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A Ser29Leu Substitution in the Cytosine Deaminase Fca1p Is Responsible for Clade-Specific Flucytosine Resistance in *Candida dubliniensis*▽

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The population structure of the opportunistic yeast pathogen *Candida dubliniensis* is composed of three main multilocus sequence typing clades (clades C1 to C3), and clade C3 predominantly consists of isolates from the Middle East that exhibit high-level resistance ($MIC_{50} \geq 128$ μg/ml) to the fungicidal agent flucytosine (5FC). The close relative of *C. dubliniensis*, *C. albicans*, also exhibits clade-specific resistance to 5FC, and resistance is most commonly mediated by an Arg101Cys substitution in the *FUR1* gene encoding uracil phosphoribosyltransferase. Broth microdilution assays with fluorouracil (5FU), the toxic deaminated form of 5FC, showed that both 5FC-resistant and 5FC-susceptible *C. dubliniensis* isolates exhibited similar 5FU MICs, suggesting that the *C. dubliniensis* cytosine deaminase (Fca1p) encoded by *C. dubliniensis FCA1* (Cd*FCA1*) may play a role in mediating *C. dubliniensis* clade-specific 5FC resistance. Amino acid sequence analysis of the Cd*FCA1* open reading frame (ORF) identified a homozygous Ser29Leu substitution in all 12 5FC-resistant isolates investigated which was not present in any of the 9 5FC-susceptible isolates examined. The tetracycline-inducible expression of the Cd*FCA1* ORF from a 5FC-susceptible *C. dubliniensis* isolate in two separate 5FC-resistant clade C3 isolates restored susceptibility to 5FC, demonstrating that the Ser29Leu substitution was responsible for the clade-specific 5FC resistance and that the 5FC resistance encoded by *FCA1* genes with the Ser29Leu transition is recessive. Quantitative real-time PCR analysis showed no significant difference in Cd*FCA1* expression between 5FC-susceptible and 5FC-resistant isolates in either the presence or the absence of subinhibitory concentrations of 5FC, suggesting that the Ser29Leu substitution in the Cd*FCA1* ORF is the sole cause of 5FC resistance in clade C3 *C. dubliniensis* isolates.

Candida dubliniensis is an opportunistic yeast pathogen that was first described in 1995 in human immunodeficiency virus-infected patients in Ireland (39). Since then the organism has been shown to have a worldwide distribution and has been recovered from other groups of immunocompromised individuals and from patients with severe underlying disease (2–4, 11, 29, 30, 36–38, 44). The population structure of *C. dubliniensis* has previously been investigated by using the species-specific complex DNA fingerprinting probe Cd25 and multilocus sequence typing (MLST) (4, 11, 15, 18). Early Cd25 fingerprinting analyses demonstrated that *C. dubliniensis* consists of two fingerprinting groups, termed Cd25 group I and Cd25 group II (15). Group I isolates comprise the majority of isolates investigated from many countries around the world and are very closely related, with an average similarity coefficient value (S_{AB}) of 0.8. Group II isolates are less closely related and have an average S_{AB} value of 0.47 (15). These results were later confirmed with a larger collection of isolates by Gee et al. (11), who also showed that Cd25 group I isolates comprised a single genotype (genotype 1) on the basis of sequence analysis of the internal transcribed spacer (ITS) region of the ribosomal DNA operon. Furthermore, Cd25 group II isolates were found to belong to three ITS genotypes (genotypes 2 to 4). In 2005, a study by Al Mosaid et al. (4) identified a third Cd25 fingerprinting group, termed Cd25 group III, which exhibited an average S_{AB} value of 0.35, among *C. dubliniensis* isolates recovered exclusively in Egypt, Saudi Arabia, and Israel, all of which belonged to ITS genotypes 3 or 4. All isolates belonging to Cd25 group III examined exhibited high-level intrinsic resistance to the antifungal drug flucytosine (5FC), apart from one Israeli isolate that was 5FC susceptible. This phenotype did not occur in isolates belonging to either Cd25 group I or Cd25 group II, including isolates from Cd25 groups I and II recovered from Egypt, Saudi Arabia, and Israel (4). Recent studies that have used MLST analysis to investigate the population structure of *C. dubliniensis* revealed the presence of three distinct clades, termed clades C1 to C3 (18). All 5FC-resistant isolates belonging to Cd25 fingerprint group III were found to cluster exclusively in MLST clade C3 (18). More recently, MLST was used to show that clade C1 *C. dubliniensis* isolates recovered from avian excrement-associated samples were genetically distinct from other clade C1 isolates that were recovered from humans (19).

The closest relative of *C. dubliniensis*, *Candida albicans*, also exhibits clade-specific resistance to 5FC, with 72.7% of isolates in MLST clade C1 (Ca3 fingerprinting clade I) exhibiting reduced susceptibility to this antifungal agent (23, 32). In *C. albicans*, the 5FC resistance patterns vary among isolates and range from reduced susceptibility (MICs, 0.5 to 2 μg/ml) to intermediate resistance (MICs, 2 to 8 μg/ml) or high-level

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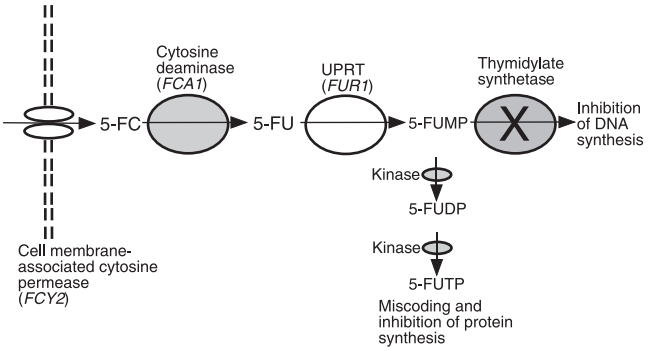

