

# A Novel Automethylation Reaction in the *Aspergillus nidulans* LaeA Protein Generates S-Methylmethionine\*

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**Background:** LaeA, a putative methyltransferase in *Aspergillus nidulans*, is a master regulator of secondary metabolism.

**Results:** LaeA automethylates at a methionine residue near the AdoMet-binding site. This modification is not required for *in vivo* function.

**Conclusion:** Automethylation of LaeA reveals a novel protein methionine methyltransferase activity.

**Significance:** Elucidating the substrate(s) of LaeA will provide insights into the physiological function of LaeA in modulating gene expression.

The filamentous fungi in the genus *Aspergillus* are opportunistic plant and animal pathogens that can adapt to their environment by producing various secondary metabolites, including lovastatin, penicillin, and aflatoxin. The synthesis of these small molecules is dependent on gene clusters that are globally regulated by the LaeA protein. Null mutants of LaeA in all pathogenic fungi examined to date show decreased virulence coupled with reduced secondary metabolism. Although the amino acid sequence of LaeA contains the motifs characteristic of seven- $\beta$ -strand methyltransferases, a methyl-accepting substrate of LaeA has not been identified. In this work we did not find a methyl-accepting substrate in *Aspergillus nidulans* with various assays, including *in vivo* S-adenosyl-[methyl-<sup>3</sup>H]methionine labeling, targeted *in vitro* methylation experiments using putative protein substrates, or *in vitro* methylation assays using whole cell extracts grown under different conditions. However, in each experiment LaeA was shown to self-methylate. Amino acid hydrolysis of radioactively labeled LaeA followed by cation exchange and reverse phase chromatography identified methionine as the modified residue. Point mutations show that the major site of modification of LaeA is on methionine 207. However, *in vivo* complementation showed that methionine 207 is not required for the biological function of LaeA. LaeA is the first protein to exhibit automethylation at a methionine residue. These findings not only indicate LaeA may perform novel chemistry with S-adenosylmethionine but also provide new insights into the physiological function of LaeA.

The Ascomycete genus *Aspergillus* represents a diverse collection of filamentous fungi. In human health, *Aspergillus* is an opportunistic pathogen and is one of the leading causes of infection-related deaths in immunocompromised patients (1, 2). Furthermore, *Aspergillus* is of significant biological, commercial, and medical importance because of the small molecule secondary metabolites it synthesizes. These include beneficial molecules such as the  $\beta$ -lactam antibiotic penicillin (3) and anticholesterol hypolipidemic agent lovastatin (4), as well as the carcinogenic aflatoxins (5) and the cytotoxic gliotoxin (6).

The fungal genes required for the biosynthesis of a secondary metabolite are organized into gene clusters composed of hallmark enzymes (5, 7), including polyketide synthases, nonribosomal peptide synthetases, terpene cyclases, dehydrogenases, esterases, and methyltransferases (8). A cluster-specific transcription factor is often required to recognize palindromic sequences and promote the transcription of an entire biosynthetic gene cluster. For example, the sterigmatocystin and aflatoxin gene clusters are controlled by the binuclear zinc finger protein AflR (9). Although gene clusters are of considerable biological and economic interest, much remains unknown about their structure, function, and regulation. In fact, of the 55 clusters predicted by the software SMURF in *Aspergillus flavus*, only six clusters have actually been characterized (10, 11).

Recent work has focused on elucidating the regulation of a secondary metabolism. In a mutagenesis screen to identify *Aspergillus nidulans* mutants with reduced sterigmatocystin, a protein was found to regulate AflR and designated LaeA<sup>4</sup> (loss of *aflR* expression  $\Delta$ ) (12). LaeA is a nuclear protein that when absent results in a decrease in secondary metabolism and virulence (13, 14). Further studies have revealed that LaeA is a master regulator, controlling the transcription of not only the genes in 13 of the 22 secondary metabolite clusters in *Aspergillus fumigatus* but also 10% of the genome itself (15).

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<sup>4</sup> The abbreviations used are: LaeA, Loss of AflR Expression A; AdoMet, S-adenosylmethionine; [<sup>3</sup>H]AdoMet, S-adenosyl-L-[methyl-<sup>3</sup>H]methionine; OPA, o-phthalaldehyde; MBP, maltose-binding protein; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol.

**TABLE 1**  
Plasmids used in this study

Plasmid	Description	Source
pKLD116	His <sub>6</sub> -MBP	Ref. 27
pGSG01	His <sub>6</sub> -MBP-LaeA	This study
pGSG02	His <sub>6</sub> -MBP-LaeA <sup>Δ1-86</sup>	This study
pGSG03	His <sub>6</sub> -MBP-LaeA <sup>Δ1-30</sup>	This study
pGSG04	His <sub>6</sub> -MBP-LaeA <sup>Δ1-42</sup>	This study
pJMP141	His <sub>6</sub> -MBP-LaeA <sup>Δ1-86, M101A, M195A</sup>	This study
pJMP143	His <sub>6</sub> -MBP-LaeA <sup>Δ1-86, M207A</sup>	This study
pJMP144	His <sub>6</sub> -MBP-LaeA <sup>Δ1-86, M213A</sup>	This study
pJMP145	His <sub>6</sub> -MBP-LaeA <sup>Δ1-86, M268A</sup>	This study
pJW53	¾ <i>pyroA</i> in <i>SspI</i> site of pBluescript II SK-	Ref. 73
pFNO3	GA <sub>5</sub> -GFP- <i>A. fumigatus pyrG</i>	Ref. 28
pJW45-4	<i>laeA(p)::laeA::laeA(t)</i> in ½ <i>trpC</i>	Ref. 12
pJMP148	<i>laeA(p)::laeA::GFP::laeA(t)</i> in ½ <i>trpC</i>	This study
pJMP150	<i>laeA(p)::laeA<sup>M207A</sup>::GFP::laeA(t)</i> in ½ <i>trpC</i>	This study
pJMP151	<i>laeA(p)::laeA<sup>Δ1-86</sup>::GFP::laeA(t)</i> in ½ <i>trpC</i>	This study
pJMP152	<i>laeA(p)::laeA::laeA(t)</i> in ¾ <i>pyroA</i>	This study
pJMP154	<i>laeA(p)::laeA::GFP::laeA(t)</i> in ¾ <i>pyroA</i>	This study
pJMP156	<i>laeA(p)::laeA<sup>M207A</sup>::GFP::laeA(t)</i> in ¾ <i>pyroA</i>	This study
pJMP157	<i>laeA(p)::laeA<sup>Δ1-86</sup>::GFP::laeA(t)</i> in ¾ <i>pyroA</i>	This study
pGST-velB	GST-VelB	Ref. 31
pJMP134	His <sub>6</sub> -veA-GST-Stag	Ref. 31

LaeA is a highly conserved protein in filamentous fungi and a conserved virulence factor in all pathogenic fungi examined to date (13, 14, 16–19). An analysis of the 374 amino acids of LaeA shows the presence of *S*-adenosylmethionine (AdoMet)-binding motifs common to seven-β-strand methyltransferases (12, 20). LaeA is known to be a nuclear protein that interacts with the VeA and VelB proteins in a heterotrimeric complex, called the velvet complex, which functions to coordinate development and secondary metabolism in response to light (21). Because LaeA has similarities to methyltransferases and is localized to the nucleus, it has been suggested that it regulates transcription by protein lysine or protein arginine methyltransferase functions (12, 22, 23). Although no direct biochemical studies on the methyl-accepting specificity of LaeA have been done, this protein has been linked to changes in chromatin structure (24).

Because of the current enigmatic nature of the molecular function of LaeA and the velvet complex, determining the methylated substrates of LaeA would be important to elucidate the global regulatory mechanism of secondary metabolism. Here, we show that the putative methyltransferase LaeA does not methylate histones or the velvet complex proteins *in vitro*. Additionally, both *in vitro* and *in vivo* biochemical analyses were unsuccessful in identifying targets of LaeA. However, in these experiments LaeA was consistently found to be automethylated at a methionine residue. In this work, we have characterized this automethylation reaction.

## EXPERIMENTAL PROCEDURES

**Construction of Plasmids**—Standard techniques were used for nucleic acid manipulations according to Sambrook and Russell (25). All plasmids used in this study are listed in Table 1 and primers used in this study are listed in Table 2. Unless noted otherwise, all plasmids were constructed using a PCR-mediated cloning approach (26). Point mutations and truncations were made using the QuikChange mutagenesis kit (Stratagene). For heterologous expression of LaeA in *Escherichia coli*, *laeA* cDNA was introduced into the 6× histidine and maltose-binding protein (MBP) expression vector pKLD116 (27). This

resulted in the construction of the full-length MPB-LaeA expression vector (pGSG01). N-terminal truncations of LaeA were made, including MBP-LaeA<sup>Δ1-86</sup> (pGSG02), MBP-LaeA<sup>Δ1-30</sup> (pGSG04), and MBP-LaeA<sup>Δ1-42</sup> (pGSG06). Subsequent methionine to alanine point mutations were constructed in MBP-LaeA<sup>Δ1-86</sup> (pGSG02) creating pJMP141, pJMP143, pJMP144, and pJMP145.

**In vivo** complementation vectors were based off of the vector pJW45-4 (12) harboring the native *laeA* gene from *A. nidulans*. First, GA<sub>5</sub>-GFP (glycine-alanine linker fused to GFP) was amplified from pFNO3 (28) and inserted in-frame at the C terminus of *laeA* yielding pJMP148. Truncation of the N-terminal 86 amino acids of *laeA* and the methionine 207 to alanine point mutant was constructed in pJMP148 to make pJMP151 and pJMP150, respectively. Finally, HindIII fragments from pJW45-4, pJMP148, pJMP150, and pJMP151 were subsequently cloned into the HindIII site of pJW53 (29) yielding the *pyroA* targeting complementation vectors of pJMP152, pJMP154, pJMP156, and pJMP157.

**Expression and Purification of Proteins**—MBP-LaeA proteins were expressed in Rosetta DE3 (Novagen) and subsequently purified as described previously (27) with the modifications indicated below. Briefly, proteins were first purified using nickel-nitrilotriacetic acid resin (Qiagen) followed by a second round of purification on amylose resin (New England Biolabs). Removal of the N-terminal 6× histidine and MBP tag was achieved by proteolytic cleavage using recombinant tobacco etch virus (30) and subsequent removal of the recombinant tobacco etch virus and MBP tags with nickel-nitrilotriacetic acid resin. Purification of VeA-GST and GST-VelB was done according to Palmer *et al.* (31).

