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The *Aspergillus nidulans* Pbp1 homolog is required for normal sexual development and secondary metabolism

Alexandra A. Soukup[1], **Gregory J. Fischer**[1], **Jerry Luo**[2], and **Nancy P. Keller**[2,3,*]

[1]Department of Genetics, University of Wisconsin-Madison, WI

[2]Department of Medical Microbiology and Immunology, University of Wisconsin-Madison, WI

[3]Department of Bacteriology, University of Wisconsin-Madison, WI

Abstract

P bodies and stress granules are RNA-containing structures governing mRNA degradation and translational arrest, respectively. *Saccharomyces cerevisiae* Pbp1 protein localizes to stress granules and promotes their formation and is involved in proper polyadenylation, suppression of RNA-DNA hybrids, and preventing aberrant rDNA recombination. A genetic screen for *Aspergillus nidulans* mutants aberrant in secondary metabolism identified the Pbp1 homolog, PbpA. Using Dcp1 (mRNA decapping) as a marker for P-body formation and FabM (Pab1, poly-A binding protein) to track stress granule accumulation, we examine the dynamics of RNA granule formation in *A. nidulans* cells lacking *pub1*, *edc3*, and *pbpA*. Although PbpA acts as a functional homolog of yeast PBP1, PbpA had little impact on either P-body or stress granule formation in *A. nidulans* in contrast to Pub1 and Edc3. However, we find that PbpA is critical for sexual development and its loss increases the production of some secondary metabolites including the carcinogen sterigmatocystin.

Keywords

stress response; RNA granule; secondary metabolism; sexual development

1. Introduction

Regulation of mRNA levels are critical processes by which cells may rapidly respond to flux in inter- or extracellular conditions. This regulation is achieved by such means as mRNA localization, mRNA stability, and mRNA translation. Recent evidence indicates that these processes can be co-regulated by segregation of nontranslating mRNA molecules and their associated proteins into distinct structures within the cytoplasm, termed mRNP granules (Buchan, 2014). These mRNP granules include both P-bodies and stress granules, which

*Corresponding author: Phone: (608) 262-9795; fax: (608) 262-8418, npkeller@wisc.edu, Address: 1550 Linden Drive, Madison WI 53706.

perform separate but overlapping functions, and share a number of constituents (Kedersha and Anderson, 2009). Formation of mRNP granules is interdependent, as that P-body formation is required for normal stress granule assembly (Buchan et al., 2008). mRNA trafficking and sequestration in fungi have been associated with such diverse processes as stress response (Keller, 2015; Ren et al., 2016; Wang et al., 2015), polar growth (Becht et al., 2006; Inglis et al., 2013; Tey et al., 2005), nutrient acquisition (Morozov et al., 2010b), and morphological switching (Göhre et al., 2013).

While both P-bodies and stress granules contribute to the regulation of translation, P-bodies are more commonly associated with mRNA decay, and are defined by an enrichment of proteins involved in this process. In yeast, these include decapping enzymes (Dcp1/2), activators of decapping (Edc3 and Lsm1–7), and an exonuclease (Xrn1). P-bodies are normally present in low numbers, but can increase after translation inhibition or exposure to certain environmental stresses (reviewed in (Parker and Sheth, 2007). In *Aspergillus nidulans*, P-bodies components have been linked to response to nitrogen sufficiency by facilitating turnover of the transcription factor AreA (Morozov et al., 2010a; Morozov et al., 2010b). Deletion of the P-body component Edc3 in this system led to severe depletion of P-bodies (as monitored by Dcp1-GFP) and a defect in the rate of both global and targeted RNA turnover, although no significant growth phenotype was noted.

Stress granules are much less common under standard cellular conditions, and normally form under strong inhibition of translation, as induced by translational inhibitors or other environmental stimuli (Kedersha et al., 2005). mRNA arrest in these stress granules can lead to reintroduction of the mRNAs back into the translating pools at a later time. Although the exact composition of stress granules can vary depending on their cause of formation and species, in *S. cerevisiae* protein components can include Poly-A binding proteins (Pab1 and Pub1), elongation factors (eIF4G, eIF4E), proteins involved in RNA destabilization (Ngr1), and Pbp1, a Poly-A binding protein (PAB1) binding protein (Buchan et al., 2008; Hoyle et al., 2007). Removal of both *pub1* and *pbp1* have been shown to limit stress granule formation in *S. cerevisiae* (Buchan et al., 2008), while the essential gene PAB1 is often used as a marker (Buchan et al., 2008; Kozubowski et al., 2011). In *A. oryzae*, various stresses led to accumulation of the PAB1 homolog (Huang et al., 2013). Presumed limitation of stress granule formation by deletion of the *pub1* homolog led to greatly increased sensitivity to stress, suggesting that the integrity of these granules may be critical to respond to adverse conditions (Huang et al., 2013).

Numerous previous studies have shown a link between stress and fungal secondary metabolism (Keller, 2015; Ren et al., 2016; Wang et al., 2015). We have reported on the applicability of using a genetic suppressor screen to look for genes involved in secondary metabolism including RsmA, a bZIP transcription factor linking both secondary metabolism and sexual development with the stress response in *A. nidulans* (Shaaban et al., 2010; Yin et al., 2012). From this same screen, we now present our identification of an *A. nidulans* homolog of the *S. cerevisiae* Pab1 binding protein Pbp1 as also playing an unexpected role in secondary metabolism and sexual development. In both yeast and mammalians cells, Ppb1 and its mammalian homolog Ataxin-2 have been shown to be involved in assembly of stress granules (Buchan et al., 2008), with mutations in *ATXN2*, the gene encoding

Ataxin-2, associated with the neurodegenerative disease spinocerebellar ataxia type 2 (Stevanin et al., 2000). Deletion of *PBP1* in *S. cerevisiae* leads to significant decreases in stress granule formation under glucose deprivation, while P-bodies are unaffected. In line with its role in stress granule assembly, *PBP1* has also been implicated in resistance to various stresses, including caffeine, cycloheximide, hydroxyurea (Kapitzky et al., 2010), and recovery from ethanol stress (Kato et al., 2011), as well as regulation of poly-A tail length (Mangus et al., 1998; Mangus et al., 2004), and cell growth (Kimura and Irie, 2013). Here we show that *AN1325*, here called *pbpA*, is a homolog of yeast *PBP1* and is required for normal sexual sporulation and has a repressive effect on secondary metabolism but has no major role in either P-body or stress granule biology in *A. nidulans*.

