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Csr1/Zap1 Maintains Zinc Homeostasis and Influences Virulence in *Candida dubliniensis* but Is Not Coupled to Morphogenesis

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The supply and intracellular homeostasis of trace metals are essential for every living organism. Therefore, the struggle for micronutrients between a pathogen and its host is an important determinant in the infection process. In this work, we focus on the acquisition of zinc by *Candida dubliniensis*, an emerging pathogen closely related to *Candida albicans*. We show that the transcription factor Csr1 is essential for *C. dubliniensis* to regulate zinc uptake mechanisms under zinc limitation: it governs the expression of the zinc transporter genes *ZRT1*, *ZRT2*, and *ZRT3* and of the zincophore gene *PRA1*. Exclusively, artificial overexpression of *ZRT2* partially rescued the growth defect of a *csr1*Δ/Δ mutant in a zinc-restricted environment. Importantly, we found that, in contrast to what is seen in *C. albicans*, *Csr1* (also called *Zap1*) is not a major regulator of dimorphism in *C. dubliniensis*. However, although a *csr1*Δ/Δ strain showed normal germ tube formation, we detected a clear attenuation in virulence using an embryonated chicken egg infection model. We conclude that, unlike in *C. albicans*, Csr1 seems to be a virulence factor of *C. dubliniensis* that is not coupled to filamentation but is strongly linked to zinc acquisition during pathogenesis.

Access to zinc is essential for organisms throughout the three domains of life. It is the only metal that occurs as a cofactor in all six classes of enzymes, from oxido-reductases to lyases (1), and the average proportion of enzymes containing zinc is 8.8% in eukaryotic proteomes (2). In pathogens, virulence-associated proteins frequently bind zinc for structural stability or catalytic activity; e.g., the Ser/Thr-protein kinase PrkC of *Bacillus anthracis*, which is essential for its pathogenicity, is regulated by zinc (3). In the pathogenic yeast *Candida albicans*, three out of six known superoxide dismutases (CaSod1, CaSod4, and CaSod6) are copper-zinc dependent. Enzymes of this class detoxify reactive oxygen species and thus contribute to virulence (4–6). Therefore, it is of particular importance for both benign and pathogenic microbes to ensure a sufficient zinc supply, especially when faced with a micronutrient-poor environment.

Exploiting this dependency, mammalian hosts manipulate levels of accessible zinc and other metals to inhibit pathogen growth and dissemination. This targeted limitation of micronutrients is known as nutritional immunity and is one of the main strategies used to defend against pathogenic microorganisms (7). To oppose zinc deprivation, pathogenic bacteria and fungi evolved specialized uptake mechanisms to obtain zinc (8, 9). For example, a high-affinity zinc transporter system is required for virulence of *Salmonella enterica* in mice (10). The intracellular zinc homeostasis is generally strictly controlled, and in *C. albicans*, the response to zinc deficiency is mediated by the transcription factor Csr1 (*Candida* suppressor of *ROK1*) (11), the ortholog of *Saccharomyces cerevisiae* Zap1 (zinc-responsive activator protein). Within the *Candida* clade, *CSR1* orthologs have been found in all sequenced species. However, to date, this transcriptional factor has been investigated only in *C. albicans* in more detail, while the function of Csr1 in other pathogenic yeasts like *Candida glabrata* or even in the closest relative of *C. albicans*, *Candida dubliniensis*, is unknown.

Both *C. dubliniensis* and *C. albicans* are harmless gastrointestinal colonizers, but they can cause diseases ranging from superficial mucosal infections to life-threatening candidemia, especially in immunocompromised individuals. Interestingly, *C. dubliniensis* is less frequently isolated from patients with nosocomial bloodstream infections than *C. albicans* (2 to 3% versus 10%, respectively) (12–14). The overall lower virulence of *C. dubliniensis* has also been confirmed in mice infection models (15) and was found to be associated with differences in species-specific pathogenicity properties, such as the ability to adhere and to form true hyphae, which allow tissue invasion (16, 17). Finding differences in the genetic setup and infection-relevant phenotypes of these two fungi is, therefore, a promising avenue to dissect virulence in pathogenic yeasts and may provide insights into the mechanisms of evolutionary rewiring of regulatory factors among related microbes.

In *C. albicans*, Csr1 is known to have dual functions: it plays the key role both in transcriptional regulation of zinc homeostasis and in biofilm formation. *C. albicans* mutants lacking *CSR1* hence cannot proliferate under low-zinc conditions and show reduced filamentation in the presence of serum (11) accompanied by al-

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TABLE 1 Strains in this study

Candida strain	Parent	Genotype[a]	Reference
SC5314		*C. albicans* wild-type strain	59
Wü284		*C. dubliniensis* wild-type strain	60
CSR1/*SAT1*A/B	Wü284	*CD36_44490*Δ::*SAT1-FLIP*/*CD36_44490*	This study
CSR1/*csr1*ΔA/B	CdCSR1M1A/B	*CD36_44490*Δ::*FRT*/*CD36_44490*	This study
*csr1*Δ/*SAT1*A/B	CdCSR1M2A/B	*CD36_44490*Δ::*FRT*/*CD36_44490*Δ::*SAT1-FLIP*	This study
*csr1*Δ/ΔA/B	CdCSR1M3A/B	*CD36_44490*Δ::*FRT*/*CD36_44490*Δ::*FRT*	This study
CdCSR1KS1A/B	*csr1*Δ/ΔA/B	*CD36_44490*Δ::*FRT*/ *CD36_44490*Δ:: *CD36_44490*-T_{ACT1}-*SAT1-FLIP*	This study
*csr1*Δ/Δ+*CSR1*A/B	CdCSR1KS1A/B	*CD36_44490*Δ::*FRT*/ *CD36_44490*Δ:: *CD36_44490*-T_{ACT1}-*FLIP*	This study
ZRT1^{OE}A/B	Wü284	*ADH1*/*adh1*::P_{ADH1}-*CD36_46440*-ca*SAT1*	This study
ZRT2^{OE}A/B	Wü284	*ADH1*/*adh1*::P_{ADH1}-*CD36_17380*-ca*SAT1*	This study
PRA1^{OE}A/B	Wü284	*ADH1*/*adh1*::P_{ADH1}-*CD36_46450*-ca*SAT1*	This study
*csr1*Δ/Δ+*ZRT1*^{OE}A/B	*csr1*Δ/ΔA/B	*CD36_44490*Δ::*FRT*/*CD36_44490*Δ::*FRT ADH1*/*adh1*::P_{ADH1}-*CD36_46440*-ca*SAT1*	This study
*csr1*Δ/Δ+*ZRT2*^{OE}A/B	*csr1*Δ/ΔA/B	*CD36_44490*Δ::*FRT*/*CD36_44490*Δ::*FRT ADH1*/*adh1*::P_{ADH1}-*CD36_17380*-ca*SAT1*	This study
*csr1*Δ/Δ+*PRA1*^{OE}A/B	*csr1*Δ/ΔA/B	*CD36_44490*Δ::*FRT*/*CD36_44490*Δ::*FRT ADH1*/*adh1*::P_{ADH1}-*CD36_46450*-ca*SAT1*	This study

[a] SAT1-FLIP denotes the SAT1 flipper cassette (33).

tered biofilm formation (18). Further analysis of genes regulated by Csr1 of *C. albicans* (CaCsr1) under biofilm-inducing conditions revealed 60 targets, including Ca*ZRT1-3*, Ca*PRA1*, and Ca*CSR1* itself (18). It is noteworthy that in biofilm-producing communities, a *C. albicans csr1*Δ/Δ mutant strain secretes smaller amounts of the quorum-sensing molecule farnesol, which contributes to an altered morphology (19).