**Creation of Mutant Fungal Strains and Fungal Physiology Experiments**—Fungal strains used in this study are listed in Table 3. All strains were maintained on glucose minimal medium (GMM) (32) at 37 °C and when appropriate were supplemented with 0.5 mM pyridoxine-HCl, 0.01 μg/ml riboflavin, 5 mM uridine, and 5 mM uracil. *In vivo* complementation of  $\Delta laeA$  strains was achieved by targeted integration of *laeA* complementation vectors at the *pyroA* locus in RJMP155.16 (*riboB2*, *pyroA4*, and  $\Delta laeA$ ). Transformation was conducted as described previously in Szweczyk *et al.* (33). Transformants were screened and verified through a combination of diagnostic PCR and Southern blots (data not shown). Strains harboring a single integration of the complementation vector at the *pyroA* locus were subsequently crossed to RJW130.1 (*pyroA4* and  $\Delta laeA$ ) and/or RJMP260.28 (*pyroA4*, *hhoA-mCherry*, and  $\Delta laeA$ ) to generate prototrophic strains. Analysis of sterigmatocystin production was done from point-inoculated cultures grown for 4 days in dark and light conditions, and sterigmatocystin was extracted and visualized via thin layer chromatography (31). Analysis of asexual and sexual sporulation was conducted as described previously (34) with the minor modification of using Champe's media for sexual developmental induction (35). Visualizing the cellular localization of LaeA-GFP fusion proteins was conducted as described previously in Palmer *et al.* (31) using a Zeiss AxioVision A10 microscope.

## LaeA Automethylates at a Methionine Residue

**TABLE 2**

Oligonucleotides used in this study

Primers	Sequence (5' to 3')	Purpose
LaeA_N_116	GCAGGTGCATGTGGACGTCCCAGGCTAGCATGTTGAGATGGGCCCGGTGGGAACCTCG	pGSG01
LaeA_C_116	CGCGCCGCAAGCTTGCCCTGCAGGCCATGTTATCTTAATGGTTTCCCTAGCCTGG	pGSG01
AnLAEAdel86F	CCACTagtGAGAATCTCTACTTCCCAAGGCTCGGATCATGAAGAGAACGGACCCCTACC	pGSG02
AnLaeAdel30F	GAGAACTCTACTTCCCAAGGCTTAGCGACAGAGGCCGGTCAAGGC	pGSG04
AnLaeAdel30R	GCCTTGACCGCCTCTGTGCGTAAGGCCTTGGAAGTAGAGATTCTC	pGSG04
AnLaeAdel42F	GAGAATCTCTACTTCCCAAGGCTTAGCGACATCCAGTCCATCACTGAACG	pGSG06
AnLaeAdel42R	CGTTCAGTGATGGACTGGATGTCGTAAGGCCTTGGAAGTAGAGATTCTC	pGSG06
JP AnLaeA M101A For	ACCATGGCTTTCGAGGGGAgcGTATTTTCTCCGTGCGATG	LaeA <sup>M101A</sup>
JP AnLaeA M101A Rev	CATCGCACGGAAGAAAATACgCtCCCTTGCAGAAAGCCATGGT	LaeA <sup>M101A</sup>
JP AnLaeA M195A For	GACTTCGAAGCGCCATGGGCCgCgGGGGGAGGATTCCTGGGATC	LaeA <sup>M195A</sup>
JP AnLaeA M195A Rev	GATCCAGGAATCCTCCCCgCgGGCCATGGCGCTTCGAAGTC	LaeA <sup>M195A</sup>
JP AnLaeA M207A For	GGGATCTAATCCATCTGCAGgCgGGTTGCGGTAGTGTCTATGG	LaeA <sup>M207A</sup>
JP AnLaeA M207A Rev	CCATGACACTACCGCAACCCgCgCTGCAGATGGATTAGATCCC	LaeA <sup>M207A</sup>
JP AnLaeA M213A For	CAGATGGGTTGCGGTAGTGTGcGGGCTGGCCAAACTTGTATC	LaeA <sup>M213A</sup>
JP AnLaeA M213A Rev	GATACAAGTTTGGCCAGCCcGcGACACTACCGCAACCCATCTG	LaeA <sup>M213A</sup>
JP AnLaeA M268A For	CTTAAACAGCGCAGCAGAGACCgCgCGGCCAATCGCCATAGCTC	LaeA <sup>M268A</sup>
JP AnLaeA M268A Rev	GAGCTATGGGCGATTGGCCGcGgGGTCTCTGCTGCGCTGTTAAG	LaeA <sup>M268A</sup>

**TABLE 3**

Strains of *A. nidulans* used in this study

Strain	Genotype	Source
RJMP103.5	Wild type	Ref. 74
RJW41A	<i>metG1</i> , $\Delta$ <i>laeA::metG</i>	Ref. 21
RJW152.1	$\Delta$ <i>laeA::metG</i> , <i>pyroA::gpdA(p)::laeA</i>	J. W. Bok, unpublished data
RJMP155.16	<i>riboB2</i> , <i>pyroA4</i> , <i>metG1</i> , $\Delta$ <i>laeA::metG</i>	This study
TJMP154.5	<i>riboB2</i> , <i>metG1</i> , $\Delta$ <i>laeA::metG</i> , <i>pyroA::laeA</i>	This study
TJMP156.1	<i>riboB2</i> , <i>metG1</i> , $\Delta$ <i>laeA::metG</i> , <i>pyroA::laeA-GFP</i>	This study
TJMP158.7	<i>riboB2</i> , <i>metG1</i> , $\Delta$ <i>laeA::metG</i> , <i>pyroA::laeA<sup>M207A</sup>-GFP</i>	This study
TJMP159.3	<i>riboB2</i> , <i>metG1</i> , $\Delta$ <i>laeA::metG</i> , <i>pyroA::laeA<sup>\Delta1-86</sup>-GFP</i>	This study
RJW130.1	<i>metG1</i> , <i>pyroA4</i> , $\Delta$ <i>laeA::metG</i>	J. W. Bok, unpublished data
RJMP254.1	$\Delta$ <i>laeA::metG</i> , <i>pyroA::laeA</i>	This study
RJMP256.3	$\Delta$ <i>laeA::metG</i> , <i>pyroA::laeA-GFP</i>	This study
RJMP258.1	$\Delta$ <i>laeA::metG</i> , <i>pyroA::laeA<sup>M207A</sup>-GFP</i>	This study
RJMP259.1	$\Delta$ <i>laeA::metG</i> , <i>pyroA::laeA<sup>\Delta1-86</sup>-GFP</i>	This study
TJMP125.1	<i>riboB2</i> , <i>pyroA4</i> , <i>pyrG89</i> , <i>hhoA::mCherry::riboB</i> , $\Delta$ <i>nkuaA</i> , <i>veA1</i>	J. M. Palmer, unpublished data
RJMP260.28	<i>pyroA4</i> , $\Delta$ <i>laeA::metG</i> , <i>hhoA::mCherry::riboB</i>	This study
RJMP260.1	$\Delta$ <i>laeA::metG</i> , <i>hhoA::mCherry::riboB</i>	This study
RJMP261.26	$\Delta$ <i>laeA::metG</i> , <i>hhoA::mCherry::riboB</i> , <i>pyroA::laeA-GFP</i>	This study
RJMP263.3	$\Delta$ <i>laeA::metG</i> , <i>hhoA::mCherry::riboB</i> , <i>pyroA::laeA<sup>M207A</sup>-GFP</i>	This study
RJMP264.5	$\Delta$ <i>laeA::metG</i> , <i>hhoA::mCherry::riboB</i> , <i>pyroA::laeA<sup>\Delta1-86</sup>-GFP</i>	This study

**Protein Extraction from *Aspergillus***—Extracts were prepared from cells in vegetative, asexual, and sexual growth stages. Vegetative growth was achieved by incubating *A. nidulans* in liquid shaking cultures (GMM, 230 rpm, 37 °C) for 24 h with an inoculum density of  $1 \times 10^6$  spores/ml. Asexual development was induced by growing mycelia as described above for vegetative growth, filtering the mycelia through sterile Miracloth (Calbiochem), and then transferring the mycelia to solid media (GMM) under constant room light for an additional 24 h. Sexual developmental was induced as described above for asexual induction except the solid medium plates were wrapped in parafilm and aluminum foil and incubated for a further 48 h in the dark. Mycelia were collected from all three growth stages and immediately frozen in liquid nitrogen. Whole cell extracts were prepared by grinding mycelia to a fine powder under liquid nitrogen, followed by vortexing in extraction buffer (100 mM Tris-HCl, pH 7.5, 250 mM NaCl, 10% glycerol, 0.1% Nonidet P-40, 1 mM EDTA, and one Roche Complete Protease inhibitor tablet without EDTA per 50 ml), and removing cellular debris by centrifugation at  $20,000 \times g$  for 20 min.

Crude nuclear extracts were prepared essentially as described previously (36). Briefly, 250 ml of liquid GMM medium was inoculated with  $1 \times 10^6$  spores/ml and incubated at 37 °C for 48 h at 250 rpm. Mycelia were collected by filtering through sterile Miracloth, frozen in liquid nitrogen, and ground

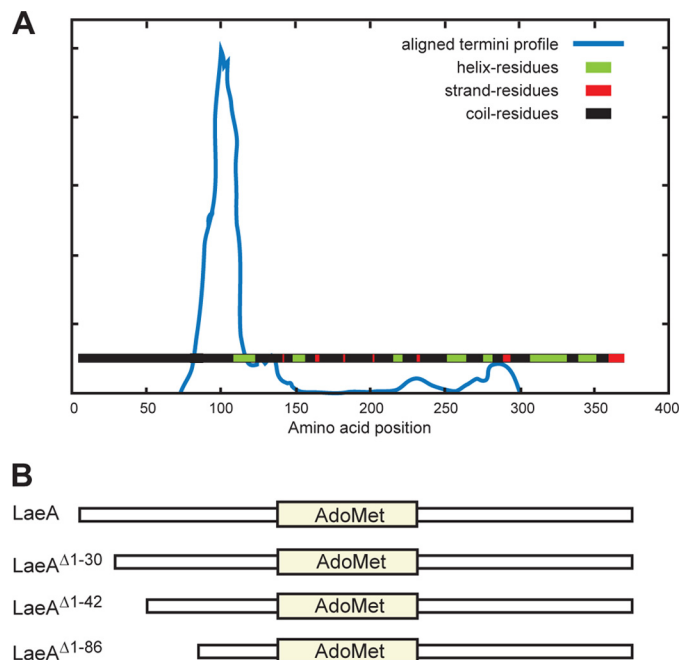
to a fine powder using a mortar and pestle. Approximately 5 g of ground mycelia was vortexed with cold nuclear isolation buffer (10 mM Tris-HCl, 1 M sorbitol, 10 mM EDTA, 0.15 mM spermine, 0.5 mM spermidine, 2.5 mM PMSF, pH 7.5). Cell debris was removed by centrifugation at  $1000 \times g$  for 10 min at 4 °C, and the supernatant was filtered through Miracloth. The supernatant was centrifuged at  $10,000 \times g$  for 15 min at 4 °C, and the pellet was subsequently washed in cold resuspension buffer (10 mM Tris-HCl, 1 M sorbitol, 1 mM EDTA, 0.15 mM spermine, 0.5 mM spermidine, 2.5 mM PMSF, pH 7.5). Crude nuclei were then pelleted by centrifugation at  $10,000 \times g$  for 15 min at 4 °C. Finally, crude nuclei were resuspended in cold ST buffer (10 mM Tris-HCl, 1 M sorbitol, protease inhibitor mixture for fungi (Sigma P8215)). All protein extracts were quantified using the protein assay according to manufacturer's recommendations (Bio-Rad).

