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Structure and Function of *fas-1A*, a Gene Encoding a Putative Fatty Acid Synthetase Directly Involved in Aflatoxin Biosynthesis in *Aspergillus parasiticus*

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A novel gene, *fas-1A*, directly involved in aflatoxin B1 (AFB1) biosynthesis, was cloned by genetic complementation of an *Aspergillus parasiticus* mutant strain, UVM8, blocked at two unique sites in the AFB1 biosynthetic pathway. Metabolite conversion studies localized the two genetic blocks to early steps in the AFB1 pathway (*nor-1* and *fas-1A*) and confirmed that *fas-1A* is blocked prior to *nor-1*. Transformation of UVM8 with cosmids NorA and NorB restored function in *nor-1* and *fas-1A*, resulting in synthesis of AFB1. An 8-kb *Sac*I subclone of cosmid NorA complemented *fas-1A* only, resulting in accumulation of norsolorinic acid. Gene disruption of the *fas-1A* locus blocked norsolorinic acid accumulation in *A. parasiticus* B62 (*nor-1*), which normally accumulates this intermediate. These data confirmed that *fas-1A* is directly involved in AFB1 synthesis. The predicted amino acid sequence of *fas-1A* showed a high level of identity with extensive regions in the enoyl reductase and malonyl/palmityl transferase functional domains in the beta subunit of yeast fatty acid synthetase. Together, these data suggest that *fas-1A* encodes a novel fatty acid synthetase which synthesizes part of the polyketide backbone of AFB1. Additional data support an interaction between AFB1 synthesis and sclerotium development.

Aflatoxins are polyketide-derived secondary metabolites that are produced by strains of the imperfect fungi *Aspergillus parasiticus* and *Aspergillus flavus*. Aflatoxins are highly toxic, mutagenic, and carcinogenic in a variety of animal species and are suspected carcinogens in humans (11). Peanuts, treenuts, corn, cottonseed, and other important crops are occasionally contaminated with aflatoxin as a result of infection by toxigenic aspergilli. An understanding of the aflatoxin biosynthetic pathway may result in the identification of strategies to inhibit aflatoxin contamination of plant-derived products at the preharvest level.

Aflatoxin biosynthesis is proposed to begin with the condensation of acetyl coenzyme A and malonyl coenzyme A via a polyketide synthetase (PKS) to form the decaketide noranthrone (4, 10). Alternatively, a six-carbon fatty acid, hexanoate, is first synthesized by a fatty acid synthetase (FAS) and then extended by a PKS to generate noranthrone (22). Noranthrone is oxidized to norsolorinic acid (NA), which is converted to aflatoxin B1 (AFB1) through a series of pathway intermediates, including averantin (AVN), averufanin, averufin, versiconal hemiacetal acetate, versiconal, versicolorin B, versicolorin A (VA), demethylsterigmatocystin, sterigmatocystin (ST), *o*-methylsterigmatocystin (OMST), and AFB1 (4, 10).

Several genes encoding enzyme activities or regulatory proteins involved in AFB1 biosynthesis in *A. parasiticus* and *A. flavus* and ST biosynthesis in *Aspergillus nidulans* have been cloned (6, 24). These genes are clustered in a 65-kb region on one chromosome in *A. parasiticus* and *A. flavus* (25, 26). Transcript mapping analysis identified three other genes in the cluster encoding large transcripts (7.5, 7.0, and 6.5 kb) which appear to be involved in AFB1 biosynthesis (25). Gene disruption and nucleotide sequence analyses of *pksA* (7.0-kb transcript) suggested that it encodes a PKS involved in synthesis of the AFB1 polyketide backbone (7, 25).

This study focuses on *fas-1A*, which encodes the 7.5-kb transcript. Analysis of *fas-1A* mutants combined with nucleotide sequence analysis strongly suggests that this gene encodes one subunit of a novel FAS directly involved in synthesis of the AFB1 polyketide backbone.

MATERIALS AND METHODS

Strains, plasmids, and culture conditions. Plasmid DNA was propagated in *Escherichia coli* DH5α (12) and purified by an alkaline lysis procedure (18). Plasmid pRZ2.8 (see Fig. 3) contains a 2.8-kb *Eco*RI subclone of cosmid NorA inserted into the *Eco*RI site of plasmid pBluescriptIISK(−). Plasmid pAPSa8 (Fig. 1) contains an 8-kb *Sac*I subclone of cosmid NorA inserted into the *Sac*I site of pBluescriptIISK(−).