The Zrt proteins belong to the ZIP (Zrt/Irt-like proteins) transporter family and facilitate zinc ion transfer across membranes into the cytosol or cellular organelles. Zrt1 of *S. cerevisiae* (ScZrt1) is a high-affinity transporter in *S. cerevisiae* that mediates zinc uptake under strong zinc depletion, but it is downregulated under low-zinc conditions. There, the low-affinity zinc transporter ScZrt2 ensures import of zinc (20, 21). These complementary uptake systems are under the control of ScZap1 (22). Tightly controlled zinc uptake mechanisms in response to extracellular zinc levels have been observed not only in *S. cerevisiae* but also in *Schizosaccharomyces pombe* and *Aspergillus fumigatus* (22–24). Finally, Pra1 is a zinc-binding protein which is part of a novel zinc uptake mechanism of *C. albicans* recently discovered by Citiulo et al. (25).

A *C. albicans csr1*Δ/Δ mutant is known to be proliferation defective during murine infections (26) and to elicit a decreased immune response in mice (27). In addition to this observation, expression of *CSR1* and some of its target genes was increased up to 10-fold during the early stage of infection with the corresponding *C. albicans* wild-type strain (27).

In the present work, we analyzed the role of the *C. dubliniensis* transcription factor Csr1 (*CD36_44490*)—a homolog of *C. albicans* Csr1—in zinc homeostasis, germ tube formation, and virulence traits.

MATERIALS AND METHODS

Strains and culture conditions. *Candida* strains were routinely propagated on YPD agar (20 g peptone, 10 g yeast extract, 20 g glucose, 15 g agar per liter) at 30°C and stored as frozen stocks in YPD medium with 15% (vol/vol) glycerol at −80°C. For zinc starvation experiments, low-zinc medium (LZM) was prepared as described previously (22). The medium was supplemented with $ZnSO_4$ as indicated (LZM0 contains no zinc; LZM25 and LZM2000 contain 25 μM and 2,000 μM $ZnSO_4$, respectively), and 25 μM $FeSO_4$ was used as a source of iron. *Candida* strains used in this work are listed in Table 1.

Germ tube assays. Strains were grown in YPD overnight (30°C and 180 rpm), washed with double-distilled water (ddH_2O), and transferred into filament-inducing medium at an optical density at 600 nm (OD_{600}) of 0.2. To stimulate filamentation, we used spider medium (1% mannitol, 1% nutrient broth, 0.2% K_2HPO_4 [pH 7.2]), liquid YPD, or H_2O plus 10% (vol/vol) fetal calf serum. Cultures were shaken (180 rpm) for 4 h at 37°C, and morphology was microscopically analyzed (Axiovert, Zeiss, Germany).

Chlamydospore formation. Chlamydospore production was induced on rice extract-Tween 80 agar (BD, Heidelberg, Germany) or Staib agar (28), both prepared as described before. The plates were incubated at 28°C for 2 to 4 days in the dark, and chlamydospore formation was monitored microscopically.

Plasmid construction. The deletion cassette for *CSR1* was constructed as follows. An ApaI-XhoI fragment with *CSR1* upstream sequences was cloned after amplification by PCR with the primers CSR1-1 and CSR1-2 (see Table S1 in the supplemental material) using genomic DNA from *C. dubliniensis* Wü284 as the template. A SacII-SacI fragment containing *CSR1* downstream sequences was obtained with the primers CSR1-3 and CSR1-4. The *CSR1* upstream and downstream fragments replaced *SSU2* upstream and downstream fragments in plasmid pSSU2M2 (29) via the introduced restriction sites, to result in pCSR1M2, in which the *SAT1* flipper is flanked by *CSR1* sequences.

The whole *CSR1* gene for the gene reconstitution was amplified using the primers CdCSR1-1 and CdCSR1-5, the ApaI/BglII-cut DNA fragment was integrated into pSAP2KS1 (30), and the *CSR1* downstream DNA element was inserted as described above.

For the generation of the *PRA1* overexpression cassette, a XhoI-BglII fragment was amplified via PCR with the primers PRA1-1 and PRA1-2. Genomic DNA from *C. dubliniensis* Wü284 was used as the template. This DNA fragment was introduced behind the *cdADH1* promoter into the pcdADH1E2 vector (31), and the plasmid was named pcdPRA1E1. The plasmids pcdZRT1E1 and pcdZRT2E1were constructed in a similar way by amplifying a XhoI-BglII fragment with the primer pair ZRT1-1 and ZRT1-2 or ZRT2-1 and ZRT2-2. The primer ZRT2-1 carried a SalI restriction site that is compatible with the XhoI overhang of the parental plasmid pcdADH1E2.

***C. dubliniensis* transformant construction.** Linear DNA fragments were transformed by electroporation into chemically competent *C. dubliniensis* cells (32), and clones were selected on YPD plates containing nourseothricin (Werner Bioagents, Jena, Germany). The usage of the SAT1 flipper strategy allowed the recycling of the selection marker, as described here (33). The insertion locus of the DNA fragment was confirmed by Southern blot analyses.

Southern blotting. A 10-μg portion of isolated genomic DNA was digested with an appropriate restriction enzyme. After DNA separation on an agarose gel (1%), DNA was stained with ethidium bromide and transferred onto a nylon membrane using a vacuum blot system. UV-linked DNA was hybridized with chemiluminescence-labeled probes and detected via the Amersham ECL direct nucleic acid labeling and detection kit (GE Healthcare, Braunschweig, Germany) according to the manufacturer's instructions (see Fig. S1 in the supplemental material).

Growth curve analyses. Proliferation under zinc depletion was evaluated via growth curve assays. Strains were pregrown overnight in YPD at 30°C and after repeated washing, cells with an OD_{600} of 0.4 were inoculated in LZM0 without additional zinc. After starvation in LZM0 for 24 h at 30°C, cells were diluted to an OD_{600} of 0.01 in LZM supplemented with various concentrations of $ZnSO_4$. Cultures were incubated at 30°C in a Magellan TECAN plate reader with shaking for 30 s, and the OD_{600} was determined every 15 min over 48 h. Changes of the OD_{600} were plotted against the incubation time.