**In Vitro Methylation Reactions and Fluorography**—Methylation reactions were performed in a final volume of 100  $\mu$ l and included [<sup>3</sup>H]AdoMet diluted from a 7.1  $\mu$ M stock solution (*S*-adenosyl-L-[methyl-<sup>3</sup>H]methionine; PerkinElmer Life Sciences, 75–85 Ci/mmol, 0.55 mCi/ml in 10 mM H<sub>2</sub>SO<sub>4</sub>/EtOH (9:1, v/v)). Recombinant proteins, whole cell extracts, nuclear extracts, or human recombinant histones H2A, H2B, H3.3, and H4 (New England Biolabs, 1 mg/ml stock) were added and incubated in a buffer of 50 mM Tris-HCl, pH 7.5, 300 mM NaCl, 5%

glycerol, and 1 mM DTT. Reactions were allowed to proceed at 37 °C for 18–20 h. The reactions were terminated by adding SDS-PAGE loading buffer (final concentration of 250 mM Tris-HCl, pH 6.8, 10% (w/v) SDS, 50% (v/v) glycerol, 5% (v/v)  $\beta$ -mercaptoethanol, and 0.05% (w/v) bromophenol blue) and heated at 100 °C for 3 min. The entire sample was loaded onto 10% Tris glycine or 10% BisTris-HCl polyacrylamide slab gels as described in Frankel and Clarke (37) and Kinoshita and Kinoshita-Kikuta (38), respectively. The samples were separated by electrophoresis for ~5 h at a constant current of 60–80 mA. The gel was stained with Coomassie (0.1% (w/v) Brilliant Blue R-250, 10% (v/v) glacial acetic acid, and 50% (v/v) methanol) for 1 h, destained in 10% (v/v) acetic acid and 5% (v/v) methanol, and imaged using an Alpha Imager 2200 (Alpha Innotech Corp.) to visualize protein bands. To identify methylated species, fluorography was performed by soaking the gels for 1 h in EN<sup>3</sup>HANCE (PerkinElmer Life Sciences) followed by a 30-min water treatment. The gel was subsequently dried for 3 h at 80 °C before being exposed to film (Denville Scientific, 5 × 7-inch HyBlot CL). Gels were exposed to film for 3 days to 9 months at –80 °C.

**High Resolution Cation Exchange Chromatography Amino Acid Analysis**—Radioactive bands of interest from dried gels were cut out and rehydrated in 1 ml of water for 1 h. The gel slice was diced into smaller pieces and placed into several 6 × 50-mm glass tubes with 100  $\mu$ l of 6 M HCl. Acid hydrolysis was carried out in a Waters Pico-Tag Vapor-Phase apparatus in a vacuum vial with an additional 500  $\mu$ l of 6 M HCl for 18 h at 110 °C. After the hydrolysates were vacuum dried, they were resuspended in 100  $\mu$ l of water, and centrifuged at 10,600 × *g* for 10 min to remove any debris. The sample was added to 360  $\mu$ l of citrate buffer (0.2 M Na<sup>+</sup>, pH 2.2) and 1  $\mu$ mol each of the following standards: DL-methionine *S*-methylsulfonium chloride (*S*-methylmethionine, Sigma M0501); *L*- $\omega$ -N<sup>G</sup>-monomethylarginine acetate salt (Sigma M7033); *L*- $\omega$ -N<sup>G</sup>,N<sup>G</sup>-asymmetric dimethylarginine dihydrochloride salt (Sigma D4268); *L*-1-( $\pi$ )-methylhistidine (Sigma M9005);  $\epsilon$ -monomethyl-lysine hydrochloride salt (Sigma M6004);  $\epsilon$ -*N*-dimethyl-lysine (Sigma 19773), and  $\epsilon$ -*N*-trimethyl-lysine hydrochloride salt (Sigma M1660).

All 500  $\mu$ l of sample was loaded onto a 0.9 × 8-cm column of PA-35 sulfonated polystyrene beads (6–12  $\mu$ m, Benson Polymeric Inc., Sparks, NV). The column was equilibrated and eluted with citrate buffer (0.35 M Na<sup>+</sup>, pH 5.27) at 55 °C and a flow rate of 1 ml/min, and 1-ml fractions were collected. The elution times of amino acids were determined by ninhydrin assay. Into a 96-well plate, 30  $\mu$ l of cation exchange fraction was added to 200  $\mu$ l of water and 100  $\mu$ l of ninhydrin reagent (20 mg/ml ninhydrin and 3 mg/ml hydrindantin in 75% (v/v) dimethyl sulfoxide and 25% (v/v) 4 M lithium acetate, pH 4.2) and heated at 100 °C for 15 min. Absorbance was measured at 570 nm using a SpectraMax M5 microplate reader with a path length of 1 cm. The radioactivity in each column fraction was quantified using a Beckman LS6500 scintillation counter and expressed as an average of three 5-min counting cycles after mixing 200  $\mu$ l of sample, 400  $\mu$ l of water, and 5 ml of scintillation reagent (Safety Solve, Research Products International, 111177).



**FIGURE 1. DomPred predicts a domain boundary of LaeA near amino acid 100.** *A*, full-length LaeA amino acid sequence was inputted to the DomPred domain prediction server. The output “aligned termini profile” predicts that LaeA has two domains split near amino acid residue 100. *B*, because the first motif of the AdoMet-binding site starts at amino acid 141, we chose to make N-terminal truncation mutants by removing 30, 42, or 86 residues.

***o*-Phthalaldehyde (OPA) Amino Acid Analysis**—Amino acid standards or cation exchange column fractions were fluorescently labeled with OPA, separated with an Agilent Eclipse AAA HPLC column (5  $\mu$ m, 4.6-mm inner diameter, and 150-mm length) at 1.5 ml/min, and detected by a Gilson Model 121 fluorometer (excitation and emission filters of 305–395 and 430–470 nm, respectively, and a setting of 0.1 relative fluorescence units) as described previously (39). During the HPLC analysis, 0.5-ml column fractions were collected and mixed with 5 ml of scintillation reagent to quantify radioactivity. The radioactivity was expressed as an average of three 10-min counting cycles.

**Multiple Sequence Alignment and Protein Modeling of LaeA**—A multiple-sequence alignment of LaeA proteins from different fungi was generated using Clustal Omega (40) and Jalview (41). Three-dimensional structure prediction of LaeA based on the primary sequence was performed using Phyre<sup>2</sup> (42). The prediction of ligands and their respective binding sites in LaeA was calculated by 3DLigandSite (43).

***In Vivo* Methylation Assay**—Mycelia were grown overnight in 50 ml of GMM. Approximately 1 g of mycelia was collected by vacuum filtration, transferred to 2 ml of fresh GMM with 125  $\mu$ l of [<sup>3</sup>H]AdoMet (7.1  $\mu$ M, 75–85 Ci/mmol) to a final concentration of 0.4  $\mu$ M, and incubated at 37 °C in a gyratory shaker at 230 rpm. After 6 h, the samples were centrifuged, and the supernatant was removed. The tissue was lyophilized overnight and ground to a powder. The sample was resuspended in extraction buffer (100 mM Tris-HCl, pH 7.5, 250 mM NaCl, 10% glycerol, 0.1% Nonidet P-40, 1 mM EDTA, and 1 Roche Complete Protease inhibitor without EDTA per 50 ml). The mycelia were vortexed, stored on ice for 10 min, and centrifuged at 20,000 × *g* for 20 min. The supernatant (“Soluble Fraction”) was