A. parasiticus SU1 (ATCC 56775, NRRL 5862) served as the aflatoxin-producing wild-type strain. *A. parasiticus* B62 (*niaD nor-1 br-1* [8]), derived from *A. parasiticus* ATCC 24690 (16), was used for isolation of mutants created by UV mutagenesis and for gene disruption experiments. B62 accumulates NA and retains the ability to produce low levels of AFB1 (five- to eightfold less than SU1).

Methods for the maintenance of fungal strains and preparation of conidial stocks and descriptions of the liquid and solid growth media used for production (YES) and analysis (coconut agar medium [CAM] [2]) of aflatoxins have been reported previously (25).

UV mutagenesis. Conidia in sterile water (10^6 per ml) were exposed to up to 10 sequential doses of UV light at 200,000 μJ per dose (UV Stratalinker; Stratagene). Mutants lacking NA synthesis were obtained after irradiation with seven doses (UVM7) or eight doses (UVM8).

Metabolite conversion studies. Metabolite conversion studies with whole cells were conducted as described by Bhatnagar et al. (5) and Adye and Mateles (1). One gram (wet weight) of washed mycelia from UVM7 or UVM8 was incubated for 12 h with constant shaking (150 rpm) at 28°C in the presence of acetate (1,000 μg), NA (10 μg), AVN (10 μg), VA (10 μg), ST (5 μg), or OMST (5 μg). Aflatoxins were analyzed by thin-layer chromatography (TLC) and quantitated by densitometry (Shimadzu dual-wavelength TLC scanner model CS9000U) as described by Bhatnagar et al. (5).

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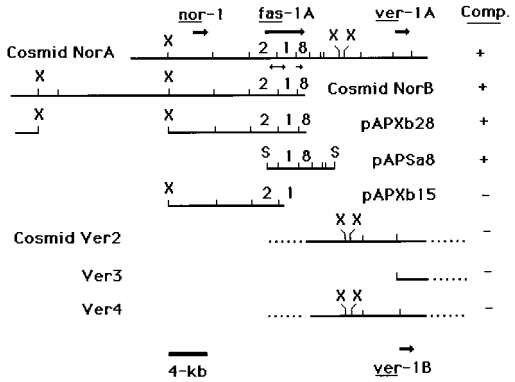

FIG. 1. DNA fragments used for complementation of UVM8. Seven DNA fragments, represented by solid lines, were used in complementation experiments. Only insert DNA is shown. Complementation (Comp.) of UVM8 is shown for each fragment. Unlabeled restriction endonuclease sites are *Eco*RI. Other sites are *Sac*I (s) and *Xba*I (x). The thick arrows represent the size and orientation of transcripts from *nor-1*, *ver-1A*, *ver-1B*, and *fas-1A*. The numbers 2, 1, and 8 on the maps for cosmids NorA and NorB and subclones pAPXb28 and pAPSa8 are *Eco*RI subclones used in nucleotide sequence analysis. The small arrows on the NorA map show the approximate length and direction of sequencing to generate the data shown in Fig. 6. The dotted lines on the maps of cosmids Ver2, -3, and -4 represent unmapped regions.

Genetic complementation: transformation of fungal protoplasts and analysis of transformant clones. Protoplasts were transformed by a polyethylene glycol procedure (21). Plasmid pSL82 contains the nitrate reductase gene *niaD* (13), used as a selectable marker for cotransformation. The cosmids used in complementation experiments (Fig. 1) were isolated in a previous study (25): cosmid NorA contains *nor-1* and *ver-1A* (17); cosmid NorB contains *nor-1* on a 21-kb DNA fragment that overlaps with NorA; and cosmids Ver2, Ver3, and Ver4 contain all or part of a 12-kb duplication of the *ver-1A aflR* region on cosmid NorA. *ver-1B*, a nearly identical copy of *ver-1A*, is contained in this duplicated region (17). Cosmids or subclones were added in 2- to 10-fold molar excess over pSL82. *niaD*$^+$ transformants, selected by growth on Czapek Dox (CZ) medium, were transferred to CAM to screen for aflatoxin and NA accumulation.

