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Analysis of Ferrichrome Biosynthesis in the Phytopathogenic Fungus *Ustilago maydis*: Cloning of an Ornithine-N^5-Oxygenase Gene

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By using a non-enterobactin-producing *enb-7* mutant of *Salmonella typhimurium* LT2 as a biological indicator, a novel screening method was developed for identifying mutants of *Ustilago maydis* defective in the biosynthesis of the siderophores ferrichrome and ferrichrome A. Two classes of siderophore mutations, both recessive, were isolated after mutagenesis of haploid cells of the corn smut fungus. Class I mutants no longer produced ferrichrome while retaining the ability to produce ferrichrome A; class II mutants were defective in the production of both ferrichrome and ferrichrome A. Genetic and biochemical data suggest that class II mutants are defective in the ability to hydroxylate L-ornithine to δ-*N*-hydroxyornithine, the first step in the biosynthesis of these siderophores. A genomic library of wild-type *U. maydis* DNA was constructed in the cosmid transformation vector pCU3, which contains a dominant selectable marker for hygromycin B resistance. Two cosmids, pSid1 and pSid2, were identified in this library by their ability to complement class II siderophore auxotrophs. The production of both siderophores was concomitantly restored in the majority of the resultant transformants. Transforming DNA could be recovered from the fungal, cosmid-containing transformants by in vitro packaging with lambda bacteriophage extracts. Alternatively, the clones could be identified by a sib selection procedure. Cotransformation was found to occur at a high frequency in the fungus and was used to determine that a 2.5-kilobase *Hin*dIII-*Nru*I fragment in pSid1 was responsible for complementing the class II siderophore biosynthetic mutation.

During iron stress, most microorganisms produce a group of low-molecular-weight compounds termed siderophores, which have a specific affinity for ferric ions (21). Ferrichrome, the first chemically defined siderophore, was discovered by Neilands in 1952 from the basidiomycete *Ustilago sphaerogena* (20). Since then, ferrichrome has been detected in many basidiomycetes and ascomycetes, including *Ustilago* spp., *Penicillium* spp., *Aspergillus niger*, and *Tilletiaria anomala* (21, 35).

Recently, a systematic investigation of the biological significance of the siderophores of the corn smut fungus *Ustilago maydis* has been initiated (15). Budde and Leong (A. D. Budde and S. A. Leong, submitted for publication) have isolated and characterized two siderophores, ferrichrome and ferrichrome A, from the fungus. The chemical structure of ferrichrome and ferrichrome A are closely related. Both are cyclic hexapeptides that contain three residues of a unique amino acid, δ-*N*-hydroxyornithine. The δ-N group is acylated by *trans*-β-methylglutaconic acid in ferrichrome A and by acetic acid in ferrichrome. The remainder of the ferrichrome A peptide moiety consists of two serines and one glycine, while ferrichrome contains three glycines. The study also examined 47 *U. maydis* strains, including laboratory strains and natural isolates collected from Wisconsin, and found that all of these strains produced both ferrichrome and ferrichrome A (Budde and Leong, submitted).

Genetic and molecular biological studies on biosynthesis and transport of fungal siderophores have been scarce. Progress on these fundamental aspects of fungal siderophore research has been impeded, in part, by the difficulty of isolation of relevant fungal mutants. In the only case reported, a mutant defective in the production of rhodotorulic acid, a hydroxamate siderophore from the basidiomycete yeast *Rhodotorula pilimanae*, has been obtained (16).

U. maydis can be readily manipulated genetically (12) and molecularly (32); this fungus offers an attractive system for evaluating the role of siderophores in fungal saprophytic growth as well as in the interaction of the pathogen with the host plant, corn. As the first step toward understanding these processes, we report here (i) the isolation of fungal mutants defective in biosynthesis of siderophores, (ii) biochemical and genetical characterization of these mutants, and (iii) the isolation and analysis of DNA clones that complement fungal siderophore auxotrophs defective in ornithine-N^5-oxygenase. During the course of this work, we have also developed a series of molecular genetic techniques useful in isolation of genes from this fungal pathogen.

MATERIALS AND METHODS

Media and strains. Potato dextrose agar was from Difco Laboratories (Detroit, Mich.). Minimal, complete, and plate mating media used in *U. maydis* studies were prepared as previously described (12), except that potassium nitrate was replaced by ammonium nitrate as the nitrogen source in the minimal medium and Casamino Acids was added at a concentration of 1% in the plate mating medium. Low-iron (LI) medium (9) and E medium (30) were prepared as described previously with Noble agar. ME medium was a modified form of medium E and was composed of two layers; the lower layer contained 20 ml of medium E, and the upper one contained about 4 ml of soft agar (0.7% Noble agar) in physiological saline and seeded with 30 μl of an overnight nutrient broth culture of the *Salmonella typhimurium* LT-2

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TABLE 1. *U. maydis* strains

