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Isogenic Strain Construction and Gene Targeting in *Candida dubliniensis*

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***Candida dubliniensis* is a recently described opportunistic fungal pathogen that is closely related to *Candida albicans* but differs from it with respect to epidemiology, certain virulence characteristics, and the ability to develop fluconazole resistance in vitro. A comparison of *C. albicans* and *C. dubliniensis* at the molecular level should therefore provide clues about the mechanisms used by these two species to adapt to their human host. In contrast to *C. albicans*, no auxotrophic *C. dubliniensis* strains are available for genetic manipulations. Therefore, we constructed homozygous *ura3* mutants from a *C. dubliniensis* wild-type isolate by targeted gene deletion. The two *URA3* alleles were sequentially inactivated using the *MPA^R*-flipping strategy, which is based on the selection of integrative transformants carrying a mycophenolic acid resistance marker that is subsequently deleted again by site-specific, FLP-mediated recombination. The *URA3* gene from *C. albicans* (*CaURA3*) was then used as a selection marker for targeted integration of a fusion between the *C. dubliniensis MDR1* (*CdMDR1*) promoter and a *C. albicans*-adapted *GFP* reporter gene. Uridine-prototrophic transformants were obtained with high frequency, and all transformants of two independent *ura3*-negative parent strains had correctly integrated the reporter gene fusion into the *CdMDR1* locus, demonstrating that the *CaURA3* gene can be used for efficient and specific targeting of recombinant DNA into the *C. dubliniensis* genome. Transformants carrying the reporter gene fusion did not exhibit detectable fluorescence during growth in yeast extract-peptone-dextrose medium in vitro, suggesting that *CdMDR1* is not significantly expressed under these conditions. Fluconazole had no effect on *MDR1* expression, but the addition of the drug benomyl strongly activated the reporter gene fusion in a dose-dependent fashion, demonstrating that the *CdMDR1* gene, which encodes an efflux pump mediating resistance to toxic compounds, is induced by the presence of certain drugs.**

Several yeast species within the genus *Candida* are opportunistic pathogens of humans, *Candida albicans* being by far the most frequently isolated and also the most pathogenic species (20). Recently, a previously unrecognized *Candida* species, *Candida dubliniensis*, has been described (28). *C. dubliniensis* is closely related to *C. albicans*, and before the phylogenetic separation of the two species was recognized and reliable phenotypic and molecular markers to distinguish them became established, *C. dubliniensis* was often misidentified as *C. albicans* (27). Whereas *C. albicans* is a member of the normal microflora in most healthy persons, *C. dubliniensis* has been isolated primarily from the oral cavities of human immunodeficiency virus (HIV)-infected individuals and AIDS patients. However, recent epidemiological studies have also identified *C. dubliniensis* in cases of oral infection in non-HIV-infected individuals and in cases of septicemia, although at a lower incidence than *C. albicans* (3, 13, 21, 23, 29). These observations still have to be confirmed on a broad scale using recently identified, easily screenable, species-specific phenotypic markers (25). However, the current evidence suggests that *C. albicans* and *C. dubliniensis* differ with respect to their epidemiology and, consequently, in their capacity to colonize and infect specific host niches. Of particular interest in this respect are the reported differences in certain phenotypic characteristics that are linked to virulence, such as adherence to epithelial cells, formation of hyphae, and proteinase production (9, 12), the relative importance of which probably depends on the colonization site and the infection stage. Of potential clinical significance also is the ability of *C. dubliniensis* to develop fluconazole resistance very rapidly in vitro, which is difficult to achieve with *C. albicans*, although fluconazole-resistant *C. albicans* is frequently isolated from patients (16). Similar mechanisms seem to be responsible for fluconazole resistance in the two species (15, 24), but the molecular basis for the higher propensity of *C. dubliniensis* compared with *C. albicans* to develop drug resistance in vitro is unknown.

Comparative analysis of two such related, but nevertheless distinct, species should facilitate a better understanding of their biology and mechanisms of pathogenicity. By revealing differences, for example, in the relative importance of virulence traits or in the regulation of the corresponding genes, insights into the specific adaptation mechanisms of each of the two species to their host should be gained. This goal can best be achieved if the full arsenal of molecular methods becomes available for both species.

The molecular genetic analysis of *C. albicans* is based mainly on the use of the *URA3* gene as a marker for the selection of prototrophic transformants of *ura3*-auxotrophic host strains. Since the *URA3* marker can be both positively and negatively

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TABLE 1. *C. dubliniensis* strains used in this study

Strain	Parent	Relevant genotype[a]	Source or reference
Wü284		Clinical isolate	19
CdUM1A	Wü284	*ura3*Δ1::*MPA^R-FLIP*/*URA3*	This study
CdUM1B	Wü284	*ura3*Δ1::*MPA^R-FLIP*/*URA3*	This study
CdUM2A	CdUM1A	*ura3*Δ1::*FRT*/*URA3*	This study
CdUM2B	CdUM1B	*ura3*Δ1::*FRT*/*URA3*	This study
CdUM3A	CdUM2A	*ura3*Δ1::*FRT*/ *ura3*Δ2::*MPA^R-FLIP*	This study
CdUM3B	CdUM2B	*ura3*Δ1::*FRT*/ *ura3*Δ2::*MPA^R-FLIP*	This study
CdUM4A	CdUM3A	*ura3*Δ1::*FRT*/ *ura3*Δ2::*FRT*	This study
CdUM4B	CdUM3B	*ura3*Δ1::*FRT*/ *ura3*Δ2::*FRT*	This study
CdMGFP1A	CdUM4A	*MDR1*/*mdr1*::P$_{CdMDR1}$-*GFP*	This study
CdMGFP1B	CdUM4B	*MDR1*/*mdr1*::P$_{CdMDR1}$-*GFP*	This study