Quantitative real-time reverse transcription-PCR (qRT-PCR). To determine gene expression rates, cells were precultured in YPD overnight (30°C and 180 rpm) and washed with phosphate-buffered saline (PBS). A total of 5×10^6 cells/ml were inoculated into 200 ml LZM plus 2,000 μM $ZnSO_4$, and the cells were grown for an additional 24 h (30°C and 180 rpm). To remove residual zinc, cultures were washed four times with ultrapure water, and all yeast cells were transferred into 200 ml LZM0 without zinc.

Cells from 20 ml of liquid culture were sampled and frozen in liquid nitrogen at 0 h, 0.5 h, 4 h, and 24 h. RNA was isolated using an RNeasy kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. A Bioanalyzer instrument (Agilent, Santa Clara, CA) was used to measure RNA quality, and RNA concentration was determined via NanoDrop (Thermo Fisher Scientific, Waltham, MA). A 700-ng portion of RNA was treated with DNase and transcribed into cDNA (enzymes by Promega [Fitchburg, WI]). Finally, a total amount of 13.3 ng cDNA was used for each qRT-PCR that included EvaGreen as fluorescent dye and ROX as an internal reference (Biosell, Feucht, Germany). The experiments were performed in a thermal cycler (Bio-Rad, Hercules, CA) and run in biological duplicates and technical triplicates. The expression rates reported here are relative to the expression values of the housekeeping gene *TEF3*. All primers are listed in Table S1 in the supplemental material.

Sequence analyses. The protein sequences of *C. dubliniensis* Cd36_44490 (CdCsr1), *C. albicans* orf19.3794 (CaCsr1), *C. glabrata* CAGL0J05060g (CgCsr1), *S. cerevisiae* YJL056C (ScZap1), *Aspergillus fumigatus* Afu1g10080 (ZafA), and *Cryptococcus neoformants* (CnZap1) were compared using NCBI PBLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi); they were aligned with the ClustalW2 multiple-sequence-alignment tool (http://simgene.com/ClustalW), and the phylogenetic tree was constructed at http://www.phylogeny.fr (34). This phylogenetic analysis includes MUSCLE (v3.7) alignment, removal of ambiguous regions with Gblocks (v0.91b), and the reconstruction of the phylogenetic tree used the maximum-likelihood method implemented in the PhyML program (v3.0 aLRT). The tree was plotted with TreeDyn (v198.3).

Pro Coffee (35) was used as a tool to align homologous promoter regions of *ZRT2* from *C. dubliniensis*, *C. albicans*, and *S. cerevisiae*.

Chicken embryo infection model. The embryonated chicken infection model was used to study virulence as described previously (36). Briefly, overnight cultures of yeasts were washed with PBS and adjusted to 10^8 cells/ml. An inoculum of 10^7 yeast cells/egg was applied to the chorioallantoic membrane at developmental day 10 via an artificial air chamber. In each experiment, the viability of 20 eggs per group (*Candida* or PBS control) was evaluated daily by candling for 7 days. Experiments were performed twice. Surviving embryos were humanely terminated by chilling on ice at the end of the experiment. All experiments were performed in compliance with the German animal protection law. According to this, no specific approval is needed for work performed on avian embryos before the time of hatching. Experiments were terminated at the latest on developmental day 18.

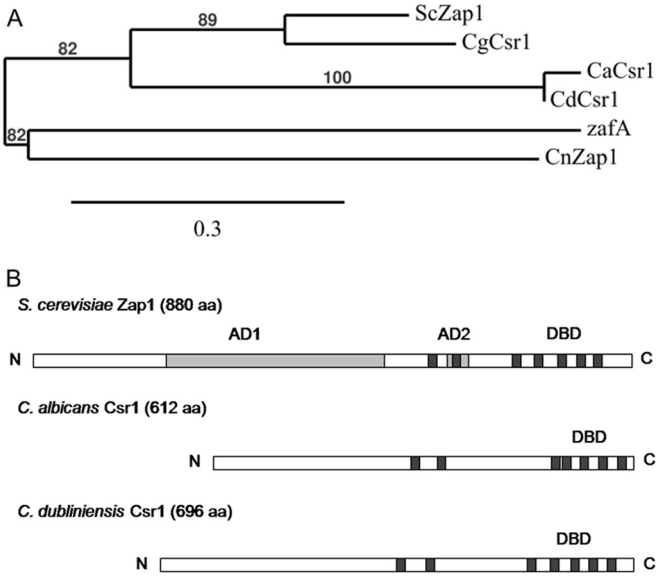

FIG 1 Structural analysis of the homologous Csr1 proteins. (A) Phylogenetic analysis of homologous Csr1/Zap1 protein sequences from distinct fungi: *Candida dubliniensis* Cd36_44490 (CdCsr1), *C. albicans* 19.3794 (CaCsr1), *C. glabrata* CAGL0J05060g (CgCsr1), *S. cerevisiae* YJL056C (ScZap1), *Cryptococcus neoformans* serotype A CNAG_05392 (CnZap1), and *Aspergillus fumigatus* Afu1g10080 (ZafA). Construction of the phylogenetic tree used the maximum likelihood method. The scale bar indicates the genetic distance, which is proportional to the number of amino acid substitutions. (B) Comparison of the Zap1/Csr1 protein domains. The protein structure was determined using the SMART analysis service. Zap1 from *S. cerevisiae* contained two activation domains (AD1 and AD2; in gray) that are absent in both *Candida* species. Seven C_2-H_2-like zinc finger domains (black) were found in all analyzed species, and comparison of the C-terminal DNA binding domain (DBD) regions showed highest similarities.

RESULTS

The transcription factors CdCsr1, CaCsr1, and ScZap1 are orthologous proteins. Protein sequence comparisons using NCBI PBLAST revealed a high similarity of the *C. dubliniensis* protein Cd36_44490 with the *C. albicans* transcription factor and zinc acquisition regulator Csr1 (also known as Zap1; 86% identities and 91% positives), the *S. cerevisiae* zinc-responsive activator protein Zap1 (37%/52%), and the Zap1 ortholog CNAG_05392 in *Cryptococcus neoformans* serotype A (43%/51%). Multiple sequence alignments showed the highest similarities for the C-terminal part of the protein sequence with a high degree of conservation of this domain. Phylogenetic tree reconstruction using the protein sequences of Csr1 homologs from the yeasts *C. dubliniensis* (CdCsr1), *C. albicans* (CaCsr1), *C. glabrata* (CgCsr1), and *S. cerevisiae* (ScZap1), from the filamentous fungus *A. fumigatus* (ZafA), and from the basidiomycete *C. neoformans* (CnZap1) shows the relationship of the Csr1 proteins in fungi (Fig. 1A). The close relationship of *C. dubliniensis* and *C. albicans* is well reflected in this analysis.

