by DNA sequencing at the Australian Genome Research Facility Ltd. (AGRF).

2.3. Protein expression under amino acid starvation-induced selective pressure on *HIS4*

Successfully transformed *P. pastoris* cells were kept under amino acid starvation by cultivation in buffered Minimal Glycerol (BMG) medium (1.34% YNB without amino acids, 100 mM potassium phosphate, pH 6.0, and 1% glycerol). Under these conditions, only successfully transformed cells are able to synthesise histidine and therefore thrive. Explicitly, continuous amino acid starvation was maintained for 10 days; re-inoculating into fresh BMG medium every 2 days (Fig. 2A). A *HIS4*⁺ colony was inoculated into 25 mL of BMG in a 250 mL baffled flask and incubated at 28˚C for 48 h with a shaking speed of 200 rpm until reaching an OD_{600}≥2 (log-phase growth) (EnSpire® Multimode Plate Reader, PerkinElmer). Subsequently, the cells were harvested by centrifugation at 3000 *g* for 5 min at room temperature. Cell pellets were resuspended in 50 mL PBS buffer (0.1 M Phosphate Buffer Saline, pH 7.4) to remove residual glycerol. Finally cell pellets were resuspended in 50 mL buffered methanol-complex (BMMY) medium (1% yeast extract, 2% peptone, 1.34% YNB, 100 mM potassium phosphate, pH 6.0, 0.5% methanol) to a starting OD_{600}=1 in a 250 mL baffled flask (Fig.2-B). To induce expression of *hIFNγ*, pure methanol was added to a final concentration of 1% (v/v) every 24 h. Culture supernatant was obtained by centrifugation at 1500 *g* after 72 h of cultivation to analyse expression of *hIFNγ*, cell pellets were used for genomic DNA extraction for qPCR and total RNA extraction for RT-qPCR (Section 2.5).

2.4. ELISA

Recombinant *hIFNγ* protein levels in supernatants were quantified using a modified indirect ELISA protocol (Abcam).

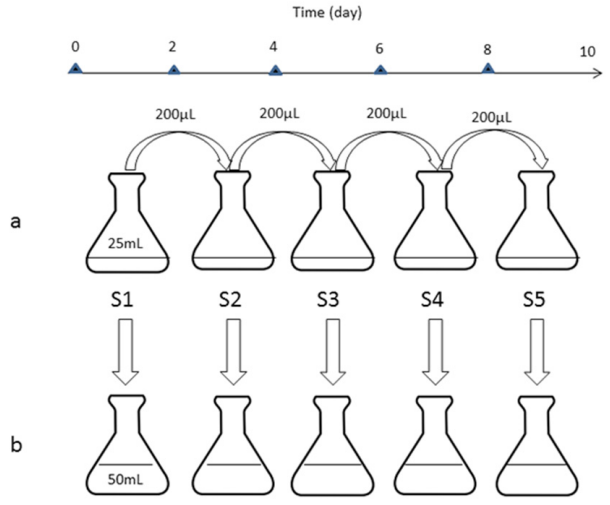

Figure 2. Diagram showing continuous amino acid starvation over 10 days in buffered Minimal Glycerol (BMG) medium (a) and protein expression in buffered methanol-complex (BMMY) medium (b). S: Serial passage

Figure 3. Dot blot showing hIFNγ-positive cultivation media of two cultures exposed to amino acid starvation (a) and supernatant of cell culture of untransformed P. pastoris GS115 (negative control) (b)

Replicated sample aliquots (50 µL) were added to each well of a polyvinyl chloride micro-titre plate and incubated overnight at 4°C. Wells were washed three times with 200 µL Tris-buffered saline (Tris-HCl 20 mM, NaCl 150 mM, pH 7.5). Protein-binding sites were blocked by adding 200 µL blocking buffer (5% Bovine Serum Albumin (BSA) in TBS) per well, followed by incubation for 1 h at 37°C in a shaking incubator chamber (HO35™ Hybridisation Oven, Ratek), followed by washing twice with TBS. 100 µL of diluted (0.5 µg·mL^{-1}) primary antibody (polyclonal rabbit-anti-*hIFNγ*, Abcam cat no. ab9657) was added to each well and incubated for 1 h at 37°C in a shaking incubator chamber. Plates were washed four times with TBS. 100 µL of conjugated secondary antibody (polyclonal goat anti-rabbit, Abcam cat no. ab98505) diluted 1/1000 in blocking buffer was added to each well and incubated for 1 h at 37°C in a shaking incubator chamber. After washing four times with TBS, 50 µL of Alkaline Phosphatase Yellow (pNPP) Liquid Substrate (P7998 SIGMA) was added per well. Absorbance at 405 nm was recorded after 30 min on a spectrophotometer (EnSpire® Multimode Plate Reader, PerkinElmer). Supernatant of cell culture of untransformed *P. pastoris* GS115was used as negative controls.