2. Materials and Methods

2.1 Sequence analysis

Genomic DNA sequence and translation of *AN1325.4* (*pbpA*) gene was obtained from the Aspergillus Genome Database (www.aspgd.org). *fabM* (*AN4000* (Marhoul and Adams, 1996)), *edc3* and *dcp1* (*AN6893* and *AN7746* (Morozov et al., 2010b), and *pub1* (*AN10164* (Huang et al., 2013)) have been previously described.

Alignment was performed using the NCBI's Conserved Domain Detection tools (Marchler-Bauer et al., 2015) and COBALT (Papadopoulos and Agarwala, 2007) with the following Genbank accession sequences: *Homo sapiens* ATXN2: AAI14547; *Aspergillus nidulans*: XP_658929; *Saccharomyces cerevisiae*: CAA97204; *Schizosaccharomyces pombe*: CAB57927; *Candida albicans*: XP_717736; and *Cryptococcus neoformans*: XP_571007. Percent identity was calculated using Clustal Omega (Sievers et al., 2011). Amino acid alignments are included in Fig. S1.

2.2 Culture conditions, Southern, and northern analysis

All strains (Table S1) were propagated at 37°C on glucose minimum medium (GMM) with appropriate supplements. Fungal DNA was isolated as previously described (Shimizu and Keller, 2001). DNA manipulations, Southern, and northern analysis were conducted according to standard procedures (Sambrook and Russell, 2001).

2.3 Yeast complementation assay

PCR was used to amplify either *ScPBP1* + 1 kb flanks or *pbpA* and *PBP1* flanks. Yeast recombineering was used to insert these fragments into the backbone of pGAD424 (Chien et al., 1991). These plasmids were transformed into BY4741 and BY4741 Δ*PBP1*. For assessment of the petite negative phenotype, overnight cultures were grown in YPD. Five-fold dilutions of these cultures were performed and plated on YPD and YPD + 40 μg/ml ethidium bromide (Hwang et al., 2007).

2.4 Construction of mutant *pbpA* strains

One kb flanks upstream and downstream of *pbpA* were amplified and fused to an *A. parasiticus pyrG – A. nidulans gpdA* fusion cassette from pJMP9 (Soukup et al., 2012b) using double joint PCR (Yu et al., 2004). The resulting knockout construct was transformed

into RJMP1.1 as previously described (Szewczyk et al., 2006). Transformants were examined for targeted replacement of the native locus by PCR and Southern blotting (Fig. S2A,B), and confirmed by northern blot of the appropriate transcript (Fig. S2C). Prototrophic overexpression strains were obtained by crossing the transformants with RTMH207.13 or DVARI (Kim et al., 2002). Desired recombinants were confirmed by PCR screening.

For *pub1* deletion strains, 1 kb of flanking regions were amplified and fused to *A. parasiticus pyrG* from pJW24 (Calvo et al., 2004) using double joint PCR. The resulting knockout construct was transformed into TJMP1.1. Transformants were examined for targeted replacement of the native locus by PCR and Southern blotting. Prototrophic deletion strains were obtained by crossing transformants with RTMH207.13 or DVARI.

For complementation of *pbpA*, the coding region and 1 kb upstream and downstream flanks were amplified using PCR and cloned into the *Not*I sites of pJW53. The resulting knockout constructs was transformed into RJMP1.59 as previously described (Szewczyk et al., 2006). Transformants were examined for integration by PCR and Southern blotting (Fig. S2D). These were crossed to TAAS110.7 to produce prototrophic *pbpA* complements.

2.5 Northern analysis

Fifty milliliter cultures of liquid GMM were inoculated with 1×10^6 spores per ml and incubated at 250 rpm and 37°C for 36 hours under light. Mycelia were harvested, lyophilized overnight, and total RNA was extracted using Isol-RNA Lysis Reagent (5 Prime) according to manufacturer's recommendations. Subsequent northern analysis was done using radiolabeled probes for the corresponding transcript (primers are listed in Table S2).

2.6 Fluorescent strain construction

For initial C-terminal tagging of *pbpA*, *dcp1*, and *fabM*, 1 kb of the 3' end of the gene and 1 kb downstream of the gene of interest were amplified and fused to either pXDRFP4 (RFP) or pFNO3 (GFP)(Yang et al., 2004) via double joint PCR (Yu et al., 2004). The resulting constructs was transformed into RJMP1.1 as previously described (Szewczyk et al., 2006). Transformants were examined for targeted integration at the native locus by PCR and Southern blotting (Fig. S4). To obtain the final florescent strains sequential crosses were performed between TAAS228.16 and PW1 to yield RAAS235.6. This was crossed to TAAS227.6 to yield RAAS236.1. RAAS236.1 (*fabM::gfp::pyrG; dcp1::rfp::pyrG; metG1; biA1*) was crossed to the appropriate transformants. Desired strains were confirmed via PCR. The fluorescent prototroph (RAAS237.2: *fabM::gfp::pyrG; dcp1::rfp::pyrG*) was compared to WT (RJMP103.5) to confirm functionality of tagged proteins (Fig. S5).

For construction of *stcS-GFP*, 1 kb of the C-terminal coding region and 1 kb downstream of *stcS* was amplified and fused to *gfp:pyroA* from plasmid pHL84 (Liu et al., 2009) via double joint PCR (Yu et al., 2004). The resulting constructs was transformed into RJMP1.59 as previously described (Szewczyk et al., 2006). Transformants were examined for targeted integration at the native locus by PCR and Southern blotting (Fig. S4). To obtain the final florescent strains subsequent crosses were performed between TAAS245.27 and PW1 or

TAAS110.1 to yield RAAS246.9 and RAAS247.1, respectively. Desired strains were confirmed via PCR.