The protein domain architecture of Csr1 in *S. cerevisiae*, *C. albicans*, and *C. dubliniensis* was then analyzed using the SMART program (34, 37). In all these homologs, the C-terminal region contains seven C_2H_2-like zinc finger domains (Fig. 1B). Both ac-

tivation domains (ADs) present in ScZap1, AD1 and AD2 (38), were not detected in *C. dubliniensis* or in *C. albicans*.

The characteristics of the *ZRT2* promoter region in *S. cerevisiae* allow both transcriptional activation and repression of *ZRT2* via Zap1 in response to zinc levels (39). An alignment of the homologous *ZRT2* promoter regions in *S. cerevisiae*, *C. albicans*, and *C. dubliniensis* using Pro Coffee (35) revealed a strong divergence of the zinc-responsive elements (ScZRE1 and ScZRE2) between the *Candida* species and *S. cerevisiae* (39). Of particular note, the repressive ScZRE3 region was entirely absent in *Candida* spp. (see Fig. S2 in the supplemental material). However, a direct comparison of *C. albicans* and *C. dubliniensis* promoter sequences revealed a high similarity between two species. This hints at a promoter type-specific, distinct regulation of the *ZRT2* genes among the different yeasts.

***CSR1* is essential for *C. dubliniensis* growth in low-zinc medium.** The aim of this study was to elucidate the functions of the transcriptional factor Cd*CSR1*, called *CSR1* here, in *C. dubliniensis*. To this end, we created both a *csr1*Δ/Δ knockout and a *CSR1*-complemented mutant. Additionally, the zinc responsive genes *ZRT1*, *ZRT2*, and *PRA1* were expressed under the control of the constitutive *ADH1* promoter both in the *C. dubliniensis* wild-type strain Wü248 and in the *csr1*Δ/Δ mutant strains. All mutants were constructed as independent duplicates and gene deletions were confirmed by Southern blot analyses (see Fig. S1 in the supplemental material).

To investigate the role of Csr1 for zinc acquisition in *C. dubliniensis*, growth of prestarved (24 h without zinc) wild-type and mutants strains was monitored for 2 days in defined medium (LZM) with no (0 μM), little (25 μM), or plentiful (2,000 μM) zinc. The prestarvation step was designed to largely deplete the internal zinc storage, so that fungal growth depended on the ability to acquire zinc from the surrounding medium.

The growth of wild-type and all mutant strains was nearly abolished when no zinc was added to the LZM (Fig. 2A). Under low-zinc conditions (25 μM $ZnSO_4$), all *C. dubliniensis* strains harboring at least one intact *CSR1* allele proliferated robustly and at a rate virtually identical to the wild-type strain Wü284, whereas most mutants lacking *CSR1* (the *csr1*Δ/Δ, *csr1*Δ/Δ+*ZRT1*^{OE}, and *csr1*Δ/Δ+*PRA1*^{OE} strains) failed to adapt and grow in the low-zinc medium (Fig. 2B). Only artificial overexpression of *ZRT2* in the *csr1*Δ/Δ mutant could largely phenocopy wild-type growth under low-zinc conditions (Fig. 2B).

Addition of 2,000 μM $ZnSO_4$ to the LZM rescued the growth defect of all *csr1*Δ/Δ mutant strains (Fig. 2C). We concluded that Csr1 is a key regulator of *C. dubliniensis* for growth in environments with low zinc. While overexpression of neither *ZRT1* nor *PRA1* in the *csr1*Δ/Δ background improved growth under conditions of low zinc, the *ZRT2* overexpression mutant displayed intermediate growth in LZM plus 25 μM $ZnSO_4$, which indicates that this zinc transporter may play an important role under conditions of low zinc.

The upregulation of *CSR1* and its target genes facilitates adaption to low zinc. In *S. cerevisiae*, more than 40 putative target genes of Zap1 are known. All of these are regulated in response to zinc levels and contain zinc-responsive elements (ZREs) to which Zap1 binds (40). Additionally, *C. albicans* Csr1 is known to control not only zinc homeostasis but also the hypha-associated gene *HWP1* under filament-inducing conditions (11) and during biofilm formation (18). To determine whether selected homologs of these target genes are also zinc responsive in *C. dubliniensis*, the transcription levels of genes encoding putative zinc transporters (*ZRT1* to *ZRT3*), the zincophore gene *PRA1*, and the hypha-associated gene *HWP1* were analyzed by quantitative real-time PCR (qRT-PCR). Cells were precultured for 24 h in LZM plus 2,000 μM $ZnSO_4$ before these LZM-adapted cells were shifted into LZM without added zinc. This ensured that changes in gene expression were solely due to zinc deficiency and not the medium *per se*. At 2,000 μM zinc in the preculture, the *csr1*Δ/Δ mutant strains proliferated at wild-type levels (Fig. 2C). The relative gene expression was normalized to *TEF3*, an established *C. dubliniensis* reference gene used for Northern blot analyses (41).

In the wild-type strain Wü284, a 10-fold increase of *CSR 1* mRNA levels was observed within the first 4 h of starvation, reflecting the transcriptional response to the absence of external zinc. The transcript levels remained highly elevated until the end of the experiment at 24 h (1,440 min) (Fig. 3A). This gives additional support to the presumptive key role for *CSR1* in the upkeep of zinc homeostasis. All *ZRT* genes were highly (>20×) upregulated at 24 h. By 4 h, the expression of the putative low-affinity zinc transporter *ZRT2* and the vacuolar zinc exporter *ZRT3* was increased 37-fold and 7-fold, respectively. In contrast, *ZRT1* (likely encoding a high-affinity zinc transporter) transcript levels slightly decreased within the first 4 h but reached a 125-fold upregulation after 24 h compared to the zero time point. The transcript level of *PRA1* reached its measured maximum after 24 h, where this zincophore-encoding gene showed the highest transcript level of all genes investigated in the wild-type strain.

Morphologically, no hypha formation was observed under zinc limitation (data not shown), in agreement with a negligible mRNA level of *HWP1* at all time points (compared to *TEF3*) in the wild type. As expected, no *CSR1* gene expression was measured in the *csr1*Δ/Δ mutant, and in addition, transcript levels of the *ZRT* genes and of *PRA1* were significantly decreased compared to those in the wild type (Fig. 3B). The absolute amount of *ZRT1* transcripts was mostly below the detection limit, showing its dependency on Csr1 during zinc depletion. A slight increase was observed for *ZRT2*, *ZRT3*, and *PRA1* mRNA levels after 24 h, suggesting that their expression is regulated by other factors in addition to Csr1. Reintroduction of one *CSR1* allele into the *csr1*Δ/Δ mutant restored the overall expression pattern of *CSR1* as well as of the other zinc-responsive genes, although the transcript amounts of *ZRT3* and *PRA1* did not fully achieve the level of the wild type (compared to *TEF3*) (Fig. 3C). Reintroduction of *CSR1* hence largely restored the transcriptional response to zinc limitation.