FIG. 1. Metabolic pathway and mode of action of 5FC in *Candida* yeasts. 5FC and 5FU are transported into the cell by cell membrane-associated cytosine-purine permeases. In *Candida* spp., these are encoded by two genes that display amino acid sequence homology with the *FCY2* gene of *S. cerevisiae* (13). Upon entry into the cell, 5FC is then deaminated to 5FU by Fca1p, encoded by *FCA1*. 5FU is then phosphorylated by UPRT, encoded by *FUR1*, yielding 5FUMP. 5FUMP inhibits thymidylate synthetase, which leads to thymidine depletion in the cell and which ultimately interrupts DNA synthesis. 5FUMP is also metabolized by two kinases, yielding fluorouridine diphosphate (5FUDP) and, subsequently, fluorouridine triphosphate (5FUTP), the latter of which is incorporated into RNA in the place of UTP, which leads to miscoding and the inhibition of protein synthesis.

resistance (MICs, ≥8 μg/ml); and a wide range of 5FC MICs for this drug have been reported among isolates (range, 0.06 μg/ml to ≥128 μg/ml) (7, 13). In *C. dubliniensis*, the resistance patterns are more clearly defined, with 5FC-susceptible isolates exhibiting 5FC MICs of ≤0.125 μg/ml and 5FC-resistant isolates exhibiting 5FC MICs of ≥128 μg/ml (4). To date, 5FC resistance in *C. dubliniensis* has been reported only in isolates from the Middle East, all of which that have been tested belong to MLST clade C3 (1, 4, 18, 29).

The antifungal action of 5FC relies on the intracellular conversion of 5FC to fluorouracil (5FU) by cytosine deaminases upon entry into fungal cells (Fig. 1). Cytosine deaminase (Fca1p) is encoded by *FCA1* in *C. albicans* and *C. dubliniensis* (Cd*FCA1*) (4, 9), and the *FCA1* genes in these two species are homologues of the *FCY1* gene in *Saccharomyces cerevisiae* (9) and in other *Candida* species, such as *Candida lusitaniae* (26). The absence of cytosine deaminases in mammalian cells prevents 5FC toxicity in humans, as the 5FC prodrug itself is nontoxic. After the conversion of 5FC to 5FU, the *FUR1*-encoded uracil phosphoribosyltransferase (UPRT) catalyzes the phosphorylation of 5FU to fluorouridine monophosphate (5FUMP) (Fig. 1). Two specific kinases catalyze the further phosphorylation of 5FUMP, eventually converting it to fluorouridine triphosphate. Fluorouridine triphosphate in turn gets incorporated into RNA, which causes miscoding, leading to the inhibition of fungal protein synthesis (Fig. 1). As a secondary method of inhibition, 5FUMP inhibits thymidylate synthetase (Fig. 1), leading to the depletion of dTTP and the misincorporation of dUTP into newly synthesized DNA, causing irreversible DNA damage and cell cycle arrest (14, 31, 41).

In haploid *C. lusitaniae* isolates, a missense T26C nucleotide mutation in the *FCY1* gene has been reported in four clinical isolates demonstrating 5FC resistance, although 5FC and 5FC-fluconazole cross-resistance has more commonly been attributed to defects in the purine cytosine permease-encoded *FCY2* gene in this species (10). In *C. albicans*, resistance to 5FC is mediated by a reduction in the activity of either the Fca1p encoded by *FCA1* or the UPRT encoded by *FUR1* (13, 31, 43). Two different research groups reported that in the majority of 5FC-resistant *C. albicans* isolates, resistance is associated with a homozygous single amino acid substitution, Arg101Cys, in UPRT (7, 13). However, other 5FC-resistant *C. albicans* isolates lack this substitution (13). One such isolate (5FC MIC, >64 μg/ml) was reported to contain a homozygous Gly28Asp substitution in the cytosine deaminase gene, and a Ser29Leu amino acid substitution was also observed in the same gene of another *C. albicans* isolate displaying intermediate 5FC resistance (5FC MIC, 4 μg/ml) (13). In *C. dubliniensis*, the DNA sequences of the *FUR1* genes encoding the UPRTs of four 5FC-resistant and five 5FC-susceptible isolates from the Middle East were determined previously, and while several single nucleotide polymorphisms (SNPs) were identified, no amino acid substitutions were observed between the isolates (4).

The purpose of the present study was to investigate the role of Fca1p in *C. dubliniensis* clade-specific 5FC resistance by the use of broth microdilution assays with 5FC and 5FU, analysis of DNA and amino acid sequences, and analysis of Cd*FCA1* expression. A tetracycline-inducible expression plasmid was used to incorporate the Cd*FCA1* gene from a 5FC-susceptible isolate (hereafter called Cd*FCA1*^{s}) into the *ADH1* locus of a 5FC-resistant isolate and the Cd*FCA1* gene from a 5FC-resistant isolate (hereafter called Cd*FCA1*^{r}) into the *ADH1* locus of a 5FC-susceptible isolate. These strains were used to determine if 5FC susceptibility or resistance could be induced in isolates upon the acquisition and expression of the respective Cd*FCA1* gene.

