urbs1, a Gene Regulating Siderophore Biosynthesis in *Ustilago maydis*, Encodes a Protein Similar to the Erythroid Transcription Factor GATA-1

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*Ustilago maydis* secretes ferrichrome-type siderophores, ferric-ion-binding compounds, in response to low iron starvation. TA2701, a non-enterobactin-producing, non-ferrichrome-utilizing mutant of *Salmonella typhimurium* LT-2, was employed as a biological indicator in a novel screening method to isolate three *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine-induced *U. maydis* mutants defective in the regulation of ferrichrome-type siderophore biosynthesis. These mutants displayed a constitutive phenotype; they produced siderophores in the presence of iron concentrations that would typically repress siderophore synthesis in wild-type strains. A 4.8-kb fragment of *U. maydis* genomic DNA capable of restoring normal regulation of siderophore biosynthesis in the constitutive mutants was identified. This segment of DNA contains an intronless open reading frame that specifies a protein of 950 amino acids containing two finger motifs similar to those found in the erythroid transcription factor GATA-1. Disruption of this open reading frame in a wild-type strain gave rise to cells that produced siderophores constitutively. Genetic studies indicated that the disruption mutation was allelic to the chemically induced mutations, confirming that the structural gene for a regulator rather than a suppressor gene had been cloned. Northern (RNA) analysis of the gene revealed a 4.2-kb transcript that is expressed constitutively at low levels in wild-type cells. The data support the hypothesis that this gene, which we designate *urbs1* (*Ustilago* regulator of biosynthesis of siderophores), acts directly or indirectly to repress biosynthesis of siderophores in *U. maydis*.

Acquisition of iron is a crucial process for most organisms, since this element is an essential component of enzymes of primary metabolism. Most organisms possess efficient systems for the uptake and transport of iron. In vertebrates, proteins such as transferrin sequester and mobilize iron (13). In microorganisms, iron acquisition is facilitated by high-affinity iron chelators called siderophores, which are actively taken up by iron-depleted cells through specific membrane receptors (3). Although iron has an essential role in cell metabolism, free iron can be deleterious to cells by forming oxygen radicals after reaction with hydrogen peroxide (19). Consequently, iron acquisition and management in microorganisms must be efficiently regulated to avoid both starvation and toxicity. In *Ustilago maydis*, the biosynthesis of siderophores is negatively regulated by iron (5). When iron is limiting in the growth medium, this fungus produces and secretes copious amounts of the cyclic peptide siderophores ferrichrome and ferrichrome A (5). The extracellular concentration of both siderophores can reach 0.5 mM after 1 week of cultivation in a low-iron medium. In contrast, ferrichrome A is not detectable and ferrichrome is barely detectable when fungal cells are cultured in the same medium containing 10 μM FeSO₄. Inhibition of siderophore biosynthesis occurs rapidly; production of siderophores is almost completely eliminated within 6 h after the addition of iron to low-iron cultures of the fungus (5).

As a first step toward understanding the mechanisms that regulate the biosynthesis of siderophores in *U. maydis*, we developed a novel approach to obtain fungal mutants defective in this regulatory process. In this communication, we describe the isolation and characterization of a class of *U. maydis* mutants that produce siderophores in the presence of high concentrations of iron. We also report the identification and characterization of *urbs1* (*U. maydis* regulator of biosynthesis of siderophores), a gene capable of complementing these constitutive mutants. DNA sequence analysis of *urbs1* revealed the presence of putative DNA binding motifs (zinc fingers) similar to those found in erythroid transcription factors of vertebrates (14, 46) and regulators of nitrogen metabolism in ascomycetes (16, 25, 30).

**MATERIALS AND METHODS**

**Bacterial and fungal cultures.** Escherichia coli DH5α [80dlacZ DM15 endA1 recA1 hsdR17 *(λ^− mK^−)* supE44 thi-1 gyrA96 λ^− F^- Δ(lacZΔM15-argF)U169)] was obtained from Bethesda Research Laboratories, Inc. (Gaithersburg, Md.). *Salmonella typhimurium* LT-2 TA2701 (emb-7 fhuA) (28) was a gift from J. B. Neilands (University of California, Berkeley). *U. maydis* strains and plasmids used in this study are listed in Table 1.

**Media, chemicals, and reagents.** Low-iron (LI) medium was prepared as described previously (18). R medium, used to screen for mutagenized colonies deregulated in siderophore production, contained 0.33% Casamino Acids (Difco), 0.33% NaCl, 0.17% yeast extract (Difco), 1% glucose, 1.5% Noble agar (Difco), 0.05% glycine, 0.05% ornithine, and 0.05% serine. Minimal and complete media
were prepared as described previously (22). Plate-mating medium was described previously (38). Nutrient broth medium and potato dextrose agar medium were from Difco. All chemicals were reagent grade. Ferrichrome, ferrichrome A, ferrichrome C, rhodotorulic acid (deferrated), malonochrome, ferricrocin, and albomycin were gifts from J. B. Neilands (University of California). Deferrated ferroxbamine B (Desf) was from Ciba-Geigy (Summit, N.J.). The ferric siderophores were deferrated by the 8-hydroxyquinoline procedure (27). The siderophore concentration was determined by using the ferric perchlorate assay (2). The isolation and characterization of U. maydis siderophores were done as described previously (5).