Strain	Relevant characteristic[a]	Source
518	Wild type; *a2b2*	R. Holliday
521	Wild type; *a1b1*	R. Holliday
227	*ade1-1 met1-2 nar1-6 rec2-1 a2b2*	R. Holliday
288	*pan1-1 ino1-3 nar1-1 rec1-1 a1b1*	R. Holliday
S1	NTG mutant from 518; Fec^- *a2b2*	This work
S2	NTG mutant from 518; Fec^- FeA^- *a2b2*	This work
S8	NTG mutant from 518; Fec^- FeA^- *a2b2*	This work
S9	NTG mutant from 518; Fec^- FeA^- *a2b2*	This work
S11	NTG mutant from 518; Fec^- FeA^- *a2b2*	This work
S15	NTG mutant from 518; Fec^- FeA^- *a2b2*	This work
S16	NTG mutant from 518; Fec^- FeA^- *a2b2*	This work
S20	Derived from cross between S15 and 521; Fec^- FeA^- *a2b2*	This work
S23	Derived from cross between S2 and 521; Fec^- FeA^- *a1b1*	This work
S23pSid1-1	S23 transformed with pSid1; Hyg^r Fec^+ FeA^+	This work
S23pSid1-2	S23 transformed with pSid1; Hyg^r Fec^+ FeA^+	This work
S23pSid1-8	S23 transformed with pSid1; Hyg^r Fec^- FeA^-	This work
S238.1-1	S23 transformed with pUC18-8.1; Hyg^r Fec^+ FeA^+	This work

[a] Abbreviations: Fec^-, no ferrichrome production; FeA^-, no ferrichrome A production; Hyg^r, hygromycin B resistant.

mutant carrying *enb-7* (26). The upper layer was poured immediately before the fungal cells were applied to the surface of the plate.

S. typhimurium LT2 carrying *enb-7* was a kind gift from J. B. Neilands. *Escherichia coli* DH5α [ϕ80d*lacZ* ΔM15 *endAL recAL hsrR17* (r_K^- m_K^+) *sup-44 thi-1* λ^- *gyrA* F^- Δ(*lacZYA-argF*)*U169*] was from Bethesda Research Laboratories, Inc. (Gaithersburg, Md.). *U. maydis* strains used in this study are shown in Table 1.

Chemicals. *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) was from Sigma Chemical Co. (St. Louis, Mo.). Hygromycin B was purchased from Calbiochem-Behring (San Diego, Calif.). Novozym 234 was obtained from Novo Laboratories (Wilton, Conn.). Restriction endonucleases and other DNA-modifying enzymes were purchased from either Bethesda Research Laboratories or New England BioLabs, Inc. (Beverly, Mass.). Gigapack Gold packaging mix was from Stratagene (San Diego, Calif.). All other chemicals were American Chemical Society reagent grade or equivalent. The water employed was deionized by using a filtration system (Millipore Corp., Bedford, Mass.) and had a resistance of 15 mΩ/cm or greater. Authentic ferrichrome and ferrichrome A were gifts from J. B. Neilands.

Isolation of fungal siderophores. Ferrichrome and ferrichrome A were detected by the ferric perchlorate assay (4). Siderophores were extracted from culture supernatants and analyzed by thin-layer chromatography or high-pressure liquid chromatography (HPLC) as described elsewhere (Budde and Leong, submitted).

Mutagenesis of *U. maydis*. Haploid cells of *U. maydis* were grown in 100 ml of liquid complete medium until early log phase (1×10^7 to 5×10^7 cells per ml), collected by using a table centrifuge at 2,000 × *g*, washed once, and suspended in 19 ml of water in a 100-ml sterile Erlenmeyer flask. After adding 1 mg of NTG, freshly dissolved in 20% ethanol solution, the cell culture was shaken at 250 rpm at 28°C for 40 min. The treatment was stopped by adding 1 ml of ice-cold 1 M Tris hydrochloride (pH 7.0). The cells were collected by centrifugation as above, washed twice with 50 mM Tris hydrochloride buffer, suspended in 10 ml of complete medium, and incubated at 28°C with shaking for 3 h. The cells were then collected by centrifugation, resuspended in 2 ml of complete medium, and maintained at 4°C in the dark for at least 2 days but not longer than 2 weeks before plating. Typically, 95 to 99% of the fungal cells were killed by this treatment.

***U. maydis* genetic techniques and DNA transformation.** Genetic crosses were carried out on maize cultivar Golden Bantam by standard procedures (12). Diploid cells were constructed as described elsewhere (27). DNA transformation and isolation were carried out as previously reported (32).

Biochemical feeding with δ-*N*-hydroxyornithine. δ-*N*-Hydroxyornithine was prepared by acid hydrolysis of rhodotorulic acid as described elsewhere (3). The concentration of δ-*N*-hydroxyornithine in the preparation was determined by the tetrazolium assay with hydroxyamine as the standard (29). Less than 0.05% of the starting rhodotorulic acid remained in these preparations, as measured by the ferric perchlorate assay. δ-*N*-Hydroxyornithine was stored in acidic solution (pH about 1.0) at −20°C and adjusted to pH 5.0 with NaOH before use. *U. maydis* was grown at 28°C in LI medium to middle-or-later log phase (optical density at 600 nm equaled 1.0 to 1.6), supplemented with δ-*N*-hydroxyornithine, and further incubated with vigorous shaking for 36 h. Culture supernatants were analyzed for siderophores as described above. To minimize the degradation of δ-*N*-hydroxyornithine, the pH of LI medium used in feeding experiments was lowered to 5.5 with HCl.

Construction of cosmid pCU3 and *U. maydis* cosmid genomic library. A 3.3-kilobase (kb) *Hin*dIII fragment of *U. maydis* transformation plasmid vector pHL1 (32) was inserted into the *Hin*dIII site of pBR322 to generate pCU2. This 3.3-kb fragment contains a transcriptional fusion of an *U. maydis hsp70* promoter with the bacterial hygromycin B phosphotransferase (10) and confers hygromycin B resistance in *U. maydis*. pCU2 was restricted at the unique *Sph*I site, and the ends were repaired with T4 DNA polymerase and then ligated to a *cos*-containing *Bgl*II fragment (25), which had been blunt ended by treatment with Klenow fragments. The resultant cosmid, pCU3, is 9.0 kb in size and contains a unique *Bam*HI site for the cloning of large (about 40-kb) DNA inserts.