## LaeA Automethylates at a Methionine Residue

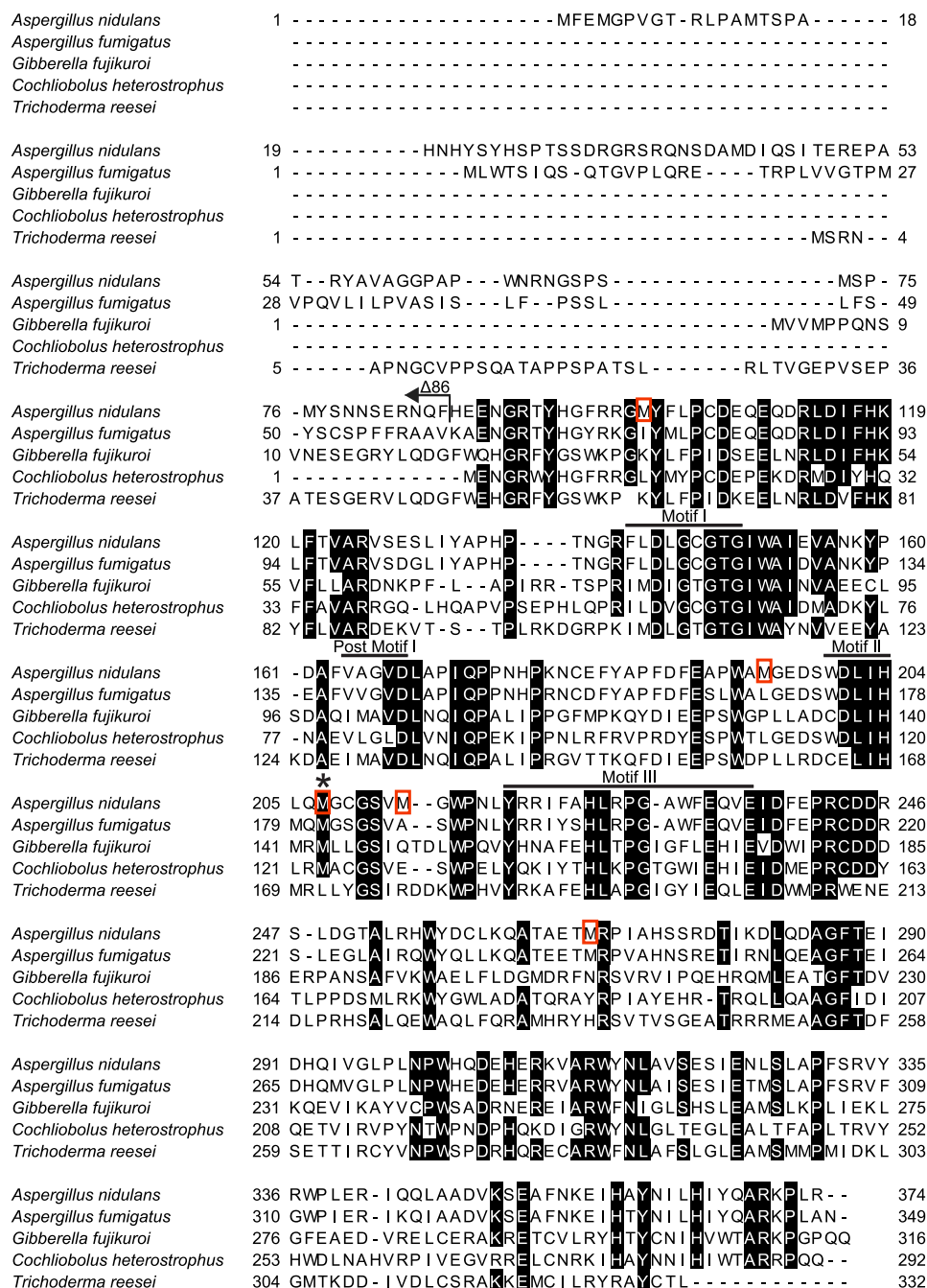


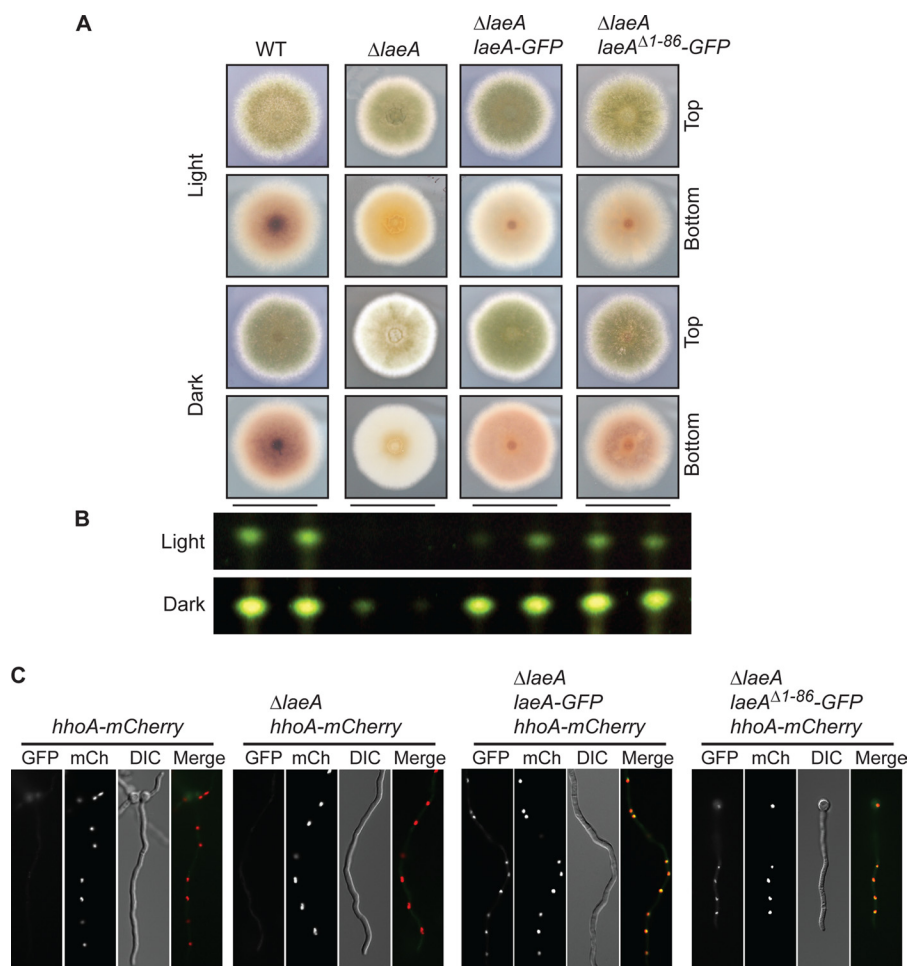
FIGURE 2. Sequence alignment of LaeA from *A. nidulans* with homologs in other fungal species. Amino acids with 80% or greater conservation are indicated in black. The arrow indicates the amino acid sequence missing at the N terminus of the LaeA<sup>1-86</sup> protein. Bars are used to denote the amino acids comprising the four common methyltransferase motifs of seven- $\beta$ -strand methyltransferases. Red boxes highlight methionine residues mutated in this study. Amino acid sequences are taken from UniProt accession numbers C8VQG9, Q6TFC7, E0WDF6, G4XKY9, and I3RU94.

removed. Both the soluble fraction and the pellet (“Insoluble Fraction”) were added to SDS-PAGE loading buffer, heated at 100 °C for 3 min, and loaded on a gel as described above.

## RESULTS

**Soluble N-terminal Truncation of LaeA Demonstrates Activity in Vivo**—To elucidate the function of LaeA, we heterologously expressed LaeA in *E. coli*. Our initial expression trial of LaeA with a 6 $\times$  histidine N-terminal tag proved to be insoluble. To improve solubility, we constructed an N-terminal maltose-

binding protein fusion protein (MBP-LaeA), which proved to be soluble and eluted as a monomer in gel filtration analysis. Although the MBP-LaeA full-length protein was soluble, upon cleavage of the N-terminal MBP tag, LaeA rapidly became insoluble. To facilitate the *in vitro* functional characterization of LaeA, partial proteolysis experiments were conducted on MBP-LaeA (data not shown). These data indicated that portions of the protein were indeed soluble. DomPred domain prediction (44) of LaeA revealed a putative domain boundary at about residue 100 (Fig. 1), and multiple sequence alignments of



**FIGURE 3. N-terminal 86 amino acids of LaeA are dispensable for function.** Complementation of  $\Delta laeA$  was accomplished by targeted integration of constructs at the *pyroA* locus. *A*, strains of *A. nidulans* were grown for 4 days under light or dark conditions on glucose minimal media where null mutants of *laeA* produce fewer conidia and reduced colony pigmentation. As expected, a carboxyl GFP tag of *laeA* complements these gross phenotypes. Additionally, truncation of the N-terminal 86 amino acids ( $LaeA^{\Delta 1-86}$ -GFP) also results in restoration of the gross phenotype of  $\Delta laeA$  strains. *B*, production of the mycotoxin sterigmatocystin was extracted and visualized via thin layer chromatography from cultures shown in *A*. Sterigmatocystin is nearly absent in  $\Delta laeA$  strains, and this phenotype is restored in both the *LaeA*-GFP and  $LaeA^{\Delta 1-86}$ -GFP complementation strains. *C*, *LaeA*-GFP localized constitutively in the nucleus as expected, and the N-terminal truncation mutant  $LaeA^{\Delta 1-86}$ -GFP also localizes exclusively in the nucleus. Histone H1 fused to mCherry (*hhoA*-mCherry) serves as a marker for nuclei. Strains used are as follows: WT (RJMP260.12),  $\Delta laeA$  (RJMP260.1),  $\Delta laeA$  *laeA*-GFP (RJMP261.26), and  $\Delta laeA$   $laeA^{\Delta 1-86}$ -GFP (RJMP264.5). DIC, differential interference contrast.

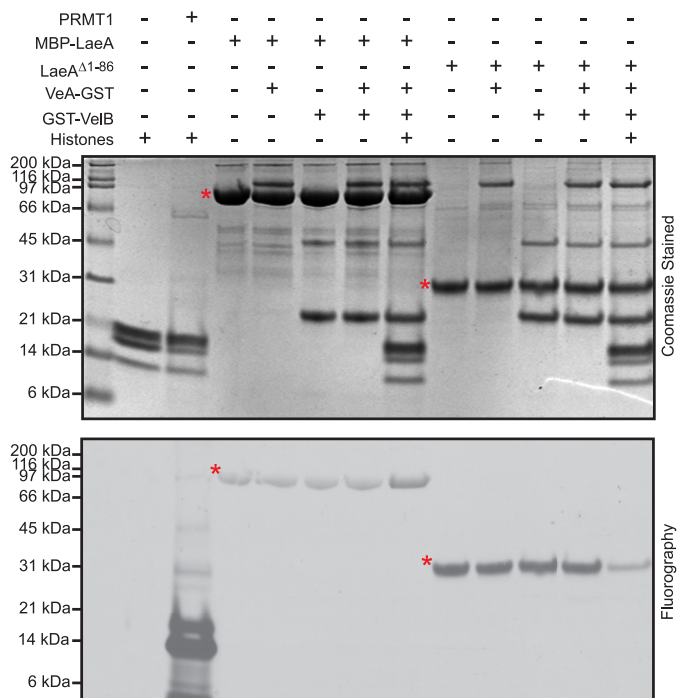
LaeA homologs indicated that the N terminus of LaeA was variable up to residue 88 (Fig. 2). We thus made several N-terminal truncations of LaeA, and we found that truncation mutants of the N-terminal 30, 42, and 86 amino acids all resulted in soluble protein (Fig. 1; data not shown).

We next determined whether the largest truncation mutant ( $LaeA^{\Delta 1-86}$ ) was biologically active and able to complement  $\Delta laeA$  *in vivo*. To simultaneously test the function of the  $LaeA^{\Delta 1-86}$  mutant and determine cellular localization, we constructed several *laeA* complementation vectors in the 3/4 *pyroA* targeting vector that are driven by the native *laeA* promoter (p) as well as terminator (t): *laeA*(p)::*laeA*::*laeA*(t), *laeA*(p)::*laeA*-GFP::*laeA*(t), and *laeA*(p)::*laeA* $^{\Delta 1-86}$ -GFP::*laeA*(t). These constructs were then targeted to the *pyroA* locus in a  $\Delta laeA$  background. Transformants were obtained and crossed to prototrophy. Importantly,  $LaeA^{\Delta 1-86}$ -GFP was capable of complementing the  $\Delta laeA$  phenotype as sterigmatocystin production and sexual development returned to levels similar to wild type (Fig. 3, *A* and *B*). Moreover,

$LaeA^{\Delta 1-86}$ -GFP localized to the nucleus (Fig. 3C). These data suggest that the N terminus of LaeA is dispensable for function and validated the use of the  $LaeA^{\Delta 1-86}$  protein for further *in vitro* methylation assays.

