ON THE COMPLEMENTATION OF *SACCHAROMYCES CEREVISIAE MET16* IN *SCHIZZOSACCHAROMYCES POMBE*

Is homology conserved in a metabolic pathway required for methionine biosynthesis?

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Abstract

Metabolically active pathways tend to be more highly conserved between ecologically similar relatives than other pathways for their critical role in life-functions. The developmental features of Schizzosaccharomyces pombe and Saccharomyces cerevisiae may have become quite divergent, but their ecological niche is still quite similar. The functional homology between S. cerevisiae's MET16p and S. pombe's was investigated through plasmid insertion and sulfate assimilation assays in S. pombe. Although the S. cerevisiae:MET16p was produced, the S. pombe colonies were unable to utilize extracellular sulfate in the synthesis of Methionine.

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1. Introduction

The yeast species *Saccharomyces cerevisiae* continues to be a model organism for its growth characteristics, phylogeny, and its long history of lab use (Drubin 1989; Knorre et al. 2005; Duina et al. 2014) ; but going back further, this yeast has another factor influencing it's popularity: commercial utilization. While not the focus of this article, the manufacturing of better fermentables through genetic alteration of yeast strains has been clear in recent literature (Cordente et al. 2009; Kim et al. 2013). Nevertheless, experiments looking to reduce hydrogen sulfide (H_2S) production in commercial *S. cerevisiae* strains have directly implicated the methionine biosynthesis pathway as important in sulfur (several forms) management in *S. Cerevisiae* (Barbosa et al. 2012) . Herein, the *S. cerevisiae MET16* gene—and its homolog in *Schizosaccahromyces pombe*—is the focus.

The *MET16* gene encodes for PAPS reductase in the yeast *S. cerevisiae*. This enzyme is critical in the biochemical *MET* pathway which produces methionine from extracellular sulfate—and for sulfate assimilation in general. The MET16p (EC 1.8.4.8) catalysis the following reaction:

Adenosine biphosphate + SO_3^{-2} + thioredoxin disulfide ⇋ phosphoadenylyl sulfate + thioredoxin

The phenotype observed for a *MET16* mutant S. *cerevisiae* strain was auxotrophy for various compounds(Villa-García et al. 2011) as well as heat sensitivity(Sinha et al. 2008), decreased fitness(Breslow et al. 2008), and reduced resistance to metals(Hwang et al. 2007) and chemicals(O'Connor et al. 2013).

The xray crystal structure for this enzyme is available at high resolution(Yu et al. 2008). Consisting of chains A, B, C, and D, the quaternary structure consist of a dimer of dimers consisting of chains A with B and C with D. The complete complex contains four active sites—one on each chain(Yu et al. 2008). Although the catalytic mechanism has not been extensively studied, the overall reaction results in oxidation of the sulfate through nucleophilic attack of the adenosine bi-phosphate. The x-ray structure also suggests no metal ion involvement for catalysis.

MET16p homologs are phylogenetically wide-spread and have been found in the bacteria *E. coli* and *Pseudomonas aeruginosa*. A BLASTP search resulted in

numerous results from throughout the Ascomycota phyllum with near perfect homology (>80%). The conservation of the enzyme function is expected due to the integral nature of PAPS-reductase in sulfate metabolism.

The series of experiments conducted here were based on a screening format which leveraged the growth phenotype to gauge complementarity. After ensuring the proper construction of plasmids containing the *S. cerevisiae URA* and *S. pombe MET16* genes under Gal1 control, including a set of controls, colonies of transformed *S. cerevisiae* were both plated on selective media and inspected via Western blot. Even though *S. pombe MET16* was expressed, it did not successfully complement in vivo since growth was not observed on sulfur-rich plates lacking methionine. Both positive and negative controls helped to strengthen this conclusion.

2. Materials and Methods

The strain of *S. cerevisiae* utilized in all aspects of this experiment was BY4742 (see Table 2-1 for genotype). For the preliminary work on classifying the phenotype of *MET* mutants on selective media and in verification by colony PCR, three strains were formed by the insertion of KAN^R gene inside the coding region of either *MET1*, *MET2* or *MET16* by our supplier.