[a] *MPA^R-FLIP* denotes the *MPA^R* flipper cassette.

selected for, it can be used as a single, recyclable marker, for example, in the construction of specific mutants by sequential, targeted gene disruptions, but also for the introduction of reporter gene fusions to analyze gene expression. The construction of a *ura3* mutant by targeted gene deletion from a *C. albicans* wild-type strain by Fonzi and Irwin was a significant milestone in *C. albicans* genetics (6). Insertion of recombinant DNA into a specific locus in the *C. albicans* genome is straightforward, since homologous recombination is very efficient in this organism. Nevertheless, at that time the construction of such a strain required considerable effort because *C. albicans* is a diploid organism without a known sexual cycle, and two rounds of mutagenesis are necessary to obtain homozygous mutants. Deletion of the first *URA3* allele would not result in a detectable phenotype and, in the absence of available dominant selection markers, the desired heterozygous mutants had to be identified and isolated by a tedious PCR-based approach combined with sibling selection (6). Subsequent inactivation of the second *URA3* allele was then more readily achieved because *ura3* mutants could be identified by selection with 5-fluoro-orotic acid (FOA) (2). The resulting *ura3* strain, CAI4, is otherwise isogenic with the clinical *C. albicans* isolate SC5314 from which it was derived, and it has since been used as a model strain for the majority of molecular work in *C. albicans*. The development of CAI4 resulted in an explosion in the number of studies addressing many aspects of *C. albicans* biology and pathogenicity and, therefore, contributed much to our present knowledge about this fungus (4, 22).

For a comparison of *C. albicans* and *C. dubliniensis* at the molecular level, a similar *ura3*-negative *C. dubliniensis* host strain for use in genetic experiments would be extremely valuable. Recently, a dominant selection marker for the transformation of *C. albicans* wild-type strains became available (30), and it has been shown that this marker, which confers resistance to mycophenolic acid (MPA), can also be used for the selection of integrative *C. dubliniensis* transformants (26), which should allow the direct selection of heterozygous mutants. Although there is no negative selection scheme for deletion of the *MPA^R* marker from heterozygous mutants to allow its use for inactivation of the second allele of the target gene, this problem was circumvented in *C. albicans* by the development of the *MPA^R*-flipping strategy (18, 31). This mutagenesis scheme is based on the use of a cassette that contains, in addition to the selection marker, a *C. albicans*-adapted *FLP* gene (*caFLP*) encoding the site-specific recombinase FLP from *Saccharomyces cerevisiae* under the control of the inducible *C. albicans SAP2* promoter. The cassette is flanked by direct repeats of the FLP recognition site such that it can be excised from the genome by induced, FLP-mediated recombination after specific chromosomal insertion, leaving behind a disrupted copy of the target gene. A second round of mutagenesis then generates the desired homozygous mutants, which are otherwise isogenic with the wild-type parent strain. The findings that the *MPA^R* marker functions in *C. dubliniensis* and that the *SAP2* promoter from *C. albicans* is regulated in the same way also in the heterologous species *C. dubliniensis* (26) suggested that the *MPA^R*-flipping strategy could be used to generate specific mutants from *C. dubliniensis* wild-type strains by sequential, targeted gene disruption. Therefore, we set out to construct a *C. dubliniensis ura3* mutant that could serve as a host strain for efficient genetic manipulations aimed at comparing various aspects in which *C. dubliniensis* may differ from *C. albicans*, such as morphogenesis, virulence, and drug resistance.

MATERIALS AND METHODS

Strains and growth media. The *C. dubliniensis* strains used in this study are listed in Table 1. For routine growth of the strains, YPD liquid medium (containing, per liter, 10 g of yeast extract, 20 g of peptone, and 20 g of glucose) was used. Cells were grown overnight in YCB-BSA (23.4 g of yeast carbon base and 4 g of bovine serum albumin per liter [pH 4.0]) to induce the *SAP2* promoter for excision of the *MPA^R* flipper from MPA-resistant transformants. To screen for MPA-sensitive derivatives, 100 to 200 CFU was plated on minimal agar (containing, per liter, 6.7 g of yeast nitrogen base without amino acids [YNB; Bio 101, Vista, Calif.], 20 g of glucose, 0.77 g of complete supplement medium [CSM; Bio 101], and 15 g of agar) containing 1 μg of MPA ml^{-1}, which resulted in the generation of large MPA^r and small MPA^s colonies, respectively (31). Uridine (100 μg ml^{-1}) was added to the media to support the growth of *ura3* mutant strains.