Morphology of *C. dubliniensis* is not coupled to *CSR1*. Previously, Kim et al. reported a filamentation defect for the *C. albicans csr1*Δ/Δ mutant in serum-containing medium (11). To test the possible relevance of *C. dubliniensis* Csr1 for initiation of germ tubes, the wild type, the *csr1*Δ/Δ mutant, and the revertant were tested for germ tube induction in water with 10% serum or in liquid spider medium at 37°C. Invariably, all strains formed proper germ tubes under these filament-inducing conditions (Fig. 4). In addition, germ tube formation was tested for the overexpressing strains *ZRT1*^{OE}, *ZRT2*^{OE}, *PRA1*^{OE}, *csr1*Δ/Δ+*ZRT1*^{OE}, *csr1*Δ/Δ+*ZRT2*^{OE}, and *csr1*Δ/Δ+*PRA1*^{OE}. No difference relative to the wild-type phenotype was detected in any strain (data not shown). These results confirm the findings from our gene expression analysis of *HWP1*, and together they demonstrate that in *C.*

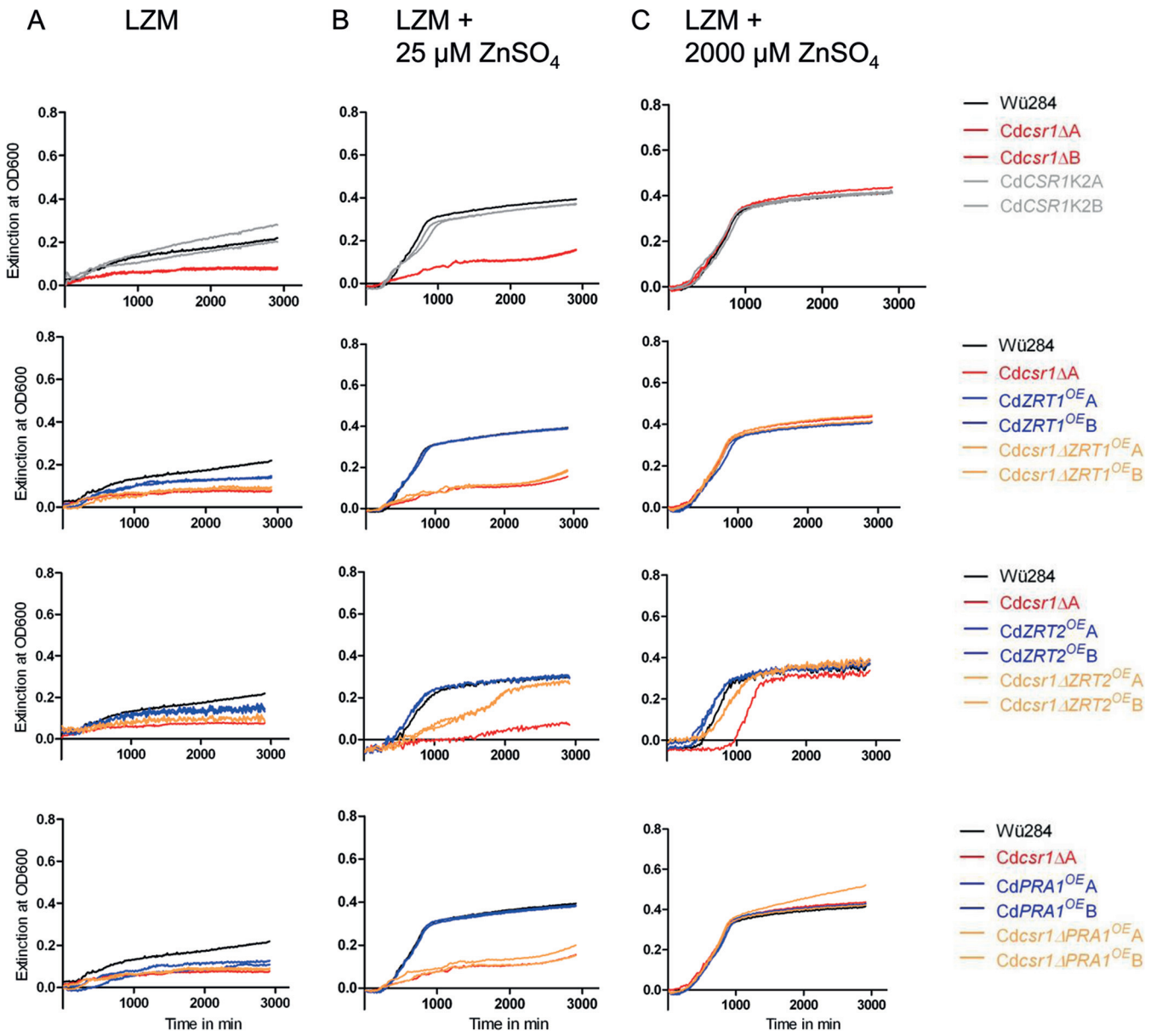

FIG 2 Growth of wild-type and mutant strains depends on extracellular zinc levels. *C. dubliniensis* Wü284 and the *csr1*Δ/Δ, *csr1*Δ/Δ+*CSR1*, *ZRT1*^{OE}, *csr1*Δ/Δ+*ZRT1*^{OE}, *ZRT2*^{OE}, *csr1*Δ/Δ+*ZRT2*^{OE}, *PRA1*^{OE}, and *csr1*Δ/Δ+*PRA1*^{OE} strains were assayed for growth. Cells were prestarved in LZM0 for 24 h at 30°C, and afterwards strains were grown in LZM without zinc (A) and with addition of 25 μM (B) or 2000 μM (C) $ZnSO_4$. At the starting point, the optical density at 600 nm was adjusted to 0.01, and changes were monitored every 15 min for 48 h.

dubliniensis, in contrast to *C. albicans*, hypha formation is not regulated by Csr1 under all our investigated conditions.

The simultaneous deletion of two zinc transporter genes *TZN1* and *TZN2* in *Neurospora crassa* caused a growth defect under zinc depletion conditions, and this double mutant strain failed to exhibit conidiation (42). In this context, we tested production of chlamydospores on Staib and rice agar under chlamydospore-inducing conditions (see Fig. S3 in the supplemental material). All strains analyzed in this study were able to produce these morphological structures in wild-type-like quality and quantity.