Strain/Structure Name	Genotype/Description
S. cerevisiae (WT)	*MATα his3-Δ1 leu2Δ0 lys2Δ0 ura3Δ0*
pBG1805.1	*URA3 AMP* Tag[b] Gal1 promoter
pYES2.1	*URA3 AMP* Tag[c] Gal1 promoter
S. cerevisiae:MET16	*MET16* coding sequence from *S. cerevisiae*
S. pombe:MET16	*MET16* coding sequence from *S. pombe*

Table 2-1. Summary of genotype and nomenclature used.

[b] Adds *His6-HA epitote-3C cytochrome-ZZ* tag to C-terminus of insert.
[c] Adds *His6-V5* tag to C-terminus of insert.

2.1 Selective Media

The media was selected in order to identify the particular gene disrupted in each of the mutant strains. First, a series of five 1:10 dilutions were first prepared of each of the four *S. cerevisiae* strains being tested: WT, *met1*, *met2*, and *met16*. These dilutions were then spotted on a series of five various formulas of media. As summarized below in Table 3-1, four of the plates were minimal media[d] (MM) with or without the indicated nutrient, and one plate was YPD media as a positive control. After incubating for 72 hours at 37° C, the plates were imaged and the colonies quantified (data not shown).

2.2 PCR

To verify the presence of the correct mutation, PCR was conducted using Taq Polymerase to amplify the suspect gene mutation in each of the colonies. Each experimental reaction used a particular *MET* gene forward primer and a *KAN* specific reverse primer. A negative control for each of the three experimental reactions was run. The products of this PCR reaction were then run on an agarose gel[e] and visualized via Ethidium Bromide.

2.3 Restriction Mapping

2.3.1 Plasmid Isolation

Two plasmids were selected for development: pBG1805 and pYES2.1 for *S. cerevisiae* and *S. pombe MET16 gene insertion*, respectively (see Table 1). Both vectors contained an origin of replication for both *Escherichia coli* and *S. cerevisiae*, ampicillin resistance (*AMP*), as well as *URA3* and a Gal1 inducible promoter. The *AMP* served as a selectable marker for *E. coli* while the *URA3* provided a selective marker in our *ura3* yeast strains.

The plasmids pBG1805 + S. *cerevisiae*:*MET16*, pYES2.1 + S. *pombe*:*MET16*, and pYES2.1 + *LacZ* (negative control) were formed by our supplier. The plasmids had been grownup with ampicillin media in *E. coli* for 72 h prior to our isolation

[d] See page(s) 47-48 of the BI204 lab manual for details on MM.
[e] Gel was 1.25% agarose and ran at 120V for 20 minutes.

via a Zyppy® miniprep kit. A NanoDrop spectrometer was used to quantify the plasmid concentrations.

2.3.2 Plasmid Characterization

To verify the proper plasmid generation, a restriction digest was carried out using the ACCI enzyme and the EAEI enzyme. After 2 h at 37° C, the reaction products were visualized on a 1.5% agar gel containing ethidium bromide.

2.4 Transformation

The transformations were carried out using stationary phase liquid cultures of *S. cerevisiae*. The protocol used to transform the cells with the plasmids was *Quick Lithium Transformation*, details for which can be found in the *Laboratory Manual*. The transformed cultures were then spot plated on YPD media in a series of 1:10 dilutions to determine transformation efficiency. A master plate of MM+Met was also formed for each of the three plasmids. All plates were grown at 30° C for 48 h.

2.4.1 Replica Plating of Transformants

Complementation was assessed via replica plating on selective media. From the master plates, a series of three selective plates were replicatively formed using velveteen transfer. The MM-Met+Galactose, MM-Met+Glucose, and MM+Met+Glucose plates were used as the experimental, negative control, and positive control media, respectively. These plates were incubated at 30° C for 72 hours to permit growth of colonies.