High-molecular-weight DNA was isolated from *U. maydis* wild-type strain 518 (32). After partial digestion with *Sau*3A, the DNA was size fractionated by centrifugation on a sucrose density step (10 to 40%) gradient (19). The fractions containing 35- to 55-kb DNA fragments were pooled together, precipitated by the addition of ethanol, and suspended in TE buffer (10 mM Tris hydrochloride [pH 7.4], 1 mM EDTA [pH 8.0]) to give a final DNA concentration of 0.5 μg/μl. After treatments with *Bam*HI and, subsequently, calf intestine alkaline phosphatase, 2 μg of pCU3 was ligated to 1 μg of the *Sau*3A-digested genomic DNA in a 20-μl volume, using T4 DNA ligase at 14°C for 18 h. A 5-μl aliquot of the ligation solution was packaged in vitro, using Gigapack Gold packaging mix. The diluted bacteriophage particles were transduced into *E. coli* DH5α according to the supplier's manual. Approximately 80,000 ampicillin-resis-

tant colonies were obtained from 1 μg of insert DNA. A total of 3,360 individual cosmid clones were stored at −70°C in LB containing 8% dimethyl sulfoxide and 50 μg of ampicillin per ml.

RESULTS

Isolation of siderophore auxotrophic mutants. Since the wild-type fungus produces two siderophores, ferrichrome and ferrichrome A (Budde and Leong, submitted), direct screening for ferrichrome auxotrophs by replica plating onto iron-deficient medium was not feasible. The *S. typhimurium* LT-2 mutant carrying *enb-7* has lost the ability to produce its own siderophore, enterobactin, and therefore shows no visible growth on ME medium. This iron-deficient medium contains 9.5 mM citrate, which strongly inhibits iron transport in this organism (26). The growth of the mutant carrying *enb-7* on ME medium can be greatly promoted by adding a small amount of ferrichrome to the medium; ferrichrome A did not have the same effect on the strain carrying *enb-7* and did not interfere with the growth-promoting effect of ferrichrome (data not shown). Spotting a *U. maydis* colony or adding $FeSO_4$ solution onto the agar surface gave rise to the same growth-promoting effect. These observations indicated that the observed growth of the strain carrying *enb-7* on the iron-deficient medium was a result of the cross-feeding of ferrichrome secreted by the fungal colony. A number of enteric bacteria, such as *S. typhimurium* and *E. coli*, possess a ferrichrome-specific outer membrane receptor (FhuA protein) and consequently can efficiently utilize this heterologous siderophore as an iron scavenging agent (18). Thus, it was assumed that a *U. maydis* mutant defective in ferrichrome biosynthesis would fail to promote the growth of the mutant carrying *enb-7*.

Originally, the screening was carried out by individually patching thousands of fungal colonies into test plates. Subsequently, mutagenized fungal cells were spread directly on medium E containing 5 mM $AlCl_3$. After 2 to 3 days of growth, the fungal colonies were visible and soft agar containing the *S. typhimurium* LT2 mutant carrying *enb-7* was overlaid. Ferrichrome diffuses rapidly in agar plates and, therefore, the key to the success of this direct screening method was the addition of a high concentration of $AlCl_3$ to the medium. Aluminum ion is able to form a complex with deferriferrichrome (2, 17) and presumably reduces the effective concentration of ferrichrome secreted by the fungus, making it unavailable for iron transport in areas in which the aluminum concentration exceeds that of the siderophore. As the result, the apparent size of the bacterial growth halo was considerably reduced. This permitted the direct screening of up to 300 colonies per plate. The colonies that could not support the growth of the bacterial indicator were singled out as candidate mutants and purified. The isolates were then grown in 100 ml of LI medium for at least 4 days, and the siderophores were extracted from the supernatant as previously described (Budde and Leong, submitted). The purified samples were dissolved in a small volume of methanol, 200 to 600 μl when samples were from wild-type isolates and less than 50 μl when samples were from siderophore auxotrophs, and subjected to thin-layer chromatography and HPLC analysis. The extracts of wild-type controls usually contained >1.0 mg of ferrichrome and >10 mg of ferrichrome A, equivalent to 28 mM and 200 mM, respectively, in a volume of 50 μl. The limit of detection by the HPLC analysis is 25 μM (about 0.9 μg of ferrichrome or 1.25 μg of ferrichrome A in 50 μl). Thus, failure to detect the siderophores in the cell culture supernatants by the HPLC analysis was considered as evidence that these cells did not produce siderophores. In all the cases examined, the fungal cells that were unable to promote growth of the strain carrying *enb-7* on ME medium failed to produce ferrichrome.