Cloning of the *CdURA3* gene. A genomic library from *C. dubliniensis* strain CD36 (5, 15) was screened with a *C. dubliniensis URA3* (*CdURA3*) gene probe, generated using primers derived from the *C. albicans URA3* (*CaURA3*) gene. A 2-kb *Spe*I-*Hin*dIII fragment from a positive clone was subcloned into pBluescript and completely sequenced. In a parallel approach, the *CdURA3* downstream region was PCR amplified with primers URA17 (5′-CTATTTACAATCTCGAGGTGGTCCTTC-3′) and URA18 (5′-CCATTAATTGCGAGCTCTGCTACTGGAG-3′), derived from the *CaURA3* sequence (http://www-sequence.stanford.edu/group/candida), using genomic DNA from *C. dubliniensis* strain Wü284 as a template. The PCR product was digested at the introduced *Xho*I and *Sac*I sites (underlined), cloned into pBluescript, and sequenced. The overlapping sequences of the genomic clone and the PCR product were combined and used to

select primers for the amplification of *CdURA3*-flanking sequences in gene disruption experiments.

Construction of *CdURA3* gene deletion cassettes. Two different deletion constructs were made to inactivate both *URA3* alleles in *C. dubliniensis*. For pSFIcdU2, *CdURA3* sequences from position −799 to +53 and from position +797 to +1712 (with respect to the start codon) were PCR amplified with the primer pair CdURA1 (5′-AGAACGCATGCCAAGTTTGATAGTACTG-3′) and CdURA2 (5′-TGTGCCCGCGGTGAAGCATGAGTCTCTGC-3′) and the primer pair URA21 (5′-ATGCTTATTTGCTCGAGACTGGCCAA-3′) and CdURA4 (5′-GATACTTGGAGAGCTCGATTTGTTGCTGGTGC-3′), respectively, thereby introducing *Sph*I, *Sac*II, *Xho*I, and *Sac*I restriction sites (underlined). The *Sph*I-*Sac*II *CdURA3* upstream fragment and the *Xho*I-*Sac*I *CdURA3* downstream fragment were then cloned on both sides of a *Sac*II-*Xho*I fragment containing the MPA^R flipper from pSFI1 (31) in the *Sph*I/*Sac*I-digested vector pBluescript to generate pSFIcdU2 (Fig. 1A, top). Similarly, for pSFIcdU4, *CdURA3* sequences from position −799 to +225 and from position +620 to +1712 were amplified with the primer pair CdURA1 and CdURA5 (5′-CTAATAATGGTTCCGCGGTAGATTCATA-3′) and the primer pair CdURA6 (5′-TATTATGACTCCTCGAGTTGGATTAGA-3′) and CdURA4, respectively (the introduced *Sac*II and *Xho*I restriction sites are underlined). The *Sph*I-*Sac*II *CdURA3* upstream fragment and the *Xho*I-*Sac*I *CdURA3* downstream fragment were substituted for the corresponding *CdURA3*-flanking regions in pSFIcdU2, resulting in pSFIcdU4 (Fig. 1A, bottom). The *Sph*I-*Sac*I fragments from pSFIcdU2 and pSFIcdU4 were used in transformation experiments.

Construction of a P_{CdMDR1}-*GFP* reporter gene fusion. A *CdMDR1* promoter fragment (positions −943 to −8 with respect to the *CdMDR1* start codon) was obtained by PCR amplification from genomic DNA of strain Wü284 with primers CdMDR5 (5′-ATATATCTAGACTTACAAGATAGGTAAAG-3′) and CdMDR6 (5′-GCATTGTCGACAATTGTGTTTTCTTTGGTTGAG-3′), thereby introducing *Xba*I and *Sal*I restriction sites (underlined). A *CdMDR1* downstream fragment (positions +1564 to +2382) was amplified with primers CdMDR7 (5′-CCCGAATATCCTGCAGCCTGGGGTAGTTC-3′) and CdMDR8 (5′-ACTCACCCCACTAGAGCTCCAGTTTACAC-3′), thereby introducing *Pst*I and *Sac*I sites (underlined). The *CdMDR1* fragments were used to replace the flanking *SAP2* sequences in the previously described plasmid pGFP41 (17), yielding pCdMGFP2 (Fig. 2A). The *Xba*I-*Sac*I fragment from this plasmid was used to transform the *C. dubliniensis ura3* mutants.

***C. dubliniensis* transformation.** *C. dubliniensis* strains were transformed by electroporation (11). Cells from a YPD preculture were diluted 10^{-4} in 50 ml of fresh YPD medium and grown overnight at 30°C to an optical density at 600 nm (OD_{600}) of 1.6 to 2.2, which yielded the best transformation efficiency in our hands. The cells were collected by centrifugation and resuspended in 8 ml of water. After addition of 1 ml of 10× TE (100 mM Tris-HCl, 10 mM EDTA [pH 7.5]) and 1 ml of 1 M lithium acetate (pH 7.5), the suspension was incubated in a rotary shaker at 150 rpm for 60 min at 30°C. A 250-μl volume of 1 M dithiothreitol was then added, and the cells were incubated for a further 30 min at 30°C with shaking. After addition of 40 ml of water, the cells were centrifuged, washed sequentially in 50 ml of ice-cold water and 10 ml of ice-cold 1 M sorbitol, resuspended in 50 μl of 1 M sorbitol, and kept on ice. The inserts from the plasmids described above were excised with appropriate restriction enzymes and purified by agarose gel electrophoresis and elution with the GeneClean kit from Bio 101. Five microliters (approximately 1 μg) of the linear DNA fragments was mixed with 40 μl of electrocompetent cells, and electroporation was carried out in a Bio-Rad Gene Pulser (0.2 cm cuvette, 1.6 kV, 200 Ω, 25 μF) with a Bio-Rad Pulse Controller included in the circuit. MPA-resistant transformants were selected on minimal agar plates containing 10 μg of MPA ml^{-1}. Single colonies were picked after 5 to 7 days of growth at 30°C, restreaked on the same medium, and, after verification of the correct allelic replacement, maintained on YPD agar plates. Uridine-prototrophic transformants of *ura3* mutants were selected on minimal agar plates without uridine and picked after 2 days of growth at 30°C. To determine the proportion of transformed cells, the number of viable cells after electroporation also was determined by plating an appropriate dilution of the cell suspension onto medium supplemented with uridine.