MATERIALS AND METHODS

Isolates and culture conditions. Twenty-one epidemiologically unrelated human *C. dubliniensis* isolates were included in the present study, including 9 5FC-susceptible isolates and 12 5FC-resistant isolates (Table 1), as reported previously (2, 4, 11, 30, 39). Previously, 5FC resistance in *C. dubliniensis* has ever been reported only in isolates from Saudi Arabia, Egypt, Israel, and Kuwait (1, 4, 29); and all but two of these have previously been investigated by MLST analysis and/or Cd25 fingerprint analysis and were shown to belong to *C. dubliniensis* MLST clade C3 and Cd25 fingerprint group III (4, 18). For these reasons, 20 of the 21 isolates chosen for study (12 5FC-resistant isolates and 8 5FC-susceptible isolates) were originally recovered in Egypt, Saudi Arabia, or Israel (Table 1). The 5FC-susceptible isolates belonged to MLST clade C1 or C2 and Cd25 fingerprint group I or II (4, 18). The *C. dubliniensis* type strain CD36, originally isolated from the oral cavity of a human immunodeficiency virus-infected individual in Ireland, was also included as a reference isolate because the complete genome sequence of this organism has been determined (http://www.sanger.ac.uk/sequencing/Candida/dubliniensis/). The *C. dubliniensis* isolates were routinely cultured on yeast extract-peptone-dextrose (YPD) agar, pH 5.6, at 37°C. For liquid culturing, isolates were grown in YPD broth (with the following per liter: 10 g yeast extract [Sigma-Aldrich Ireland Ltd., Wicklow, Ireland], 20 g peptone [Oxoid, Basingstoke, Hampshire, England], and 20 g D-glucose, pH 5.5) at 37°C in an orbital incubator (Gallenkamp, Leicester, United Kingdom) at 200 rpm. *Escherichia coli* strain DH5α (12) [F^- ϕ80d*lacZ*Δm15 Δ(*lacZYA-argF*)*U169 endA1 recA1 hsdR17*(r_K^- m_K^+) *supE44 thi-1 d*$^-$ *gyrA96 relA1*] was routinely grown on Luria-Bertani agar, pH 7.4, at 37°C or in Luria-Bertani broth, pH 7.4, at 37°C with shaking at 200 rpm for liquid culture.

Susceptibility testing. The MICs for 5FC and 5FU (Sigma-Aldrich) were determined by the method described in document M27-A2 of the Clinical and Laboratory Standards Institute (CLSI; formerly the National Committee for Clinical Laboratory Standards) by using RPMI 1640 medium (6). Both drugs were titrated from a concentration of 128 μg/ml to one of 0.25 μg/ml for the preliminary analysis of all 21 clinical isolates, as well as for analysis of the

TABLE 1. *Candida dubliniensis* isolates used to investigate the molecular mechanism of 5FC resistance and their susceptibilities to 5FC and 5FU[a]

Isolate	Country of origin	Yr of isolation	Sample	Cd25 fingerprint group	ITS genotype	MIC_{50} (μg/ml)		Reference
						5FC	5FU	
CD36[b]	Ireland	1988	Oral	I	1	≤0.25	32	4, 11, 39
SA101	S. Arabia	2002	Oral	I	1	≤0.25	16	4
SA105	S. Arabia	2002	Oral	I	1	≤0.25	32	4
SA115	S. Arabia	2002	Oral	I	1	≤0.25	32	4
Eg203	Egypt	2002	Oral	I	1	≤0.25	16	4
Eg206	Egypt	2002	Oral	I	1	≤0.25	32	4
p7276	Israel	1999	RT	II	3	≤0.25	8	4, 11
p6785	Israel	1999	Urine	II	3	≤0.25	16	4, 11, 30
p7718	Israel	1999	Wound	III	4	≤0.25	16	4, 11
Eg200	Egypt	2002	Oral	III	4	≥128	8	4
Eg201	Egypt	2002	Oral	III	4	≥128	32	4
Eg202	Egypt	2002	Oral	III	4	≥128	32	4
Eg207	Egypt	2002	Oral	III	4	≥128	32	4
SA100	S. Arabia	2002	Oral	III	3	≥128	32	4
SA103	S. Arabia	2002	BAL	III	3	≥128	32	4
SA107	S. Arabia	2002	Oral	III	3	≥128	32	4
SA108	S. Arabia	2002	Oral	III	3	≥128	32	4
SA109	S. Arabia	2002	Oral	III	3	≥128	8	4
SA113	S. Arabia	2002	Oral	III	4	≥128	32	4
SA118	S. Arabia	2002	Oral	III	3	≥128	32	4
SA121	S. Arabia	2002	Oral	III	4	≥128	32	4

[a] Abbreviations: S. Arabia, Saudi Arabia; RT, respiratory tract; BAL, broncheoalveolar lavage fluid.
[b] *C. dubliniensis* type strain.

recipient isolates and transformant derivatives from cloning experiments. These were determined as the lowest concentrations of the drug that reduced the turbidity by 50% relative to the turbidity of the growth of the drug-free controls. All isolates were tested in duplicate and on two separate occasions.

DNA extraction. Isolates were grown overnight in 5 ml of YPD broth as described above. Cells were harvested from 1.5 ml of culture by centrifugation at 14,000 × *g*, and the DNA was extracted from the resulting pellet by using a DNeasy blood and tissue kit, according to the manufacturer's instructions (Qiagen Science, Crawley, West Sussex, United Kingdom) and resuspended in a final volume of 200 μl. Nucleic acids were ethanol precipitated and resuspended in 50 μl of molecular-grade Milli-Q Biocel-purified water (resistivity, 18.2 MΩ/cm; Millipore, Carrigtwohill, Cork, Ireland).

PCR amplification, sequencing, and sequence analysis of Cd*FCA1*. The complete open reading frame (ORF) of the *C. dubliniensis* Cd*FCA1* gene was amplified from 12 5FC-resistant isolates (Table 1) and 9 5FC-susceptible isolates (Table 1) by using oligonucleotide primers FCA1F and FCA1R, which incorporated SalI and BglII restriction endonuclease recognition sites, respectively (Table 2). The reaction mixtures contained 100 ng of purified template DNA, 1× Expand high-fidelity buffer, 2.5 U of Expand high-fidelity PCR system enzyme mixture (Roche Applied Science, Mannheim, Germany), 0.2 mM concentrations of each deoxynucleoside triphosphate (Promega Corporation, Madison, WI), and 0.2 μM concentrations of each oligonucleotide (Sigma Genosys Biotechnologies Europe Ltd., Pampisford, Cambridgeshire, United Kingdom). The reaction mixtures underwent an initial denaturation step of 94°C for 10 min, followed by 35 cycles of 94°C for 30 s, 52°C for 30 s, and 72°C for 1 min and a final elongation step of 72°C for 7 min. The 550-bp products were purified with a GenElute PCR cleanup kit (Sigma-Aldrich), and both strands were sequenced by using the same primers that had been used for amplification. DNA sequencing reactions were performed commercially by Cogenics (Essex, United Kingdom) with an ABI 3730xl DNA analyzer. Multiple DNA and amino acid sequence alignments of Cd*FCA1* genes and their encoded proteins from 5FC-susceptible and 5FC-resistant *C. dubliniensis* isolates were carried out by using the CLUSTAL W sequence alignment computer program (40), available at the EMBL-EBI website (http://www.ebi.ac.uk/).