2.5 SDS-PAGE Gel

In all three strains the inserted gene, either a *MET16* or *LacZ*, was placed under the control of a galactose inducible promoter; therefore, six cultures were grown consisting o 2 mL of MM+raffinose for 48 hours at 30°C. The suspended cells were then pelleted and the media was replaced with 2 mL MM-uracil with either galactose or glucose (repressor), see discussion for example. The cultures were then incubated a further 24 hours at 30° C.

The induced cultures were then lysed with NaOH. A pellet was then formed and resuspended in TAE buffer and boiled to denature the proteins. The protein extract was then ready.

An SDS-PAGE gel was prepared consisting of a stacking and resolving gel. The stacking gel consisted of a final concentration of 5% acrylamide:bis-acrylamide (37.5:1), 0.2% APS, and 0.5X buffer[f]. The gel was activated through TEMED. The resolving gel consisted of 12% acrylamide:bis-acrylamide (37.5:1) solution, 0.25X buffer[g], 0.1% APS, and was again activated through TEMED. The gel was run for 40 minutes at 200 V.

A simply-blue stain was then used to soak the gel in over night. The stained gel was then rinsed with H_2O and imaged.

2.6 Western Blot

A Western blot was conducted using the same SDS-PAGE gel formula expressed above. The SDS-PAGE gel was run at 220 V for 1.5 hours. The completed gel was then placed in contact with a PVDF membrane. The protein was transferred at 125 V over a period of 30 minutes. The exposed membrane was then rocked in a blocking solution of 5% non-fat milk for 24 hours before the primary antibody was added. The primary, monoclonal antibody identified the HA epitope protein in the *pBG1805.1*. After washing the membrane with H_2O, the secondary antibody was added and rocked for 1 hour which bound to the primary antibody V5 region as well as the *S. pombe* gene-construct at the V5 epitope. The membrane was then washed in H_2O, and the antibodies were developed with 3,3′-5,5′-tetramethyl benzide.

3. Results

3.1 Selective Media

An image of the spot plates and a table summarizing the growth results are provided in Figure 3-1 and Table 3-1, respectively. The first strain, *met16*, was able to grow with MM + SO_3 indicating that the affected gene mutation occurred

[f] See Lab Manual for Buffer recipe.
[g] See Lab Manual for Buffer recipe.

within *PAPS reductase,* a critical step in methionine biosynthesis. The *S. cerevisiae* strain *met1* was able to gown in MM + Cys but not in MM + SO_3 indicating an inability to reduce sulfite as a sulfur source. The final strain, *met2* was unable to grown with sulfite. A *met2* mutant is unable to synthesize O-acetyl homoserine, which is required when an external sulfur source is used for methionine biosynthesis.

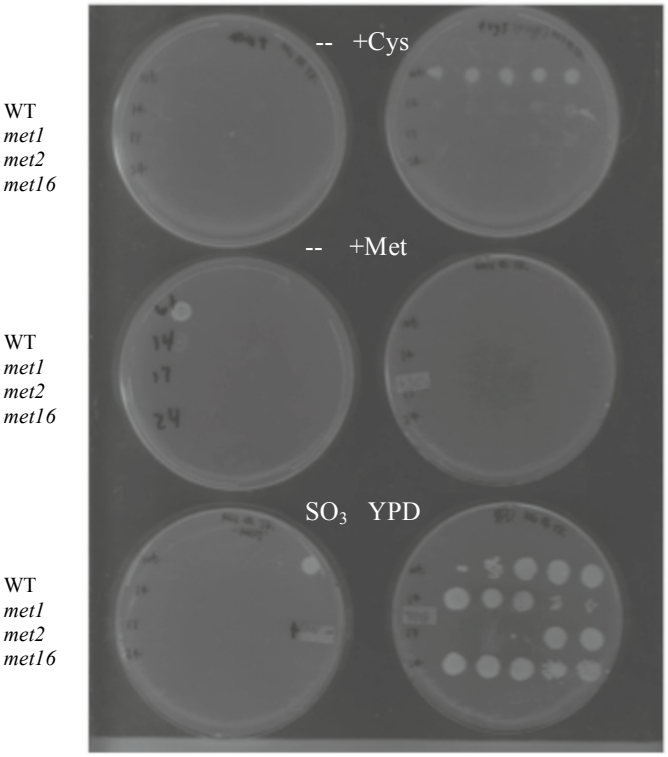

Figure 3-1. Spot plating of WT, met1, met2, and met16 S. cerevisiae on MM with indicated nutrient. Incubated for 72 h at 37° C.