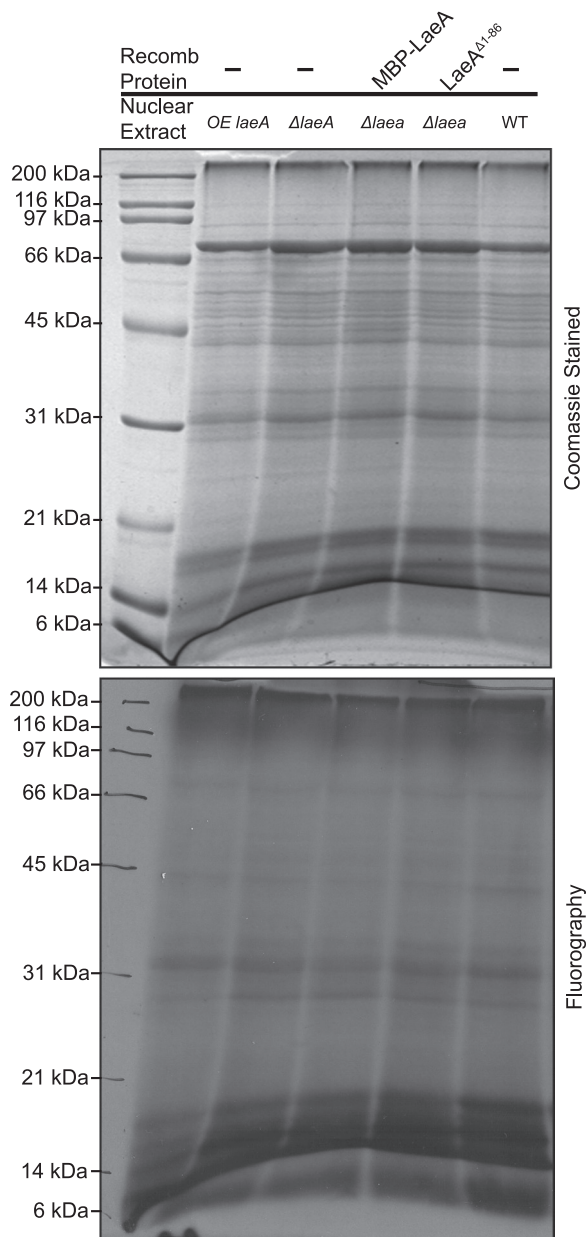
*Is LaeA a Protein Methyltransferase?*—From the presence of the four appropriately spaced sequence motifs (I, post-I, II, and III) associated with the AdoMet binding domain of seven- $\beta$ -strand methyltransferases present in the amino acid sequence of LaeA, a catalytic function in methyl transfer is indicated (Fig. 2) (12, 20). Such a function has been supported by the loss of LaeA function *in vivo* in a double mutation in motif I (23). Furthermore, it has been shown that LaeA can bind AdoMet *in vitro* (31). These data all suggest that LaeA could be a *bona fide* methyltransferase. Although BLAST searches of the LaeA sequence reveal it contains common motifs shared by seven- $\beta$ -strand methyltransferases (Fig. 2), this group of enzymes is involved in modifying such a variety of DNA, RNA, protein, lipid, and small molecule substrates that it is impossible to infer potential LaeA methyl-accepting substrates.

## LaeA Automethylates at a Methionine Residue



**FIGURE 4. Recombinant LaeA<sup>Δ1-86</sup> or MBP-LaeA does not methylate purified VeA, VelB, or histone proteins.** *In vitro* methylation reactions were prepared with 1.7  $\mu\text{M}$  [<sup>3</sup>H]AdoMet and incubated for 20 h at 37 °C with methyltransferases and potential methyl-accepting substrates, including 5  $\mu\text{g}$  each of recombinant human histone H2A, H2B, H3.3, and H4, 1  $\mu\text{g}$  of purified PRMT1 (positive control), 27.4  $\mu\text{g}$  of MBP-LaeA,  $\sim$ 5  $\mu\text{g}$  of VeA,  $\sim$ 5  $\mu\text{g}$  of VelB, and 8.8  $\mu\text{g}$  of LaeA<sup>Δ1-86</sup>. The samples were separated on a 10% BisTris gel with MES running buffer and stained with Coomassie. Polypeptide molecular weight markers (Bio-Rad broad range,  $\sim$ 3  $\mu\text{g}$  of each protein, catalogue number 161-0317) were electrophoresed in a parallel lane and shown on the left, and include myosin (200 kDa),  $\beta$ -galactosidase (116 kDa), phosphorylase *b* (97 kDa), serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21 kDa), lysozyme (14 kDa), and aprotinin (6 kDa). Fluorography was performed by treating the gel with EN<sup>3</sup>HANCE and exposing the dried gels to film for 5 days at  $-80$  °C as described under "Experimental Procedures." Red asterisks indicate the position of MBP-LaeA and LaeA<sup>Δ1-86</sup>.

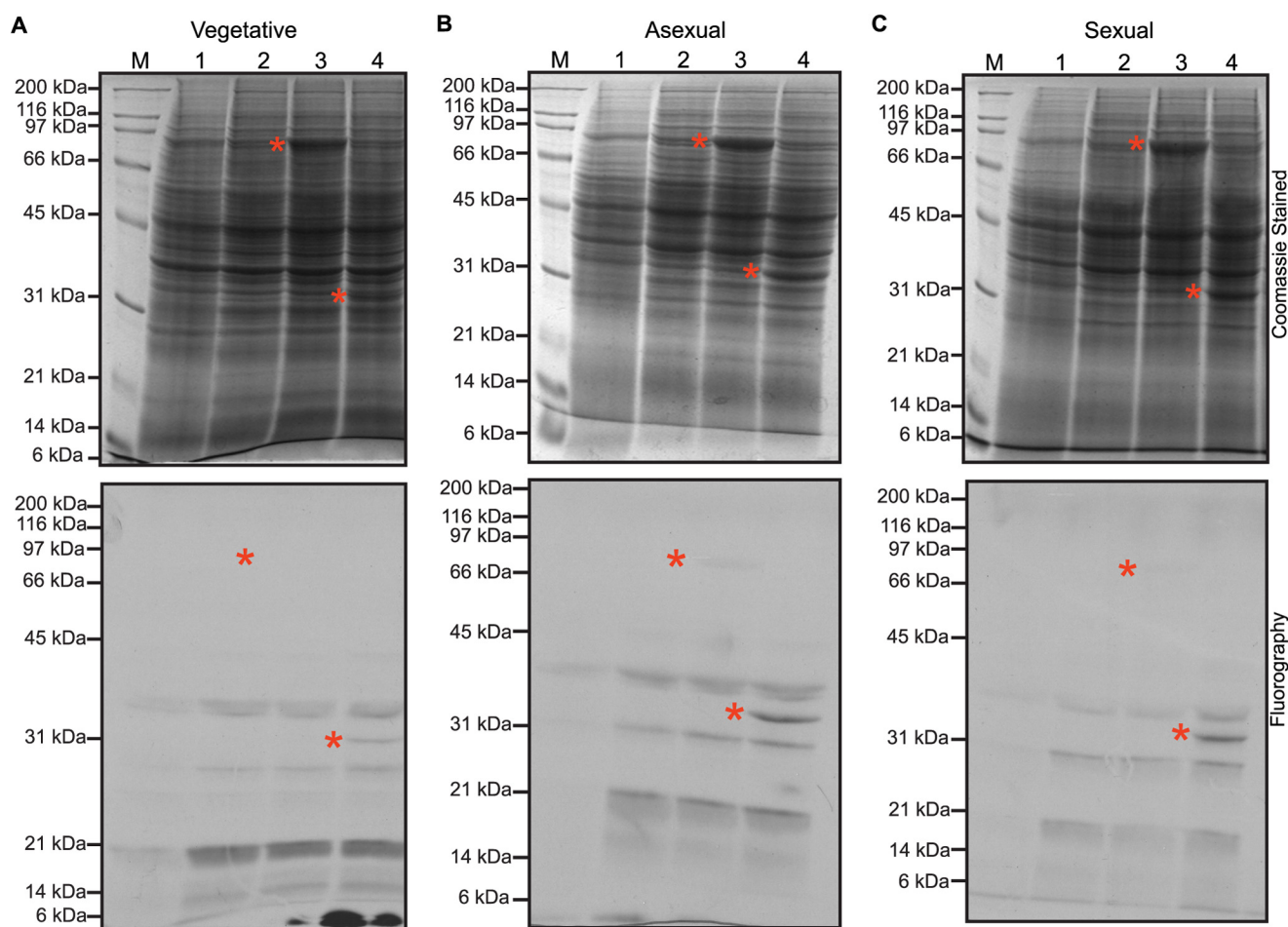
However, the function of LaeA in controlling gene expression suggested the possibility that LaeA could be a protein methyltransferase similar to those histone methyltransferases involved in transcriptional activation and repression (45–47). LaeA is known to transcriptionally regulate as much as 10% of the transcriptome (15) and subsequently has been linked to changes in chromatin structure (24). However, it is unknown whether this is a direct or indirect effect. To specifically address the possibility that LaeA methylates histone proteins or is required to be in a protein complex to methylate a substrate, we incubated MBP-LaeA or LaeA<sup>Δ1-86</sup> with human recombinant histone proteins (H2A, H2B, H3.3, and H4) or with proteins associated with the velvet complex (VeA and VelB). The reaction components were separated by gel electrophoresis and analyzed by fluorography (Fig. 4). As a control, we showed that the protein arginine methyltransferase PRMT1 robustly methylates histones (48). Although we did not observe any methylation of recombinant histones or the velvet complex in these experiments, we were quite surprised to find that both MBP-LaeA and LaeA<sup>Δ1-86</sup> automethylated (Fig. 4). The extent of automethylation appeared to be  $\sim$ 10-fold greater for



**FIGURE 5. *In vitro* methylation assay using wild type, *laeA* overexpressing, and  $\Delta laeA$  nuclear extracts.** Approximately 110  $\mu\text{g}$  of crude nuclear protein from each strain was isolated as described under "Experimental Procedures" and incubated with 100 nM [<sup>3</sup>H]AdoMet for 4 h at 37 °C. When added, MBP-LaeA and LaeA<sup>Δ1-86</sup> were present at 4  $\mu\text{g}$  of protein. The samples were electrophoresed on a 10% Tris glycine polyacrylamide gel with Tris glycine running buffer and Coomassie-stained. Fluorography was performed by treating the gel with EN<sup>3</sup>HANCE and exposing the dried gel to film for 9 months at  $-80$  °C. The positions of Bio-Rad broad range marker proteins are shown on the left. *Recomb*, recombinant.

LaeA<sup>Δ1-86</sup> than for MBP-LaeA, suggesting a potential regulatory role of the N terminus.