Isolation of chromosomal DNA and Southern hybridization. Genomic DNA from *C. dubliniensis* strains was isolated as described by Millon et al. (14). DNA (10 μg) was digested with *Eco*RI, separated on a 1% (wt/vol) agarose gel, and, after ethidium bromide staining, transferred by vacuum blotting onto a nylon membrane and fixed by UV cross-linking. Southern hybridization with enhanced chemiluminescence (ECL)-labeled probes was performed with the ECL labeling and detection kit from Amersham (Braunschweig, Germany) according to the instructions of the manufacturer.

Drug stock solutions. Fluconazole (a kind gift from Pfizer UK) was dissolved in water (5 mg ml^{-1}), and benomyl (Sigma-Aldrich Chemie, Taufkirchen, Germany) was dissolved in dimethyl sulfoxide (DMSO; 10 mg ml^{-1}).

Fluorescence microscopy. Cells from an overnight culture were inoculated into fresh YPD medium and grown at 30°C to mid-log phase, when the indicated amount of drug was added. Aliquots of the cultures were taken at various times of growth in the absence or presence of drugs and spotted on microscope slides. Fluorescence was detected with a Zeiss Axiolab microscope equipped for epifluorescence microscopy with a 50-W mercury high-pressure bulb and the Zeiss fluorescein-specific filter set 09.

Nucleotide sequence accession numbers. The sequence of *CdURA3* obtained from the genomic library of strain CD36 and the *CdURA3* downstream sequence obtained from strain Wü284 have been deposited in the EMBL and GenBank nucleotide sequence databases under accession no. AJ302032 and AF328138, respectively.

RESULTS

Cloning of the *C. dubliniensis URA3* gene. Initial attempts to disrupt the *CdURA3* gene using a mutagenesis cassette in which the MPA^R flipper was flanked by upstream and downstream sequences of the *CaURA3* gene were unsuccessful, presumably because of sequence divergence between the two species (26; also unpublished data). Therefore, we cloned and sequenced the *CdURA3* gene and flanking sequences in order to identify suitable sequences for the targeted integration of the MPA^R flipper cassette into the *CdURA3* locus by homologous recombination. PCR primers based on the *CaURA3* gene sequence successfully amplified a fragment comprising 1 kb of sequence downstream of the *CdURA3* gene of Wü284; however, we were unable to amplify upstream sequences using *C. albicans*-derived primers. Therefore, a PCR product containing 390 bp of the *CdURA3* gene was used as a probe to identify a clone carrying the complete *CdURA3* gene from a genomic library of *C. dubliniensis* strain CD36 (5), and a *Spe*I-*Hin*dIII fragment comprising the *CdURA3* coding region plus an 807-bp upstream sequence and a 410-bp downstream sequence was completely sequenced. The *CdURA3* sequences obtained from the library clone and the PCR fragment overlapped by 292 bp in the downstream region. These sequences were completely identical, despite the fact that they were derived from two unrelated *C. dubliniensis* strains. The *CdURA3* coding region exhibited 93% similarity with the corresponding *C. albicans* sequence obtained from the genome sequencing project (http://www-sequence.stanford.edu/group/candida), with 98% identity at the amino acid level. However, the flanking regions showed a much higher sequence divergence, with 88% similarity within the sequenced 1,062-bp downstream region and only 70% similarity within the cloned 807-bp upstream region. This sequence divergence explains our failure to specifically integrate a *C. albicans*-based disruption construct into the *C. dubliniensis* genome and to amplify the *CdURA3* upstream region with primers derived from the *C. albicans* sequence.

Construction of *C. dubliniensis ura3* mutants by targeted gene disruption. To facilitate the genetic manipulation of *C. dubliniensis* we constructed a *ura3* mutant of the previously characterized strain Wü284 (19, 25, 26) by targeted gene disruption using the MPA^R-flipping strategy (31). Two different deletion constructs were made for inactivation of the two *URA3* alleles. In plasmids pSFIcdU2 and pSFIcdU4, the *CdURA3* coding sequence from position +54 (with respect to the start codon) to +796 (15 bp in front of the stop codon), or