Media	*S. cerevisiae WT*	*S. cerevisiae met1*	*S. cerevisiae met2*	*S. cerevisiae met16*
MM	N	N	N	N
MM + SO_3	Y	N	N	Y
MM +cys	Y	Y	N	Y
MM + met	Y	N	N	N
YPD	Y	Y	Y	Y

Table 3-1. Summary of growth for three mutant strains and wild-type on selective media containing different sulfur sources after 48 h incubation at 37° C.

3.2 PCR

The results of the agarose gel are included as Figure 3-2. The first lane is the ladder followed by the six PCR reactions and consisting of the materials indicated in the *Materials and Methods 2.2*. See Table 3-2 for lane assignmens. The presence of a band indicated PCR product formation and therefor the presence of the specified sequence. Based on this data, *met16*, *met1*, and *met2* disruptions were all confirmed.

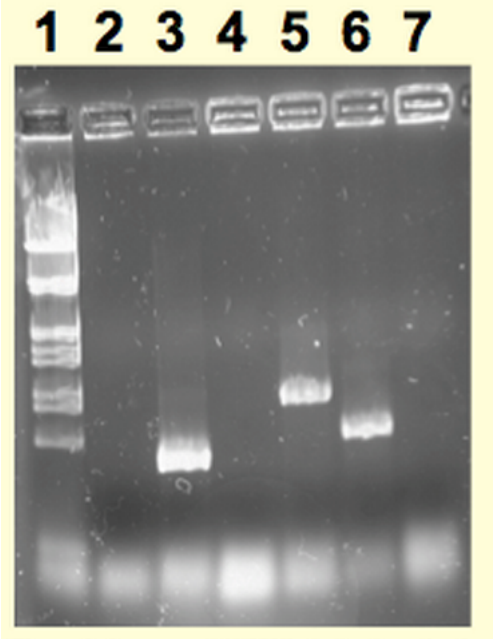

Figure 3-2. Colony PCR results on a 1.5 % agarose gel stained with ethinium bromide. The composition of reaction and lane assignments per Table 3.

Lane	Length (bp)	Strain	Forward primer	Reverse primer
1	---	ladder	---	---
2	---	YMP14	met16	met16
3	51	YMP14	met16	KAN primer B
4		YMP17	met2	met2
5	165	YMP17	met2	KAN primer B
6	90	YMP24	met1	KAN primer B
7		YMP24	met1	met1

Table 3-2. Colony PCR reactions and lane assignments. This was used to verify the identity of the strains YMP14, 17, and 24.

3.3 Restriction Map

3.3.1 Plasmid Isolation

The plasmids were isolated from 1.5 mL of liquid culture and yielded the final concentrations via NanoDrop as seen below (Table 4) after the Zyppy kit.

Plasmid	Concentration (nM/μL)
pYES2.1 + *LacZ*	27.8
pYES2.1 + *S.pombe*:*MET16*	18.9
pBG1805 + *S.cerevisiae*:*MET16*	22.2

Table 3-3-1. Nanodrop results for the isolated plasmids grown up in E. *coli*.

3.3.2 Plasmid Characterization

The ACCI enzyme was chosen since the fragment lengths would best determine the identity of the plasmids based on their suspected sequence. Table 3-3-2 provides both the expected and observed fragment lengths. The construction of the plasmids aligned with the expected results.

	pBG1805 + S.*cerevisiae:MET16*		pYES2.1 + S.*cerevisiae:MET16*		pYES2.1 + *LacZ*	
Band	**Expected**	**Observed**	**Expected**	**Observed**	**Expected**	**Observed**
1	500	387	505	369	505	310
2	700	762	670	434	625	479
3	6000	3278	5500	4337	2600	2600
4	*NA*	4399	*NA*	*NA*	5200	3927

Table 3-3-2. The expected and observed fragment lengths generated from the restriction digest of the three plasmids in order to confirm gene insert. See Figure 3 for gel image.