2.7 Phenotypic characterization and SM analysis on solid media

Secondary metabolite production was assessed by thin-layer chromatography (TLC). For TLC, 10 μl of 1×10^3 spore/μl was point-inoculated on the center of glucose minimal medium (GMM) and cultured for 72 hr at 37°C. An agar plug of the center of colonies was removed and SMs extracted with ethyl acetate according to the Smedsgaard's method (Smedsgaard, 1997). Extracts (10 μl/sample) were loaded onto silica TLC plates (Whatman, PE SIL, Maidstone, Kent, England) and metabolites were separated in the developing solvent toluene:ethyl acetate:glacial acetic acid (TEA, 8:1:1). Images were taken following exposure to UV radiation at 366 nm.

2.8 Analysis of spore production

Quantification was performed on overlay inoculated cultures set up by pipetting 1×10^6 conidia into GMM or CHAMPS medium with 0.75% molten agar that was subsequently poured over 1.5% solid agar petri dishes of the corresponding medium. Cultures were incubated at 37°C in the dark for 3 or 5 days and agar cores were taken from the plates with a 1 cm cork borer. After homogenization, ascospores and conidia were quantified using a hemacytometer and represented as spores per square millimeter. 3 replicates were performed for each strain and condition.

2.9 Antibacterial bioassay

For the penicillin bioassay, 50 milliliter cultures of liquid GMM were inoculated with 1×10^6 spores per ml and incubated at 250 rpm and 37°C for 72 hours under light. 10 ml culture was used to perform the bioassay against *Micrococcus luteus* as described in Bok and Keller (2004).

2.10 Microscopy

Ten milliliters of ammonium minimal media (AMM) was inoculated with *A. nidulans* spore suspensions at a concentration of 10^4 spores/mL in a 60 mm diameter petri dish containing a sterile 1.5 mm coverslip. Cultures were allowed to grow for 17 hours at room temperature. To induce glucose stress, 17 hours post inoculation media was exchanged for 10 mL AMM lacking glucose and left at room temperature for one hour. For cycloheximide treatment during glucose starvation, cycloheximide (Sigma) was added to a final concentration of 200 μg/mL as previously described (Huang et al., 2013) 30 minutes before media exchange. At 17 hours post inoculation, 10 mL of AMM lacking glucose containing 200 μg/mL cycloheximide was used to exchange media. Cultures were placed at room temperature for one hour before imaging.

Coverslips were removed from the petri dishes, mounted on a glass slide, and sealed using clear fingernail polish. Images were collected using a Nikon Eclipse *Ti* inverted microscope equipped with a 60x Plan Apo VC Oil DIC N2 objective. Images were analyzed using the Nikon NIS Elements Advanced Research software package (v. 4.30.01) and scale bars in all figures represents 20 um. Stress granule and P body counts (n=10) were standardized to

germling length and analyzed for significance using the Student's T Test within the Graphpad Prism statistical package. For P body quantification, a fluorescent threshold value was used to develop a binary image from which P bodies were counted. Specifically, images were loaded into ImageJ and a color threshold specified using the IsoData thresholding method within ImageJ, with the brightness threshold values set to range from 115–255. Binary images were then used to count the number of P bodies (Fig. S6). Error bars represent standard error. *p=<0.05 **=p<0.01 ***=p<0.001 ns= not significant

3. Results

3.1 AN1325 encodes a homolog of *S. cerevisiae* PBP1

We previously described a genetic screen where we identified *A. nidulans* mutants that impacted secondary metabolism as determined by pigmentation of the mutant strain on common growth media (details in (Shaaban et al., 2010). Two proteins characterized from this screen included the previously mentioned RsmA, as well as EsaA, a histone 4 acetyltransferase (Soukup et al., 2012a). A third gene identified by this screen is *AN1325*. BLAST analysis (Altschul et al., 1997) of the predicted product of *AN1325* revealed a 29.3% identity to *S. cerevisiae* Pbp1 (Mangus et al., 1998), 28.3% identity to *Schizosaccharomyces pombe* Ath1 (Wang et al., 2012), 31.3% to *Cryptococcus neoformans* Pbp1 (Park et al., 2016) and 25.5% identity to the mammalian ortholog Ataxin-2 (ATXN2 (Ralser et al., 2005). *AN1325* (*pbpA*) encodes a 1047 amino acid long protein containing the expected ataxin-2 domain. All orthologs contain an N-terminal Lsm associated domain, and the majority (with the exception of *C. neoformans*) contain the above mentioned Ataxin 2 similar domain (Fig. 1A and S1).

3.2 Complementation of *S. cerevisiae* Δpbp1

Previous studies have shown the *S. cerevisiae PBP1* deletant to have a petite negative phenotype (Dunn and Jensen, 2003), which is observed as an inability to survive loss of the mitochondrial genome on rich medium. In order to determine whether PbpA could serve to functionally complement a *S. cerevisiae PBP1* deletion and its petite negative phenotype, we constructed complementation vectors containing either yeast *PBP1* or *pbpA* genomic sequence under the yeast *PBP1* promoter and selectable *URA3* marker. We chose to examine growth of wild type and a *Δpbp1* strain transformed with these vectors on YPD medium with and without 40 μg/ml ethidium bromide (EtBr), which induces mitochondrial loss (Hwang et al., 2007). As expected, *Δpbp1* shows significantly decreased growth in the presence of EtBr, which can be restored by the addition of *PBP1* (Fig. 1B). Introduction *of pbpA* also led to normal growth on this medium, suggesting that *A. nidulans pbpA* can functionally complement *Δpbp1* and prevent mitochondrial loss under these conditions.

3.3 Deletion of pbpA does not suppress ΔfabM lethality

In both *S. cerevisiae* and *A. nidulans, PAB1* and its homolog *fabM* are essential genes (Marhoul and Adams, 1996; Sachs et al., 1987). In yeast, the requirement for *PAB1* is bypassed by deletion of both *PAB1* and *PBP1*. We therefore sought to determine if this was also the case in *A. nidulans. A ΔpbpA* strain was constructed through transformation and homologous recombination of the *pbpA* gene with *the pyrG* gene of *A. parasiticus* and

confirmed via Southern blot (Fig S1A). The construct to *delete fabM* by replacement with the *A. fumigatus riboB* gene was then transformed into both wild type and *ΔpbpA* backgrounds. Multiple transformants were obtained in both cases, with many displaying slow growth and sparse hyphal density (Fig. S3B). Southern blots were performed to confirm the successful integration of the deletion construct (Fig. S3 A). In all transformants, regardless of genetic background, bands indicating either (i) the presence of wild type *fabM* or (ii) wild type *fabM* and the deletion construct were seen, suggesting that all of the transformants which correctly integrated the deletion construct were heterokaryons with respect to *fabM*. Use of the heterokaryon rescue technique (Osmani et al., 2006) confirmed that *fabM* was essential in both backgrounds (data not shown).