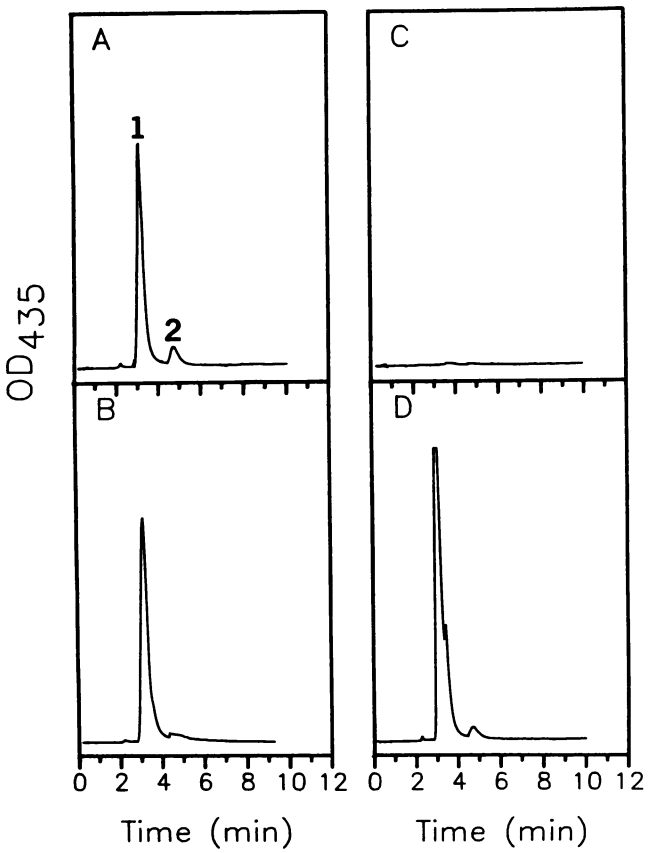

FIG. 1. HPLC analysis of extracts of *U. maydis* defective in the biosynthesis of the siderophores and of siderophores of complemented *U. maydis* transformants. All samples were extracted from 4-day-old culture supernatants of cells grown in 100 ml of LI medium with vigorous shaking and were analyzed by HPLC as described elsewhere (Budde and Leong, submitted). The ordinate of the chromatogram represents optical density at 435 nM; the abscissa represents retention time in minutes. (A) Extract of wild-type isolate 518. A 1.5-μl sample of a total sample volume of 0.6 ml was analyzed. Peak 1 represents ferrichrome A, and peak 2 represents ferrichrome. (B) Extract of the class I siderophore mutant S1. A 1-μl sample of a total sample volume of 0.4 ml was analyzed. (C) Extract of the class II siderophore mutant S2. A 1-μl sample of a total sample volume of 0.05 ml was analyzed. (D) Extract of S23pSid1-1, a complemented transformant carrying cosmid clone pSid1. A 1-μl aliquot of a total sample volume of 0.4 ml was analyzed.

A total of 11 *U. maydis* isolates defective in ferrichrome synthesis were obtained after screening more than 35,000 fungal colonies. Ten of them appeared to grow normally in LI and minimal media. One mutant, S3, showed marginal growth on ME medium and failed to grow in minimal medium. Normal growth and biosynthesis of both ferrichrome and ferrichrome A were restored when L-ornithine (200 mg/liter) was added to the medium.

Analysis of culture supernatants of the other 10 ferrichrome mutants demonstrated that 4 mutants contained ferrichrome A (Fig. 1). Mutants which had a ferrichrome-minus (Fec^-) phenotype were thereafter referred to as class I siderophore mutants. The remaining six isolates had lost

TABLE 2. Meiotic segregation of siderophore auxotrophic traits in basidiospores[a]

Cross	No. of Fec^-/Fec^+ segregants[b]	No. of Fec^- FeA^- segregants/no. of Fec^- segregants tested[c]
288 × S2	349[d]/358[e]	15/15
521 × S2	240/260	ND[f]
288 × S9	94/102	12/12
288 × S11	105/91	12/12
288 × S16	78/92	9/9

[a] Fec^+, ferrichrome production. Other abbreviations are the same as in footnote *a* of Table 1.
[b] Determined by bioassay with the strain carrying *enb-7*.
[c] Fec^- segregants were inoculated into 10 ml of LI liquid medium and vigorously shaken at 28°C for 4 days. The resultant culture supernatants were mixed with the ferric perchlorate solution at a 1:1 ratio by volume, and the solutions were measured at an optical density of 495 nm with a spectrophotometer.
[d] A total of 150 Fec^- segregants were examined for the auxotrophic markers *ino* and *pan*. The number of cells carrying the following genotypes was 35 (*ino pan*), 39 (ino^+ *pan*), 32 (*ino* pan^+), and 44 (ino^+ pan^+).
[e] A total of 150 Fec^+ segregants were examined for the auxotrophic markers *ino* and *pan*. The number of cells carrying the following genotypes was 32 (*ino pan*), 33 (ino^+ *pan*), 39 (*ino* pan^+), and 46 (ino^+ pan^+).
[f] ND, Not determined.

TABLE 3. Conversion of δ-*N*-hydroxyornithine to siderophores by class II mutants[a]

Strain	Supplement[b] (mg) to LI medium	Amt[c] (mg) of:	
		Ferrichrome	Ferrichrome A
518	None	0.084	0.41
S2	HO (2)	0.26	0.84
S2	Orn (20)	ND	ND
S8	HO (5)	0.43	3.58
S8	Orn (20)	ND	ND
S9	HO (5)	0.92	3.10
S11	HO (5)	0.36	2.32
S11	Orn (20)	ND	ND
S20	HO (5)	0.52	1.02
S23	HO (5)	0.76	3.31
S23	Orn (20)	ND	ND
S3	Orn (50)	0.36	1.17
S3	None	NG	NG
S1	HO (5)	0.00	5.10

[a] LI medium cultures (100 ml) were supplemented as indicated. After 36 h of growth, the siderophores were extracted from the supernatant as described elsewhere (Budde and Leong, submitted).
[b] Abbreviations: HO, δ-*N*-hydroxyornithine; Orn, L-ornithine.
[c] Total amount of siderophores in 100 ml of spent supernatant, as characterized and quantitated by HPLC analysis. ND, not detected by ferric perchlorate assay. NG, no growth.

the ability to produce ferrichrome A as well as ferrichrome (Fig. 1). We refer to these six isolates with the phenotype Fec^- FeA^- as class II siderophore mutants.