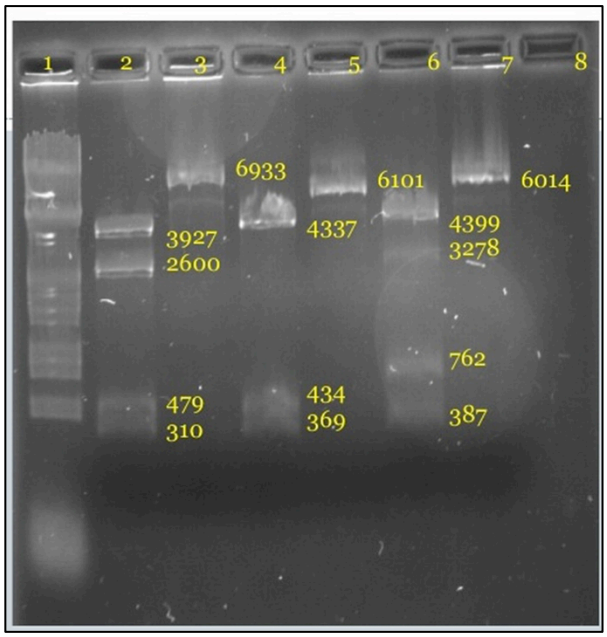

Figure 3-3. Restriction digest products run on a 1.5% agarose gel and stained with ethinium bromide. The lanes are: Ladder, pBG1805.1:*MET16* + ACCI, pBG1805.1:*MET16*, pYES2.*1:MET16* + *ACCI*, pYES2.*1:MET16*, pYES2.*1:LacZ* + *ACCI*, pYES2.*1:LacZ*. See Table 5 for expected fragment lengths.

3.4 Transformations

The results of the transformation platting provided a method of estimating the transformation efficiency. The YPD dilution spot platting provided the total number of viable cells after transformation while the master plate (on selective media) colonies provided the number of successfully transformed cells. Unfortunately, the spot plate failed to grow our cultures so a colleague's plate

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was used for estimation purposes. It was found that the transformation's efficiency ranged from 1.11 x 10^{-5} to 9.9 x 10^{-5} for all three of the supplied cultures (data not shown); and therefore, we expect ours had similar efficiency (~5 x 10^{-5}).

3.4.1 Replica Plating/Complementation

There was growth under the MM+met+glu for all three strains which served as a positive control. See Figure 3-4 for plate images. While the *S. cerevisiae* colonies had access to another carbon source the mutant *met* gene did not inhibit growth. Growth was also observed for the pBG1805 + *MET16* colonies for MM-met+gal (inducer) which suggests that complementation occurred for S. *cerevisiae* + *met16* with the pBG8501 + *Cerevisiae MET16* plasmid. Furthermore, no growth was seen for pYES2.1 + *LacZ* or pYES2.1 + *MET16* transformants on the MM-met+gal media suggesting that no complementation occurred for either the *lacZ* plasmid or the *S. pombe* plasmids, respectively. The lack of growth for all three transformants on the MM-met+glu (repressor) media further confirmed the results as a negative control. Summary of growth observations and predictions are provided in Table 3-4.