Tetracycline-inducible Cd*FCA1* expression in *C. dubliniensis*. The tetracycline-inducible gene expression plasmid pNIM1, developed by Park and Morschhauser (27), was adapted to investigate the inducible expression of the *C. dubliniensis* Cd*FCA1*^{s} and Cd*FCA1*^{r} genes in both 5FC-susceptible and 5FC-resistant *C. dubliniensis* isolates. The pNIM1 cassette was originally designed to integrate into the *C. albicans* alcohol dehydrogenase encoding gene *ADH1*. The DNA

TABLE 2. Oligonucleotide primers used in this study

Oligonucleotide	Sequence (5′–3′)[b]	Function
ADH1F	ATGCAAGCAAGCTTATTCA	PCR screening of transformants[a]
cartTA	CGGCATACTATCAGTAGTAG	
SAT	CAATGCCGCCGAGAGTAAAG	
ADH1R	CCCAAGATCTTACCTTCTTCCATT	
FCA1F	GACGCGTCGACGATATCAACGATGACATTT	Cd*FCA1* cloning and PCR screening
FCA1R	CGGGATCCAGATCTTTATTCTCCAATATCTTC	
RTFCA1F	AAACGCAGGAAGATTGCCAG	Gene expression analysis
RTFCA1R	TGGCCCCTGTACACATACTACATG	
RTACT1F	AGCTCCAGAAGCTTTGTTCAGACC	
RTACT1R	TGCATACGTTCAGCAATACCTGGG	

[a] The regions of the pNIM1-Cd*FCA1* cassette and background *ADH1* locus amplified during PCR screening of transformants are displayed in Fig. 2.
[b] The SalI and BglII restriction endonuclease recognition sites incorporated into the FCA1F and FCA1R primer sequences, respectively, are underlined.

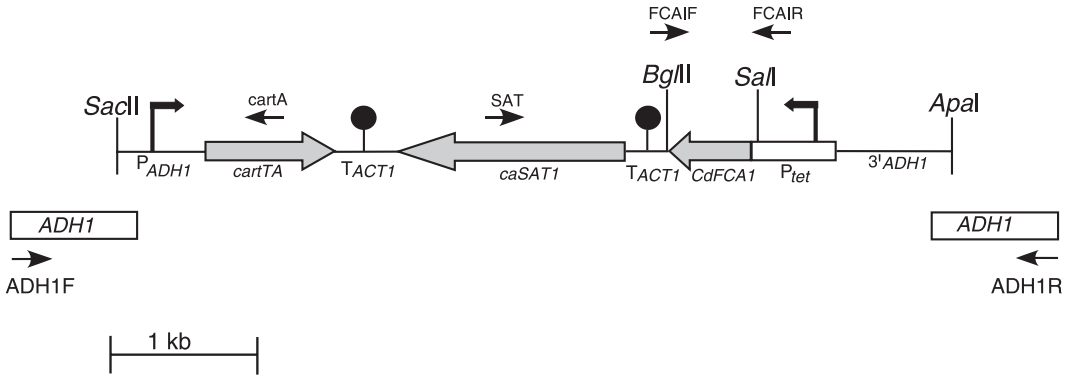

FIG. 2. Structure and *ADH1* integration site of the pNIM1-Cd*FCA1* cassette used in the tetracycline-inducible expression transformation studies. The restriction sites used for the excision of the *C. albicans GFP1* gene (replaced by the Cd*FCA1* gene) and the excision of the entire pNIM1-Cd*FCA1* cassette from the pNIM1 plasmid (27) are indicated. Transcription start sites and the directions of transcription are displayed by right-angled arrows. Terminator sequences are displayed as black hairpin loops and function in the termination of transcription of the *Candida*-adapted reverse tetracycline-dependent transactivator (*cartTA*) and the Cd*FCA1* target gene. The primers used in the PCR screening of the pNIM1-Cd*FCA1* transformants are indicated by labeled arrows and are listed in Table 2.