Because LaeA can translocate into the nucleus, we looked for methylated substrates in wild type,  $\Delta laeA$ , and overexpressing *laeA* crude nuclear extracts incubated with [<sup>3</sup>H]AdoMet. Analysis of the polypeptides separated by SDS-PAGE indicated no differences in the pattern of Coomassie staining or in the fluorography of <sup>3</sup>H-methylated proteins (Fig. 5). We then asked whether we could find potential LaeA substrates in whole cell extracts from wild type and  $\Delta laeA$  strains derived from vegeta-



**FIGURE 6. *In vitro* methylation of wild type and  $\Delta laeA$  extracts of *A. nidulans* at vegetative, asexual, and sexual growth phases do not reveal LaeA methyl-accepting substrates.** Whole cell extracts from wild type or  $\Delta laeA$  strains in vegetative (A), asexual (B), or sexual (C) growth stages were prepared as described under "Experimental Procedures." Samples analyzed were as follows: lane 1, 300  $\mu$ g of wild type whole cell extract; lane 2, 300  $\mu$ g of  $\Delta laeA$  whole cell extract; lane 3, 300  $\mu$ g of  $\Delta laeA$  whole cell extract mixed with 10  $\mu$ g of purified MBP-LaeA, and lane 4, 300  $\mu$ g of  $\Delta laeA$  whole cell extract mixed with 10  $\mu$ g of purified LaeA $^{\Delta 1-86}$ . Each sample was incubated with 100 nM [ $^3$ H]AdoMet for 3 h at 37  $^{\circ}$ C, separated by 10% Tris glycine polyacrylamide gels with Tris glycine running buffer, and stained with Coomassie. The gels were treated with EN $^3$ HANCE, and the dried gels were exposed to film for 1 month at  $-80^{\circ}$ C. The positions of Bio-Rad broad range marker proteins (M) are shown on the left. Red asterisks denote the position of MBP-LaeA or LaeA $^{\Delta 1-86}$  in the Coomassie-stained gel and in the fluorograph.

tive, asexual, and sexual growth stages. Coomassie staining and fluorography of extracts incubated with [ $^3$ H]AdoMet and separated by SDS-PAGE showed no significant differences in the pattern of polypeptides or methylated species (Fig. 6). Finally, we *in vivo* labeled wild type,  $\Delta laeA$ , and *OE laeA* strains with [ $^3$ H]AdoMet. Gel electrophoresis and fluorography revealed no significant protein or methylation differences between the three different strains (Fig. 7). Interestingly, out of all the potential methylation substrates, LaeA automethylation represented the predominant methylated species when the enzyme was incubated with any of the extracts.

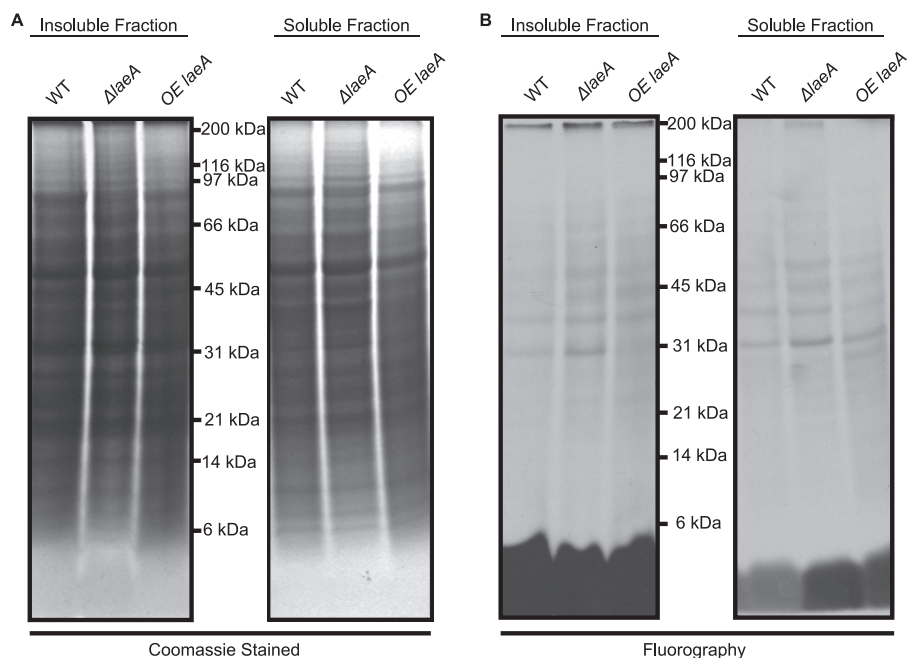
**LaeA $^{\Delta 1-86}$  and MBP-LaeA Are Automethylated at Methionine 207**—Because a number of protein arginine and lysine methyltransferases have been shown to be automethylated at the same types of residues as their substrates (49–52), we decided to identify the automethylation sites of LaeA in the expectation that it might lead us to the residue of methylation in its substrate. To determine the modified residue, the radioactive LaeA $^{\Delta 1-86}$  species was excised from the gel, acid-hydrolyzed, and characterized for possible neutral and basic

$^3$ H-methylated amino acid products by high resolution cation exchange chromatography. We found that there was no incorporation of  $^3$ H radioactivity in peaks co-migrating with added basic amino acid standards of mono-, di-, and tri-methylated lysine, mono-methylated histidine, and mono- and di-methylated arginine (Fig. 8). However, we did note a significant radioactive peak migrating between the methylated histidine and arginine standards near the peak of ammonium ion, which results from the hydrolysis of the polyacrylamide gel. We thus considered the possibility that the radioactivity was associated with *S*-methylmethionine, another basic amino acid derivative.

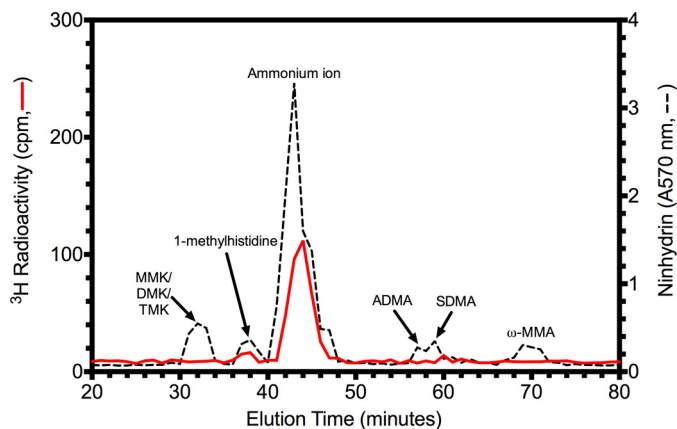
We then repeated the high resolution chromatographic analysis with the hydrolysate of  $^3$ H-automethylated MBP-LaeA $^{\Delta 1-86}$  and a standard of *S*-methylmethionine greatly in excess of the amount of ammonium ion. We found that the radioactivity eluted just prior to the standard, in a position that would be expected from the tritium isotope effect previously observed with methyl derivatives of lysine, histidine, and arginine (Fig. 9B) (53–56). To confirm the identification of *S*-methyl[ $^3$ H]methionine, we fluorescently labeled the cation



## LaeA Automethylates at a Methionine Residue



**FIGURE 7. *In vivo* protein methylation of wild type, *laeA*-overexpressing, and  $\Delta$ *laeA* *A. nidulans* strains separated into insoluble and soluble fractions.** *Aspergillus* mycelia was labeled with [ $^3$ H]AdoMet as described under "Experimental Procedures." Lyophilized mycelia were ground to a powder and resuspended in 100 mM Tris-HCl, pH 7.5, 250 mM NaCl, 10% glycerol, 0.1% Nonidet P-40, and 1 mM EDTA. The resulting lysate was vortexed, kept at 0 °C for 10 min, and centrifuged at 20,000  $\times$  *g* for 20 min at 4 °C. The supernatant *Soluble Fraction* and the pellet *Insoluble Fraction* were fractionated by SDS-PAGE as described under "Experimental Procedures" on a 10% BisTris polyacrylamide gel with MES-SDS running buffer. *A*, Coomassie-stained gel. *B*, fluorograph of EN $^3$ HANCE-treated gels after 1 month at  $-80$  °C. The positions of Bio-Rad broad range marker proteins are indicated.



**FIGURE 8. A novel *LaeA* automethylated amino acid residue.** 30  $\mu$ g of purified *LaeA* $^{\Delta 1-86}$  was incubated with 2.8  $\mu$ M [ $^3$ H]AdoMet for  $\sim$ 20 h at 37 °C as described under "Experimental Procedures." Reaction mixtures separated by 10% BisTris PAGE were stained, destained, and treated with EN $^3$ HANCE. After fluorography, the [ $^3$ H]AdoMet-labeled *LaeA* protein band was excised, rehydrated with water, and acid-hydrolyzed as described under "Experimental Procedures." After mixing with standards of methylated amino acids (1  $\mu$ mol each) including 1-( $\pi$ )-methylhistidine,  $\omega$ - $N^G$ -monomethylarginine ( $\omega$ -MMA),  $\omega$ - $N^G$ , $N^G$ -dimethylarginine (ADMA),  $\omega$ - $N^G$ , $N^{S^7}$ -dimethylarginine (SDMA),  $\epsilon$ - $N$ -monomethyllysine hydrochloride (MMK),  $\epsilon$ - $N$ -dimethyllysine (DMK), and  $\epsilon$ - $N$ -trimethyllysine (TMK), high resolution cation exchange chromatography was performed as described under "Experimental Procedures." The positions of the standard amino acids were determined by ninhydrin assay as described under "Experimental Procedures" and indicated by a dashed line. Radioactivity was determined in 200  $\mu$ l of each fraction and is shown by a red solid line.