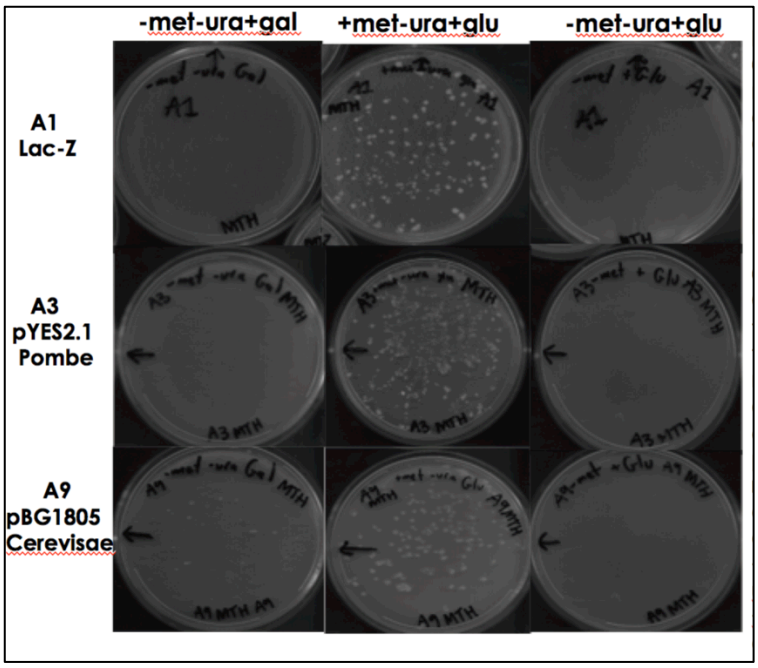

Figure 3-4. Selective plating results of indicated *S. cerevisiae* strains on media with either with or without methione and under plasmid induction or repression (Galactose or Glucose). The plates were incubated for 48h at 30° C.

Media	MM-met+gal	MM+met+glu	MM-met+glu
pYES2.1:*LacZ*	-/-	+++/+++	-/-
pBG1805.1:*MET16*	?/-	+++/+++	-/-
pYES2.1:*MET16*	+/+	+++/+++	-/-

Table 3-4. Expected and observed (exp/obs) growth on selective media of the transformants. The plates were incubated for 48h at 30° C.

3.5 SDS-PAGE Gel

The SDS-Page gel was loaded according to Table 3-5 with 15 µL of sample. Figure 3-5 is an image of the gel post staining with simply blue. Although the resolution of the gel was not fine, banding was obvious indicating the presence of protein in the samples. The absence of a large, differentially expressed band(s) between the samples may be indicative of the weak, galactose promoter being used. The Lane A bands are not defined enough to confidently place the molecular weight markers, and therefore the gel is merely qualitative.

Lane	Sample
A	Molecular Weight Standard
B	S. *pombe*(pYES2.1:*LacZ*) + glu
C	S. *pombe*(pYES2.1:*LacZ*) + gal
D	S. *pombe*(pYES2.1: *MET16*) + glu
E	S. *pombe*(pYES2.1: *MET16*) + gal
F	S. *cerevisiae*(pBG1805.1:*MET16*) + glu
G	S. *cerevisiae*(pBG1805.1:*MET16*) + gal

Table 3-5. Lane and Sample summary for SDS-PAGE gel.