A standard curve 0, 1.25, 2.5, 5, 10 µg.L^{-1} (R^2 = 0.993 and y = 12.169*x*-1.1321) was prepared by serial dilution of the recombinant *hIFNγ* (Abcam cat no. ab51240).

2.5. Immuno-blotting

Immunoblotting (dot blot) was performed to qualitatively detect the presence of *hIFNγ* in the medium supernatant, following standard procedures described by Abcam. In brief, a nitrocellulose membrane (pore size 0.2 µm N7892 SIGMA**)** was gridded and 2 µL of samples were spotted onto the nitrocellulose membrane at the centre of each grid square. The membrane was left to dry for 30 min. Unspecific binding sites were blocked with 1% BSA in TBS-T (Tris buffered saline-TWEEN 20 0.05%) for 30 min at room temperature on a rocking shaker (VSR-50® Laboratory Platform Rocker). The membrane was then incubated with the primary antibody (polyclonal rabbit anti-*hIFNγ*, Abcam cat no. ab9657) (0.1 µg·mL^{-1}) dissolved in TBS-T over night at room temperature. The membrane was washed for 5 min three times with TBS-T. Thereafter, the membrane was incubated for 2 h at room temperature with the secondary antibody (polyclonal goat-anti-rabbit, Abcam cat no. ab98505), conjugated to alkaline phosphatase. Finally, the membrane was washed twice for 5 min with TBS-T and incubated with SIGMA *FAST*™ BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium) dissolved in 10 mL deionised water, and left until colour developed (Fig. 3). Supernatant of cell culture of untransformed *P. pastoris* GS115 was used as negative controls.

2.6. qPCR, RNA EXTRACTION & RT-qPCR

QuantiTect SYBR® Green PCR Kit (Qiagen cat no. 204141) was used for both real time PCR (qPCR; assessing gene copy number) and two-step reverse transcription-PCR (RT-qPCR; assessing transcription level of the RNA) using primers as per Table 1. A set of primers was designed to amplify 168 bp of the *hIFNγ* sequence (Section 2.1).

Genomic DNA of each serial passage was extracted at the end of the experiment, and 50 ng of DNA (NanoDrop®, ND-1000 Spectrophotometer) for each serial passage was used in qPCR. Total RNA was extracted using the PureLink® RNA Mini Kit (Life Technologies cat no. 12183018A), followed by DNase treatment and reverse transcription to cDNA using the QuantiTect Reverse Transcription Kit (Qiagen cat no. 205310). For each serial passage, 500 ng of cDNA was quantified by NanoDrop based on the optical absorbance at OD_{260}. Two replicated RT-qPCRs were performed using the same primer set as for qPCR (Table 1). Genomic DNA and total RNA of untransformed *P. pastoris* GS115 was used as negative controls for qPCR and RT-qPCR, respectively.

Table 1. Primer design for qPCR /RT-qPCR.

	Sequence	Length [bp]	T_m	GC%
Forward primer	5′ ACTTCAACGCTGGTCACTC 3′	19	57.71	52.63
Reverse primer	5′ CGGACTTCTGGATGGACTG 3′	19	57.25	57.89

Standard curves for qPCR were prepared with purified DNA amplicons (section 2.2) [699 bp amplicon containing 501 bp *hIFNγ* plus secretion signal and parts of the *AOX* gene promoter and transcription terminator]. Dilution series of DNA amplicons according to mass concentration (ng/per total volume of reaction) were used to make standard curves with, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} and 0 ng DNA (R^2 = 0.998, *Overall efficiency* = 101.1% and $y = 11.149e^{-0.699x}$).