Genetic analysis of siderophore genes. The siderophore mutants, derivatives of strain 518 (*a2b2*), were crossed with the compatible strain 521 (*a1b1*), wild type, or 288 (*a1b1 ino1-3 pan1-1 nar1-1 rec1-1*). These crosses resulted in the introduction of defective siderophore genes into strains carrying various auxotrophic markers and mating types. The resultant strains were later used to construct diploid cells. Segregation of the ferrichrome auxotrophic traits from these crosses was recorded and is shown in Table 2. In these crosses, the segregation patterns gave a ratio of 1:1 for segregation of the siderophore mutation versus the wild-type allele. A number of segregants were grown in LI medium, and the culture supernatants were examined for production of ferrichrome and ferrichrome A. Neither siderophore was detected from strains that were unable to restore growth of the mutant carrying *enb-7* (Table 2).

Conversion of δ-*N*-hydroxyornithine to ferrichrome and ferrichrome A by the class II mutants. To further characterize the nature of the class II siderophore mutations, biochemical complementation experiments were conducted. The siderophore mutants were cultured for 36 h in 100 ml of LI medium supplemented with δ-*N*-hydroxyornithine, and the culture supernatants were analyzed for siderophores by HPLC. All the class II siderophore mutants, S2, S8, S9, S11, S20, and S23, were capable of converting δ-*N*-hydroxyornithine to both ferrichrome and ferrichrome A (Table 3). Addition of L-ornithine did not restore the production of either siderophore.

The feeding tests were also extended to characterize the other siderophore mutants (Table 3). As mentioned earlier, normal growth of the ornithine auxotroph S3 was restored as well as the ability to synthesize both siderophores when the growth medium was supplemented with L-ornithine. For the class I mutant S1, which no longer produces ferrichrome but still synthesizes ferrichrome A, supplementing the medium with δ-*N*-hydroxyornithine did not restore the biosynthesis of ferrichrome (Table 3). It is conceivable that the class I mutation blocks later step(s) in the biosynthesis of ferrichrome, after the hydroxylation of ornithine. Previous studies have indicated that δ-*N*-hydroxyornithine is a common precursor of both ferrichrome and ferrichrome A and that the biosynthetic pathways of these two siderophores branch afterwards (7).

Genetic complementation of siderophore mutants. *U. maydis* is a heterothallic fungus, and diploid strains carrying siderophore mutations can be constructed by fusing haploid cells of compatible mating type, which carry complementing auxotrophic markers on plate mating medium. The original siderophore mutants were first crossed to strain 288 (*pan1-1 ino1-3 nar1-1 rec1-1 a1b1*) in planta. The basidiospore segregants of these crosses were screened for isolates carrying the siderophore mutation and with the genotype of *pan1-1 ino1-3 a1b1*. The resultant isolates were then crossed with strain 227 (*ade1-1 met1-2 nar1-6 rec2-1 a2b2*) and screened for haploid segregants carrying the siderophore mutation and with the genotype of *ade1-1 met1-2 a2b2*. Diploids constructed between these complementary haploids were tested for pathogenicity to confirm that they were diploids and not prototrophic revertants. The diploids were then tested for their ability to support growth of the *S. typhimurium* LT2 strain carrying *enb-7* on ME medium. The results indicated that both the class I and class II mutations are recessive (data not shown).

Identification of clone pSid1 that complements *U. maydis* siderophore auxotroph S23. The above genetic and biochemical data argue that the class II mutants are defective in the ability to hydroxylate L-ornithine to δ-*N*-hydroxyornithine, the first committed step in biosynthesis of the siderophores (7). An integrative DNA transformation system (32) has been established for *U. maydis* which provides a general and direct approach to isolate genes from this plant pathogen. Two ways of recovering vector DNA from fungal transformants have been described. Yelton et al. (36) discovered that cosmid clones in *Aspergillus nidulans* can be packaged in vitro by lambda bacteriophage packaging extract and recovered in *E. coli* HB101 transfected with the phage particles. In *Neurospora crassa*, a sib-selection procedure has been employed to identify complementing clones (31).

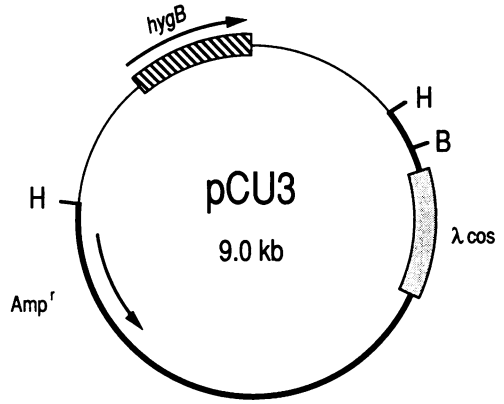

FIG. 2. Cosmid pCU3. Abbreviations: *hygB*, coding region of the bacterial hygromycin B phosphotransferase of pLG90 (11); *cos*, bacterial phage lambda *cos* site; H, *Hin*dIII; B, *Bam*HI. The thick line represents the DNA sequence of pBR322, and the thin line represents a *U. maydis* DNA insert containing a portion of an *hsp70* gene of this fungus (32).