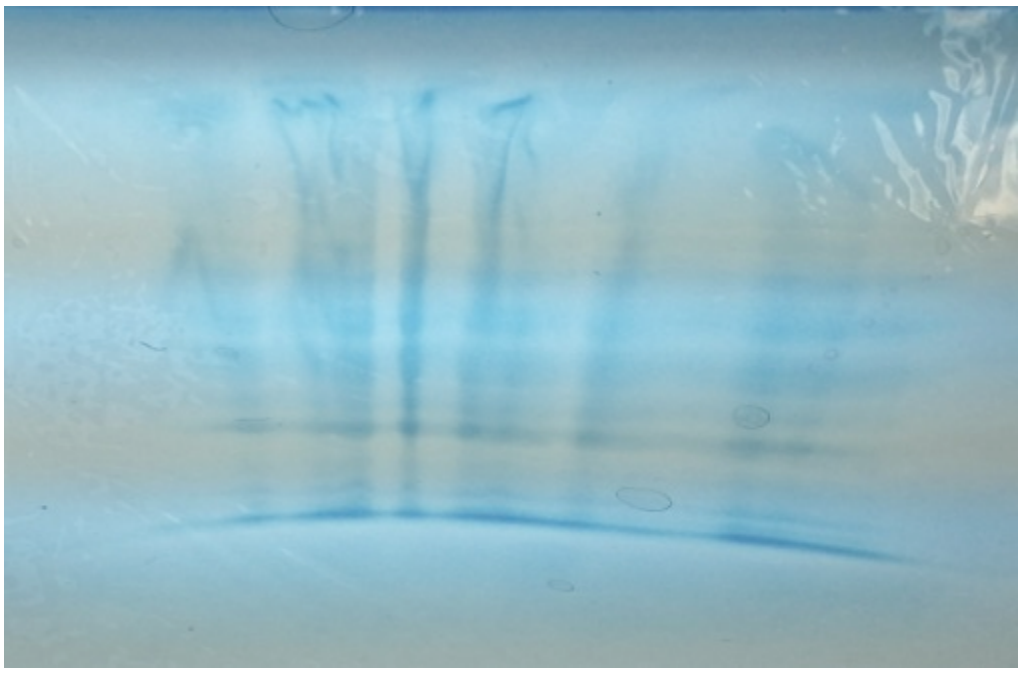

Figure 3 SDS-PAGE gel with Standard in Lane A and samples in Lanes B-G. It was run at 200 V for 40 minutes and stained with Simply-Blue for 24 hours.

Since *S. cerevisiae's* preferred carbon source is glucose rather than galactose, a high growth rate was expected for the glucose-incubated cultures.

3.6 Western Blot

Before the liquid cultures were lysed with NaOH, the optical densities of 1:10 dilutions were taken on 100 µL aliquots. The results of the NanoDrop spectrometer are recorded in Table 3-6. All cultures displayed similar optical density values suggesting a minimal effect between a media rich in glucose verses a media rich in galactose as the primary carbon source—even though glucose is the preferred carbon source for both S. *pombe* and S. *cerevisiae*.

Sample (plasmid:gene + inducer)	OD600
S. *pombe*(pYES2.1:*LacZ*) + glu	0.083
S. *pombe*(pYES2.1:*LacZ*) + gal	0.062
S. *pombe*(pYES2.1: *MET16*) + glu	0.096
S. *pombe*(pYES2.1: *MET16)* + gal	0.062
S. *cerevisiae*(pBG1805.1:*MET16*) + glu	0.075
S. *cerevisiae*(pBG1805.1:*MET16*) + gal	0.045

Table 3-6. NanoDrop results of the six cultures after 24 hours of induction with either glucose (glu) or galactose (gal).

The Western blot was setup analogous to the first SDS-PAGE gel (above). The lane assignments are in agreement with those in **Table 3-5**. After running the acrylamide gel and transferring the proteins to the PVDF membrane, a primary antibody was added which bound to the pBG1805.1:*MET16p* in the HA epitope. A secondary antibody was then used to amplify this signal and to bind to the pYES2.1:*MET16* V5 epitope. The membrane was then visualized through horseradish peroxidase activity and is seen in **Figure 3-6**.

An issue with the gel apparatus leading to a steep *frown* in the gel-front; and therefore, only tentative conclusions should be drawn from **Figure 3-6**.

The presence of two dark bands in lanes E and G of the Western blot correspond to a protein of about 25 kD. Since this band is only seen on the S. pombe + pYES2.1:*MET16* and S. cerevisiae + pBG1805.1:*MET16* under galactose-promotion, it supports the prospect that the protein of interest was produced in both transformants. Furthermore, the molecular weight is close to the expected molecular weigh of 30 kD for *MET16p* even if the Western's integrity was compromised by the poor electrophoresis run.