Analyses of aflatoxin production in transformants by TLC and ELISA. Aflatoxin and NA produced by the recipient strain B62 and *fas-1A* disruptants were quantitated by TLC and enzyme-linked immunosorbent assay (ELISA) by the method of Trail et al. (23) except that cells were cultured for 65 h instead of 72 h.

Genomic DNA isolation and Southern analysis. Genomic DNA was prepared by a published modification (14) of a phenol-chloroform protocol developed for mammalian DNA (3). Restriction endonucleases were purchased from New England Biolabs or Boehringer Mannheim Biochemicals. ^{32}P-labeled DNA probes were generated with the Random Primed DNA Labelling Kit from Boehringer Mannheim Biochemicals. Southern hybridization analyses were conducted by standard procedures (3).

Sclerotium production. Approximately 10^3 conidia were center inoculated onto petri plates containing 20 ml of CAM. Plates were incubated in the dark at 28°C for 7 days. Sclerotia were harvested and counted by a modification (19) of the method of Cotty (9). Sclerotial diameters were measured with a Java video analysis system (Jandal Corp., Corte Madera, Calif.).

TABLE 1. Conversion of metabolites to AFB1 by whole cells of two mutant strains of *A. parasiticus*

Metabolite added	Amt added (μg)	Mean AFB1 produced[a] (μg/mg of mycelium [wet wt])	
		UVM7	UVM8
None		ND	ND
Acetate	1,000	ND	ND
NA	10	0.39 ± 0.06	0.23 ± 0.08
AVN	10	2.1 ± 0.6	1.6 ± 0.4
VA	10	3.4 ± 0.9	4.1 ± 0.6
ST	5	3.1 ± 0.2	2.8 ± 0.4
OMST	5	4.6 ± 1.0	5.1 ± 0.8

[a] Mean of two experiments with two replicates each. ND, none detected.

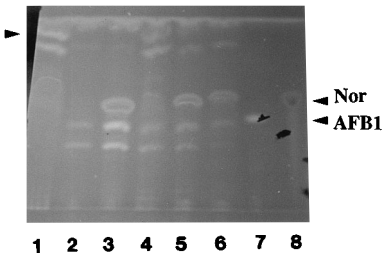

FIG. 2. TLC analysis of cell extracts from UVM8 complementation experiments. Lanes: 1, UVM8; 2, UVM7; 3, B62; 4, UVM8 transformed with pAPXb28; 5 and 6, two UVM8 isolates transformed with pAPSa8; 7, NA standard (arrow labeled Nor); 8, AFB1 standard (arrow labeled AFB1). The band immediately below AFB1 in lanes 2, 3, 4, 5, and 6 is AFG1.

Nucleotide sequence analyses. Nucleotide sequence analyses were conducted by DNA Technologies, Inc., Gaithersburg, Md., on three cosmid NorA subclones containing *fas-1A* (clones 2, 1, and 8 [Fig. 1]) (25). Nucleotide sequence data were analyzed with the Wisconsin Genetics Computer Group (GCG) software package. The locations of introns and open reading frames were predicted by using GCG programs Frames, TestCode, and CodonPreference. Comparisons of the predicted amino acid sequence of *fas-1A* with sequences in the EMBL and GenBank databases were conducted with TFastA and Gap.

Nucleotide sequence accession number. The accession number for *fas-1A* is L48183.

RESULTS

Isolation of UV mutants UVM7 and UVM8. UVM7 and UVM8, derived from *A. parasiticus* B62, no longer accumulated NA (red-orange pigment) or AFB1 (blue fluorescence) on CAM. UVM7 produced nonpigmented mycelia, and UVM8 produced a bright yellow mycelial pigment that was secreted into the growth medium. Loss of AFB1 and NA synthesis was confirmed by TLC analysis (data not shown). Inability to grow on CZ medium indicated that these mutants retained a nonfunctional *niaD* allele.