pCU3 (Fig. 2) is a derivative of pBR322 and confers ampicillin, but not tetracycline or hygromycin B, resistance in *E. coli*. The 3.3-kb *Hin*dIII fragment of the *U. maydis* transformation vector pHL1 (32) was incorporated into this cosmid and serves as a strong dominant selectable marker for *U. maydis*. The cosmid library, prepared from nuclear DNA of strain 518, includes 3,360 clones containing an average insert size of about 41 kb. Assuming *U. maydis* has a genome size of 21,000 kb (R. Duran, Abstr. 52nd Annu. Meet. Mycol. Soc. Am., p. 141, 1987), our library is sufficient to represent the fungal genome with >99.8% confidence.

U. maydis S23 (Table 1), one of the class II siderophore mutants, was used as the transformation recipient. A simple screening procedure was designed to identify fungal cells that were restored in ferrichrome production. S23 was separately transformed with cosmid DNA from 24 pools of 96 clones, representing about two-thirds of the total genomic library. Approximately 5 μg of DNA was used in each transformation. After 4 to 5 days of incubation, the hygromycin B-resistant transformants of each pool (200 to 1,000) were scraped separately from the plates into tubes containing 2 ml of liquid complete medium. The cells were suspended, diluted with H_2O, and spread on ME plates. After 16 to 24 h of incubation at 28°C, the plates were examined for fungal colonies that displayed a surrounding zone of bacterial growth. Six ferrichrome-producing fungal colonies were identified among the transformants of cosmid library pool number 13. This pool was broken down into 12 subpools of 8 clones, and DNA from these subpools was employed to transform S23. Subpool 3 gave rise to transformants that could promote the growth of the *S. typhimurium* LT2 mutant carrying *enb-7* on medium E. Finally, among individual clones from the subpool, one clone (13-3-8) was identified as the complementing clone in this pool. This clone was designated pSid1.

About 90% of the hygromycin B-resistant transformants harboring the pSid1 clone were found to be positive in the bioassay using the *S. typhimurium* LT2 strain carrying *enb-7*; namely, the transformants were capable of promoting the growth of the strain carrying *enb-7* on medium E to the same degree as the wild-type strain 518 did (data not shown). Two randomly selected transformants, S23pSid1-1 and S23pSid1-2, were inoculated into 100 ml of LI medium. Four days after incubation, the culture supernatants were extracted and analyzed for siderophores. Both ferrichrome and ferrichrome A were present in these two cultures. As a control, recipient strain S23 and a transformant of S23 carrying pCU3 were also subjected to the analysis and no siderophores were found in these culture supernatants. Representative results from these experiments are shown in Fig. 1. The two complemented transformants S23pSid1-1 and S23pSid1-2 were also cultured under iron-replete conditions by supplementing the LI medium with 10^{-5} M $FeSO_4$. Like wild-type strain 518, no measurable amounts of either ferrichrome or ferrichrome A were detected in 4-day-old cultures of either strain. This indicated that siderophore production in these two strains was under the same regulatory constraints as in the wild-type strain.

pSid1 was then used to transform S2, as well as three other independently isolated *U. maydis* class II siderophore auxotrophs. Like S23, these four mutants, S2, S9, S11, and S20, also failed to produce ferrichrome and ferrichrome A and may be defective in the conversion of L-ornithine to δ-*N*-hydroxyornithine. As for S23, among the hygromycin B-resistant transformants of the four mutants, the majority (77 to 90%) were able to promote growth of the *S. typhimurium* LT2 strain carrying *enb-7* (data not shown).

In vitro packaging of transformant DNA. Direct rescue of cosmid DNA from fungal transformants by in vitro packaging was successfully conducted in *A. nidulans* (36) but failed to work in *N. crassa* because the transforming DNA was substantially rearranged (6, 24). To assess the usefulness of this approach in *U. maydis*, we chose two transformants to test this method. One was S23pSid1-1, the complemented transformant of S23, and the other was S23pSid1-8, which failed to produce siderophores.

DNA isolated from S23pSid1-1 was packaged into lambda phage particles and used to transfect *E. coli* DH5α. Approximately, 60,000 ampicillin-resistant colonies were recovered from about 1 μg of DNA. For S23pSid1-8, the process of packaging was less efficient and only about 400 ampicillin-resistant colonies per μg of fungal DNA were obtained. Twelve ampicillin-resistant colonies were studied further; six carried S23pSid1-1 DNA, and the other six harbored cloned S23pSid1-8 DNA. The cosmid DNAs from the 12 strains were isolated and digested with *Hin*dIII or *Bam*HI, and the restriction patterns were compared with that of original clone pSid1. No alteration in the fragment banding pattern was detected in any of these 12 clones when compared with pSid1 (data not shown).