sequence for the *C. albicans ADH1* gene (GenBank accession no. CaO19.3997) was used in a BLAST search against the *C. dubliniensis* genome sequence database (http://www.sanger.ac.uk/sequencing/Candida/dubliniensis/) in order to identify a homologue in *C. dubliniensis*. A high level of sequence homology (94%) is shared by the *ADH1* ORFs of *C. albicans* and *C. dubliniensis*. The tetracycline-inducible promoter $P_{tet,}$ included in pNIM1 (27), was used to drive expression of the Cd*FCA1*^{s} and Cd*FCA1*^{r} genes individually in the *ADH1* locus of a *C. dubliniensis* isolate with the opposite 5FC phenotype. The complete coding regions of the Cd*FCA1* gene from 5FC-susceptible *C. dubliniensis* isolate p7276 and 5FC-resistant *C. dubliniensis* isolates SA113 and SA109 were amplified from genomic DNA with the FCA1F-FCA1R primer pair (Table 2). The amplimers were ligated into pGEM T-Easy vector I (Promega) vector DNA and transformed into *E. coli* strain DH5α. Plasmids were recovered from the transformants by using a GenElute plasmid miniprep kit (Sigma-Aldrich), and the cloned DNA was sequenced. The complete Cd*FCA1* ORF was digested from the pGEM T-Easy plasmids by using the SalI and BglII restriction endonuclease recognition sites, which were introduced upstream and downstream of the ORF, respectively, and the FCA1F-FCA1R primer pair. The Cd*FCA1* fragments were then gel purified with a Wizard SV Gel and PCR cleanup system (Promega) and separately cloned between P_{tet} and T_{ACT1} in SalI-BglII-digested pNIM1, incorporating the Cd*FCA1* ORFs into the pNIM1 cassette in the place of the *GFP1* gene (27), (Fig. 2). The resulting plasmids, pNIM1-Cd*FCA1*^{s} and pNIM1-Cd*FCA1*^{r}, were transformed into *E. coli* strain DH5α for replication of the plasmid, prior to purification with the GenElute plasmid miniprep kit (Sigma-Aldrich), SacII-ApaI linearization of the cassette, gel purification, and transformation into *C. dubliniensis* as described previously (35). 5FC-resistant isolates SA113 and SA109 were transformed with pNIM1-Cd*FCA1*^{s}, and 5FC-susceptible isolate p7276 was transformed with pNIM1-Cd*FCA1*^{r} (Table 1). All three of these *C. dubliniensis* isolates were also transformed with the pNIM1 cassette containing the *GFP1* gene instead of the Cd*FCA1* gene as a control for the disruption of the *ADH1* locus. In order to confirm the correct integration of the complete pNIM1-Cd*FCA1* cassette into the *ADH1* locus in transformant derivatives, a number of PCR amplifications were carried out. The primer pairs used in these PCR amplifications were ADH1F and cartTA, SAT and FCA1R, FCA1F and ADH1R, and SAT and ADH1R (Table 2). These stepwise amplifications revealed the presence and the correct integration of the full pNIM1-Cd*FCA1* cassette in transformant derivatives. This was further confirmed by Southern hybridization analysis with two separate probes: a Cd*FCA1*-directed probe and a pNIM1-directed probe which was directed toward the *cartTA* transactivator region and the background *ADH1* locus. The probes were labeled with digoxigenin (DIG) by using a DIG DNA labeling and detecting kit (Roche). The prehybridization, hybridization, wash, and detection steps were carried out according to the manufacturer's instructions.

Cd*FCA1* expression analysis. To monitor the relative gene expression of the Cd*FCA1* gene in 5FC-resistant wild-type *C. dubliniensis* isolates (isolates SA113, SA109, and Eg202) and 5FC-susceptible wild-type *C. dubliniensis* isolates (isolates Eg204 and p7276), as well as in the doxycycline (DOX)-inducible transformant derivatives (strains SA113T1, SA109T1, and SA109T2), quantitative real-time PCR was carried out according to standard protocols. In brief, RNA was extracted from isolates and transformant derivatives that were grown in YPD broth in the presence of DOX (15 μg/ml) only or YPD broth in the presence of DOX (15 μg/ml) and subinhibitory concentrations of 5FC (6.4 ng/ml). The RNAs were extracted with an RNeasy minikit (Qiagen) and were treated with DNeasy (Ambion; Applied Biosystems, Warrington, United Kingdom), according to the manufacturer's instructions. The RNA samples were then reverse transcribed to cDNA by using a Superscript II reverse transcriptase kit (Invitrogen, Biosciences Ltd., Dun Laoghaire, Dublin, Ireland). Quantitative real-time PCR was carried out with two pairs of reverse transcription-PCR primers; one pair amplified the Cd*FCA1* gene, and the second pair amplified the *ACT1* gene as an internal expression control (Table 2). The comparative amplification efficiencies of these primers were assessed, prior to reverse transcription-PCR, by using primer amplification efficiency plot analysis, as described previously (28). Quantitative real-time PCRs were carried out with 0.3 μM of each primer and SYBR green master mixture in an ABI 7500 real-time PCR system (Applied Biosystems), according to the manufacturer's recommended protocols. Data analysis was carried out as described by Schmittgen and Livak (34), and the $2^{-\Delta CT}$ values were calculated from the average threshold cycle (C_T) values acquired from three replicates for both the Cd*FCA1* and the *ACT1* genes.

RESULTS

In vitro susceptibility testing. Nine *C. dubliniensis* isolates previously reported to be 5FC susceptible and 12 *C. dubliniensis* isolates previously reported to be 5FC resistant by Al Mosaid et al. (4) (Table 1) were tested for their susceptibilities to 5FC by broth microdilution assays. All nine isolates previously reported to be 5FC susceptible were confirmed as such and exhibited 5FC MICs of ≤0.25 μg/ml. Similarly, the 12 previously reported 5FC-resistant isolates exhibited 5FC MICs of ≥128 μg/ml. In an attempt to localize potential blocks or lesions in the 5FC metabolic pathway in 5FC-resistant isolates which may contribute to resistance, broth microdilution assays were also carried out with 5FU instead of 5FC (Table 1). We hypothesized that if a block in the 5FC metabolic pathway occurred at the level of Fca1p, then 5FC-resistant isolates should be susceptible to 5FU (Fig. 1). All 9 5FC-susceptible isolates and 12 5FC-resistant isolates used in the study (Table 1) were tested for their susceptibilities to 5FU by using a range of concentrations (0.25 to 128 μg/ml). All 21 isolates exhibited 5FU MICs in the range of 8 to 32 μg/ml (Table 1). There was no correlation between the 5FU MIC and susceptibility to

5FC. These findings indicated that a block(s) in the 5FC metabolic pathway occurred in resistant isolates at the level of cytosine deaminase or upstream of this enzyme (Fig. 1).