***CSR1* is crucial for full virulence of *C. dubliniensis in vivo*.** To study the role of *CSR1* during an infection with *C. dubliniensis*, we used the embryonated chicken egg model (36). We compared the virulence of the *C. dubliniensis* wild type, the *C. dubliniensis* *csr1*Δ/Δ mutants, and the respective complemented strains. To allow a better estimate of *C. dubliniensis*' virulence, we analyzed the *C. albicans* wild-type strain SC5314 in parallel. *C. albicans* is known to generally have a higher virulence than *C. dubliniensis* (16), which was confirmed in our study. The average survival rate 7 days after *C. albicans* infections *in ovo* was 14%, whereas *C. dubliniensis* infections were survived by 44% of the embryonated eggs at the end of the experiment (Fig. 5). One of the independent *C. dubliniensis* *csr1*Δ/Δ deletion mutants showed a significantly decreased mortality rate (33%) versus the *C. dubliniensis* wild type (56%) and both reconstituted strains (62% and 67%). The second *csr1*Δ/Δ mutant (*csr1*Δ/ΔB) similarly exhibited a clear, but not

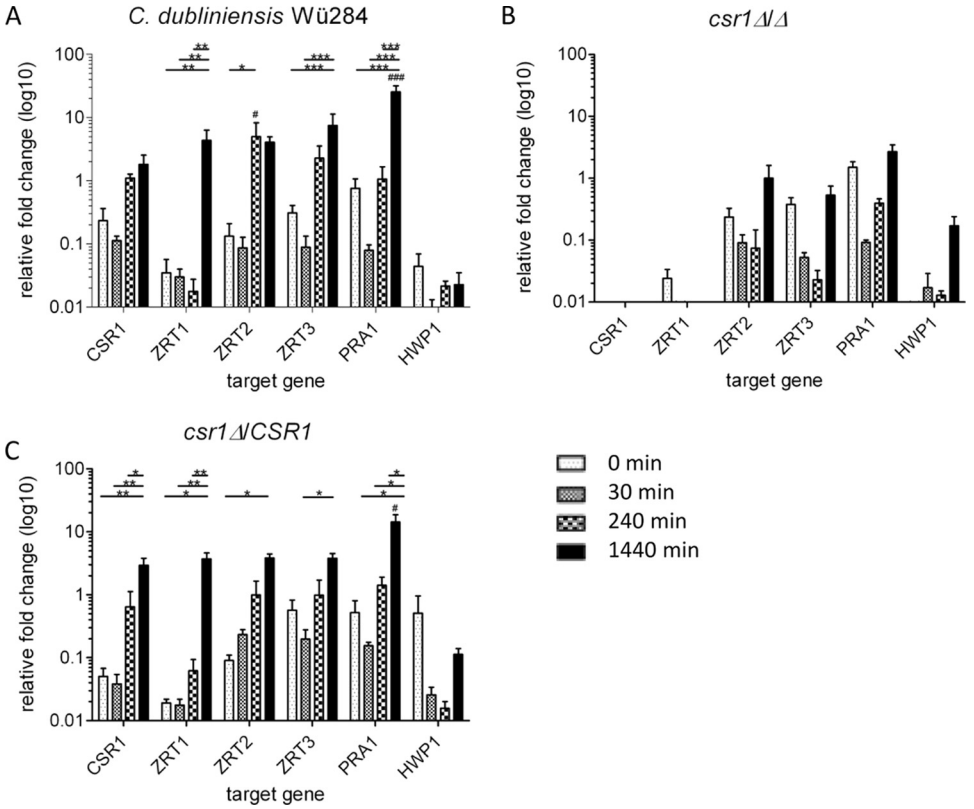

FIG 3 qRT-PCR gene expression analysis of putative Csr1 target genes in the wild-type strain Wü284 (A), the *csr1*Δ/Δ knockout strain (B), and the *csr1*Δ/*CSR1* revertant strain (C). The analyzed genes (*CSR1*, *ZRT1*, *ZRT2*, *ZRT3*, *PRA1*, and *HWP1*) are putatively regulated by Csr1. The cells were grown in LZM0 medium, and RNA samples were taken directly after inoculation in LZM0 and 30 min, 240 min, and 1,440 min postinoculation. The bars represent the relative change in expression normalized to expression of the housekeeping gene *CdTEF3*, and the results are the means and standard deviations (SD) from two biological and three technical replicates. The change in expression was significant (one-way analysis of variance [ANOVA], $P < 0.05$) within one strain (*) or compared to the expression level in the *csr1*Δ/Δ knockout strain at the same sampling time point (#).

statistically significant, attenuated virulence with a mortality rate of 46%. The reintegration of *CSR1* into the knockout strains restored the virulence pattern of the wild type. These observations indicate an important role for *CSR1* during *in vivo* infections by *C. dubliniensis*.

DISCUSSION

C. dubliniensis is an important emerging pathogen but is generally considered less virulent than *C. albicans* (43, 44). While the two fungi share many similarities, genetic, regulatory, and/or phenotypic differences must exist between them to explain this gap in virulence potential (16). The very close evolutionary relationship between *C. dubliniensis* and *C. albicans* can thus provide us with important tools to investigate the genetic basis of virulence in fungi.

One important aspect of host-pathogen interaction is the struggle for micronutrients like iron and zinc (45). In this study, we hence focused on the role of the transcriptional factor Csr1 and other putative zinc-responsive genes in zinc homeostasis of *C. dubliniensis*. Between *C. dubliniensis* and *C. albicans*, the transcriptional regulators CdCsr1 and CaCsr1 share a high sequence similarity. Both differ in the N-terminal zinc-responsive activation domains from their *S. cerevisiae* homolog, the zinc-dependent regulator Zap1 (46). Both *Candida* species lack AD1 and AD2 (11 and this study). In *S. cerevisiae*, AD1 binds multiple Zn(II) ions and is required for proper catalytic function (47). The absence of the ADs indicates differences in the structure of this zinc-responsive regulator between *S. cerevisiae* and the *Candida* species. In support of that, multiple zinc finger domains were predicted in the C-terminal region of both CdCsr1 and CaCsr1, which could allow zinc binding even in the absence of the ADs. Two out of seven C_2H_2 domains in both Csr1 proteins were predicted with low confidence, and other authors thus describe only five zinc finger domains in CaCsr1 (11, 48).

More than 270 genes are known to have lower transcription levels in a *C. albicans csr1*Δ/Δ mutant compared to the wild type during biofilm formation (18). The largest differences in expression were found for the zinc homeostasis genes *PRA1*, *CSR1*, *ZRT2*, and *ZRT1*. Our data indicate that *C. dubliniensis* Csr1 shares these target genes with *C. albicans*, as all four genes were not upregulated during zinc limitation in the *csr1*Δ/Δ knockout strain.