Metabolite conversion studies. UVM7 and UVM8 converted VA, AVN, ST, and OMST to AFB1 but could not convert acetate to NA or AFB1 (Table 1), suggesting that they were blocked prior to *nor-1* (the product of *nor-1* converts NA

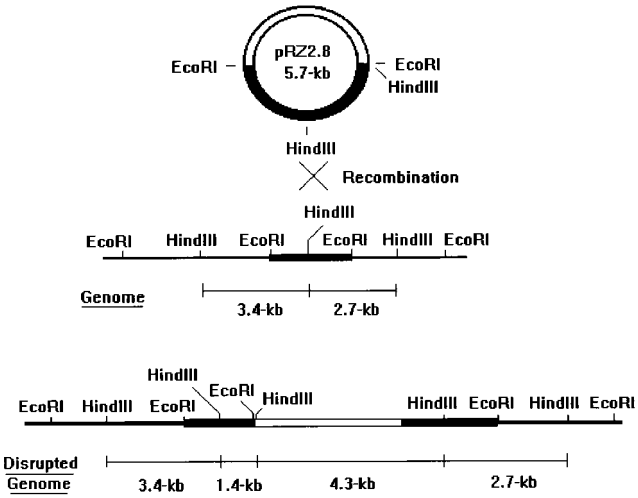

FIG. 3. Gene disruption of *fas-1A*. Recombination between the 2.8-kb insert in pRZ2.8 (solid area) and the homologous region in *fas-1A* (solid box) in the genome generates *Hin*dIII restriction fragments of the sizes indicated on the map labeled disrupted genome (the probe is the 2.8-kb insert). Nondisrupted strains have *Hin*dIII fragments of the sizes indicated on the map labeled genome.

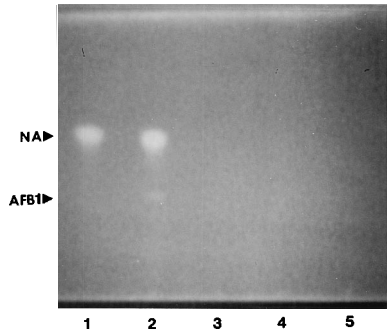

FIG. 4. TLC analysis of cell extracts from Dis1, -2, and -3. Lanes: 1, NA standard; 2, extract from B62; 3, 4, and 5, extracts from Dis1, -2, and -3, respectively.

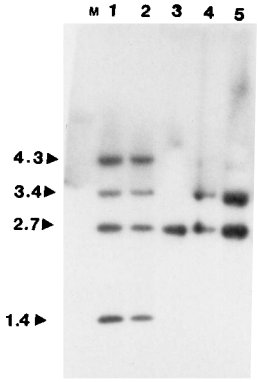

FIG. 5. Southern hybridization analysis of Dis1, -2, and -3. Genomic DNAs, digested with *Hin*dIII, were subjected to Southern hybridization analysis with the 2.8-kb *Eco*RI insert from pRZ2.8 as a probe. Arrowheads show the sizes of expected hybridizing fragments (in kilobases). Lanes: M, molecular size markers; 1, 2, and 3, genomic DNA isolated from Dis1, -2, and -3, respectively; 4 and 5, genomic DNA from *niaD*$^+$, NA-accumulating transformants (nondisrupted strains).

to AVN). The mutants converted five- to eightfold more AVN to AFB1 than NA, suggesting that they retained the *nor-1* genotype and were therefore blocked at two sites in the AFB1 pathway, *nor-1* and *fas-1A*.

Complementation of UVM8. UVM8 was cotransformed with plasmid pSL82 plus one of five cosmids or cosmid subclones (Fig. 1). Cosmids NorA and NorB complemented both pathway mutations in approximately 1% of the *niaD*$^+$ transformants in two separate experiments, resulting in synthesis of AFB1. Cosmids Ver2, Ver3, and Ver4 and plasmids pAPXb15, containing a 15-kb subclone of cosmid 698 (8), and pSL82 (control) failed to complement UVM8.