**Genetic methods.** Haploid cells of U. maydis were mutagenized with N-methyl-N'-nitro-N-nitrosoguanidine (NTG) as previously described (49). Genetic crosses, construction of diploids, and pathogenicity tests were performed as described previously (24).

**Assay for siderophores.** For routine checking for the constitutive phenotype, U. maydis colonies were grown overnight on fresh R medium plates at 28°C. Each colony was overlaid with 50 μl of a 2.3 mg/ml solution of ferric perchlorate. The color of the fungal colonies was examined after incubation for an additional 16 to 20 h at 28°C. Constitutive mutants producing large amounts of siderophores displayed an orange color (color of ferrisiderophores), while the wild-type and complemented colonies remained white. During this study, we observed that U. maydis strains producing siderophores constitutively grew as orange colonies on potato dextrose agar supplemented with 1 mM FeSO₄ or FeCl₃. By contrast, wild-type colonies were white on this medium. Subsequently, we used this characteristic to screen colonies for the constitutive phenotype. Siderophore production in liquid medium was tested by using previously described ferric perchlorate and thin-layer chromatography assays (5).

**Identification of regulatory mutants.** The following TA2701 bioassay was developed to screen for fungal mutants that produce siderophores constitutively. About 300 NGT-treated fungal cells were spread evenly on a 10-cm diameter petri dish containing R medium and incubated at 28°C for 36 to 48 h until the fungal colonies were visible. Each plate was then overlaid with 3 to 5 ml of soft agar (0.7% Noble agar in 0.85% NaCl solution) at 42°C containing 15 μl of an overnight nutrient broth culture of S. typhimurium TA2701. After incubation at 28°C for 14 to 20 h, growth of TA2701 around fungal colonies was examined. Fungal colonies that inhibited growth of TA2701 were purified on potato dextrose agar medium amended with 100 μg of penicillin per ml to eliminate the indicator bacteria and then were retested for the ability to inhibit TA2701 on R medium. Prospective mutants were also grown in liquid R medium for 24 to 36 h, and the culture supernatants were tested for siderophores with the ferric perchlorate assay.

**U. maydis transformation.** Spheroplasts of U. maydis were prepared as described previously (50). Polyethylene glycol-mediated DNA intake into spheroplasts (50) was modified as follows. Two hundred microliters of spheroplasts was mixed in a microcentrifuge tube with 100 μg of polyethylene glycol (polyethylene glycol 3350 [Sigma, St. Louis, Mo.] at 66% [wt/vol] in 25 mM CaCl₂-25 mM Tris-HCl [pH 7.5]), 2 μl of β-mercaptoethanol, 5 μl of dimethyl sulfoxide, and up to 100 μl of DNA (0.5 to 1.0 μg) in Tris-EDTA buffer or water so that the final concentration of polyethylene glycol was between 15 and 20%. After 20 min of incubation at room temperature, the protoplasts were washed twice by adding 1 ml of buffer II (25 mM CaCl₂, 25 mM Tris-HCl [pH 7.5], 1 M sorbitol) and then centrifuged for 3 min at 1,500 × g. Protoplasts were incubated for 3 h at room temperature in 200 μl of complete medium supplemented with 1 M sorbitol while the microcentrifuge tube was gently rocked. These mixtures were then gently spread onto complete medium containing 1 M sorbitol and 300 μg of hygromycin B (Calbiochem, La Jolla, Calif.) per ml.

**DNA procedures.** DNA was isolated from U. maydis transformants following lysis of protoplasts (50) to obtain high-molecular-weight DNA and by the rapid procedure for Saccharomyces cerevisiae (12) to obtain DNA for Southern hybridization analysis. Plasmid DNA was routinely isolated from E. coli by an alkaline lysis method (32). For DNA sequencing, plasmid DNA was purified by multiple differential precipitation with ammonium acetate (26). Digestion of DNA and analysis of fragments were performed by standard methods (39). Subcloning of DNA fragments was routinely done by following an in-gel protocol using low-melting temperature SeaPlaque agarose (EMC BioProducts, Rockland, Maine) (9). Fragment isolation for radioactive labeling with ³²P and for transformation was performed with a Gene Clean kit (Bio 101, Inc., La Jolla, Calif.). Labeling of DNA fragments was performed with a random oligolabeling kit (Pharmacia, Piscataway, N.J.), a nick translation kit (Bethesda Research Laboratories), or, for end labeling, polynucleotide kinase (New England BioLabs, Beverly, Mass.).