Identification of pSid2 through direct rescue of cosmid DNA from transformants. Guided by the success of the in vitro packaging experiment, a second approach was explored to identify clones that complemented S23. Cosmid DNA was isolated from another set of 4,000 clones from the *U. maydis* gene library. The DNA from the whole library was then used to transform S23. The transformation protocol was scaled up to accommodate 2×10^8 spheroplasts and 50 μg of input DNA. The resultant hygromycin B-resistant transformants were then screened for fungal colonies complemented for siderophore production as described above. Two fungal colonies were able to promote growth of the *S. typhimurium* LT2 strain carrying *enb-7*. DNA from one clone was packaged in vitro with Gigapack Gold and used to transfect *E. coli* DH5α. Cosmid DNA isolated from the transfected bacteria was designated pSid2. Comparison of the restriction pattern of pSid2 with that of pSid1 revealed that a number of

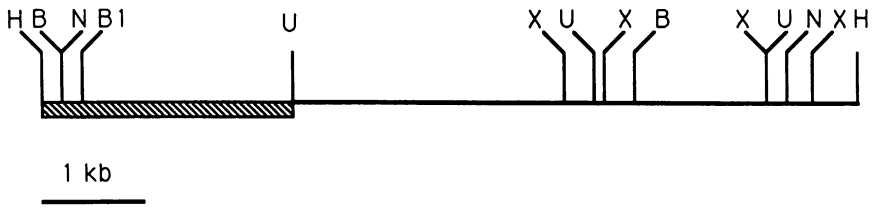

FIG. 3. Restriction map of the 8.1-kb *U. maydis* *Hin*dIII insert of pUC18-8.1. Abbreviations: H, *Hin*dIII; B, *Bgl*II; N, *Nco*I; B1, *Bgl*I; U, *Nru*I; X, *Xho*I. The 2.5-kb *Hin*dIII-*Nru*I fragment that restores siderophore biosynthesis in class II mutants (▨) is shown.

bands of similar size were present in the two complementing cosmids (data not shown).

Cotransformation and identification of a 2.5-kb *Hin*dIII-*Nru*I fragment responsible for complementing S23. High-frequency cotransformation is a common phenomenon in fungi (13, 34, 36). We tried this technique as a means of locating the region in pSid1 that was responsible for complementation of the class II siderophore mutation. The cosmid clone pSid1 has a fungal DNA insert of 45.9 kb with no *Bam*HI, *Hin*dIII, and *Xho*I restriction sites within the 3.3-kb *Hin*dIII selectable marker fragment. pSid1 was separately digested to completion with these three restriction enzymes. After phenol-chloroform extraction, the restricted cosmid DNA (2.5 μg) was used to transform the siderophore auxotroph S23. The hygromycin B-resistant transformants were first selected and then screened for ferrichrome production by the bioassay using the strain carrying *enb-7*. The number of Fec^+ transformants and the total number of transformants tested for each restriction endonuclease were as follows: 8 of 29 with *Hin*dIII, 39 of 64 with *Bam*HI, and 8 of 22 with *Xho*I. Since the restriction fragments (8 for *Hin*dIII, 4 for *Bam*HI, and 10 for *Xho*I) were present in equal quantity in the digests, this experiment demonstrates that cotransformation occurs at high frequency in *U. maydis*. This prompted us to take advantage of this technique and use the information from the cotransformation experiments to locate and subclone the DNA fragment that is responsible for complementing class II siderophore mutants.

pSid1 was digested with *Hin*dIII, and the restriction fragments were ligated into pUC18. The resultant clones were screened for clones containing *U. maydis* DNA inserts of 2.5, 8.1, 9.2, and 13.5 kb in size. These four *Hin*dIII fragments were shared by both pSid1 and pSid2, and one of them presumably carries the siderophore biosynthetic gene. DNA (2 μg) from four clones, pUC18-2.5, pUC18-8.1, pUC18-9.2, and pUC18-13.5, carrying the 2.5-, 8.1-, 9.2-, and 13.5-kb *Hin*dIII fragments of pSid1, respectively, were then individually cotransformed into S23 with the *U. maydis* transformation vector pHL1, which provided the dominant selectable marker. For a control, 1 μg of pHL1 was transformed into S23. Ferrichrome-producing transformants were only detected among the transformants carrying pUC18-8.1. Specifically, the number of Fec^+ transformants and the number of transformants tested were as follows: 0 of 48 for pHL1, 0 of 21 for pHL1 and pUC18-2.5, 32 of 67 for pHL1 and pUC18-8.1, 0 of 48 for pHL1 and pUC18-9.2, and 0 of 48 for pHL1 and pUC18-13.5. The HPLC analysis of the culture supernatants of the transformants carrying pUC18-8.1 revealed that the biosynthesis of both ferrichrome and ferrichrome A had been restored (data not shown). Further subcloning experiments have located the putative ornithine-N^5-oxygenase gene on a 2.5-kb *Hin*dIII-*Nru*I fragment (Fig. 3) on pUC18-8.1.

DISCUSSION

In this article, we have described a novel approach for isolation of fungal auxotrophs defective in siderophore biosynthesis. The approach employs the *S. typhimurium* LT2 mutant carrying *enb-7* as a biological indicator to screen for *U. maydis* ferrichrome auxotrophs. The technique is simple, and large numbers of cells can be easily screened with limited effort. The bioassay with the *S. typhimurium* LT2 mutant carrying *enb-7* does not seem to be influenced by components of the medium, provided that the concentration of iron is low. Besides ferrichrome, the strain carrying *enb-7* is also able to utilize other fungal siderophores as iron chelators (18). Therefore, this screening method can be readily adopted to isolate siderophore auxotrophs from fungal species which produce these siderophores.