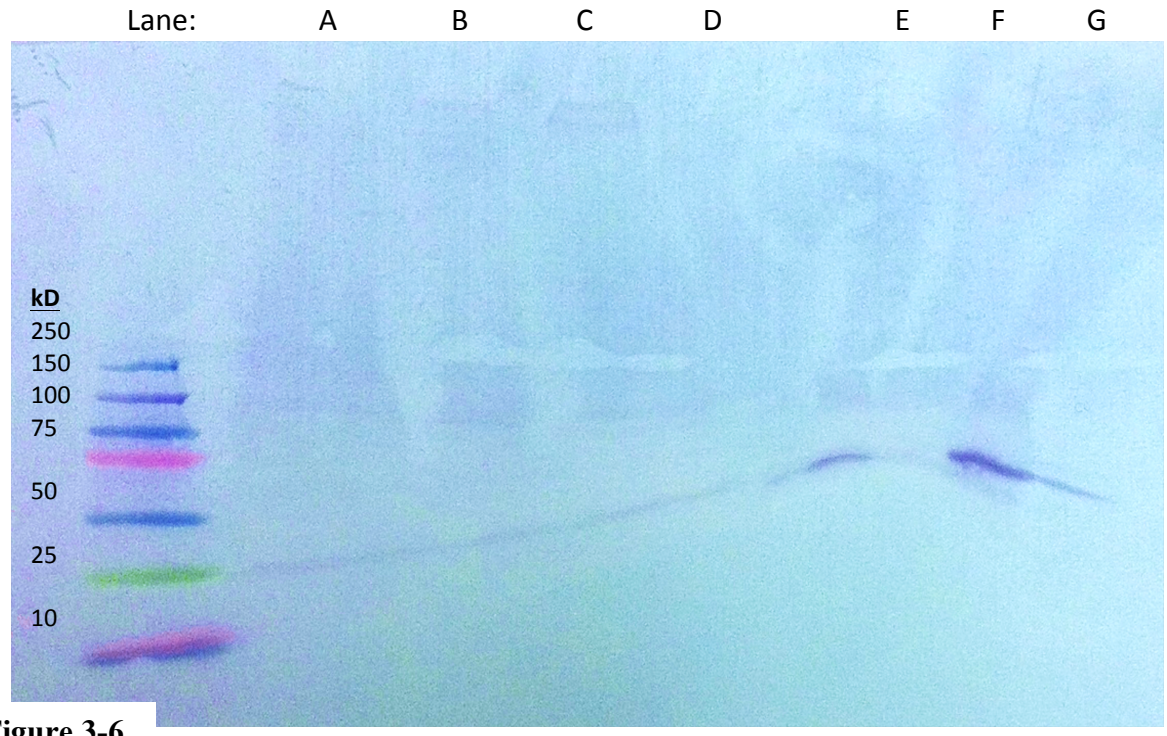

Figure 3-6. Nitrocellulose membrane after exposure to horseradish peroxidase. The protein transfer proceeded at 200 V for 20 minutes.

4. Discussion

The Western blot in conjunction with the restriction digest, which verified the plasmid construction, provided evidence that the *S. pombe MET16* gene was inducibly expressed. Yet even with the protein present in the cell, no growth was observed when cultured on MM-met indicating either a remaining disruption in the methionine biosynthesis pathway or no complementation. Since the *MET16* knockout was the only intended mutation in any of the MET genes, complementation of the *S. pombe MET16* gene in *S. cerevisiae* did not occur.

The *S. pombe MET16p* may not have been functional for a variety of reason, some of which involve the intracellular differences between *S. pombe* and *S. cerevisiae* and their divergent signaling mechanisms, but others may have been

generated from experimental design. The large antibody epitope tags placed on the C-terminus of the coding region may have negatively impacted enzyme function and regulation. The use of the Gal1 promoter, a well-known yet weak promoter, prevented overexpression but also may have limited the expression, and therefore growth, of the transformants. An *in vitro* assay of protein function for the *MET16p* would have gone a long way in ensuring viability of the pYES2.1:*MET16* construct.

Bioinformatic modeling may also provide clues to the lack of complementarity seen in these transformants. Structural modeling of the active sites and regulatory domains of the two flavors of MET16 might have assisted in understanding potential complications. Phylogeny and a multiple sequence alignment would also assist in both determining evolutionary divergence as well as suggesting possible intermediates to attempt complementation from.

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