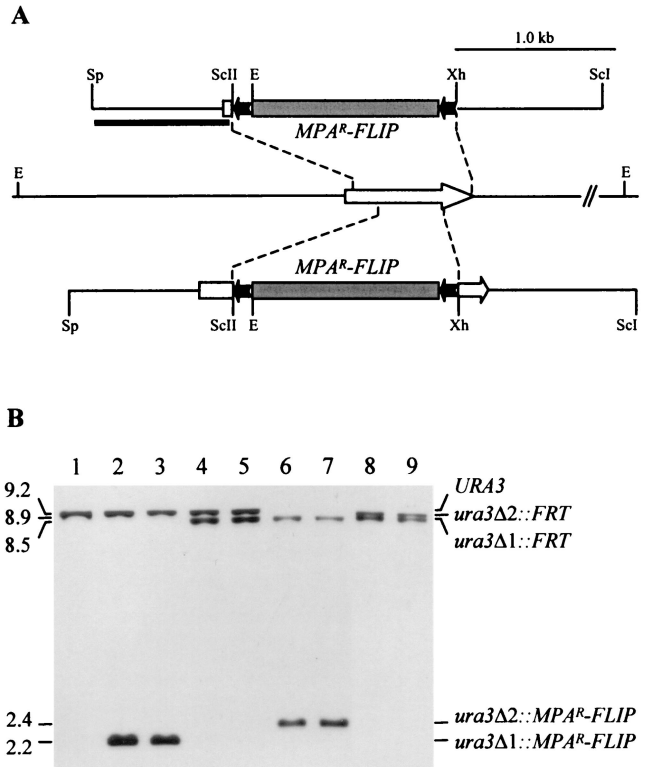

FIG. 1. Inactivation of the *CdURA3* gene by MPA^R flipping. (A) Structure of the *CdURA3* locus in strain Wü284 and allelic replacements using the inserts from pSFIcdU2 (upper part) or pSFIcdU4 (lower part). Open arrow, *CdURA3* coding region; solid lines, *CdURA3* upstream and downstream sequences. Only relevant restriction sites are shown: E, *Eco*RI; ScI, *Sac*I; ScII, *Sac*II; Sp, *Sph*I; Xh, *Xho*I. The 5.6-kb MPA^R flipper, details of which have been presented elsewhere (31), is not drawn to scale. Solid bar, DNA fragment used as a probe to verify the correct allelic replacements by Southern hybridization. (B) Southern hybridization of *Eco*RI-digested genomic DNA of the *ura3* mutants using the 5′ *CdURA3* fragment from pSFIcdU2 as a probe. The identities of the fragments are shown to the right of the blot, and molecular sizes are given on the left. Lanes: 1, Wü284 (*URA3*/*URA3*); 2, CdUM1A (*ura3*Δ1::MPA^R-*FLIP*/*URA3*); 3, CdUM1B (*ura3*Δ1::MPA^R-*FLIP*/*URA3*); 4, CdUM2A (*ura3*Δ1::*FRT*/*URA3*); 5, CdUM2B (*ura3*Δ1::*FRT*/*URA3*); 6, CdUM3A (*ura3*Δ1::*FRT*/*ura3*Δ2::MPA^R-*FLIP*); 7, CdUM3B (*ura3*Δ1::*FRT*/*ura3*Δ2::MPA^R-*FLIP*); 8, CdUM4A (*ura3*Δ1::*FRT*/*ura3*Δ2::*FRT*); 9, CdUM4B (*ura3*Δ1::*FRT*/*ura3*Δ2::*FRT*). *ura3*Δ1 and *ura3*Δ2 indicate the deletions generated using the inserts from pSFIcdU2 and pSFIcdU4, respectively.

from position +226 to +619, respectively, was replaced by the MPA^R flipper, such that the flanking *CdURA3* upstream and downstream sequences were of different lengths in the two plasmids (Fig. 1A). Strain Wü284 was first transformed with the insert from pSFIcdU2, and MPA-resistant transformants were analyzed by Southern hybridization. In the parent strain, Wü284, an *Eco*RI fragment of about 9.2 kb hybridized with the *CdURA3* probe (Fig. 1B, lane 1). In 11 out of 12 transformants tested, a single new *Eco*RI fragment of 2.2 kb appeared, suggesting correct replacement of one of the *CdURA3* alleles by the deletion cassette. In the remaining transformant an ectopic integration had occurred in addition to the desired allelic replacement. Two independent transformants that exhibited the new *Eco*RI fragment of 2.2 kb, termed CdUM1A and CdUM1B (Fig. 1B, lanes 2 and 3), were used to excise the MPA^R flipper by induced, FLP-mediated recombination as described previously (31), resulting in strains CdUM2A and CdUM2B, in which the 2.2-kb *Eco*RI fragment was replaced by an expected 8.5-kb fragment, 673 bp smaller than the original wild-type fragment (Fig. 1B, lanes 4 and 5). The insert from pSFIcdU4 was then used to delete the remaining *CdURA3* wild-type allele in strains CdUM2A and CdUM2B. MPA-resistant transformants were selected and screened for uridine auxotrophy. About half of the MPA-resistant transformants were auxotrophic, demonstrating that in both heterozygous parent strains, integration of the deletion cassette occurred with equal efficiency into the already disrupted or the remaining wild-type *URA3* allele. Southern hybridization analysis demonstrated the correct replacement of the second *CdURA3* allele in strains CdUM3A and CdUM3B (Fig. 1B, lanes 6 and 7), which is evident from the appearance of a 2.4-kb *Eco*RI fragment instead of the 9.2-kb fragment. Excision of the MPA^R flipper from these strains resulted in strains CdUM4A and CdUM4B, in which the 2.4-kb *Eco*RI fragment was replaced by an expected 8.9-kb fragment, 335 bp smaller than the original wild-type fragment (Fig. 1B, lanes 8 and 9). All 10 tested MPA-sensitive derivatives of the two parent strains exhibited this hybridization pattern, demonstrating that loss of the MPA^R marker was caused exclusively by intrachromosomal recombination of the *FRT* sites flanking the mutagenesis cassette and not by mitotic interchromosomal recombination of *FRT* sites on homologous chromosomes, which would have produced a different fragment (about 170 bp smaller).