Recently, Schwyn and Neilands (28) have reported a universal chemical assay for detecting siderophores. The method has been successfully applied to several microorganisms, mostly gram-negative bacteria, to isolate mutants defective in the synthesis, regulation, or transport of siderophores. This method should permit the isolation of other classes of siderophore mutants in *U. maydis*, mutants that are unable to produce ferrichrome A but which remain able to synthesize ferrichrome and mutants which produce siderophore constitutively. Unfortunately, hexadecyltrimethylammonium bromide, an ingredient required for the preparation of the screening medium of Schwyn and Neilands, is toxic to *U. maydis* (unpublished observation), and modifications of this assay will probably be necessary.

Two classes of *U. maydis* siderophore auxotrophs have been isolated in this study. The class I mutants are defective in the biosynthesis of ferrichrome but still are capable of producing ferrichrome A. The class II mutants fail to produce both ferrichrome and ferrichrome A. Genetic studies of the class II mutants suggest that in each mutant the lesion is caused by a defect in a single locus or in tightly linked genes, since (i) the mutation segregated 1:1, and (ii) the phenotype Fec^- FeA^- always cosegregated in the basidiospore segregants. Results of the feeding experiments substantiate this conclusion and indicate rather convincingly that the siderophore mutation occurs at the hydroxylation step of L-ornithine. The genetic complementation tests revealed that both class I and class II siderophore auxotrophs are recessive mutations. This implies that these mutations result in loss of functional gene products. By combining both genetic and biochemical data, we conclude that the class II mutants carry a genetic lesion in a single locus which is responsible for the first step of ferrichrome and ferrichrome A biosynthesis, the N hydroxylation of L-ornithine.

We have designed an effective screening procedure for fungal cells restored in ferrichrome production. This is achieved by spreading transformants directly on ME me-

dium, thus obviating the tedious task of picking and examining transformants individually. Cosmid clones capable of complementing class II siderophore mutants were identified from a genomic DNA library of a wild-type strain of *U. maydis*. The subcloning experiments show the complementing activity on a 2.5-kb *Hin*dIII-*Nru*I fragment. Although we have yet to prove that either clone actually carries the ornithine-N^5-oxygenase gene, the fact that pSid1 specifically complements S23 as well as other class II siderophore mutants implies that complementation was not caused by a suppressor gene. Moreover, all of the basidiospore segregants obtained from a cross between the two complemented transformants and wild-type strain 521 were capable of feeding the *S. typhimurium* indicator strain carrying *enb-7* (unpublished results), suggesting that the transforming DNA had integrated at or was closely linked to the original mutation.

We have noticed that a small portion (10 to 30%) of pSid1 transformants fail to complement the siderophore-minus recipient S23. This phenomenon is not due to gross rearrangement of cosmid DNA sequences, since the transforming DNA recovered from one such transformant, S23pSid1-8, showed the same restriction pattern as the original pSid1 clone. On the other hand, the copy number or sites where the transforming DNA integrated may reduce the expression of the siderophore gene in these transformants. Such position effects could be due to integration of the cosmid DNA into a transcriptionally inert domain of the genome as Kinsey and Rambosek (13) speculated. In the plasmid transformation system, the majority of *U. maydis* transformants were shown to carry the vector DNA integrated into the genome at sites other than the resident *hsp70* locus that was used in the construction of the marker gene of the vector (32). We have yet to determine if this is still the case for cosmid transformants.

Our work has delineated a general strategy to isolate genes from *U. maydis*. By using the cosmid genomic library constructed in pCU3, it is now straightforward to clone genes of interest in this plant pathogenic fungus. Our studies have revealed that cotransformation occurs at high frequency, about 30 to 60%, in *U. maydis*. Vector DNA can be recovered from *U. maydis* transformants through in vitro packaging with lambda phage extracts.

On the basis of data obtained from studies on the incorporation of isotopically labeled precursors into ferrichrome and ferrichrome A in *U. sphaerogena*, Emery (7) proposed that L-ornithine and δ-*N*-hydroxyornithine are common precursors of the two siderophores and the first step of siderophore biosynthesis is N hydroxylation of L-ornithine. Our results are consistent with this scheme. The early steps in the biosynthesis of ferrichrome and rhodotorulic acid may be identical. Both involve the hydroxylation of L-ornithine and acetylation of δ-*N*-hydroxyornithine (1, 7). It is interesting that the fungal siderophore biosynthetic pathway shares some features with that for aerobactin, a bacterial hydroxamate siderophore, in which the first biosynthetic step is the hydroxylation of lysine (11). The *iucD* gene, which encodes an oxygenase that catalyzes this reaction, has been isolated as part of the aerobactin operon (11).

Hydroxylation of L-ornithine to δ-*N*-hydroxyornithine is a unique enzymatic reaction, which does not occur in plant or animal cells. An active ornithine-N^5-oxygenase is required for the synthesis of both siderophores in *U. maydis*, and it may also play an essential role in production of other hydroxamate-type fungal siderophores, many of which contain δ-*N*-hydroxyornithine as a major component. Some of these fungi are noted plant or mammalian pathogens (35). The inhibition of this enzyme by an effective inhibitor may offer a novel means of control for these pathogens.

Iron can have an important impact on the severity of mammalian infection (8) and the proliferation of neoplastic cells (33). Siderophore-mediated high-affinity iron transport systems are virulence factors in many mammalian pathogens (5). By comparison, very little is known about the impact of iron on plant diseases (14, 23). The cloning of the ornithine-N^5-oxygenase gene will allow construction of null mutants in this fungus by replacing the wild-type gene with a defective one, mutagenized in vitro. Mutants of this kind are expected to provide definitive information regarding the biological function of siderophores in reference to disease development, as such mutants will be nonleaky and nonreversible.

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