In *C. albicans*, the *csr1*Δ/Δ mutant shows impaired growth under zinc limitation (11, 18). We observed a similar growth defect of the *C. dubliniensis csr1*Δ/Δ mutant. However, in *C. albicans csr1*Δ/Δ, the overexpression of the zinc transporter genes *ZRT1* and *ZRT2* is known to improve growth of the mutant during zinc depletion (18), while overexpression of *ZRT1* or *PRA1* in *C. dubliniensis csr1*Δ/Δ did not lead to any phenotypic rescue. The artificial expression of this zinc transporter or the zinc scavenger pro-

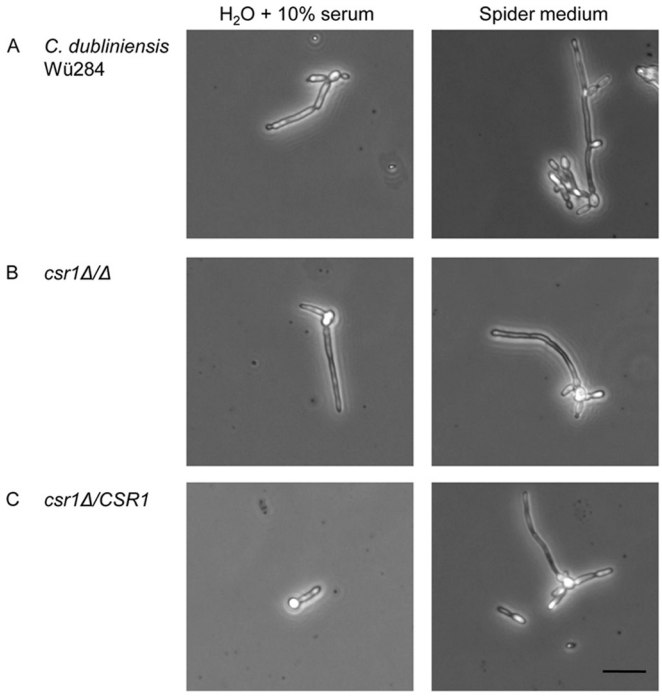

FIG 4 Filamentation of the wild-type strain Wü284, the *csr1*Δ/Δ mutant, and the *csr1*Δ/*CSR1* revertant in 10% (vol/vol) serum and spider medium. Cells were grown overnight in YPD at 30°C and shifted to germ tube-inducing medium for 4 h at 37°C. Germ tube induction was tested for Wü284 (A), the *csr1*Δ/Δ mutant (B), and the *csr1*Δ/*CSR1* strain (C) in water plus 10% serum and liquid spider medium. Cell morphology was documented via differential interference contrast microscopy. The bar represents 20 μm.

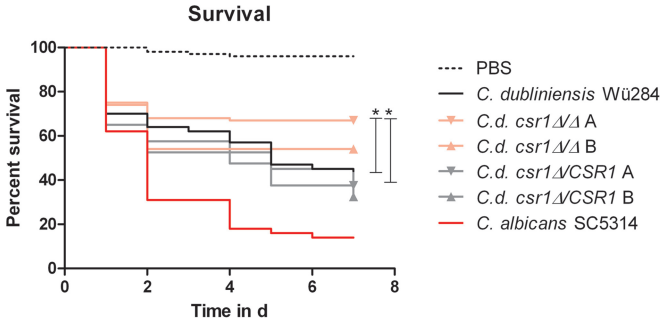

FIG 5 Virulence of the wild-type strain Wü284, the *csr1*Δ/Δ mutant, and the *csr1*Δ/*CSR1* revertant in infected chicken embryos. Survival after infection is depicted as Kaplan-Meyer plots. There were 20 chicken embryos per group per experiment, and the combined results of two independent experiments are shown. The mutant *csr1*Δ/ΔA exhibited significantly attenuated virulence ($P < 0.01$) compared with the wild type and the reconstituted mutant *csr1*Δ/*CSR1*A, as calculated by the log-rank (Mantel-Cox) test.

tein is evidently not sufficient to allow efficient zinc uptake by *C. dubliniensis*. Possibly, Cd*ZRT1* is generally less efficient in zinc uptake than Ca*ZRT1*. Alternatively, both partners of the zincophore uptake system are required in *C. dubliniensis* for zinc acquisition. In *C. albicans*, zinc uptake can occur via a zincophore system comprising both Pra1 to sequester extracellular zinc ions and Zrt1 to transport zinc into the fungal cell (25). Based on their close genetic relationship, we expect a similar mechanism to be present in *C. dubliniensis*. Possibly, *C. albicans*, but not *C. dubliniensis*, has sufficient remaining transcriptional activation of *PRA1* and *ZRT1* in the absence of *CSR1* to compensate for growth defects during artificial expression of only one reaction partner under low-zinc conditions.

On the other hand, overexpression of *ZRT2* in the *C. dubliniensis csr1*Δ/Δ mutant allowed growth under low-zinc conditions, which hints at an important role for this transporter in such environments. In *S. cerevisiae*, Zrt1 is known to be the high-affinity extracellular zinc transporter (21). Comparisons of the ScZrt1 protein with the *C. dubliniensis* proteome revealed a higher similarity with CdZrt2 (43% identities and 59% positives) than with CdZrt1 (27%/48%), in agreement with a recent report showing a rather distant relationship between ScZrt1 and its homologous proteins in several *Candida* species (49). Furthermore, regulatory ZREs were not detected in the promoter sequence of the two *Candida* species, which points to differences in the transcriptional regulation between *Candida* spp. and *S. cerevisiae*. Therefore, we hypothesize Zrt2, rather than Zrt1, to be the high-affinity zinc transporter in *C. dubliniensis*.

The zinc transporters Zrt1 and Zrt2 of *C. dubliniensis* exhibit only 30% amino acid sequence identity, suggesting nonredundant functions. Eide showed that in *S. cerevisiae*, the regulation of *ZRT1* and *ZAP1* transcription differs from that of *ZRT2*, with the first two being downregulated at higher zinc concentrations (50). This indicates, for baker's yeast, the presence of both a high-affinity zinc uptake system, comprising the regulator Zap1 and the transporter Zrt1, and a low-affinity zinc uptake system mediated by Zrt2 (20). Here, we measured the expression levels of putative zinc-responsive genes in a *C. dubliniensis csr1*Δ/Δ mutant and noticed a strong dependency of *ZRT1* on *CSR1*.

Overall, data on *ZRT2* gene expression in *S. cerevisiae* are contradictory. Bird et al. showed a peak in *ZRT2* mRNA accumulation at 300 to 1,000 μM zinc (39). In a different study, a β-galactosidase activity assay demonstrated *ZRT2* promoter activity under low-zinc conditions, which was reduced under conditions of increased zinc abundance (250 μM or more) (46). In our experiments, we observed a strong upregulation of *C. dubliniensis ZRT2* during zinc depletion. *ZRT2* transcription is hence in agreement with a role for Zrt2 as a high-affinity zinc transporter in *C. dubliniensis*.

A detailed study on the structural basis of the transcriptional regulation of *ZRT2* in *S. cerevisiae* revealed that one of three ZREs (ZRE3) is located inside the promoter region. Zinc deprivation results in repressional binding of Zap1 to ZRE3, which inhibits the initiation of *ZRT2* transcription (39). As we observed a significant upregulation of *ZRT2* in the absence of zinc, promoter regions of *ZRT2* in both *Candida* species were aligned with sequences from *S. cerevisiae*. The lack of the repressing ZRE3 domain in *Candida* species supports our finding that *ZRT2* was upregulated during zinc limitation. These differences in the promoter sequence seem to be clade specific, as Bird et al. reported a conserved *ZRT2* promoter region for different *Saccharomyces* species (39).