3.4 PbpA does not localize to stress granules in *A. nidulans* under glucose deprivation

Pbp1, Ath1, and their mammalian ortholog, Ataxin-2, accumulate in stress granules upon glucose deprivation or arsenite stress, respectively (Buchan et al., 2008; Nonhoff et al., 2007; Wang et al., 2012). In order to determine if this was also the case in *A. nidulans*, we constructed fluorescent tagged versions of stress granule and P-body components based on previous studies (Buchan et al., 2008; Huang et al., 2013; Kozubowski et al., 2011; Morozov et al., 2010b), and confirmed normal vegetative growth of these strains (Fig. S5). Dcp1-RFP was used as a marker for P-bodies, and FabM-GFP as a marker for stress granules. RFP and GFP tagged PbpA were generated, and crossed into the appropriate backgrounds for comparison (see methods).

Under normal growth conditions, Dcp1-RFP localizes to multiple cytoplasmic foci (Fig 2A), which we will refer to as P-bodies. Addition of stress through glucose increases the number of these foci approximately twofold. In contrast, FabM-GFP is distributed throughout the cytoplasm, with very few foci of high intensity under normal growth conditions. Upon shifting to media lacking glucose, both the number and intensity of these stress granule foci increase, as seen in yeast (Buchan et al., 2008). PbpA-GFP is present throughout the cytoplasm under low stress conditions (Fig. 2B), with no clear foci present. Glucose deprivation does not change the intensity or distribution of the GFP signal. No clear colocalization is seen with PbpA and either Dcp1 or FabM. Therefore, under the conditions tested, PbpA does not accumulate in either P-bodies or stress granules upon stress.

3.5 Deletion of *pub1*, but not *pbpA*, affects stress granule formation in *A. nidulans*

Previous studies in *S. cerevisiae* have shown that P-bodies help promote formation of stress granules, although mutations that affect stress granule assembly generally do not alter P-body formation (Buchan et al., 2008), although the requirements for assembly components of both types of granules vary amonst different species (Wang et al., 2012). In order to determine the requirements for mRNP particle formation in *A. nidulans*, we examined the effects of deleting *edc3, pub1*, and *pbpA* on stress granule and P-body formation. Edc3 (enhancer of decapping) has been shown to deplete P-body assembly under certain conditions in *S. cerevisiae* (Decker et al., 2007) and *A. nidulans* (Morozov et al., 2010b), but not *S. pombe* (Wang et al., 2013). Pub1 (Poly-uridylate binding protein) binds polyadenylated RNA and colocalizes with FabM in numerous fungi, although its requirement for stress granule and P-body formation varies (Anderson et al., 1993; Buchan

et al., 2008; Wang et al., 2012; Zhang et al., 2014). Loss of Pbp1 homologs has been shown to either deplete P-body formation (Buchan et al., 2008), or have no effect (Wang et al., 2013).

Shown in Fig. 2, under normal growth conditions the presence of P-bodies is apparent as indicated by Dcp1-RFP. FabM-GFP localized throughout the cell, with very few foci of high intensity. Upon stress by glucose deprivation, the number of P-bodies approximately doubled, and several FabM containing stress granules formed per germling (Fig. 3). Deletion of *pub1* almost completely ablated formation of stress granules, and caused a decrease in P-body formation under both normal and stressed conditions. A decrease in P-body formation was also seen in *Δedc3* strains under stress conditions. Deletion of *pbpA* did not cause a significant decrease in either P-bodies or stress granules under the conditions tested, although a minor increase in P-bodies was seen under normal conditions. Addition of cycloheximide, which traps mRNAs in polysomes due to a translational block, abolishes formation of both P bodies and stress granules (Fig. S7; (Kedersha et al., 2005). These data suggest *A. nidulans* mRNP granule formation presents both unique and shared characteristics with *S. pombe* and *S. cerevisiae*.

3.6 Deletion of *pbpA* greatly reduces meiotic spore production and alters secondary metabolism

Yeast PBP1 has been shown to contribute to resistance to a number of stresses in *S. cerevisiae*, including caffeine, cycloheximide, hydroxyurea (Kapitzky et al., 2010), and recovery from ethanol stress (Kato et al., 2011). We examined the effects of both *pbpA* deletion and overexpression on resistance to a number of different stresses, including sodium chloride, menadione, cycloheximide, sorbitol, and hydroxyurea. Under all conditions tested, no significant differences were seen in relative growth rates among strains (Fig. S8). However, we noted that under normal growth conditions, *ΔpbpA* showed increased pigmentation (the phenotype from the original suppressor screen) as well as altered sporulation patterns (Fig. 4A), while *Δpub1* and *Δedc3* did not have any noticeable macroscopic phenotypes (data not shown).

We thus compared asexual spore production, sexual spore production and secondary metabolite output of the *ΔpbpA* mutant to wild type, its complement, and an overexpression strain. Whereas the null strain showed no difference in the production of the asexual spores (conidia), it was greatly impacted in sexual spore production, only producing ~3% as many ascospores as wild type (Fig. 4B,C).

Sexual development is frequently linked with changes in secondary metabolism (Calvo and Cary, 2015), and thus we next investigated any potential changes in secondary metabolism of the *pbpA* mutant. TLC analysis of mutant strain extracts revealed that *pbpA* loss led to increased intensity of numerous bands, including that corresponding to sterigmatocystin (ST) and ST precursors (Fig. 4D). Examination of StcS-GFP (a p450-monooxygenase required for converting the precursor versicolorin A to ST, (Keller et al., 1995) showed an increased intensity of StcS foci in the *ΔpbpA* background, supporting increased production of sterigmatocystin (Fig. 4D). In order to determine if antibacterial secondary metabolites were impacted *by pbpA* loss, an assay was performed using culture supernatants against

Micrococcus luteus (Fig. 4F). An increased zone of inhibition seen in the samples suggests that there are increased levels of penicillin, or another antibacterial, present in the *ΔpbpA* supernatant.