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**TABLE 1. List of U. maydis strains**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant characteristics</th>
<th>Source and/or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>518</td>
<td>a2 b2</td>
<td>R. Holliday (22)</td>
</tr>
<tr>
<td>521</td>
<td>a1 b1</td>
<td>R. Holliday (22)</td>
</tr>
<tr>
<td>289</td>
<td>a1 b1 pan1-1 inos-3 nar1-1 rec1-1</td>
<td>R. Holliday (22)</td>
</tr>
<tr>
<td>227</td>
<td>a2 b2 adel1-1 met1-2 nar1-6 rec2-1</td>
<td>R. Holliday (22)</td>
</tr>
<tr>
<td>UM031</td>
<td>a1 b2</td>
<td>Kronstad and Leong (24)</td>
</tr>
<tr>
<td>UM032</td>
<td>a2 b1</td>
<td>Kronstad and Leong (24)</td>
</tr>
<tr>
<td>UMC002</td>
<td>a2 b2 urbs1-1 mutant of 518</td>
<td>This study</td>
</tr>
<tr>
<td>UMC005</td>
<td>a2 b2 urbs1-2 mutant of 518</td>
<td>This study</td>
</tr>
<tr>
<td>UMC007</td>
<td>a2 b2 urbs1-3 mutant of 518</td>
<td>This study</td>
</tr>
<tr>
<td>UMC013</td>
<td>a2 b2 urbs1::Hyg'</td>
<td>This study</td>
</tr>
<tr>
<td>UMC014</td>
<td>a1 b1 urbs1::Hyg' adel1-1</td>
<td>This study</td>
</tr>
<tr>
<td>d2102</td>
<td>diploid UMC021 × UMC002</td>
<td>This study</td>
</tr>
<tr>
<td>d2105</td>
<td>diploid UMC021 × UMC005</td>
<td>This study</td>
</tr>
<tr>
<td>d2107</td>
<td>diploid UMC021 × UMC007</td>
<td>This study</td>
</tr>
</tbody>
</table>

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7092 VOISARD ET AL. MOL. CELL. BIOL.
Transfer of DNA to Nytran membranes (Schleicher & Schuell, Keene, N.H.) and hybridization of membranes were performed as described previously (21).

**RNA procedures.** Procedures recommended for RNA work were followed (39). RNA was isolated from *U. maydis* by the glass bead procedure developed for *S. cerevisiae* (23). A fresh culture of cells (optical density at 600 nm of 1.0 to 1.5) was quick cooled to 0 to 2°C by immersion of a 125-ml culture aliquot in a dry ice-ethanol bath for 1.5 min followed by centrifugation at 4°C for 5 min at 2,500 × g. Cells were resuspended in 10 ml of sodium acetate buffer (50 mM, 10 mM EDTA [pH 5]) and transferred to a 50-ml tube containing 12 ml of phenol-chloroform (1:1, 0.5% 8-hydroxyquinoline; equilibrated with sodium acetate buffer [pH 5]), 1 ml of 10% sodium dodecyl sulfate, and 11 g of acid washed glass beads (0.4-mm diameter; Sigma). Cells were vortexed at 30-s intervals over 5 min and maintained at 65°C. Phase separation was achieved by centrifugation at 2,500 × g for 10 min at 4°C. The supernatant was further extracted with phenol-chloroform and chloroform-isoamyl alcohol (24:1, vol/vol). Nucleic acids were precipitated at −20°C with 3 volumes of ethanol and 1/10 volume of 3 M sodium acetate (pH 5.2) for a few hours or overnight and collected by centrifugation at 2,500 × g and 4°C for 15 min. The pellets were resuspended at 65°C in a small volume of diethylpyrocarbonate-treated water. RNA was separated from DNA contaminants by CsCl density gradient centrifugation (31). Poly(A) mRNA selection for the cDNA bank construction was done by using the PolyATtract isolation system from Promega (Madison, Wis.) according to the supplier's protocol.

**Construction of a cDNA library.** A cDNA library was constructed with the ZAP-cDNA synthesis kit (Stratagene, La Jolla, Calif.), and poly(A) mRNA was extracted from cells grown in LI medium. Amplification of the cDNA library was performed by following the instructions included in the cDNA synthesis protocol.

**DNA sequencing and analysis.** Nucleotide sequencing of double-stranded DNA fragments cloned in pUC18 was achieved with the Sequenase kit (U.S. Biochemicals, Cleveland, Ohio) (4′-thio-dGTP oligonucleotides) were synthesized by the Biotechnology Center of the University of Wisconsin-Madison and purified before use (40). DNA sequence data were analyzed with the software of the University of Wisconsin Genetics Computer Group (10). Data base searches were done by using Mail-FASTA (17, 37).

**Analysis of urbs1 for the presence of introns.** The sequence of the open reading frame of the genomic urbs1 was analyzed for any evidence of introns by using the DNA Inspector program version 3.13 (Textco, West Lebanon, N.H.). Additionally, two cDNA clones of 1.2 kb were sequenced and their sequences were compared with the genomic urbs1 sequence. Since the cDNAs were not full-length, additional analyses were performed. The first analysis involved comparing restriction endonuclease profiles of polymerase chain reaction (PCR) products derived from templates of a reverse transcription reaction of total RNA treated with RNase-free DNase (Promega) and PCR products derived from a template of genomic urbs1 DNA. Custom oligonucleotides were used as primers (see Fig. 3). PCR amplifications were performed by following the protocol supplied with the AmpliTaq (Perkin-Elmer Cetus, Norwalk, Conn.) Taq polymerase. The incubation conditions consisted of 35 cycles of 1 min of denaturation at 95°C, 1 min of annealing at 55°C, and 1 min of extension at 72°C, followed by a single 5-min incubation at 72°C to complete any partial extensions. PCR products were extracted with phenol-chloroform and then digested with several restriction endonucleases having 4-bp recognition sequences. Full-length and digested PCR products were sized and compared by electrophoresis through a mixture of 3% NuSieve and 1% SeaKem LE agarose (FMC BioProducts).

The second analysis, termed 5′ RACE (rapid amplification of cDNA ends [15]), was performed to determine whether the extreme 5′ region of the transcribed DNA contained an intron. This analysis was performed by using the modification described by Mei et al. (29) and the downstream primers P3 and P7 (see Fig. 3) attached to a 10-nucleotide EcoRI adapter. Total RNA treated with RNase-free DNase (Promega) was used in first-strand synthesis.