For calculation of approximate gene copy number the following equation was used, based on the fact that 699 bp dsDNA amplicon weighs ~$75.33*10^{-10}$ ng.

Gene copy number = Initial concentration of detected DNA amplicon (ng) / $75.33*10^{-10}$ ng.

Each 50 µL reaction contained 25 µL (2x) QuantiTec SYBR GreenPCR Master Mix, 10 µM forward and reverse primers with final concentration of 0.3 µM (5 µL each), 10 µL sample (genomic DNA or cDNA) and 5 µL RNase-free water. qPCR reactions were run on a Peltier Thermal Cycler-200 (BioRad) under the following conditions: PCR initial activation step 95˚C for 15 min, followed by 45 cycles of denaturation at 94˚C for 15 s and annealing at 57˚C for 30 s, and extension at 72°C for 30 s.

2.7. Statistical analysis

Data on *hIFNγ* protein secretion levels and C(t) of RT-qPCR were statistically analysed via one-way ANOVA using Microsoft Office Excel, Data Analysis. Homogeneity of variances was confirmed using Levene's Test. Critical value was set to ($\alpha = 0.05$) and results were deemed statistically significant at $p \leq 0.05$. If statistical significance was detected, Tukey-Kramer HSD post-hoc tests were performed to identify samples significantly different to each other.

3. RESULTS

3.1. Transformation and confirmation of integration

Six clones were retained and their phenotypic *HIS4*$^+$ status was confirmed on Minimal Dextrose and *Mut*$^+$ status on Minimal Methanol agar plates. The successful integration of the *hIFNγ* was confirmed by PCR using 5′ and 3′ primers of the *AOX1* and α-factor partial sequence, respectively (Section 2.2). Agarose gel electrophoresis of *HIS4*$^+$ *Mut*$^+$ transformants confirmed PCR products between 500-1000 bp according to a DNA ladder (EasyLadder I, Bioline), while negative controls (untransformed *P. pastoris*) showed no band. Results of DNA sequencing at the Australian Genome Research Facility Ltd. (AGRF) confirmed that amplicons sequences match the optimised *hIFNγ* (Section 2.1).

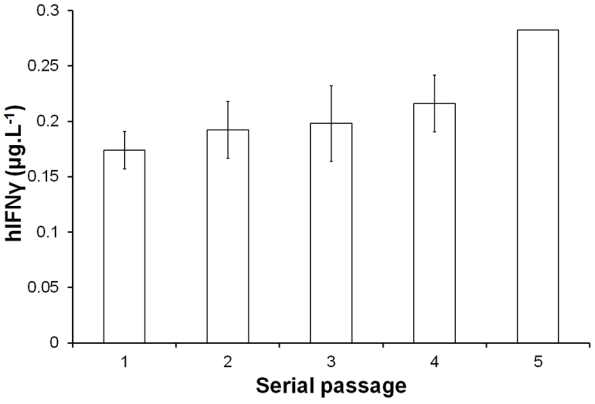

Figure 4. Amino acid starvation-induced levels of secreted hIFNγ over 5 serial passages of P. pastoris GS115 transformed with hIFNγ and HIS4 (Mean ± SD, n = 2)

3.2. Protein expression under amino acid starvation-induced selective pressure on *HIS4*

The strain (C6) yielded the strongest agarose gel signal and was used for protein expression and secretion studies (Section 2.3). The amount of secreted *hIFNγ* was assessed 72 h after induction in BMMY medium by immunoblots (Fig. 3) and ELISA, with the latter detecting secreted yields of 0.18 to 0.28 µg.L^{-1} of *hIFNγ* (Fig. 4). Culture supernatants of the untransformed *P. pastoris* were used as negative controls. Immunodot blots using culture supernatant of the untransformed *P. pastoris* yielded no positive signal (Fig. 3), further demonstrating that the construct, *pPIC9-hIFNγ*, was successfully expressed in *P. pastoris*.

Levene's test for determining homogeneity of variances validated the assumption of equal variances ($p=4.95*10^{-75}$). A one-way ANOVA showed that there was a significant difference in levels of *hIFNγ* secretion between one or more pairs of serial passages (Table 2). A significant difference between serial passages 1 and 5 was detected (Tukey-Kramer HSD test: $p = 0.029$), while serial passages 2-4 were not significantly different to either serial passage 1 or serial passage 5 ($p > 0.05$).