4. Discussion

Genetic screens remain powerful tools for identifying genes involved in the feature of interest. The results can be unexpected and provide valuable insight to cellular processes involved in particular genetic traits. In genetic screens for genes affecting secondary metabolism, we have characterized a bZIP transcription factor RsmA, as well as two proteins, EsaA and MvlA, involved in chromatin remodeling in the genetic model *Aspergillus nidulans* (Bok et al., 2013; Shaaban et al., 2010; Soukup et al., 2012a). Along with altering secondary metabolite synthesis, both RsmA and EsaA also affected sexual spore production, highlighting the close linkage of these two features. Isolated from the same screen as RsmA and EsaA, we now demonstrate that PbpA also is involved in the regulation of both sexual and chemical development in *A. nidulans*.

Aspergillus nidulans PbpA shares a number of similarities with its *S. cerevisiae* homolog PBP1, as suggested by its similar domain structure and ability to functionally complement the petite negative phenotype of *the pbp1* mutant (Hwang et al., 2007) and restore growth on medium containing ethidium bromide (Fig. 1). Although *ΔpbpA* was unable to rescue the lethality of a *fabM* deletion in *A. nidulans*, unlike rescue of *Δpab1* by a *Δpbp1* deletion in *S. cerevisiae* (Mangus et al., 1998), heterokaryon exhibited phenotypic alterations including a decrease in pigmentation (Fig. S3). As pigment production and stress response have previously been linked in a number of fungi (Atanasova et al., 2013; Avalos and Carmen Limón, 2015; Rangel et al., 2006; Yang et al., 2013), this may suggest that $fabM^{+/-}$ $\Delta pbpA^{+/-}$ heterokaryons, while unable to completely suppress lethality, undergo a decreased stress response relative to $\Delta fabM^{+/-}$ heterokaryons.

Consistent with what has been seen in other species, P-bodies containing Dcp1-RFP are present at low levels under normal growth conditions, and are induced upon stress (Jung and Kim, 2011; Kozubowski et al., 2011), as has previously been shown in *A. nidulans* (Morozov et al., 2010b). FabM, the Pab1 homolog, is primarily distributed throughout the cytoplasm under normal growth conditions, but also localizes to distinct stress granules upon stress. Unlike the primary stress granule localized near the hyphal tip seen in *A. oryzae* (Huang et al., 2013), multiple stress granules form and are distributed throughout the cell. Also, in contrast to other fungi, clear co-localization of PbpA and FabM in stress granules is not apparent (Buchan et al., 2008; Jung and Kim, 2011; Park et al., 2016; Wang et al., 2012). PbpA is also present in areas lacking FabM signal, suggesting that PbpA may have functions independent of FabM such as its significant impact on sexual development.

In both *S. cerevesiae* and *C. albicans*, deletion of *edc3* decreases P-body formation (Buchan et al., 2008; Jung and Kim, 2011). Our studies show a similar phenotype where the *edc3* deletion strains show a decreased number of P-bodies present after glucose deprivation (Fig. 3), and a subsequent decrease in stress granules. However, unlike these two other studies, P-body numbers did not change under normal growth conditions in the *edc3* mutant. This is

similar to what has been seen in *S. pombe*, where Edc3 was not essential for P-body assembly (Wang et al., 2013). In *A. nidulans*, an increased number of P-bodies is seen in ammonium rather that nitrate containing medium (Morozov et al., 2010b). These results reflect different requirements for P-body assembly that are both species and condition dependent.

In *A. oryzae*, deletion of *pub1* led to growth defects under stress conditions (Huang et al., 2013), potentially due to a disruption in stress granule formation. Here we show not only a drastic decrease of stress granules in the *Δpub1* mutant under stress conditions, but also a decrease in P-body formation under both normal and stress conditions. This decrease in stress granules assembly in the *pub1* mutant has also been documented in *S. cerevisiae*, although not *S. pombe*. Thus, *A. nidulans* displays a novel combination of requirements for both P-body and stress granule assembly.

Although studies in yeast suggest that P-body formation is independent of stress granule formation, but not vice versa (Buchan et al., 2008), work on a human cell line suggest that transcripts are first sent to stress granules for sorting, followed by trafficking to P-bodies (Kedersha et al., 2005). The body of work investigating the interrelatedness of these structures is ongoing (Stoecklin and Kedersha, 2013). In our study, mutants affecting either P-body or stress granule formation resulted in defects in the other category. This may reflect response to the particular stress of glucose deprivation, or may be generalized to other forms of stress as well. Under these conditions, deletion of *pbpA* did not affect formation of either category of mRNP granule, although a minor increase in P-body number was seen under normal growth conditions. This may reflect the particular nature of the response to glucose deprivation, where PbpA is not required. Previous studies in mammalian cells show altered responses of both P-bodies and stress granules to different types of stress (reviewed in (Kedersha and Anderson, 2009), and we propose that PbpA aids in coordinating development in response to unique stresses. As granules can have different activities based on their protein components (Shah et al., 2016), and germlings likely differ in their constitution from more differentiated cell, cellular responses to stress could well vary during the life of the cell.

Deletion of *pbpA* led to numerous developmental responses in *A. nidulans*, including a severe reduction in meiotic spores and increased production of several characterized and uncharacterized secondary metabolites. Analysis of the ST pathway by both TLC analysis and imaging of an enzyme required for synthesis showed increased production of this common metabolite in the deletion strain. Increases in additional metabolites or sterigmatocystin intermediates are also seen in the mutant, suggesting an increase in multiple secondary metabolite pathways. An antimicrobial assay also showed increased production of antibacterial activity as seen by inhibition of the bacterium *Micrococcus luteus*. This enhanced production was partially ablated by the addition of penicillinase suggesting that penicillin synthesis was increased in the mutant (data not shown).