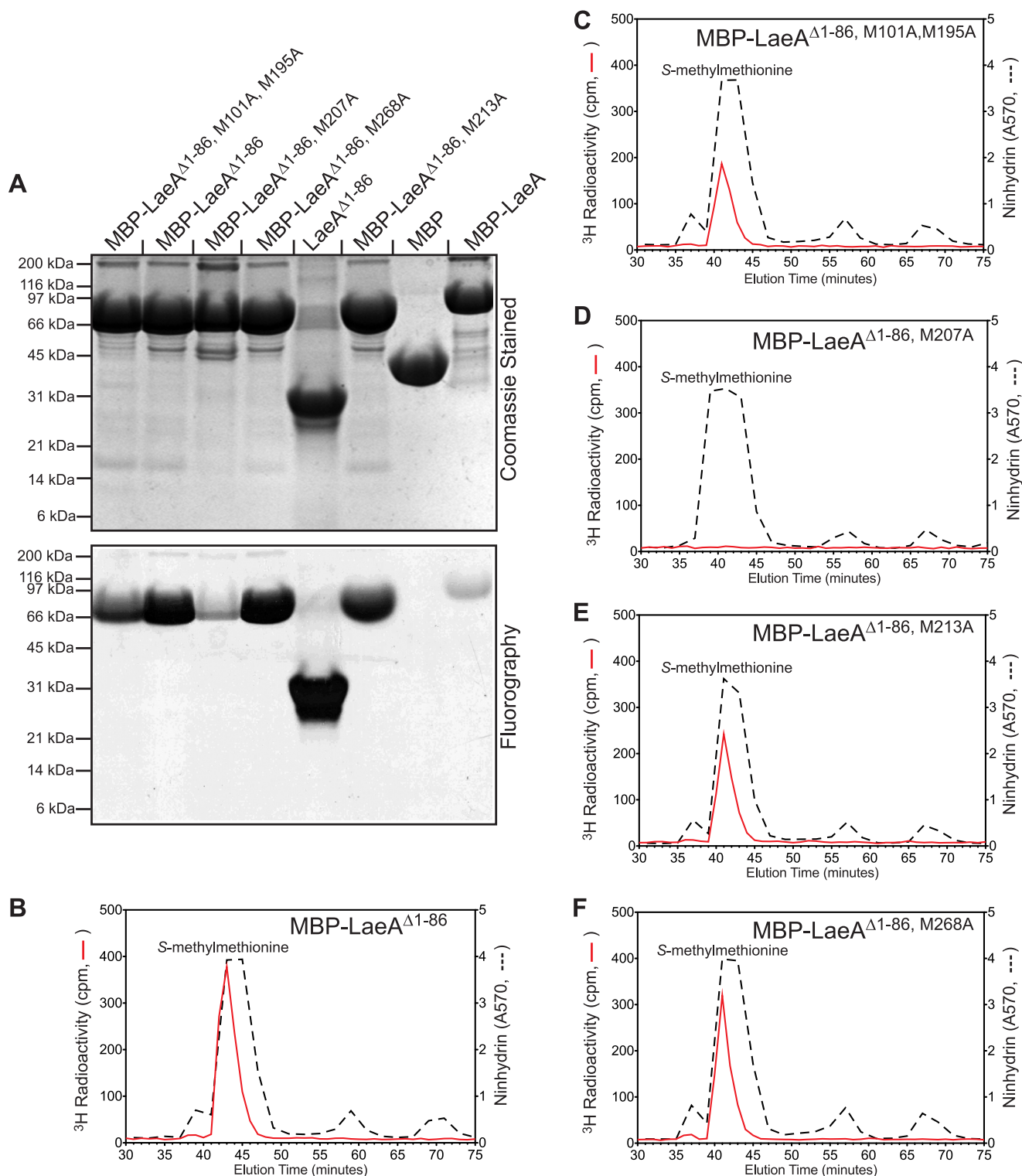
exchange chromatography fractions with OPA and analyzed the components by reverse phase HPLC and fluorometry. Analysis of the cation exchange fraction containing *S*-methylmethionine identified the fluorescently modified amino acid and confirmed its co-elution with the radioactivity (Fig. 10). These

results show that *LaeA* is automethylated on a methionine residue and that the function of the enzyme may be as a protein methionine methyltransferase.

We then prepared MBP-*LaeA* $^{\Delta 1-86}$ , where each of the five methionine residues was replaced by an alanine residue. Fluorography was used to determine the automethylation state (Fig. 9A). Compared with the control of MBP-*LaeA* $^{\Delta 1-86}$ , purified protein with substitutions at methionine 213 or methionine 268 did not have a significant reduction in automethylation. When methionines 101 and 195 were substituted to alanine, automethylation was slightly reduced. However, most of the automethylation was abolished with the methionine 207 substitution (Fig. 9A).

To confirm the site of methylation, the [ $^3$ H]AdoMet-labeled *LaeA* protein species from the fluorograph in Fig. 9A were acid-hydrolyzed and fractionated by high resolution cation exchange chromatography. We found that the  $^3$ H peak at the position of *S*-methylmethionine did not decrease significantly, or only slightly decreased, in the methionine to alanine substitutions at positions 101, 195, 213, and 268 (Fig. 9, *B*, *C*, *E*, and *F*). However, complete abolishment of methionine methylation only occurs when methionine 207 is substituted with alanine (Fig. 9D). These results suggest that the methionine 207 is the only site of automethylation. The small amount of automethylation seen in the methionine 207 mutants after gel electrophoresis (Fig. 9A) may be explained by automethylation of an alternative nucleophile in the protein or by residual binding of [ $^3$ H]AdoMet.

Methionine 207 is located in the amino acid sequence of *LaeA* immediately after motif II (Fig. 2). In the three-dimensional structures of a number of seven- $\beta$ -strand family mem-



**FIGURE 9. Direct demonstration of LaeA automethylation at methionine 207 by gel electrophoresis and high resolution cation exchange chromatography.** Constructs were made to express His-tagged MBP fusion proteins of LaeA $\Delta$ 1-86 where each methionine residue in the LaeA $\Delta$ 1-86 sequence was replaced individually by an alanine residue. 150  $\mu\text{g}$  of each of the purified LaeA proteins, with the exception of MBP-LaeA (lane 8, 82  $\mu\text{g}$ ), were incubated with 1.4  $\mu\text{M}$  [ $^3\text{H}$ ]AdoMet for 20 h at 37  $^\circ\text{C}$ , and the reaction products were separated by 10% BisTris PAGE with MES running buffer as described under "Experimental Procedures." **A**, gel was stained with Coomassie. The position of Bio-Rad broad range marker proteins are shown on the left. Controls included the unmutated MBP-LaeA $\Delta$ 1-86 (2nd lane), unmutated LaeA $\Delta$ 1-86 (5th lane), MBP alone (7th lane), and unmutated MBP-full-length LaeA (8th lane). Fluorography was performed by treating the gel with EN $^3$ HANCE and exposing the dried gel to film for 4 days at  $-80^\circ\text{C}$ . The portion of the EN $^3$ HANCE-treated gel containing the radiolabeled LaeA protein was excised, rehydrated with water, and acid-hydrolyzed as described under "Experimental Procedures." **B–F**, after mixing with standards (1  $\mu\text{mol}$  each, with the exception of *S*-methylmethionine with 5  $\mu\text{mol}$ ) of methylated amino acids, including 1-( $\pi$ )-methylhistidine, *S*-methylmethionine,  $\omega$ - $N^G$ -monomethylarginine, and  $\omega$ - $N^G, N^G$ -dimethylarginine, high resolution cation exchange chromatography was performed as described under "Experimental Procedures." The positions of the standard amino acids were determined by ninhydrin assay as described under "Experimental Procedures" and indicated by a dashed line. Radioactivity was determined in 200  $\mu\text{l}$  of each fraction and is shown in solid red lines.

## LaeA Automethylates at a Methionine Residue

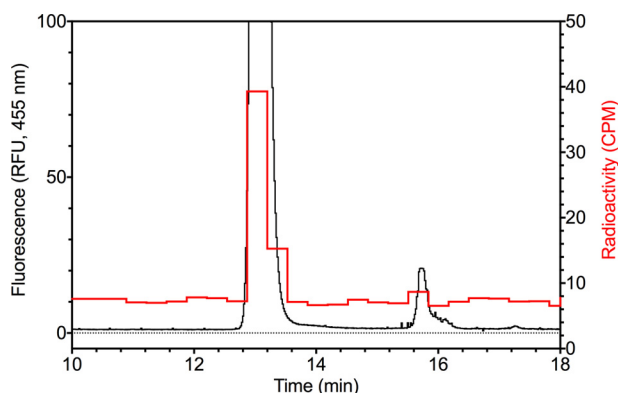


FIGURE 10. OPA amino acid analysis verifies *S*-methylmethionine is the radioactive product of LaeA automethylation. Cation exchange chromatography fractions from the experiment shown in Fig. 9B were fluorescently labeled with OPA reagent and analyzed by reverse phase HPLC coupled to a fluorometer as described under "Experimental Procedures." Fractions of 0.5 ml were collected, and radioactivity was quantified as described under "Experimental Procedures" (red line). The fluorescence HPLC trace (black line) represents relative fluorescence units (RFU); the OPA-derivative of *S*-methylmethionine elutes at 13.1 min.

bers, residues in this region interact with methyl-accepting substrates and are close to the methyl group of AdoMet (57). Because no crystal structure exists for LaeA, the three-dimensional structure was modeled using Phyre<sup>2</sup> (42). The ligand prediction server 3DLigandSite was used to identify and to place a binding site of AdoMet into LaeA (43). Eight different AdoMet binding conformations were predicted in LaeA. The transferable methyl group of AdoMet ranged from 5.2 to 7.4 Å away from the sulfur group of methionine 207 in these structures, and no other residues were found between them (Fig. 11). This result is consistent with the reaction of the methionine thioether and the methyl group of AdoMet to produce *S*-methylmethionine.

**Methionine 207 Is Not Required for Function *in Vivo***—To determine the functional role of methionine 207, we constructed a LaeA<sup>M207A</sup> point mutant *in vivo*. We found that the M207A mutant was capable of complementing the  $\Delta$ laeA allele based on initial colony morphology (Fig. 12A). We also found that LaeA<sup>M207A</sup> could restore production of sterigmatocystin (Fig. 12A) and that LaeA<sup>M207A</sup> is localized in the nucleus (Fig. 12B). These results indicate that methionine 207 is not required for these functions of LaeA. Finally, because null mutants of *laeA* are deficient in the production of both asexual spores (conidia) and sexual spores (ascospores), we measured the sporulation capacity of the complementation strains. We found that LaeA<sup>M207A</sup> restores sporulation to the same extent as the complemented control strain (Fig. 12, C and D). We noted, however, that neither the wild type nor the M207A constructs restored sporulation levels back to wild type. This observation is common as rarely does complementation at an alternative genetic locus result in the full restoration of the mutant phenotype. Taken together, these data suggest that methylation of methionine 207 is not required for LaeA function *in vivo*.