**Primer extension mapping of the 5′ end of urbs1 mRNA.** Primer extension with 32P-end-labeled primer P3 (see Fig. 3) and total RNA from strain 518 grown in complete medium was performed as described previously (52). Products were sized against a sequencing ladder by using primer P3 and pSC8 as a template.

**Nucleotide sequence accession number.** The nucleotide sequence of the 3,987-bp fragment containing urbs1 and its flanking regions has been assigned GenBank accession number M80547.

**RESULTS**

**Inhibition of *S. typhimurium* TA2701 by fungal siderophores.** The uptake of siderophores by microorganisms requires the presence of membrane receptors that are specific for a given siderophore (51). In addition to the high-affinity iron transport system for the endogenous siderophore enterobactin, *S. typhimurium* LT-2 is capable of employing the fungal siderophore ferrichrome as an iron scavenger (28). *S. typhimurium* TA2701 is defective in the ability to transport ferrichrome (*fluA*) as well as in the biosynthesis of enterobactin (ent); consequently, it is no longer able to use exogenous ferrichrome as an iron carrier and will grow only when enterobactin or free iron is present in the medium. This feature makes TA2701 an excellent candidate for a biological indicator to screen for fungal mutants that are constitutive for siderophore biosynthesis. Such mutants secrete large amounts of siderophores under iron-sufficient growth conditions. These siderophores in turn chelate free iron in the medium and thus inhibit the growth of the bacterial indicator, which cannot transport ferrichrome.

Seven deferented siderophores as well as the sideromycin, albomycin (33), were tested for the ability to inhibit the growth of *S. typhimurium* TA2701 in R medium, an iron-replete medium. Desferroxamine is produced by *Streptomyces pilosus* and a number of other streptomyces, and albomycin is produced by *Actinomyces subtropicus* (33). The remaining six siderophores, ferrichrome, ferrichrome A, ferrichrome C, rhodotorulic acid, ferricrocin, and mali-nochrome, are of fungal origin (31). All of these deferented compounds were inhibitory to TA2701 (48). By contrast, the iron complexes of ferrichrome and ferrichrome A had no effect on the growth of the bacterium. Similarly, colonies of *U. maydis* wild-type strain 518, which does not produce detectable amounts of siderophores on R medium, did not inhibit the growth of TA2701. These results suggested that *U. maydis* mutants deregulated for siderophore production might inhibit the growth of the bacterial indicator.

**Identification of regulatory mutants.** Three constitutive siderophore-producing mutant strains, UMC002, UMC005, and UMC007, were identified among 300,000 colonies screened by using the TA2701 bioassay. All three mutants...
secreted siderophores in liquid R medium, giving an orange-brown color after treatment of culture supernatant with ferric perchlorate (representative data for UMC005 are shown in Table 2). UMC002, UMC005, and UMC007 were incubated in potato dextrose broth for 2 days, and the culture supernatant was extracted and analyzed for ferrichrome and ferrichrome A by thin-layer chromatography. Both siderophores were easily detected in the culture supernatants (data not shown). By contrast, the wild-type parental strain produced very little ferrichrome and a barely measurable amount of ferrichrome A under the same experimental conditions.

**Genetic analysis of constitutive mutants.** The constitutive mutant UMC002, derived from strain 518 (a2 b2), was crossed to the compatible U. maydis prototrophic wild-type isolate 521 (a1 b1) and to the auxotrophic mutant 288 (a1 b1 pan1-1 insol-3 nar1-1 rec1-1); basidiospore segregants were then examined for the production of siderophores on R medium plates by using the S. typhimurium TA2701 bioassy. Fifty-nine basidiospore segregants from the cross between UMC002 and 521 produced siderophores constitutively, and 52 displayed the wild-type phenotype. The cross between UMC002 and 288 gave similar results: 39 segregants were constitutive and 35 were wild type for regulated siderophore production. These results (P > 0.1 for chi-square analysis of a 1:1 model) suggest that the constitutive phenotype is controlled by a single gene lesion. UMC002-2883 (insol-1 3 pan1-1 a1 b1), a constitutive progeny from a cross between strains UMC002 and 288, was fused with strain 227 (a2 b2 ade1-1 met1-2 nar1-6 rec2-1) on plate mating medium to form diploid cells. The resultant diploids, after purification on minimal medium, were pathogenic. Diploid colonies were tested for siderophore production by using the TA2701 bioassy and by chemical analysis of culture supernatants of cells grown in liquid R medium. In both tests, the diploids behaved as the haploid wild-type cells. Constitutive production of siderophores was not observed, suggesting that the constitutive mutation in UMC002 is recessive and the wild-type allele is dominant.