Pbp1 and its homologs have previously been associated with sexual reproduction through regulation of mating type switching in *S. cerevisiae* (Tadauchi et al., 2004), through post-transcriptional regulation of *HO* endonuclease mRNA. More recently, *Cryptococcus*

neoformans pbp1 mutants have been shown to be impaired in pheromone production, as well as impaired in sexual development (Park et al., 2016). We confirm this requirement for effective sexual reproduction in *A. nidulans*. Additionally, this study confirms the integration of sexual development and secondary metabolite production as seen in numerous other mutants isolated from screens identifying regulators of secondary metabolism (Bok et al., 2013; Ramamoorthy et al., 2012; Shaaban et al., 2010; Soukup et al., 2012a).

Secondary metabolites have already been shown to be associated with sexual development as pigments of fruiting body and/or sexual spores (Brown et al., 2012; Schindler and Nowrousian, 2014; Studt et al., 2012; Szewczyk et al., 2008). This coordination of metabolite production and development may be mediated through overlapping transcription factors, as has been seen to be the case in asexual spore development (Lim et al., 2014). Alternatively, trafficking of signaling molecules, precursors, or intermediates in other pathways may all be impacted coordinately. A previous study has highlighted the impact of misregulation of primary metabolism and its impact on sexual development (Palmer et al., 2010), demonstrating pleiotropic effects of metabolism on development.

Although Pbp1 and its homologs have been implicated in a plethora of processes, including regulation of poly-A tail length (Mangus et al., 1998; Mangus et al., 2004), resistance to stress (Kapitzky et al., 2010; Kato et al., 2011; Park et al., 2016), survival without mitochondrial DNA (Dunn and Jensen, 2003), sexual development (Park et al., 2016), and elongation or termination of translation (Tadauchi et al., 2004), little is known about the precise mechanism through which Pbp1 functions. Further studies have shown deletions of *pbp1* to suppress defects and or/lethality associated with mutations of other key genes (Kimura and Irie, 2013; Mangus et al., 1998; Woolstencroft et al., 2006). This is particularly interesting in light of the lack of severe phenotypes seen in *pbp1* mutants (Kimura and Irie, 2013). Given Pbp1's role in regulating HO translation (Tadauchi et al., 2004), and pheromone production (Park et al., 2016), it is a formal possibility that PbpA is required for proper expression and/or translation of mating type (*MAT*) genes. A previous study demonstrated that deletion of the *MAT* loci ablated ascospore production, while allowing formation of cleistothecia, and not affecting vegetative growth or asexual sporulation, phenotypes also seen in the *ΔpbpA* strain. Future experiments investigating mRNA levels and localization of key sexual regulators will shed further light upon the mechanism of action coordinating these critical developmental events.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

AnpbpA encodes a functional homolog of *Saccharomyces cerevisiae* PBP_1

Requirements for mRNA granule synthesis vary among species

AnpbpA is required for normal sexual reproduction and secondary metabolism

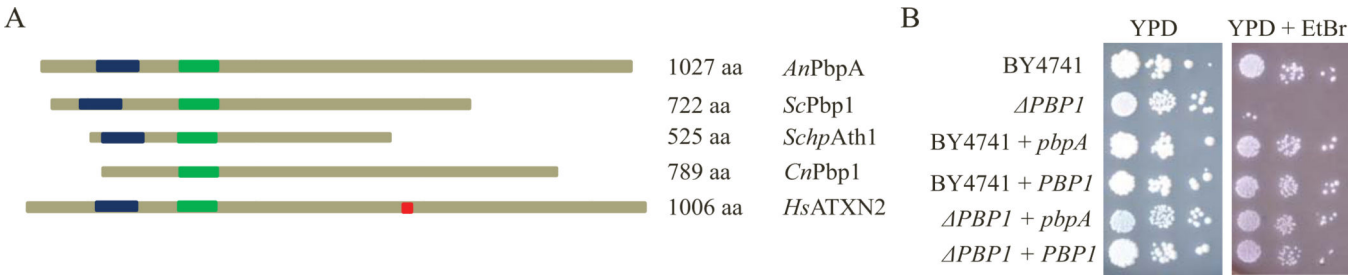

Fig. 1.

A. Diagram of PbpA orthologs and their protein domain structure. Blue: Ataxin 2 like domain. Green: Lsm Associated Domain. Red: PAB1 binding domain. *An: A. nidulans. Sc: S. cerevisiae. Schp: S. pombe. Cn: C. neoformans. Hs: H. sapiens*. B. PbpA functionally complements a *S. cerevisiae PBP1* deletion in a petite negative assay. Colonies represent 10 fold dilutions. BY4741, the *ΔPBP1* parental strain, grows normally on both YPD and YPD + 40 μg/ml ethidium bromide, which induces loss of the mitochondrial genome. Lower densities of the *ΔPBP1* strain are unable to survive this loss. Addition of either *PBP1* or *pbpA* does not affect growth or survival in a wild type background, but restores normal growth to the *ΔPBP1* parent.