Cloning of *fas-1A*. A 28-kb *Xba*I subclone of cosmid NorB (pAPXb28 [Fig. 1]), which carried the 21-kb overlap between these clones, complemented *fas-1A* and *nor-1* or *fas-1A* alone in strain UVM8, resulting in transformants which produced AFB1 (6 of 160 *niaD*$^+$ transformants) or NA plus small quantities of AFB1 (five- to eightfold less than SU1) (1 of 160 *niaD*$^+$ transformants), respectively. Comparison of the restriction endonuclease maps of cosmids NorA and NorB and plasmids pAPXb28 and pAPXb15 localized *fas-1A* to three contiguous *Eco*RI subclones of cosmid NorA (clones 2, 1, and 8 [Fig. 1]). An 8-kb *Sac*I subclone of cosmid NorA (pAPSa8) containing clones 1 and 8 and part of clone 2 was used with plasmid pSL82 to cotransform strain UVM8. Two of 30 *niaD*$^+$ transformants accumulated NA on CAM, suggesting that pAPSa8 complemented *fas-1A* (and not *nor-1*) in UVM8. Control transformants (pSL82 only) did not produce NA on CAM.

TLC analysis of the recipient strain and transformed isolates (Fig. 2) determined that UVM8 failed to produce detectable AFB1 or NA, whereas UVM8 transformed with pAPXb28 produced AFB1. UVM8 transformed with pAPSa8 produced NA and AFB1 at levels similar to those of the *nor-1* mutant *A. parasiticus* 24690. UVM7 produced low levels of AFB1 and aflatoxin G1 (AFG1), suggesting that the mutations in UVM7 and UVM8 are not allelic.

Gene disruption of *fas-1A*. Transcript mapping analysis previously localized a 7.5-kb transcript to the *fas-1A* locus (25) (Fig. 1). A 2.8-kb *Eco*RI cosmid NorA subclone (pRZ2.8 [Fig. 3]) from the middle of the 7.5-kb coding region was used to disrupt *fas-1A* in strain B62, an NA-accumulating strain. Approximately 4% of the *niaD*$^+$ transformants failed to produce detectable NA or AFB1 when grown on CAM, suggesting that they were *fas-1A* disruptants. All pSL82 transformants (control) produced NA on CAM. No transformants were obtained in the absence of plasmid DNA.

Three putative *fas-1A* disruptant clones, Dis1, Dis2, and Dis3, were subjected to TLC (Fig. 4) and direct competitive ELISA analyses. Dis1, -2, and -3 did not produce detectable NA or AFB1, whereas B62 transformed with pSL82 (control) accumulated NA and low levels of AFB1. Direct competitive ELISA confirmed that B62 transformed with pSL82 (control) produced 300-fold more AFB1 (3 to 5 μg/ml) than Dis1, Dis2, and Dis3 (0.002 to 0.015 μg/ml; near the limit of detection). Strain SU1 (wild type) produced approximately 6,000-fold more AFB1 (100 μg/ml) than Dis1, -2, and -3 and approximately 20-fold more AFB1 than strain B62.

The disruption of *fas-1A* in Dis1, -2, and -3 was confirmed by Southern hybridization analysis with the ^{32}P-labeled 2.8-kb *Eco*RI fragment of pRZ2.8 as a probe (Fig. 5). Genomic DNA isolated from Dis1 and Dis2 contained the expected 1.4- and 4.3-kb *Hin*dIII fragments (Fig. 3) in addition to the 3.4-kb and 2.7-kb fragments present in the recipient strain B62. Dis3 contained only the 2.7-kb DNA fragment, suggesting that genetic recombination between tandem copies of *fas-1A* resulted in the deletion of part of both copies of *fas-1A* plus the vector sequences in between. The deletion was not due to a precise excision of pRZ2.8 because the 3.4-kb *Hin*dII fragment was deleted and because *fas-1A* remained nonfunctional. The same filter was reprobed with ^{32}P-labeled pBluescriptIISK(−) (data not shown), which hybridized to a 4.2-kb *Hin*dIII fragment, as expected, in Dis1 and Dis2 but not in Dis3, consistent with the hypothesized deletion event. The vector DNA hybridized to a 2.8-kb *Hin*dIII fragment in all three disruptants, as expected, because of the integration of pSL82 (which contains pUC19) at the *niaD* locus. Southern hybridization analyses on identical

TABLE 2. Sclerotium production in *A. parasiticus*

Strain[a]	No. of sclerotia[b]	Avg diam[b] (μm)
B62	600	470 ± 130
SU1 (Afl$^+$)	2,400	470 ± 90
Dis1	3,000	490 ± 140
Dis2	2,100	460 ± 100
Dis3	5,400	460 ± 100

[a] B62 is the recipient strain used in the *fas-1A* disruption experiment. SU1 is an aflatoxin-producing wild-type strain. Dis1, -2, and -3 are three *fas-1A* disruptants selected for further study.
[b] Means of two experiments.