Sequence analysis of Cd*FCA1* in *C. dubliniensis*. The DNA sequence of the *C. albicans FCA1* gene, which encodes Fca1p (GenBank accession no. U55194), was used in a BLAST search against the *C. dubliniensis* genome sequence (http://www.sanger.ac.uk/sequencing/Candida/dubliniensis/) in order to identify its homologue in *C. dubliniensis*. In *C. dubliniensis*, Fca1p is encoded by the Cd*FCA1* gene, which shares 89% sequence identity with *FCA1* from *C. albicans*, and both contain an internal intron of 81 bp. In order to investigate whether a mutation(s) or a deletion(s) was present in the Cd*FCA1* gene encoding Fca1p, the DNA sequences of the Cd*FCA1* ORF were determined for all 12 5FC-resistant isolates (Table 1) and all 9 5FC-susceptible isolates (Table 1) investigated in the study. For each isolate, the Cd*FCA1* sequence was compared with that of the Cd*FCA1* consensus sequence of 5FC-susceptible *C. dubliniensis* type strain CD36 (http://www.sanger.ac.uk/sequencing/Candida/dubliniensis/). Three separate SNPs were identified in the Cd*FCA1*-coding sequences of the 21 *C. dubliniensis* isolates investigated. Two synonymous SNPs were observed; one of these (position 264, A → T transition) occurred in all isolates sequenced, with the exception of six of the nine 5FC-susceptible isolates (isolates CD36, Eg203, Eg206, SA101, SA105, and SA115), and the second (position 390, T → C transition) occurred in only one of the 5FC-susceptible isolates (isolate p7276). The third SNP was nonsynonymous (position 86, C → T transition), resulting in an amino acid substitution (Ser29Leu) in all 12 of the 5FC-resistant isolates tested, but was not present in the Cd*FCA1* gene of the 9 5FC-susceptible isolates sequenced.

Tetracycline-inducible expression of Cd*FCA1* in *C. dubliniensis*. Transformation of the pNIM1-Cd*FCA1*^{r} cassette into 5FC-susceptible isolate p7276 yielded several transformants with the correct integration of the pNIM1-Cd*FCA1*^{r} cassette into the *ADH1* locus, as determined by Southern hybridization and PCR analysis. Broth microdilution assays were carried out with these transformants by using serial dilutions of 5FC from a concentration of 0.25 to one of 128 μg/ml. All of the transformants were 5FC susceptible (5FC MICs ≤ 0.25 μg/ml). This experiment was replicated with the addition of the tetracycline derivative DOX to the RPMI 1640 broth microdilution medium at a final concentration of 15 μg/ml in order to induce the expression of the pNIM1-Cd*FCA1*^{r} cassette. All of the DOX-induced transformants remained 5FC susceptible (5FC MICs ≤ 0.25 μg/ml). Transformation of pNIM1-Cd*FCA1*^{s} DNA into 5FC-resistant isolates SA113 and SA109 yielded several transformants with the correct integration of the pNIM1-Cd*FCA1*^{s} cassette into the *ADH1* locus, as determined by Southern hybridization and PCR analysis (Table 3). All of these transformants were 5FC resistant (5FC MICs ≥ 128 μg/ml). In contrast, in a parallel series of experiments, replicate broth microdilution assays were carried out with the addition of DOX (15 μg/ml) to the RPMI assay medium. This resulted in a dramatic change in the 5FC resistance phenotype of these transformants (isolates SA109T1, SA109T2, and SA113T1) from being 5FC resistant (5FC MICs ≥ 128 μg/ml) to being 5FC susceptible (5FC MICs ≤ 0.25 μg/ml) (Table 3). Similar results were obtained in separate broth microdilution experiments with DOX at a final concentration of 30 μg/ml (Table 3). As transformation controls, 5FC-resistant parental isolates SA109 and SA113 and 5FC-susceptible parental isolate p7276 were also transformed with the pNIM1 cassette containing the *GFP1* gene instead of the Cd*FCA1* gene (Table 3). These control pNIM1-*GFP1* transformants were also examined by broth microdilution assays in the presence and absence of DOX (15 μg/ml). The presence or absence of DOX in the broth microdilution medium had no effect on the 5FC MICs, and all the transformant derivatives tested exhibited 5FC MICs similar to those of their respective parental isolates (Table 3). These results strongly suggest that expression of the Cd*FCA1*^{s} gene by the transformants harboring the entire pNIM1-Cd*FCA1*^{s} cassette (i.e., isolates SA109T1, SA109T2, and SA113T1; Table 3) is responsible for the DOX-inducible 5FC susceptibility exhibited by these transformants.

TABLE 3. Susceptibilities of *C. dubliniensis* isolates and pNIM1-*FCA1*/*GFP1* transformant derivatives in the presence or absence of DOX

Isolate or transformant	5FC MIC_{50} (μg/ml) with DOX at:		
	None	15 μg/ml	30 μg/ml
C. dubliniensis clinical isolates			
SA113	≥128	≥128	≥128
SA109	≥128	≥128	≥128
p7276	≤0.25	≤0.25	≤0.25
C. dubliniensis pNIM1-Cd*FCA1*^{r} transformants			
p7276T1	≤0.25	≤0.25	≤0.25
p7276T2	≤0.25	≤0.25	≤0.25
p7276T3	≤0.25	≤0.25	≤0.25
C. dubliniensis pNIM1-Cd*FCA1*^{s} transformants			
SA109T1	≥128	≤0.25	≤0.25
SA109T2	≥128	≤0.25	≤0.25
SA113T1	≥128	≤0.25	≤0.25
C. dubliniensis pNIM1-*GFP1* transformants			
SA113-GFP1T1	≥128	≥128	≥128
SA109-GFP1T1	≥128	≥128	≥128
SA109-GFP1T2	≥128	≥128	≥128
p7276-GFP1T1	≤0.25	≤0.25	≤0.25