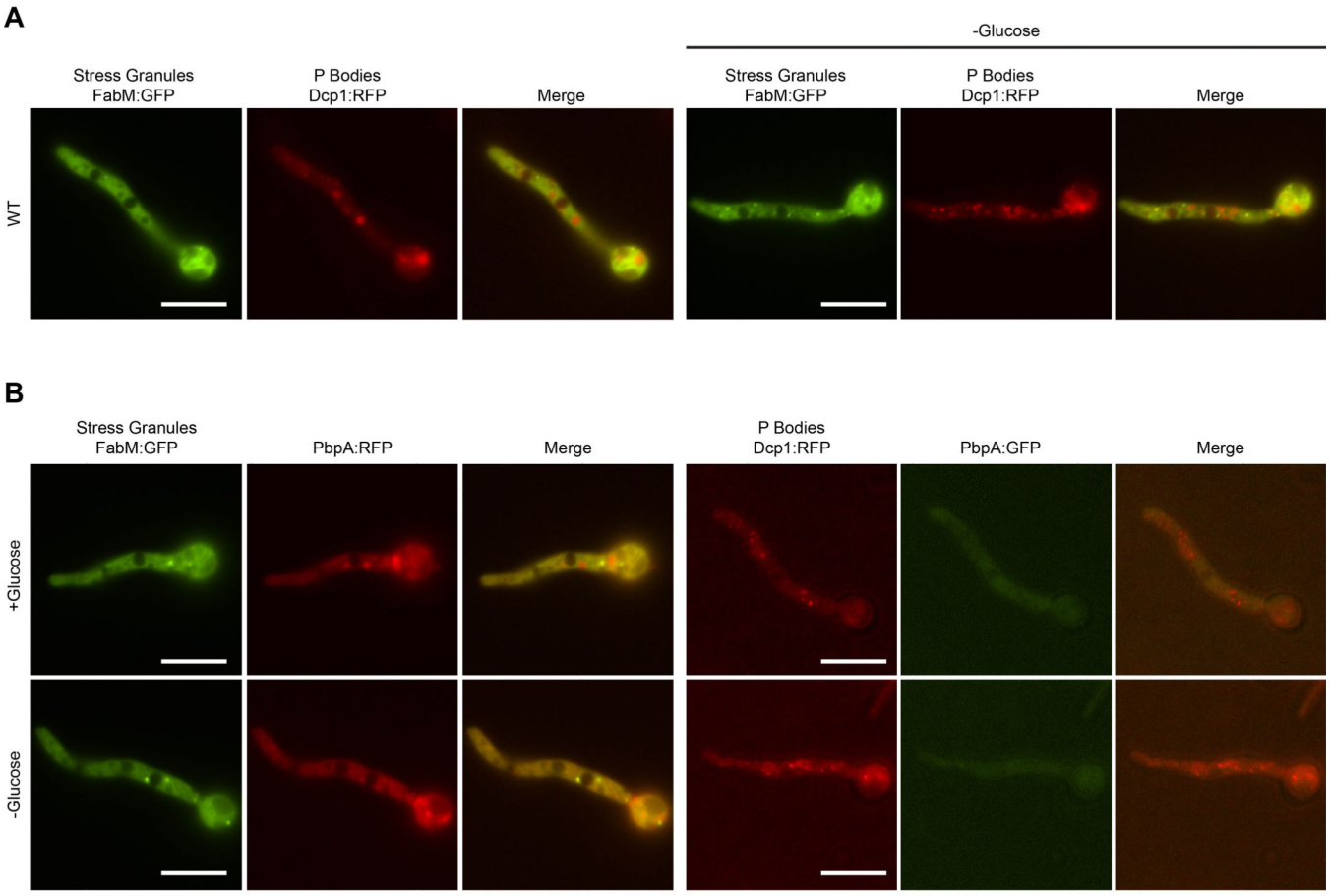

Fig. 2.

A. Visualization of P-body and stress granule assembly. Dcp1-RFP serves as a marker for P-bodies, while FabM-GFP depicts stress granule assembly. B. Strains were imaged under normal growth conditions and after 1 hour glucose deprivation. Fluorescently labelled PbpA is present throughout the cytoplasm and does not coalesce into P-bodies or stress granules under the conditions tested. Scale bar represents 20 μm.

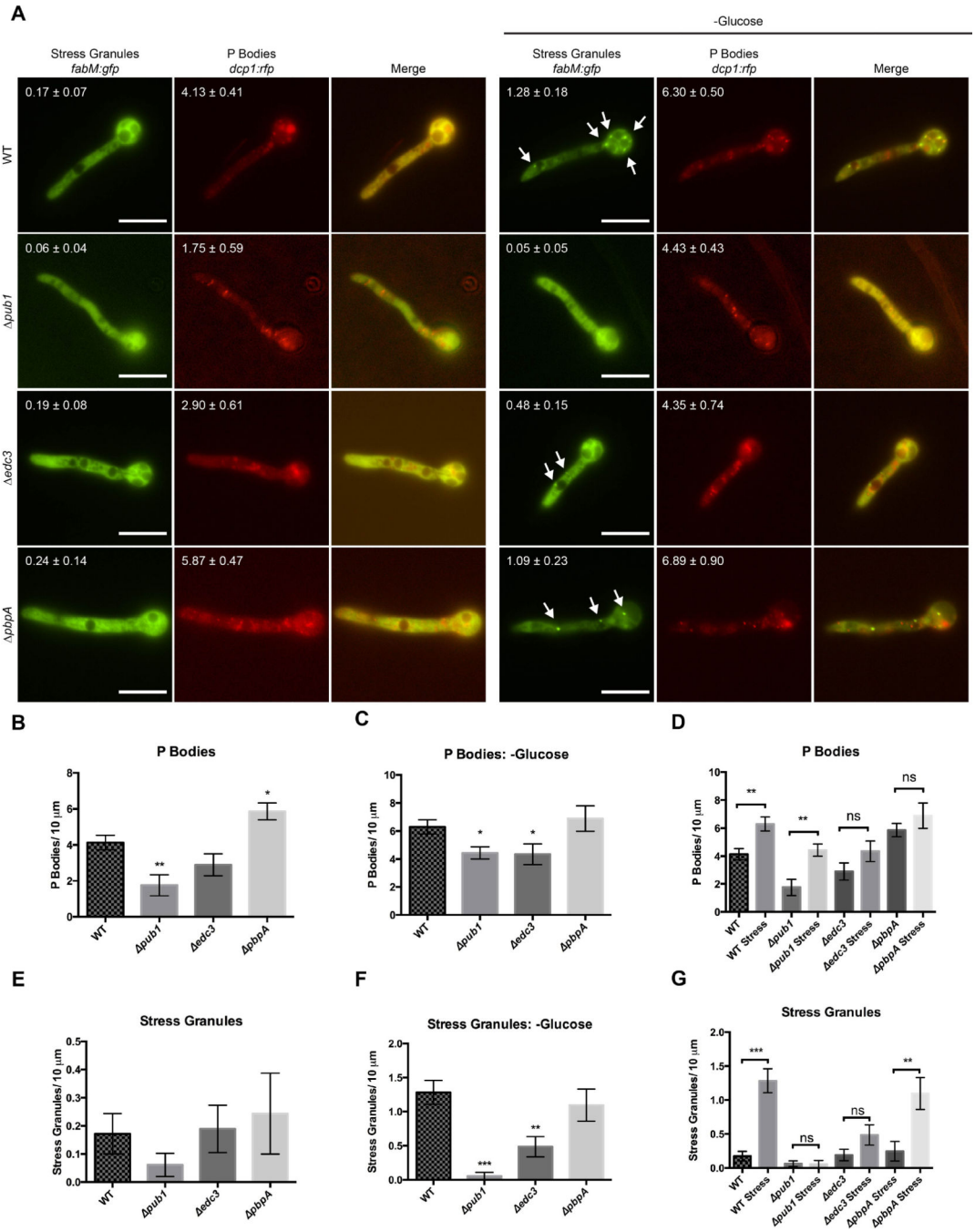

Fig. 3.