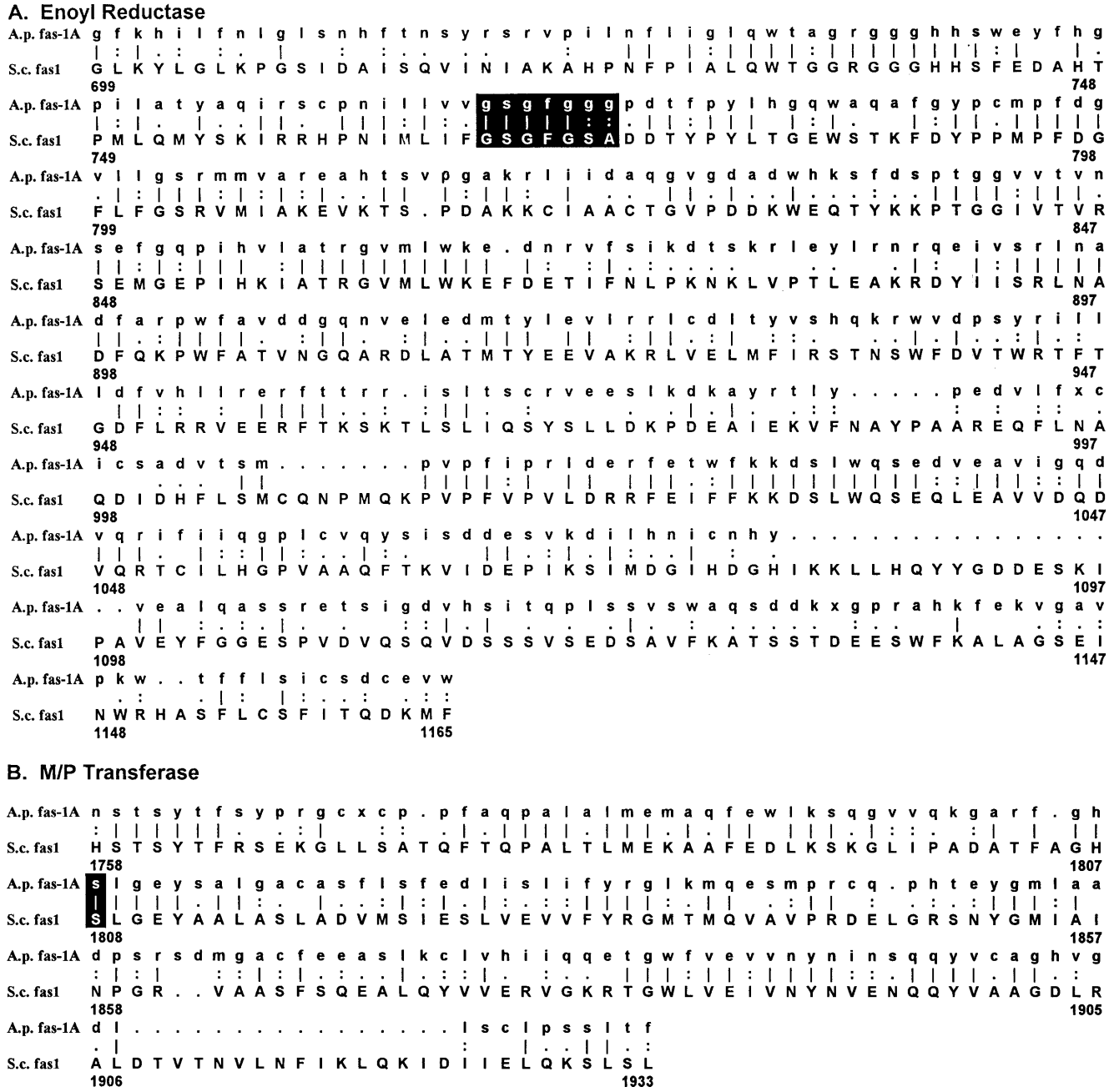

FIG. 6. Gap (GCG) comparison of *A. parasiticus fas-1A* and *S. cerevisiae FAS1* products (15). Predicted amino acid sequences encoded in two regions of *fas-1A* were compared with functional domains in the yeast *FAS1* gene product by using the GCG software Gap. (A) Gap analysis of the enoyl reductase functional domain. The putative active-site motif is highlighted. (B) Gap analysis of malonyl/palmityl (M/P) transferase functional domain. The putative active-site residue (serine) is highlighted. Numbers in the yeast amino acid sequence are those reported by Kottig et al. (15). Vertical lines between residues in the comparison represent identity, two dots represent more highly conserved substitutions, and a single dot represents less highly conserved substitutions.

genomic DNAs digested with *Sca*I confirmed the *Hin*dIII data (not shown).