**Identification of complementing cosmid restoring iron-regulated siderophore production.** U. maydis constitutive mutant UMC002 was transformed with a cosmid library of genomic DNA of U. maydis 518 in pCU3 (49). Cosmid DNA was recovered from transformants as described previously (49). One of the cosmids, pSidCB2, was found to complement all three constitutive mutants, UMC002, UMC005, and UMC007. An 11-kb BamHI genomic DNA fragment was derived from pSidCB2 and subcloned in pCU3. The resulting plasmid, pSidCB2-2, restored normal siderophore regulation when transformed into UMC002. The 11-kb BamHI fragment was isolated, labeled with 32P, and used as a probe in Southern hybridizations with genomic DNA of wild-type strain 518 digested with either BamHI or BamHI-XbaI. The results of the experiments revealed the presence of an 11-kb BamHI fragment and 4.8- and 5.9-kb BamHI-XbaI fragments in the genome of U. maydis. These results matched those obtained when the probe was hybridized to cosmid DNA digested with BamHI or BamHI-XbaI, verifying that no DNA rearrangements had occurred during the cloning procedure (data not shown).

**Complementation of constitutive mutations in trans.** Both XbaI-BamHI fragments of 4.8 and 5.9 kb derived from pSidCB2-2 were cloned in the autonomously replicating vector pCM54 (47) to test the complementation of these clones in trans in the U. maydis constitutive strain UMC005. Plasmid pSC3 contains the 4.8-kb fragment, and pSC4 contains the 5.9-kb fragment. Transformants obtained with both pSC3 and pSC4 were tested on potato dextrose agar containing 1 mM FeSO4 by overlaying colonies grown on R medium containing ferric perchlorate solution and by measuring the production of siderophores in L3 medium containing 10 μM FeSO4 and 300 μg of hygromycin B per ml after 2 days at 28°C with the ferric perchlorate assay. In each test, only pSC3 was able to restore the wild-type phenotype (Fig. 1). Three deletion derivatives of pSC3 were constructed by subcloning various parts of the BamHI-XbaI insert in pCM54 (Fig. 1). The complementation test showed that pSC8 was able to fully complement the constitutive mutation (Fig. 1 and Table 2), while pSC11 and pSC9 gave partial complementation (Fig. 1). Similar patterns of complementation with these plasmids were noted with the other constitutive strains, UMC002 and UMC007 (data not shown).

**Gene disruption.** The 4.8-kb XbaI-BamHI fragment of pSC3 was cloned in pUC18 to obtain a SaI fragment of pCU3 carrying the hygromycin B resistance gene (50) was inserted in the unique XhoI site of pSC6 to create pSC13 (Fig. 1). On the basis of the location of the XhoI site within the complementing DNA, the insertion of the hygromycin B resistance cassette at this site was expected to disrupt the putative gene. The SotI fragment encompassing the disrupted site was transformed into strain 518 (a2 b2). Eight hygromycin B-resistant (Hyg+) transformants were obtained: three transformants retained the wild-type phenotype, and five transformants produced siderophores constitutively. Southern hybridization of XbaI-BamHI-digested genomic DNA of the constitutive transformants with the SotI fragment as a probe showed the correct exchange of the wild-type fragment with the mutated allele containing the hygromycin B resistance cassette (data not shown); strain UMC013, representing this class of transformants (Table 2), was used in further experiments. Similar results were obtained after transformation of the Hyg'-carrying fragment into another wild-type haploid strain, 521 (a1 b1) (data not shown). Using turbidity or colony size as a measure of cell growth, we found no indication that the urbs1 disruption mutant UMC003 was different in ability to grow compared with the wild type while UMC005, a primary Ntg-induced urbs1 mutant, did grow poorly in low-iron medium (data not shown). By contrast, sid1 disruption mutants of Ustilago, which are unable to produce sidero-

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**TABLE 2. Siderophore production by U. maydis urbs1 mutants and complemented derivatives**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Siderophore production* (A₄₈₅)</th>
<th>− Fe</th>
<th>+ Fe</th>
</tr>
</thead>
<tbody>
<tr>
<td>518</td>
<td>0.22 (0.05)</td>
<td>0.01 (0.01)</td>
<td></td>
</tr>
<tr>
<td>UMC013</td>
<td>0.15 (0.03)</td>
<td>0.17 (0.07)</td>
<td></td>
</tr>
<tr>
<td>UMC005</td>
<td>0.17 (0.01)</td>
<td>0.12 (0.01)</td>
<td></td>
</tr>
<tr>
<td>UMC005/pSc8</td>
<td>0.13*</td>
<td>0.01 (0.00)</td>
<td></td>
</tr>
<tr>
<td>UMC005/pCM54</td>
<td>0.14*</td>
<td>0.09 (0.01)</td>
<td></td>
</tr>
<tr>
<td>d2102</td>
<td>0.11 (0.02)</td>
<td>0.17 (0.02)</td>
<td></td>
</tr>
<tr>
<td>d2105</td>
<td>0.09 (0.01)</td>
<td>0.12 (0.04)</td>
<td></td>
</tr>
<tr>
<td>d2107</td>
<td>0.17 (0.01)</td>
<td>0.14 (0.01)</td>
<td></td>
</tr>
</tbody>
</table>

* Cells were grown for 48 h at 28°C in LI medium with or without 10 μM FeSO₄. Culture supernatants were extracted and analyzed for ferrichrome and ferrichrome A by thin-layer chromatography. Both siderophores were easily detected in the culture supernatants (data not shown). By contrast, the wild-type parental strain produced very little ferrichrome and a barely measurable amount of ferrichrome A under the same experimental conditions. **
phores, grow very poorly in low-iron medium relative to the wild type (29a).