Cd*FCA1* expression analysis. A comparison of Cd*FCA1* gene expression by the 5FC-susceptible isolates (isolates Eg204 and p7276) and 5FC-resistant isolates (isolates SA113, SA109, and Eg202) and the pNIM1-Cd*FCA1*^{s} transformant derivatives (isolates SA113T1, SA109T1, and SA109T2) was undertaken following exposure to DOX (15 μg/ml) or following exposure to DOX (15 μg/ml) and a subinhibitory concentration (6.4 ng/ml) of 5FC. The expression of Cd*FCA1* was analyzed by quantitative real-time PCR, and the data were normalized to the level of *ACT1* expression, which was used as an internal control. Two-tailed Student's *t* tests were carried out on the Cd*FCA1* expression values obtained from 5FC-susceptible and 5FC-resistant clinical isolates, and no significant differences in Cd*FCA1* expression were observed between the two groups of isolates in either the presence ($P = 0.47$) or the absence ($P = 0.16$) of 5FC in the growth medium. This suggests that alter-

ations in Cd*FCA1* expression do not play a significant role in mediating 5FC resistance. Upon exposure to 5FC (6.4 ng/ml), the level of Cd*FCA1* expression increased in both the 5FC-susceptible isolates (range, 14.5- to 25-fold) and the 5FC-resistant isolates (range, 4- to 18-fold). Two-tailed Student's *t* tests confirmed the significance of these Cd*FCA1* expression increases upon addition of 5FC to the growth medium ($P < 0.001$). On exposure to DOX, transformant derivatives SA109T1, SA109T2, and SA113T1 all showed significant ($P < 0.001$) increases in their levels of Cd*FCA1* expression (range, 5- to 26-fold) in comparison to those of parental isolates SA109 and SA113. These transformant derivatives also showed significant ($P < 0.001$) increases in their levels of Cd*FCA1* expression (range, 5- to 22-fold) in comparison to those of their parental isolates, isolates SA109 and SA113, in the presence of a subinhibitory concentration of 5FC as well as DOX.

DISCUSSION

Studies of pyrimidine salvage pathways (Fig. 1) and 5FC resistance mechanisms in yeast species have previously been undertaken with *S. cerevisiae* (9, 17, 25, 42), *C. albicans* (9, 13, 43), and *C. lusitaniae* (5, 22, 26). Investigations with *S. cerevisiae* have shown that the disruption of the *FCY2* or the *FUR1* gene can play a role in 5FC resistance, but only the *FCA1* gene is absolutely required for the mediation of 5FC susceptibility (24, 25). In *C. lusitaniae*, Papon et al. (26) reported that inactivation of either the *FCA1* or the *FCY2* gene mediates 5FC resistance (5FC MICs, 128 μg/ml and 64 μg/ml, respectively) and promotes cross-resistance to 5FC and fluconazole (MICs, 4 μg/ml to 32 μg/ml), and the authors suggested that this was due to the competitive inhibition of fluconazole uptake by extracellular 5FC (5, 22, 26). Further analysis has identified a nonsense mutation in the *FCY2* gene that resulted in a truncated purine cytosine permease in seven such isolates. In addition to this finding, a missense mutation (Met9Thr transition) has been identified in the *FCY1* genes of four clinical *C. lusitaniae* isolates that also exhibited 5FC and 5FC-fluconazole cross-resistance (10). In *C. albicans*, two different research groups (7, 13) identified a homozygous Arg101Cys amino acid substitution in the *FUR1*-encoded UPRT to be the most common cause of high-level 5FC resistance (5FC MICs, 8 to >64 μg/ml). Isolates that were heterozygous for this transition exhibited reduced 5FC susceptibility (5FC MICs, 0.5 to 1 μg/ml) (13). Furthermore, a homozygous Gly28Asp substitution in Fca1p, encoded by *FCA1*, was suggested by Hope et al. to be an alternative method of resistance in a *C. albicans* isolate that did not harbor the UPRT-associated Arg101Cys substitution (13). Finally, Hope et al. (13) also described a *C. albicans* isolate with a Ser29Leu substitution in Fca1p which exhibited an intermediate level of 5FC resistance (5FC MIC, 4 μg/ml). In the light of the findings of the previous studies, we hypothesized that the *C. dubliniensis* pyrimidine salvage pathway very likely retains structural and functional homology with the pyrimidine salvage pathways in other *Candida* species, as *C. dubliniensis* is the closest relative to *C. albicans* in the genus *Candida*. Therefore, we investigated the Cd*FCA1*-encoded Fca1p (Fig. 1) as a possible cause of *C. dubliniensis* clade-specific 5FC resistance. Initially, broth microdilution assays were carried out with both 5FC and 5FU to determine whether the deamination step in the 5FC metabolic pathway was responsible for 5FC resistance. If the deamination step was responsible for 5FC resistance, bypassing its requirement in the metabolic pathway by exposing 5FC-resistant cells to 5FU should result in susceptibility to 5FU (see Fig. 1). Both 5FC-susceptible and 5FC-resistant isolates exhibited similar 5FU MICs (Table 1), indicating that Fca1p is very likely responsible for the clade-specific 5FC resistance in *C. dubliniensis*. In order to investigate this possibility further, DNA sequence analysis of the Cd*FCA1* genes from 12 5FC-resistant and 9 5FC-susceptible *C. dubliniensis* isolates was undertaken. This identified a homozygous Ser29Leu substitution that occurred exclusively among 5FC-resistant isolates. This radical substitution results in the replacement of a hydrophilic polar amino acid (serine) with a hydrophobic nonpolar residue (leucine) in the β1 strand of the cytosine deaminase enzyme and is closely linked to an active-site residue, according to the yeast cytosine deaminase structure defined by Ko et al. (16). This amino acid substitution may disrupt the quaternary structure of the enzyme, distorting the active site and inhibiting the conversion of the 5FC prodrug to its toxic form, 5FU. As mentioned above, a similar amino acid substitution was reported by Hope et al. in a *C. albicans* isolate; however, that isolate exhibited intermediate resistance (5FC MIC, 4 μg/ml) to 5FC (13), in comparison to the high levels of 5FC resistance observed in the *C. dubliniensis* isolates displaying the Ser29Leu substitution in the present study. The differences in the levels of resistance to 5FC exhibited by the *C. albicans* isolate reported by Hope et al. and the 5FC-resistant *C. dubliniensis* isolates reported here, all of which harbored the same Ser29Leu substitution in Fca1p, may be due to the fact that different 5FC MIC determination methods were used in the two studies: the EUCAST method (33) was used in the previous study for the *C. albicans* isolates, and the CLSI method (6) was used in the present study for the *C. dubliniensis* isolates. Alternatively, differences in Cd*FCA1* and *FCA1* gene expression or posttranscriptional or posttranslational modifications may be responsible for the differences between the two species in the levels of resistance to 5FC exhibited by isolates harboring the Fca1p Ser29Leu substitution.