Furthermore, Eide suggested an at least partial independency of *ZRT2* transcription from Zap1 in *S. cerevisiae* (50). We observed the same phenomenon in *C. dubliniensis* with a delayed and reduced but measurable upregulation of *ZRT2* even in the *csr1*Δ/Δ background. Interestingly, in addition to *ZRT2*, *ZRT3* and *PRA1* also remained responsive to zinc starvation in a *csr1*Δ/Δ deletion mutant. Hence, additional factors besides Csr1 likely contribute to expression of zinc-responsive genes in *C. dubliniensis*. Finally, it is

known that *ZRT1* and *PRA1* share the same intergenic promoter region in *C. albicans*, which allows efficient zinc assimilation by their coregulation (25). The synteny of this *PRA1-ZRT1* locus is conserved in *C. dubliniensis*, and we detected largely synchronous shifts in gene expression during zinc starvation as long as *CSR1* was present.

S. cerevisiae stores zinc intracellularly under zinc-replete conditions via the vacuolar importer Zrc1. Under conditions of low extracellular zinc availability, this intracellular storage is accessed via the vacuolar zinc exporter Zrt3 (51). We observed a clear upregulation of *ZRT3* in *C. dubliniensis* within 4 h of zinc starvation. Likely, the cells had filled their vacuolar storage during the adaption phase in 2,000 μM zinc, which was then used to maintain zinc homeostasis under starvation. We found *ZRT3* upregulation to be dependent on Csr1, as *ZRT3* expression never exceeded the initial levels in the *csr1*Δ/Δ mutant. This is in agreement with the Zap1-mediated upregulation of *ZRT3* in *S. cerevisiae* (51).

A highly interesting aspect of *Candida* pathobiology is that human infections with *C. dubliniensis* occur much less frequently than those with *C. albicans*. *C. dubliniensis* is also far less able to disseminate into the kidney and liver in oral-intragastrically infected mice. Histological analyses of these organs revealed that *C. dubliniensis* remained as yeast cells *in vivo*, whereas *C. albicans* formed true hyphae and caused major tissue damage (17). Due to their potential role as a pathogenicity factor differentiating *C. albicans* and *C. dubliniensis*, we characterized the ability of a *C. dubliniensis csr1*Δ/Δ deletion mutant to produce hyphae *in vitro*.

A filamentation defect has been observed for the *C. albicans csr1*Δ/Δ deletion mutant in inducing medium, accompanied by impaired gene expression of the hypha-associated *HWP1* gene (11). Similar hypha formation defects were observed in *in vitro*-grown biofilms and *in vivo* using a rat intravenous catheter model (18). In the same study, expression of hypha-associated genes like *HYR1*, *HWP1*, *IHD1*, and *RBT1* were found to be positively regulated by Csr1, while the yeast-specific *YWP1* was downregulated in a *C. albicans* wild-type biofilm (18).

Therefore, we investigated the capacity of *C. dubliniensis csr1*Δ/Δ to induce germ tubes and found no differences relative to the wild-type strain. Hence, in contrast to *C. albicans*, hypha induction is not coupled to the zinc-responsive transcription factor Csr1 in *C. dubliniensis*. This constitutes a species-specific phenotype which may help to explain the different *in vivo* morphologies of the two fungi. In fact, one of the main differentiation criteria between *C. dubliniensis* and *C. albicans* is the differences in regulation of true hypha formation (52). Compared to the common ancestor, *C. dubliniensis* underwent reductive evolution and pseudogenization, which affected several virulence factors, including genes known to be hypha associated in *C. albicans*. This includes the disappearance of members of the *SAP* gene family, *ALS3* and *HYR1*, and a strong divergence in the *HWP1* gene, among others (53). Interestingly, the latter two are also targets of Csr1 in *C. albicans* (18), which might contribute to the filamentation defect in the absence of *CSR1*. This offers a possible explanation for the filamentation of *C. dubliniensis* even with a *csr1*Δ/Δ background. Interestingly, a *C. albicans csr1*Δ/Δ mutant was also shown to produce less of the quorum-sensing molecule farnesol during biofilm formation (19). As farnesol is also able to block hypha formation in *C. dubliniensis* (54), our data hint at possible species-specific differences in the relation of CdCsr1 and CaCsr1 to farnesol production and/or detection.

A supply of micronutrients like zinc is essential for a microbial pathogen to survive and disseminate during an infection. Previous studies have shown that orthologs of Csr1 are essential for pathogenicity of different fungal pathogens: A murine infection with *zap1* and *zafA* knockout strains resulted in milder forms of cryptococcosis and aspergillosis, respectively (48, 55). Very recently, the effect of a *C. albicans csr1*Δ/Δ deletion on virulence in mice and the associated transcriptome changes were assayed (27). In the present work, we used the embryonated egg infection model (36) for the first time to examine the virulence of wild type and mutant *C. dubliniensis*. This alternative infection model reflected the species-specific differences in virulence observed in human infections with *C. albicans* and *C. dubliniensis*. The survival rate of chicken embryos infected with *C. dubliniensis* Wü284 (44%) was more than three times higher than after infection with *C. albicans* SC5314 (14%) and paralleled previously published data on murine infections (68% versus 19% survival) (56).

The attenuated virulence of the *C. dubliniensis csr1*Δ/Δ strains is of special interest, as hypha formation was still intact *in vitro*, and these results thus hint at an important role for zinc homeostasis during *C. dubliniensis* infections. This is also in agreement with data for *CSR1* in *C. albicans* obtained by infection experiments in mice, where *csr1*Δ/Δ cells were strongly depleted in infected kidneys (26, 27). However, in *C. albicans*, a lack of filamentation by the *CSR1* mutation may have played an additional or even dominant role besides the defect in zinc supply, although the *C. albicans csr1*Δ/Δ mutant showed no reduction in hypha-associated gene expression during kidney invasion (27). Likely, important differences exist in hypha-related gene regulation by CdCsr1 and CaCsr1 (Zap1) *in vitro* and *in vivo*. Thus, our data provide an important hint at an independent contribution of the zinc supply to the success of fungal infection. Interestingly, the few virulence-associated genes verified in *C. dubliniensis* are generally associated with hypha formation, e.g., via calcineurin signaling (57) or telomere-associated open reading frames (ORFs) (58). Csr1, in contrast, seems be a virulence factor that is not mandatorily linked to a global filamentation defect.

In conclusion, we found that zinc homeostasis regulation by Csr1 seems to be generally conserved among *C. dubliniensis*, *C. albicans*, and *S. cerevisiae*, although there are important differences, especially with regard to its role in hypha formation. Furthermore, we identified Csr1 as a virulence factor in *C. dubliniensis*, which underlines the general relevance of micronutrient supply during fungal infections.

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