**Genetic analysis of the disrupted mutant.** The disruption mutant UMC013 (a2 b2) was crossed with wild-type strain 521 (a1 b1) to determine whether hygromycin B resistance was genetically linked to the constitutive mutation and to assess the segregation pattern of the mutant and corresponding wild-type alleles. Among 107 basidiospore segregants tested, 46 Hyg<sup>+</sup> colonies exhibited the constitutive phenotype and 61 Hyg<sup>−</sup> colonies had the wild-type phenotype, indicating tight linkage of the constitutive mutation and resistance to hygromycin B. This segregation pattern is consistent with the 1:1 ratio expected for segregation of alternate alleles at a locus (P = 0.14).

**Allelism test.** Diploids were constructed with NTG-generated mutants UMC002, UMC005, and UMC007 and an auxotrophic strain carrying a disrupted urbs1 gene in order to investigate whether the lesions in the gene-disrupted strain and the NTG-induced mutants are allelic. An urbs1::Hyg<sup>−</sup> a1 b1 strain termed UMC014 was identified among the progeny of a cross between UMC013 (a2 b2 urbs1::Hyg<sup>−</sup>) and 521 (a1 b1). The auxotrophic marker ade1-1 of strain 227 (a2 b2 ade1-1 met1-2 nar1-6 rec2-1) was then introduced in the UMC014 (a1 b1 urbs1::Hyg<sup>−</sup>) background by crossing 227 and UMC014 to obtain UMC021 (a1 b1 urbs1::Hyg<sup>−</sup> ade1-1). Diploids were constructed by crossing UMC021 with UMC002 (a2 b2 urbs1-1), UMC005 (a2 b2 urbs1-2), or UMC007 (a2 b2 urbs1-3) and then selecting on minimal medium plates containing 300 μg of hygromycin B per ml. Three diploids, d2102 (UMC021 × UMC002), d2105 (UMC021 × UMC005), and d2107 (UMC021 × UMC007), were grown in LI medium supplemented with 10 μM FeSO<sub>4</sub> and 300 μg of hygromycin B per ml for 2 days at 28°C, and the supernatants were tested for the production of siderophores with the ferric perchlorate assay. All three diploids produced siderophores in this medium (Table 2), indicating that the NTG-induced mutations in UMC002, UMC005, and UMC007 are allelic to the mutation caused by the insertion of the Hyg<sup>−</sup> fragment into urbs1. Moreover, all (hygromycin-resistant and -sensitive) basidiospore segregants obtained from teliospores of galls derived from d2105 or d2107 displayed the constitutive phenotype (data not shown).

**Analysis of the DNA sequence and the translated protein.** A nucleotide sequence of 3,987 bp from the 4.8-κb XbaI-BamHI complementing genomic DNA fragment (Fig. 1) was determined for both strands. A large open reading frame of 2,850 bp starting at base 692 of the sequenced DNA encodes a putative protein of 950 amino acids (Fig. 2). We designated this open reading frame urbs1 (*Ustilago* regulator of biosynthesis of siderophores). The sequence (AΔTCATGGC) surrounding the putative translational start matches in 7 of 9 nucleotides with the consensus sequence (CΔCCATGGC) of filamentous fungi (4). Furthermore, the two most conserved nucleotides, the A at position −3 and the C at position +5 (underlined in the sequences above) relative to the ATG start codon, are present in the *urbs1* sequence.

The nucleotide sequence as well as the deduced protein were compared with sequences in GenBank and EMBL.

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**FIG. 1.** Complementation of the UMC005 mutation with derivatives of pSidCB22. Various segments of the 4.8-κb XbaI-BamHI fragment containing urbs1 were cloned in pCM54, an autonomously replicating vector in *U. maydis*. Full complementation is indicated by a plus sign; a plus/minus sign indicates partial complementation. pSC13 is a derivative of pSC6 in which a hygromycin B resistance cassette has been inserted at the unique *Xho*I site; the resulting 4.4-κb *Stu*I fragment was used to disrupt the urbs1 wild-type allele in UMC001 by gene exchange. The urbs1 open reading frame found by DNA sequence analysis is indicated by the horizontal arrow.
This search revealed two regions of *urbsl* with 60 to 70% identity at the nucleotide and amino acid levels with the vertebrate erythroid transcription factors GATA-1 from humans (45), rats (46), and chickens (14) as well as the nitrogen regulatory genes GLN3 of *Saccharomyces cerevisiae* (30), DAL80 of *S. cerevisiae* (8), nit-2 of *Neurospora crassa* (16), and areA of *Aspergillus nidulans* (25) (Fig. 3). The regions of identity in these proteins correspond to a putative zinc finger DNA binding motif. The vertebrate transcription factors contain two such motifs, while the ascomycete factors have only one motif. Like the vertebrate protein, the *urbsl* protein contains two motifs; however, in the *urbsl* protein these are separated by 100 amino acids. By contrast, the finger motifs in the vertebrate proteins follow each other.
VOL. 13, 1993
REGULATOR OF SIDEROPHORE PRODUCTION IN U. MAYDIS

The amino acid sequence lying between these two motifs in urbsl includes five cysteines. A similar sequence repeated six times is found in actin (11).

The urbsl protein has long stretches of identical residues. At the N terminus, a sequence composed of 4 histidines, 10 serines, and 6 alanines is present. Similar homopolymeric sequences have been found in a number of DNA-binding proteins, such as the caudal gene of Drosophila melanogaster (31) and the ADR6 protein of S. cerevisiae (35). At the C terminus of the urbsl protein, within a stretch of 28 amino acids, are 21 histidine residues. Since histidine residues in proteins are often involved in metal binding (1), this region is a potential site for the binding of iron.