The independently constructed *ura3* mutants CdUM4A and CdUM4B had identical growth characteristics in YPD medium supplemented with uridine. In addition, reintroduction of a *URA3* gene (see below) restored growth in medium without uridine to the same level as that of the original parent strain, Wü284.

Integration specificity in *C. dubliniensis ura3* mutants using *CaURA3* as a selection marker. We next evaluated whether the *URA3* gene could be used for selection of prototrophic transformants of the *C. dubliniensis ura3* mutants and for targeted integration of recombinant DNA into a specific genomic locus by homologous recombination. The heterologous *CaURA3* gene was used for this purpose, since it is widely used for *C. albicans* molecular genetics and many already existing genetic constructs could serve as the basis for future genetic manipulations of *C. dubliniensis* without the necessity to exchange the marker. Strains CdUM4A and CdUM4B were transformed by electroporation with a linear DNA fragment containing a *C. albicans*-adapted green fluorescent protein (*GFP*) gene under the control of the *CdMDR1* promoter (see below), the *CaURA3* selection marker, and *CdMDR1* downstream sequences. The flanking *CdMDR1* sequences served for integration of the reporter gene fusion into one of the *CdMDR1* alleles by allelic exchange (Fig. 2A). Uridine-prototrophic transformants of the two parent strains used were obtained with similar frequencies: using approximately 1 μg of the linear DNA fragment, we obtained 300 transformed cells out of 5.4×10^7 viable cells for CdUM4A (5.5×10^{-6}) and 950 transformed cells out of 1.36×10^8 viable cells for CdUM4B (7.0×10^{-6}). For each parent strain, six independent transformants were analyzed by Southern hybridization. All 12 transformants

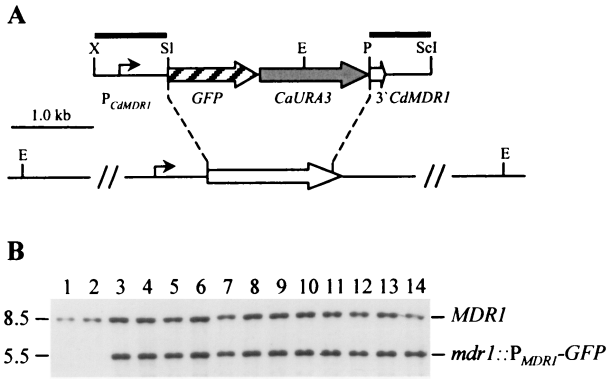

FIG. 2. Integration of the P_{CdMDR1}-*GFP* reporter gene fusion into the *MDR1* locus of strains CdUM4A and CdUM4B. (A) Genomic structure of the *MDR1* locus in strain Wü284 and its *ura3* derivatives and structure of the inserted reporter gene fusion from plasmid pCdMGFP2. Open arrow, *MDR1* coding region; solid lines, flanking upstream and downstream sequences. The position of the *CdMDR1* promoter (P_{CdMDR1}) and the direction of transcription are indicated by the solid arrow. Only relevant restriction sites are shown: E, *Eco*RI; P, *Pst*I; ScI, *Sac*I; Sl, *Sal*I; X, *Xba*I. Solid bars indicate the DNA fragments used as probes for verification of the correct allelic replacement by Southern hybridization. (B) Southern hybridization of *Eco*RI-digested genomic DNA of the parent strains CdUM4A (lane 1) and CdUM4B (lane 2) and transformants carrying the P_{CdMDR1}-*GFP* reporter gene fusion inserted into one of the *MDR1* alleles using the P_{CdMDR1} fragment as a probe. Lanes 3 to 8 transformants of CdUM4A; lanes 9 to 14, transformants of CdUM4B. The identities of the fragments are shown to the right of the blot, and molecular sizes are given on the left.

had correctly integrated the reporter fusion into the *CdMDR1* locus, as shown by the appearance of a new 5.5-kb *Eco*RI fragment in addition to the 8.5-kb wild-type fragment after hybridization with a probe from the *CdMDR1* upstream region (Fig. 2B). The correct allelic replacement was also confirmed by hybridization with a probe from the *CdMDR1* downstream region, which produced a 4.0-kb fragment in addition to the wild-type fragment in all 12 transformants (data not shown). These results demonstrate that the *CaURA3* gene can be used for efficient and targeted integrative transformation of the *C. dubliniensis ura3* mutants.