## DISCUSSION

Based on its primary amino acid sequence that resembles seven- $\beta$ -strand methyltransferases, its nuclear localization, and its large scale effects on gene expression, LaeA has been postulated to be a histone methyltransferase. However, no direct bio-

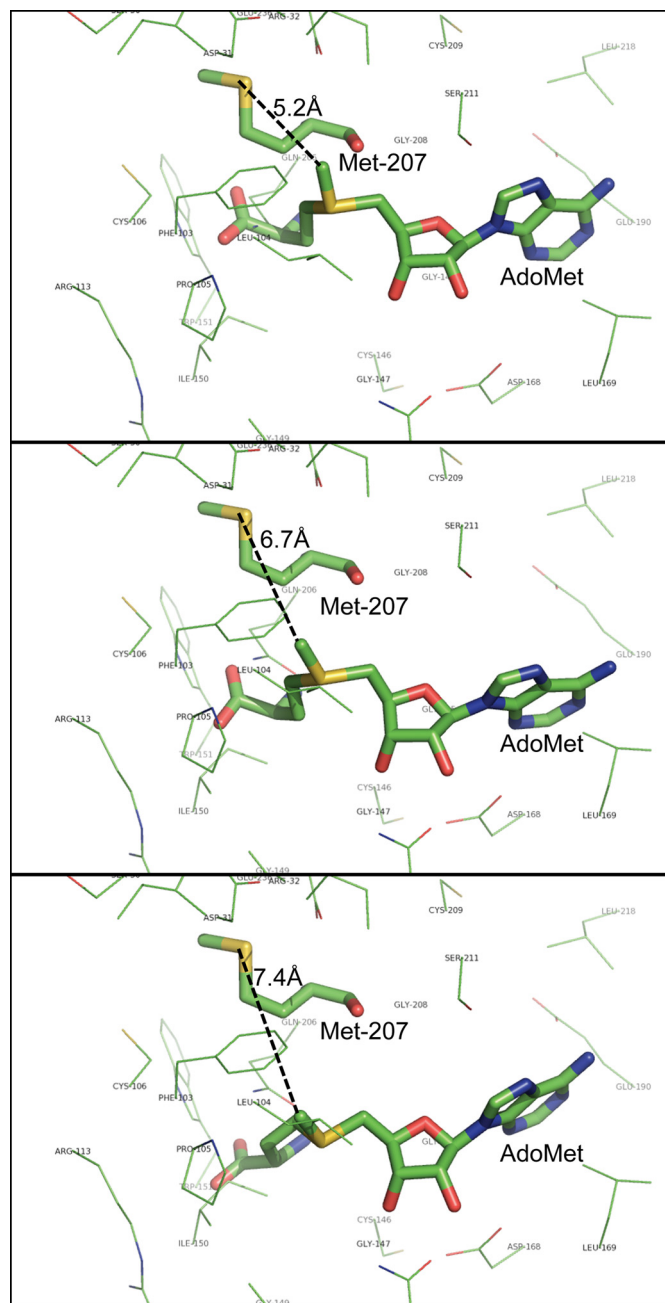
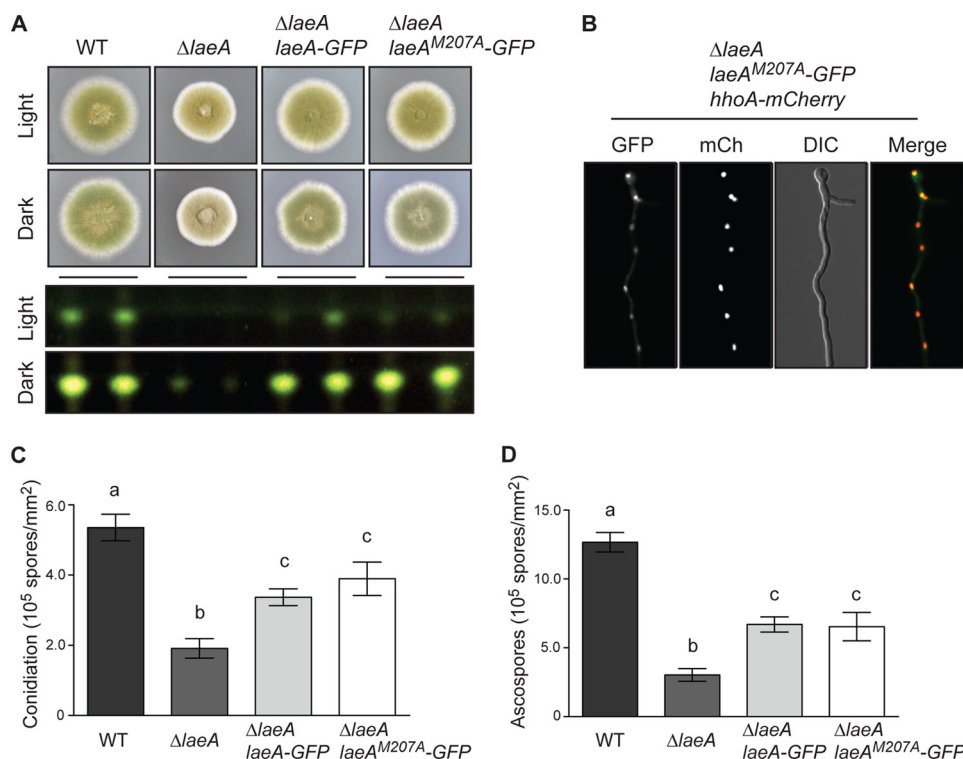


FIGURE 11. Modeling of the LaeA structure suggests that the sulfur atom of methionine 207 is close to the position of the transferable methyl group of bound AdoMet. The three-dimensional structure of full-length LaeA was based on the primary sequence (UniProt accession number C8VQG9) and derived from Phyre<sup>2</sup> modeling; the position of the ligand AdoMet was modeled by 3DLigandSite. The positions of three possible conformations of AdoMet are shown that encompass the extent of the eight different ligand structures obtained. LaeA side chain residues within 4 Å of AdoMet are shown as thin lines with the exception of methionine 207, which is shown as a thick line with the sulfur atom in yellow. The bound AdoMet is also shown as a thick line. The distance of this sulfur atom to the transferable methyl group of AdoMet is 5.2, 6.7, and 7.4 Å in these three simulated conformations and is represented by black dashed lines.

chemical studies on the possible substrates of LaeA have been described. In this study, we attempted to identify substrates of LaeA using *in vitro* and *in vivo* biochemical and molecular biology techniques. Because LaeA automethylation is readily observed in *in vitro* experiments with [<sup>3</sup>H]AdoMet (Figs. 4 and



**FIGURE 12. Methionine 207 of LaeA is not required for function *in vivo*.** A methionine 207 to alanine point mutation was constructed and integrated at the *pyoA* locus in a  $\Delta laeA$  strain of *A. nidulans*. *A*,  $LaeA^{M207A}$ -GFP strains restored the gross phenotype of  $\Delta laeA$  strains as well as production of sterigmatocystin in both light and dark growth conditions. *B*, using fluorescent microscopy,  $LaeA^{M207A}$ -GFP was found to be located in nuclei. *C*, quantification of asexual sporulation (conidiation) was measured on plates grown for 4 days under light conditions. Null mutants of *laeA* produce fewer conidia under these conditions. Complementation strains partially restore the production of conidia, although not to wild type levels. *LaeA*-GFP and  $LaeA^{M207A}$ -GFP produce similar amounts of conidia. *D*, production of sexual spores (ascospores) is also reduced in  $\Delta laeA$  strains compared with wild type. This phenotype is partially restored in both *LaeA*-GFP and  $LaeA^{M207A}$ -GFP complementation strains. Statistical differences were measured by analysis of variance using Prism 5.0 (GraphPad) and different letters represent statistical differences  $p < 0.001$ . Strains used in *A*, *C*, and *D* were WT (RJMP103.5),  $\Delta laeA$  (RJW41A),  $\Delta laeA laeA$ -GFP (RJMP256.3), and  $\Delta laeA laeA^{M207A}$ -GFP (RJMP258.1). RJMP263.3 ( $\Delta laeA$ , *hhoA*-mCherry, *pyoA::laeA^{M207A}-GFP) was used in *B*.*

6), we expected that methyl group transfer to other substrates would also be readily detected. However, this was not the case in our *in vitro* (Figs. 5 and 6) and *in vivo* (Fig. 7) labeling experiments. Taken together, these results suggest that either LaeA is not a protein methyltransferase, that it modifies substrates at levels undetectable by our methods, or that specific conditions are required for target methylation.

Importantly, we consistently observed LaeA automethylation at a methionine residue located close to the AdoMet-binding site. This finding is significant because it demonstrates a novel type of automethylation.

Although methyltransferases are generally characterized by their action on exogenous methyl-accepting substrates, some have been previously shown to self-methylate. The first such enzyme was the bovine protein repair L-isoaspartyl-(D-aspartyl) methyltransferase, which self-methylates at a modified asparaginyl and at an aspartyl residue (58, 59). Several protein arginine methyltransferases, including mammalian PRMT1 (60, 61), mammalian and trypanosomal PRMT6 (51, 62), and *Arabidopsis* PRMT10 (63) also demonstrate automethylation. In these cases, automethylation does not appear to have an effect on enzymatic activity. However, there are several cases in which the automethylation of an enzyme may have an influence on function. In the SET domain family, the automethylation of *Chlamydia* nuclear effector methyltransferase and human histone-lysine-N-methyltransferase (SETMAR or Metnase) is

hypothesized to increase methylation activity toward histone substrates (64) or to repress methyltransferase-associated TopoII $\alpha$  decatenation (65), respectively. Finally, automethylation roles involving transcription and pre-mRNA splicing (50), the inactivation of unused methyltransferases (66), and auto-regulation (49) have been suggested for CARM1/PRMT4, the DNA methyltransferase Dnmt3a, and PRMT8, respectively.

The site of automethylation can also provide insights into the substrates of methyltransferases. Although the SETMAR protein lysine methyltransferase automethylates both lysine and arginine residues (65), PRMT1 (61), CARM1/PRMT4 (50), and PRMT8 (49) automethylation is specific for arginine residues. Furthermore, in the lysine methyltransferase G9a, the sequence context of an automethylated lysine residue (an ARKT motif) is identical to the site of G9a modification on lysine 9 of histone H3 (52). Therefore, the presence of the automethylated methionine residue in LaeA may indicate that other potential substrate(s) could be methylated at a methionine residue, perhaps in a similar sequence context. The only prior evidence for methionine protein methylation is from a single report to a still unidentified methyltransferase that modifies cytochrome *c* in *Euglena gracilis* (67). A BLAST search of LaeA against the proteins encoded by the *E. gracilis* genome reveals no homologs.

Although this study showed the LaeA-dependent methylation of a methionine residue in a protein, free S-methylmethionine has been extensively studied for its role in plant stress

## LaeA Automethylates at a Methionine Residue

tolerance (68) and as a methyl donor for a select few methyltransferases, such as YagD in *E. coli* (69), the human betaine-homocysteine S-methyltransferase 2 (70), and the yeast Mht1 methyltransferase (71). It is thus possible that the methyl group may be transferred from the methylmethionine residue in LaeA to another acceptor.

As illustrated by our three-dimensional modeling of LaeA (Fig. 11), the sulfur group of methionine 207 is close to the transferable methyl group of AdoMet, ranging between ~5.2 and 7.4 Å. This is similar to the 6-Å distance between the sulfhydryl group of cysteine and the methyl group of AdoMet in the previously characterized automethylation of Dnmt3a (66). The close positioning of methionine 207 to the AdoMet-binding site appears to be important as this residue in LaeA is conserved in other filamentous fungi, with the notable exception of *Trichoderma reesei* LAE1, which interestingly does not complement  $\Delta laeA$  strains of *A. nidulans* (Fig. 2) (72). Despite the conservation of methionine 207, the LaeA<sup>M207A</sup> point mutant fully complements  $\Delta laeA$  *in vivo*. These data suggest that automethylation of methionine 207 could be occurring in the absence of substrate. However, it is also possible that LaeA automethylation could be serving as a self-regulator, as evident by how mutating the automethylation site of CARM1 does not affect enzymatic activity but does impair CARM1-activated transcription and pre-mRNA splicing (50).

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