Table 2. Summary of one-way ANOVA results for 5 serial passages of transformed P. pastoris producing hIFNγ

Source of Variation	*SS*	*df*	*MS*	*F*	*F crit*	*P-value*
Between Groups	0.015841	4	0.00396	6.961538	5.192168	0.028203
Within Groups	0.002844	5	0.000569			
Total	0.018685	9				

Table 3. Approximate hIFNγ gene copy number and hIFNγ DNA amplicon concentration [ng] of serial passages 1, 3 and 5 of hIFNγ –HIS4^{+} Mut^{+} P. pastoris transformants under amino acid starvation

Content	C(t)	Initial Concentration of *hIFNγ* DNA amplicons (ng)	Approx. gene copy number
Serial passage 1	13.93	$65.84*10^{-5}$	$\sim 87.34*10^{3}$
Serial passage 3	13.79	$72.61*10^{-5}$	$\sim 96.37*10^{3}$
Serial passage 5	13.97	$64.02*10^{-5}$	$\sim 85.96*10^{3}$

Table 4. C(t) values of RT-qPCR for quantification of hIFNγ RNA and calculated initial concentration of the cDNA amplicons (Mean ± SD, n = 2)

Content	C(t)	Initial Concentration of cDNA amplicons (ng)
Serial passage 1	24.79 ± 0.035	$3.34*10^{-07}\pm 0.08*10^{-07}$
Serial passage 3	25.02± 0.162	$2.89*10^{-07}\pm 0.33*10^{-07}$
Serial passage 5	23.28± 0.028	$9.55*10^{-07}\pm 0.19*10^{-07}$

3.3. Gene quantification and gene copy number analysis

The results of the qPCR for determining concentration of target gene showed consistent and similar amounts of amplified the 168 bp amplicon, suggesting equal *hIFNγ* gene numbers across serial passages (Table 3).

3.4. Transcriptional analysis of *hIFNγ* RNA

Levene's test for determining homogeneity of variances validated the assumption of equal variances ($p = 1.3*10^{-41}$). A one-way ANOVA on C(t) values showed that there was a significant difference between one or more pairs of serial passages ($p = 0.0007$). A Tukey-Kramer HSD test revealed no significant differences between serial passages 1 and 3 ($p > 0.01$), but a significant difference between serial passages 1 and 5 ($p < 0.01$) and serial passages 3 and 5 ($p < 0.001$). These results conformed to the protein expression/secretion results obtained by ELISA (section 3.2).

4. DISCUSSION

Studies in model organisms propose that the expression levels of most genes change and evolve under stabilising selective pressure which has been proposed to be the dominant mode of evolutionary changes in gene expression (Gilad et al., 2006). Gene expression in yeast has also been shown to change in response to environmental stress; for example, the expression of a significant number of genes (1372) was altered distinctively when *S. cerevisiae* was cultivated for either five or twenty five generations under microgravity to compared controls cultured under identical conditions in normal gravity (Sheehan et al., 2007). Therefore, as a driving force of evolution, the outcomes of selective pressures are generally well documented, while the linkage to direct genetic effects are less understood.

Much of the work on regulatory networks has focused on the yeast *S. cerevisiae*, for which data are most copious (Babu et al., 2004). To the best of our knowledge, the effects of amino acid starvation-induced selective pressure on *HIS4* and the transcriptional co-regulation of recombinant adjacent genes in *P. pastoris* has not been documented to date. As predicted in our study, amino acid starvation-induced selective pressure on *HIS4* increased expression of the adjacent *hIFNγ* gene by ~55%., suggesting co-regulation, as increased secretion levels were positively correlated with RNA transcription levels. Investigation of gene copy number of *hIFNγ* in every other serial passage showed no variation (Table 3), suggesting that increased level of protein expression and RNA transcription is not due to "gene duplication" making transcriptional co-regulation between *hIFNγ* and the adjacent *HIS4* gene highly likely.