In order to obtain direct evidence that the Ser29Leu substitution present in Fca1p from 5FC-resistant *C. dubliniensis* isolates was responsible for the 5FC-resistant phenotype in these isolates, the gene encoding cytosine deaminase from 5FC-susceptible *C. dubliniensis* isolate p7276 (Cd*FCA1*^{s}), which was originally recovered in Israel and which lacked the Ser29Leu substitution, was introduced into the *ADH1* locus of two separate 5FC-resistant Saudi Arabian isolates, SA109 (ITS genotype 3) and SA113 (ITS genotype 4), by the tetracycline-inducible cassette pNIM1 (27). Three transformant derivatives (isolates SA109T1, SA109T2, and SA113T1) harboring the complete pNIM1-Cd*FCA1*^{s} cassette integrated into the *ADH1* locus were tested and exhibited DOX-inducible 5FC susceptibility on the acquisition and expression of the Cd*FCA1*^{s} gene (Table 3). In contrast, transformant derivatives of 5FC-susceptible *C. dubliniensis* isolate p7276 harboring the complete pNIM1-Cd*FCA1*^{r} cassette encoding the *FCA1* gene with the Ser29Leu substitution from 5FC-resistant *C. dubliniensis* isolate SA113 integrated into the *ADH1* locus remained 5FC susceptible following DOX induction (Table 3). These findings provided convincing evidence that the Ser29Leu substitution in

Fca1p from 5FC-resistant isolates was responsible for 5FC resistance in these isolates, but they also showed that the 5FC resistance mutation is recessive and, thus, that 5FC resistance is not expressed in the presence of a wild-type, functional *FCA1* allele.

No significant difference in Cd*FCA1* expression was detected between the 5FC-susceptible and the 5FC-resistant *C. dubliniensis* isolates tested by quantitative real-time PCR analysis in either the presence or the absence of subinhibitory concentrations of 5FC in the growth medium. These results indicate that the lack of expression or the reduced expression of Cd*FCA1* in 5FC-resistant *C. dubliniensis* isolates following exposure to subinhibitory concentrations of 5FC was not responsible for the 5FC resistance in 5FC-resistant isolates and that the Ser29Leu substitution is very likely the sole method of Cd*FCA1*-mediated 5FC resistance in the *C. dubliniensis* isolates investigated. Previous studies have shown that free pyrimidines often present in peptones present in some brands of culture media can antagonize the activity of 5FC (8). Antagonism was not observed in the present study with the YPD-grown cultures used in the expression studies. Following the addition of a subinhibitory concentration of 5FC (i.e., 6.4 ng/ml) to YPD-grown cultures, the quantitative real-time PCR experiments consistently showed that both 5FC-susceptible and 5FC-resistant *C. dubliniensis* isolates exhibited significant upregulation of *FCA1* expression: 14.5- to 20-fold and 4- to 18-fold, respectively.

All Cd25 fingerprint group III *C. dubliniensis* isolates, apart from one (isolate p7718; Table 1), tested so far exhibit high-level 5FC resistance; all were originally recovered in Saudi Arabia or Egypt; and all belong to MLST clade C3 (4, 18). The close genetic relationship shared by these isolates is reflected by their identical Cd*FCA1* DNA sequences and high-level resistance to 5FC. It is highly likely that an identical mechanism is used to mediate 5FC resistance in all of these isolates. MLST C3 clade (Cd25 fingerprint group III) *C. dubliniensis* isolates can be subdivided into ITS genotypes 3 and 4 on the basis of the nucleotides sequence of the ITS region of the ribosomal DNA operon (4), although clade C3 isolates of both ITS genotypes exhibit high-level 5FC resistance. In the present study, clade C3 *C. dubliniensis* isolates SA109 (ITS genotype 3) and SA113 (genotype 4) were both transformed with the pNIM1-Cd*FCA1*^{s} cassette, and both yielded transformant derivatives (isolates SA109T1, SA109T2, and SA113T1) that exhibited DOX-inducible 5FC susceptibility. These findings support our view that clade-specific 5FC resistance in *C. dubliniensis* is mediated by a common molecular mechanism, i.e., the presence of the Ser29Leu substitution in Fca1p.

This is not the first report of a clade-specific SNP that has resulted in the alteration of a protein involved in antifungal drug resistance in *C. dubliniensis*. In 2002, Moran et al. (20) reported that 58% of ITS genotype 1 *C. dubliniensis* isolates (Cd25 group I, MLST clade C1) harbored a TAG nonsense mutation in the *CDR1* gene encoding an ABC transporter. In *C. albicans*, the upregulation of *CDR1* is the most common mechanism of fluconazole resistance, whereas in *C. dubliniensis*, the most common mechanism of fluconazole resistance involves the overexpression of the *MDR1* gene encoding a multidrug transporter (21). These studies highlight the fact that despite the close phylogenetic relationship between *C. dubliniensis* and *C. albicans*, resistance to particular antifungal drugs can be due to different mechanisms in the two species.

In conclusion, the results of this study demonstrate that the presence of a Ser29Leu substitution in Fca1p in *C. dubliniensis* isolates is responsible for clade-specific resistance to 5FC. Isolates belonging to *C. dubliniensis* clade C3 have been recovered only from individuals of Arab ethnicity in Saudi Arabia, Egypt, and Israel (4, 18). Resistance to 5FC has not yet been reported in *C. dubliniensis* isolates from other countries around the world, apart from Kuwait. In 2004, Ahmad et al. reported the recovery of two 5FC-resistant isolates of *C. dubliniensis* from Kuwait (1). Because of Kuwait's close proximity to Saudi Arabia, it is likely that these isolates also belong to *C. dubliniensis* MLST clade C3.

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