Expression of the *CdMDR1* gene is induced by benomyl but not by fluconazole. The *MDR1* gene encodes a membrane transport protein of the major facilitator superfamily and is involved in the resistance of *C. albicans* and *C. dubliniensis* to fluconazole and several other, structurally unrelated drugs (1, 15, 24). Fluconazole-susceptible *C. albicans* strains do not significantly express the *MDR1* gene in standard media in vitro, but *MDR1* is constitutively activated in many fluconazole-resistant isolates (7, 8). Similar data have also been obtained for *C. dubliniensis* (15). Although fluconazole itself does not influence *CaMDR1* expression, other drugs, such as benomyl, induce *CaMDR1* transcription in fluconazole-susceptible *C. albicans* (10). Therefore, we investigated a possible induction of the *CdMDR1* gene by the presence of these drugs using the *C. dubliniensis* strains with the chromosomally integrated P_{CdMDR1}-*GFP* reporter gene fusion. After growth in YPD medium, no fluorescence of the reporter strains was observed, demonstrating that, as in *C. albicans*, the *MDR1* gene was not significantly expressed in *C. dubliniensis* in the absence of drugs (Fig. 3). The addition of 100 μg of fluconazole ml^{-1} to the culture medium did not result in detectable fluorescence (Fig. 3), and the same result was obtained with a higher fluconazole concentration (200 μg ml^{-1}) and during extended times of growth in the presence of the drug (data not shown). In contrast, benomyl induced the *CdMDR1* promoter, resulting in maximal fluorescence of the reporter strains within 1 h following addition of the drug. *CdMDR1* induction by benomyl was dose dependent, with a higher benomyl concentration resulting in stronger fluorescence of the reporter strains. This fluorescence was not caused by autofluorescence due to possible benomyl-induced cell damage, since the parent strain Wü284, which did not carry the *GFP* gene, produced no detectable fluorescent signal under the same conditions (Fig. 3).

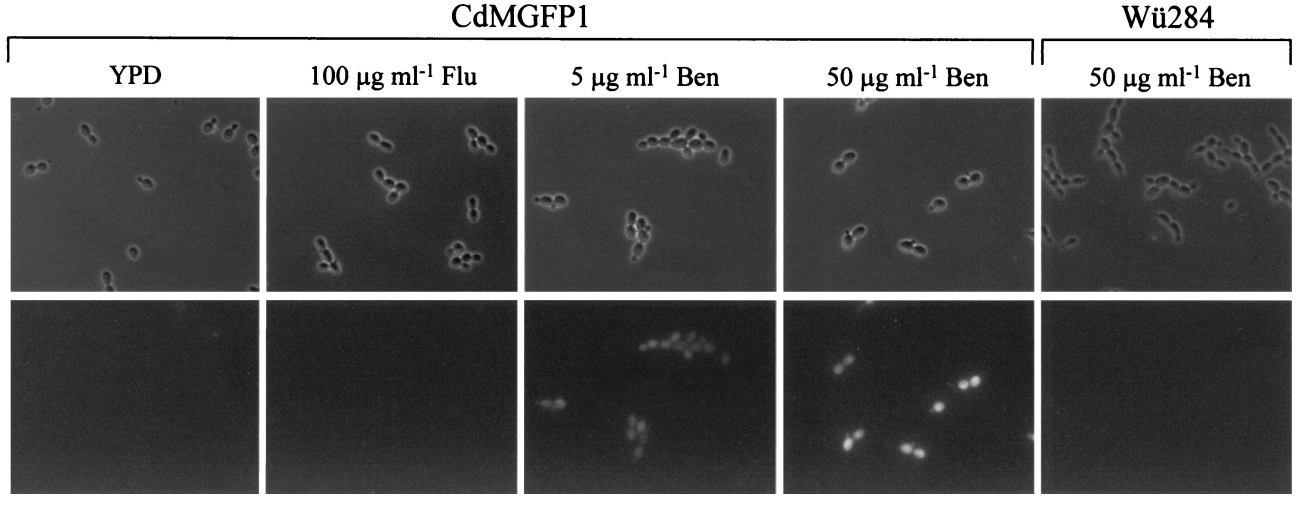

FIG. 3. Expression of the P_{CdMDR1}-*GFP* reporter gene fusion in *C. dubliniensis*. Shown are phase-contrast and corresponding fluorescence micrographs of the parent strain, Wü284, or the reporter strains grown for 1 h in the absence or presence of the indicated drugs. Identical results were obtained with strains CdMGFP1A and CdMGFP1B. Flu, fluconazole; Ben, benomyl.

DISCUSSION

Until recently, the generation of specific mutants of *C. albicans* wild-type strains by targeted gene deletion was a laborious task and, to our knowledge, has been performed only once, to construct the widely used *ura3* mutant strain CAI4 and similar derivatives from the clinical isolate SC5314 (6). The use of a dominant marker for the positive selection of integrative transformants, combined with its subsequent excision by FLP-mediated, site-specific recombination has made such an approach much more straightforward, and genes can now specifically be inactivated in any *C. albicans* strain, relieving the dependency on auxotrophic host strains for genetic manipulations (31). In the present work, using the same strategy, we successfully constructed a *C. dubliniensis ura3* mutant by targeted gene replacement, thereby providing the first available auxotrophic *C. dubliniensis* strain. Our results suggest that in *C. dubliniensis* the MPA^R marker is an even better tool than in *C. albicans* to direct the integration of recombinant DNA into a specific genomic locus. Since the MPA^R marker is derived from the *C. albicans IMH3* gene, MPA-resistant *C. albicans* transformants are frequently obtained that have not undergone the desired targeted recombination event but instead have presumably only integrated the marker by homologous recombination into the *IMH3* locus (reference 30 and unpublished data). In contrast, almost all MPA-resistant *C. dubliniensis* transformants analyzed in this study had specifically integrated the MPA^R flipper into one of the *CdURA3* alleles. The more-specific integration of the mutagenesis cassette into the target locus was probably due to the divergence between *C. albicans* and *C. dubliniensis* sequences, which very likely prevented integration of the MPA^R marker into the *IMH3* locus in the heterologous host. Sequence divergence between the two species also caused our failure to specifically direct the integration of a reporter gene fusion or of the MPA^R flipper into the *C. dubliniensis* genome when flanking sequences from *C. albicans* were used (26). These results are in line with a recent report demonstrating that even minor allelic differences within a given strain strongly bias integration specificity (32). Therefore, the *C. albicans*-derived MPA^R marker appears to be a powerful tool for the genetic engineering of *C. dubliniensis*. This result also suggests that a similar marker derived from *C. dubliniensis* (or another species) should facilitate targeted integrations in *C. albicans* wild-type strains in the same way.