Effect of various mutants on P-body and stress granule assembly. A. Strains were imaged under normal growth conditions and after 1 hour of glucose deprivation. Values represent average number of foci over a 10 micrometer distance of hyphae +/- standard error (n=10). B. Quantification of Dcp1-RFP foci. Images were subjected to color thresholding (see Material and Methods) to consistently quantify P body foci (Supplemental Figure S4). Edc3 cells contain approximately half the number of P-bodies. C. Quantification of Dcp1-RFP foci after 1 hour without glucose. D. Comparison of number of P-bodies in strains with and

without stress. E. Quantification of FabM-GFP foci. F. Quantification of FabM-GFP foci after 1 hour without glucose. G. Comparison of number of stress granules in strains with and without stress. Error bars represent standard error and scale bar represents 20 μm. *p=<0.05 **=p<0.01 ***=p<0.001 ns= not significant. Arrows have been used to identify stress granules which were only identified under glucose starvation.

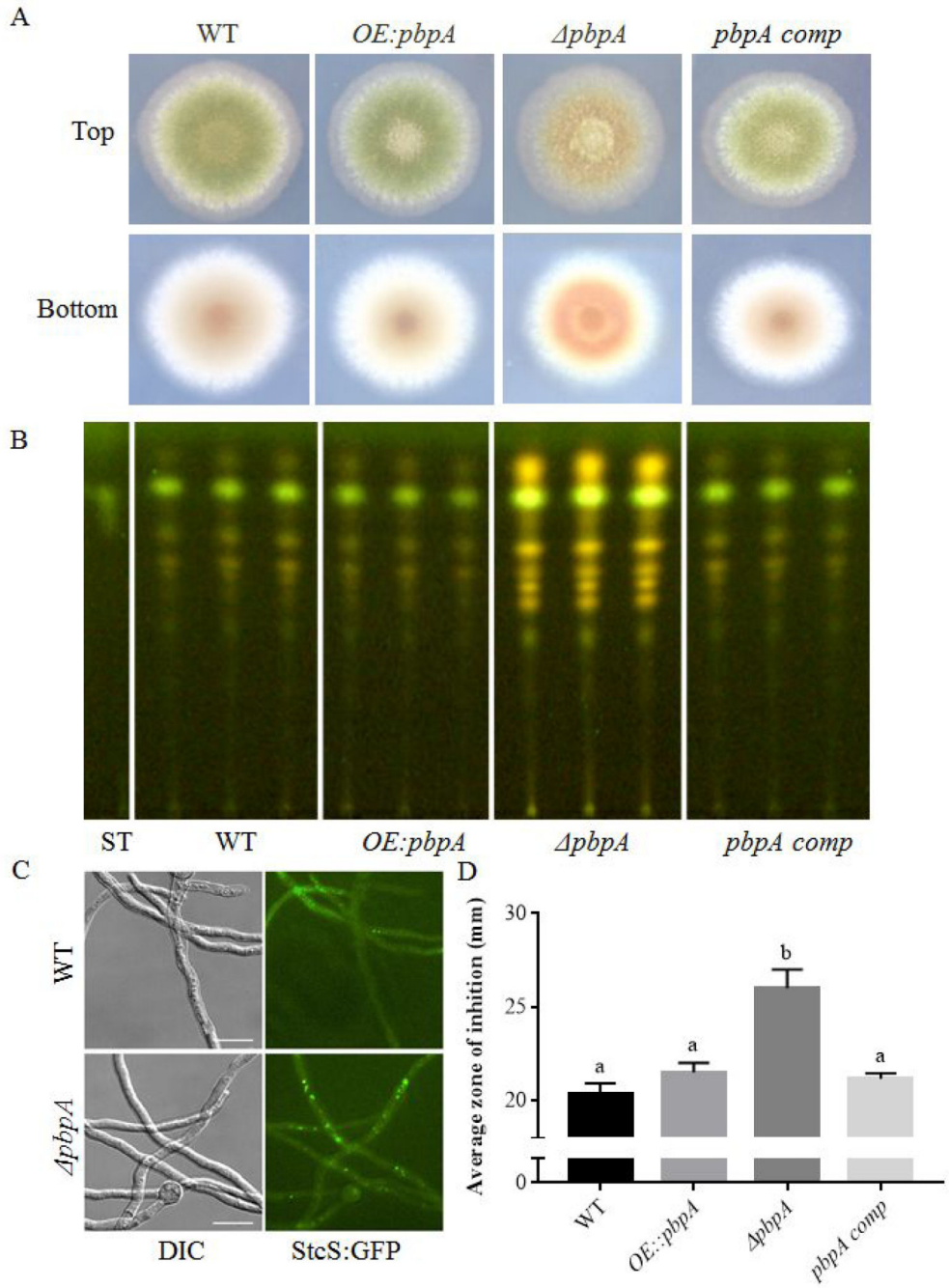

Fig. 4.

Loss *of pbpA* results in alterations in sexual development and secondary metabolism. A. Phenotypes of strains grown for 3 days at 37°C on GMM. *ΔpbpA* displays increased pigmentation and altered sporulation patterns. B. *pbpA* deletion mutants fail to produce ascospores. Phenotypically, no significant differences are seen among among strain under conditions promoting sexual (CHAMPS medium, 5 days growth in dark) or asexual (GMM, 3 days growth in light). C. When spore number is quantified under each condition, Δ*pbpA* strains display a significant decrease in ascospore number, producing ~3% WT numbers. D.

TLC analysis of organic extracts. Increased intensity of numerous bands, including ST, is seen in the *pbpA* mutant. E. Localization of StcS-GFP in WT and *pbpA* mutant backgrounds. F. Penicillin assay quantifying the zone of inhibition produced from culture supernatants against *Micrococcus luteus*.