The protein encoded by urbsl also contains a large number of S(T)-PXX motifs, a sequence that is frequently found in regulatory proteins (43). A total of 20 S(T)-PXX motifs, with the majority (17 motifs) represented by the type SPX, are scattered throughout the urbsl sequence; this corresponds to a frequency of 2.1 × 10⁻³, which is among the highest scores observed (43).

Analysis of urbsl mRNA. Total RNA, isolated from cells grown in low- and high-iron medium, was analyzed by Northern (RNA) blot hybridization by using probes corresponding to different segments of the 4.8-kb XbaI-BamHI insert of pSC6 (Fig. 4). The 1.5-kb XbaI-PstI fragment did not give a clear signal, while the PstI-XhoI, XhoI-EcoRI, and EcoRI-BamHI fragments hybridized to a transcript of 4.2 kb in RNA isolated from strain 518 cells grown in low- or high-iron medium (Fig. 4). The EcoRI-BamHI fragment also hybridized to an RNA of 2.6 kb. This RNA may be homologous to a portion of the probe DNA which is downstream of the urbsl transcription unit, may represent a second transcript originating from this region that overlaps that of urbsl, or may represent another RNA with limited sequence identity to that of urbsl. An exposure time of 7 days was necessary to detect the signal, indicating that the urbsl
In a search for introns in the portion of urbs1 that was not represented by the less-than-full-size cDNAs, other analyses of the mRNA were undertaken. Total RNA was DNase treated and used as a template for reverse transcription reactions using, as primers, oligomers (Fig. 2) that correspond to regions of the urbs1 open reading frame. The resulting cDNAs and additional upstream primers were used in PCR amplifications. The products of these amplifications were found to be indistinguishable in size and endonuclease cleavage profiles from those involving the same primers and, as the template, pSC6 (pUC18 containing the 4.8-kb XbaI-BamHI genomic urbs1 segment). With 5′ RACE, cDNAs resulting from extension with primer P3 were amplified by PCR and cloned into pUC18. Among the 28 cDNA clones sequenced, 23 had 5′ ends mapping within a 59-nucleotide region corresponding to nucleotides 76 to 134 (Fig. 3). Three additional clones had 5′ ends mapping downstream (nucleotides 144, 149, and 155), and two had 5′ ends mapping upstream (nucleotides 5 and 27). In all cases, the sequence of the cDNA clones matched the genomic sequence. These data, in conjunction with the cDNA sequence data, indicate that no introns exist within the transcribed region of urbs1.

Since the 5′ ends of the RACE products varied, primer extension was used to determine the 5′ end of the urbs1 mRNA. Two major transcriptional starts (corresponding to nucleotides 82 and 133; Fig. 2) were determined with primer P3. These starts lie within the 59-nucleotide region in which most of the 5′ ends of the cDNA clones mapped.

### DISCUSSION

A class of *U. maydis* mutants defective in the regulation of siderophore biosynthesis has been isolated. The procedure described here is straightforward and effective and allows for the analysis of large numbers of fungal cells with limited time and labor. This is particularly useful since in *U. maydis* the frequency of constitutive mutants isolated was relatively low, about 1 in every 100,000 mutagenized surviving cells. Besides ferrichrome and ferrichrome A, several other fungal and bacterial siderophores were also capable of preventing the growth of the bacterial indicator *S. typhimurium* TA2701, thus extending the bioassay to the isolation of mutants deregulated for siderophore biosynthesis in other organisms.

Molecular complementation of the *U. maydis* mutants defective in the regulation of siderophore biosynthesis yielded urbs1, a gene whose features strongly suggest that the protein it encodes acts as a regulator. The translated protein shows the presence of structures [zinc fingers and S(T)PXX motifs] which are typical of transcriptional regulatory proteins (14, 16, 25, 43, 46). Together with the results of dominance and allelism tests and the constitutive production of siderophores by both the NTG-induced and urbs1 disruption mutants, regulation involving a repressor seems likely. With the available data, however, we cannot yet conclude whether Urbs1 acts directly or indirectly to effect repression of siderophore biosynthesis.

The presence of putative zinc finger motifs in the urbs1 protein sequence similar to those found in the vertebrate erythroid transcription factors GATA-1 (14, 45, 46) and in the ascomycete nitrogen regulatory proteins Gln3 (30), Nit-2 (16), Dal80 (8), and AreA (25) allows one to hypothesize a DNA binding site for Urbs1. For the vertebrate transcription factors and the ascomycete nitrogen regulators, the DNA

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**FIG. 4.** Northern blot analysis of urbs1 mRNA. Total RNA was isolated from *U. maydis* strain 518 grown in LI medium without (−) or with (+) 10 μM FeSO₄. Ten micrograms of total RNA was glyoxylated, electrophoresed through a 1.0% agarose gel, and transferred to a Nytran membrane. The resulting blot was probed with various 32P-labeled fragments derived from the 4.8-kb XbaI-BamHI insert of pSC6. The same blot was used in each hybridization experiment, and a 7-day exposure of the membrane to X-ray film was employed. The blot was also probed with DNA probes representing the iron-regulated ornithine-NO₂-oxynase (*sidl*) gene (29) and the constitutively expressed GAPDH gene (42) of *U. maydis*. In these latter cases, the X-ray film was exposed to the membrane for 24 and 1 h, respectively.