At least three mechanisms have been proposed to explain adjacent gene co-regulation:

1. *Localised chromatin modification;* where there is a correlation between histone *H4* acetylation domains and genome-wide histone *H3K14* acetylation which correlate with transcriptionally co-expressed genes in budding yeast (Deng et al., 2010). When a gene is being transcribed, the localised chromatin is forming a more open permissive transcriptional state (Sproul et al., 2005), which can affect the transcription of genes in adjacency (Ebisuya et al., 2008).
2. *Local DNA sequence looping;* which has been observed between genes on the same and different chromosomes in yeast (Duan et al., 2010), where adjacent genes can be silenced via a localised loop of DNA sequences when the promoter of the adjacent gene is in physical contact with silencing factors (Valenzuela et al., 2008).
3. *Adjacent gene co-regulation through sub-nuclear compartmentalisation;* where transcriptionally active sets of genes are lodged at the nucleolar periphery upon activation (Berger et al., 2008). Active genes have been seen to associate with 'transcription factories', which are the spot of nascent RNA production and associated transcription factors (Osborne et al., 2004). As a result, if one gene gains entry to an active sub-nuclear compartment, the adjacent gene could hypothetically follow the same regulatory process (Arnone et al., 2012).

In the study here, while gene co-regulation through DNA looping are possible scenarios for other non-investigated genes, expression of *HIS4* would have been activated by amino acid starvation (as shown by (Hinnebusch, 2005)), and, since increased transcription (mRNA) and expression/secretion was also improved, co-regulation of these two genes would need to be achieved through either mechanism 1 or 3. It is much harder to differentiate between the potential roles of localised chromatin modifications and sub-nuclear compartmentalisation in the co-regulation of *HIS4* and *hIFNγ*, because these two mechanisms are not mutually exclusive in operation. There is some evidence supporting "localised chromatin modifications" playing 'a' greater role in gene co-regulation in eukaryotes e.g. yeast (Babu et al., 2004). Latest evidence also suggests that the environment can stably affect the establishment of the epigenome which is referred to as "transgenerational epigenetic inheritance" (Daxinger & Whitelaw, 2010). The role of localised chromatin modifications as an epigenomic co-regulatory mechanism would require investigating the stable inheritance of *HIS4* and *hIFNγ* expression when the selective pressure of amino acid starvation is removed. This should be ideally conducted simultaneously with experiments aiming to identify transcriptional localisation of *HIS4* and *hIFNγ* within the nucleus to examine the potential for a contribution of nucleolar transcriptional factories.

It could be equally argued that gene co-expression might be regulated by simple regulatory networks, which do not necessarily require any of the above regulatory mechanisms. For example, it was shown that external conditions e.g. stress induced topologically simple regulatory networks, characterised by involving a limited number of steps and transcription factors in yeast (Babu et al., 2004). In relevance to the study here, amino acid starvation activated the transcription factor Gcn4p, resulting in transcriptional induction of almost all genes involved in amino acid biosynthesis (Hinnebusch, 2005), including *HIS4*. Additionally, a wide array of genes unrelated to amino acid biosynthesis, i.e. close to one tenth of the yeast genome was activated (Natarajan et al., 2001), which designates a role for Gcn4p as a "master regulator" for gene expression (Hinnebusch & Natarajan, 2002). Thus involvement of Gcn4p in regulation of both *HIS4* and *hIFNγ* can be hypothesised as a probable scenario explaining the increased level of *hIFNγ* under amino acid starvation.

5. CONCLUSION

This study showed that the adjacent localisation of *hIFNγ* and *HIS4* genes result in co-regulation of *hIFNγ* expression and secretion, a first step for potential improvement of *hIFNγ* yields using this expression system. Additionally, the recombinant system developed should lend itself for detailed studies regarding the underpinning nature of the regulatory mechanism.

ACKNOWLEDGEMENTS

KH acknowledges project support by the Advanced Manufacturing Cooperative Research Centre (AMCRC), funded through the Australian Government's Cooperative Research Centre Scheme, grant number 2.3.4. The funders had no role in study design, data collection and analysis or preparation of the manuscript and have provided permission to publish. This research is part of the MBD Energy Research and Development program for Biological Remediation of Methane from Underground Coal Mine Ventilation Air. Ali Razaghi was supported by an AMCRC PhD scholarship.

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