Sclerotium development. Dis1 and Dis2 produced four- to fivefold and Dis3 produced approximately ninefold more sclerotia than B62 (Table 2). The number of sclerotia produced by the aflatoxin-producing strain SU1 was similar to that produced by Dis1 and Dis2; however, Dis3 produced twofold more sclerotia than SU1.

DISCUSSION

Because *fas-1A* is necessary for synthesis of NA, this argues that *fas-1A* encodes either noranthrone oxidase (10) or an activity involved in polyketide backbone synthesis. The large size of the *fas-1A* transcript suggested that it might encode a multifunctional protein, similar to *pksA* (7, 25). Nucleotide sequence analysis was conducted on two extensive regions of *fas-1A* to determine if predicted amino acid sequence data

might provide clues about *fas-1A* function (Fig. 6). Comparison of the predicted amino acid sequence of the *fas-1A* product with proteins in the GenBank and EMBL databases with the TFastA program detected a high level of identity with FAS1 proteins from *Saccharomyces cerevisiae* and *Yarrowia lipolytica* (15). *FAS1* encodes the beta subunit of FAS, a protein which contains four functional domains typical of FASs, including (from amino terminus to carboxyl terminus) acetyltransferase, enoyl reductase, dehydratase, and malonyl/palmityl transferase (15). A 435-amino-acid region in the *fas-1A* product displayed 40% identity and 58% similarity with the enoyl reductase domain in FAS1, while a 159-amino-acid region displayed 47% identity and 69% similarity to the malonyl/palmityl transferase domain (including the active-site residues). These two domains appeared in the same relative position and order in the *fas-1A* product as in FAS1. These data strongly suggest that *fas-1A* encodes the beta subunit of a yeast-like FAS1 and support our new designation for this gene, *fas-1A* (formerly *uvm8*).

Townsend et al. (22) proposed that hexanoate, a six-carbon fatty acid, was the starting molecule for polyketide synthesis because NA, the first stable intermediate in AFB1 synthesis, contains a six-carbon "tail" in which two keto groups are completely reduced to hydrocarbon. Hexanoate was proposed to be extended to noranthrone, without further ketoreduction, by a PKS. Our data support this model; the *fas-1A* product is proposed to synthesize hexanoate (or a similar fatty acid starter unit), while the *pksA* product extends hexanoate to noranthrone.

Trail et al. (25) reported that the *fas-1A* transcript accumulates under aflatoxin-inducing conditions with the same pattern as *nor-1* and *ver-1* (20), suggesting that *fas-1A*, like *nor-1* and *ver-1*, is involved in secondary metabolism. Disruption of *fas-1A* in the current study had no apparent effect on the growth of *A. parasiticus* on CZ, a defined minimal growth medium that contains no added fatty acids. Together, the data suggest that *fas-1A* is involved in AFB1 synthesis and not in the synthesis of fatty acids required for growth.

Disruption of *fas-1A* also enhanced sclerotium development compared with the parental strain B62, a phenotype similar to *pksA* disruptants (25). Since no AFB1 pathway intermediates accumulate in Dis1, Dis2, or Dis3 or *pksA* disruptants, the accumulation of certain pathway intermediates (i.e., NA, AVN, and VA) appears to downregulate sclerotium development. This hypothesis is supported by previous observations. Strain CS10 (*ver-1 wh-1 pyrG*), which accumulates VA (an intermediate near the middle of the AFB1 pathway), produces few sclerotia on CAM at 30°C (19). Complementation of CS10 with *ver-1* restores wild-type levels of AFB1 synthesis and sclerotium production. The nature of the interaction between AFB1 synthesis and sclerotium development remains unclear and deserves further study.

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