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transcript is expressed at a low level. By contrast, hybridization of the same membrane with the *sidl* gene of *U. maydis* (29) revealed a 2.3-kb mRNA in low-iron medium-grown cells that was detectable within 24 h (Fig. 4). As expected (29), no hybridization signal was observed for this probe with mRNA isolated from cells grown in high-iron medium. Hybridization of the membrane with the constitutively expressed glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene of *U. maydis* (42) gave strong signals with both preparations of mRNA after 1 h of exposure of the X-ray film to the membrane.

A cDNA library of about 10⁶ recombinant clones was hybridized with an urbs1 DNA probe. Two partial cDNA clones, pSC25 and pSC26, carrying 1.2-kb inserts were isolated. The nucleotide sequences of both clones were determined and found to correspond to nucleotides 2605 to 3802 of the sequenced genomic DNA (Fig. 2). No introns were found within this region of DNA. A putative polyadenylation signal (AATAAC) is located at bp 4002 in the DNA sequence (Fig. 2).

An aliquot of the same cDNA library was hybridized with the GAPDH and *sidl* genes (29, 42) of *U. maydis*. The GAPDH probe hybridized to 103 plaques, while the *sidl* gene identified 52 plaques in the 5 × 10⁶ plaques screened. These data support those obtained by Northern hybridization analysis and indicate that the abundance of the urbs1 message is orders of magnitude lower than that of these metabolic genes.
binding site is characterized by the core sequence GATA (6, 16, 20), so it is reasonable to expect that the DNA binding site for Urbs1 will contain this sequence. This feature could be exploited to isolate genes directly regulated by Urbs1. In fact, sid1 of U. maydis, which encodes the first committed enzyme for siderophore biosynthesis, contains two GATA motifs directly upstream of the transcriptional initiation site (29). This gene is transcriptionally regulated by iron and is constitutively expressed in the urbs1 disruption mutant UMC013 (29). Experiments to investigate binding of Urbs1 to the promoter region of sid1 are under way.

In E. coli, a class of recessive mutants termed fur (ferric ion uptake regulation) are derepressed for synthesis of all iron-regulated outer membrane proteins and show enhanced rates of transport and biosynthesis of siderophores (3). The fur gene has since been isolated and sequenced (41). The gene product Fur exhibits properties of a typical gene repressor which, in the presence of ferrous or certain divalent cations, can specifically bind to the promoter region of iron-regulated operons, turning off their transcription (3). Interestingly, the Fur palindromic target sequence, also called the iron box, 5'-GATAATGATAATGATAATGATA (3), contains the core sequence GATA that is the target for the GATA-1 family of proteins. In addition, the Fe$^{2+}$ binding domain of Fur has been identified within its C terminus (7), in which a Cys-Leu-Asp-Cys-X-36-Cys-Ala-Glu-Gly-Asp-Cys motif shows a distant but obvious relationship with the GATA-1 zinc finger motif Cys-X-Asn-Cys-X-17-Cys-Ala-Cys (20). Thus, there is the possibility of an evolutionary relationship among Fur of E. coli, Urbs1 of U. maydis, and the GATA-1 type transcription factors of vertebrates. In addition, all of these regulatory proteins are involved in iron metabolism. For Fur and the urbs1 protein, the link with iron metabolism is obvious, and for the GATA-1 proteins, it must be emphasized that these proteins control the expression of the globin gene family whose protein products account for 75% of the total iron present in vertebrates (13).

Northern hybridization analysis indicated that urbs1 is expressed constitutively. Thus, the activity of Urbs1 may not be regulated at the level of transcription but rather through allosteric interaction with iron in cells. This would indicate the presence of a domain in Urbs1 that is capable of sensing iron. Recent work by Omichinski et al. (36) has shown that a synthetic 66-amino-acid peptide, which includes the second finger and DNA binding domain of chicken GATA-1, can utilize Fe$^{2+}$ as well as Zn$^{2+}$ to assume a conformation that is able to bind target DNA sequences. In fact, the Fe$^{2+}$-peptide complex binds to target DNA somewhat better than the Zn$^{2+}$-peptide complex, raising the possibility that Urbs1 and some members of the GATA-1 family of transcription factors may use iron to stabilize their active DNA binding structures. Interestingly, preliminary analysis of the DNA sequence of urbs1 from UMC002 and UMC007 has revealed single base changes in the second finger of each mutant’s DNA (28a). Another sequence that could serve as an iron sensor is the 28 amino acids between residues 753 and 780, which are rich in histidine. The imidazole of histidine is involved in binding iron in a number of proteins, such as heme proteins and transferrins (13). In addition, the in vitro DNA binding ability of the Fur protein, the repressor of iron-regulated genes in E. coli, requires divalent metal ions, such as Fe$^{2+}$ (3). As with Urbs1, Fur is rich in histidine residues (41) and contains a histidine pentapeptide, which is suspected to be a site for iron attachment (34). Other potential sequences in Urbs1 which could participate in sensing iron are the tyrosine and arginine residues which are scattered throughout the amino acid sequence and the region of 29 amino acids (residues 400-429) located between the two zinc fingers which contain five cysteine residues (11). Future studies of Urbs1 will investigate the metal content of the native protein and sites with which these metals associate.

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