The MPA^R-flipping strategy has now also been successfully used for inactivation of the *MDR1* gene in a fluconazole-resistant, *MDR1*-overexpressing *C. dubliniensis* isolate to assess the contribution of this efflux pump to drug resistance (S. Wirsching et al., unpublished data), suggesting that the method is generally applicable for *C. dubliniensis*. Nevertheless, the availability of a *ura3* mutant that is otherwise isogenic to a clinical isolate has several advantages for future genetic manipulations. First, using *URA3* as a selection marker, prototrophic transformants can be recovered after only 2 days of selection on uridine-deficient medium, whereas the primary isolation of MPA-resistant transformants usually requires about 1 week, with additional time needed for clone purification. Therefore, the generation of mutants by sequential gene disruptions is significantly accelerated when the *URA3* gene can be used as the selection marker. Second, many already available *URA3*-based genetic constructions that have been used for the molecular analysis of *C. albicans* can serve as the basis for analogous experiments in *C. dubliniensis* without the necessity for marker exchange; only homologous sequences from *C. dubliniensis* have to be substituted for the corresponding *C. albicans* fragments when genomic integration is required. Finally, the MPA^R marker has also been used as a reporter gene in *C. albicans* (A. Strauß et al., submitted for publication). Introduction of such a reporter construct into a host strain requires a second marker like *URA3* for the selection of transformants, and this approach is now feasible also for *C. dubliniensis*.

The two *CdURA3* alleles were inactivated with different deletion constructs, such that any interchromosomal recombination between the two alleles would be recognized by the appearance of new bands. This experimental design ensured that the deletion of the two *URA3* copies had occurred by independent, specific allelic replacements in our *ura3* mutants. However, the ability to differentiate between the two *URA3* alleles is also important with respect to future genetic manipulations, for example, when one is using the *URA3*-flipping strategy (18) to knock out additional genes. Since an *FRT* site is present in each of the disrupted *URA3* alleles, FLP-mediated recombination could also involve these target sequences. Due to the different extent of the deletions on both sides of the *FRT* sites present in the inactivated *URA3* alleles, any undesired recombination involving the *URA3* locus would be easily detected.

It has been suggested that *C. dubliniensis* can develop fluconazole resistance in vitro more readily than *C. albicans* (16). As with many fluconazole-resistant clinical *C. albicans* isolates, resistance in in vitro-generated fluconazole-resistant *C. dubliniensis* derivatives seems to be caused most frequently by constitutive activation of the *MDR1* gene, which also mediates resistance to several other compounds. The results of the present study do not provide an explanation for the higher propensity of *C. dubliniensis* to develop fluconazole resistance in vitro. As previously shown for *C. albicans* (7, 10), fluconazole itself did not induce the *MDR1* promoter in *C. dubliniensis*. However, as described for *C. albicans* (10), the addition of benomyl to the culture medium resulted in strong activation of the *CdMDR1* promoter. Therefore, certain drugs induce expression of the *MDR1* gene in both species. One could envisage that there might be more regulatory pathways controlling *MDR1* expression in *C. dubliniensis* than in *C. albicans*, which in turn would provide more targets for mutations that result in constitutive activation of the gene. The availability of reporter strains allowing easy detection of *MDR1* expression will help to unravel these differences, for example, by screening for substances that induce *MDR1* expression in *C. dubliniensis* but not in *C. albicans*.

In spite of the differences in the *URA3* regulatory region, the *CaURA3* gene was adequately expressed in *C. dubliniensis*, since we did not detect any difference in the growth of uridine-prototrophic transformants compared with that for the original *C. dubliniensis* wild-type isolate, Wü284. The transformation efficiency in the experiments described in this study, which required a double crossover event resulting in allelic replacement, was the same as we usually obtain in similar transformations of *C. albicans* strain CAI4 and derivatives. We also developed a *URA3*-based integrative vector for *C. dubliniensis* that requires only a single crossover for targeted insertion into

the genome. Using this linearized plasmid, the transformation rate is enhanced about 10-fold (unpublished data). This transformation efficiency will allow the directed integration of genomic libraries, for example, to clone *C. albicans* genes that influence *C. dubliniensis*-specific characteristics or to complement mutant phenotypes generated by nonspecific mutagenization of a *ura3* strain.

In conclusion, the full arsenal of molecular tools used in *C. albicans* genetics is now also available for its close relative *C. dubliniensis*. By taking advantage of species-specific differences, important questions regarding the biology and host adaptation mechanisms of these organisms can now be addressed at the molecular level.

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