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

Bettina Böttcher, a Katja Palige, b Ilse D. Jacobsen, a–d Bernhard Hube, a,c Sascha Brunke a–e

Department of Microbial Pathogenicity Mechanisms, Leibniz Institute for Natural Product Research and Infection Biology—Hans Knoll Institute Jena, Jena, Germany b; Microfluidic ChipShop GmbH, Jena, Germany c; Friedrich Schiller University, Jena, Germany d; Research Group Microbial Immunology, Leibniz Institute for Natural Product Research and Infection Biology—Hans Knoll Institute Jena, Jena, Germany e; Center for Sepsis Control and Care, Jena, Germany e

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 csr1Δ/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 csr1Δ/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 eu- karyotic 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 *Salmo- nella 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-
tered biofilm formation (18). Further analysis of genes regulated by Csr1 of *C. albicans* (CaCsr1) under biofilm-inducing conditions revealed 60 targets, including CaZRT1-3, CaPRA1, and CaCSR1 itself (18). It is noteworthy that in biofilm-producing communities, a *C. albicans* csr1 ΔΔ 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* (SscZrt1) is a high-affinity transporter in *S. cerevisiae* that mediates zinc uptake under strong zinc depletion, but it is downregulated under itself (18). It is noteworthy that in biofilm-producing communities, a *C. albicans* csr1 ΔΔ mutant strain secretes smaller amounts of the quorum-sensing molecule farnesol, which contributes to an altered morphology (19).

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A *C. albicans* csr1 ΔΔ mutant is known to be proliferative 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 ZnSO₄ as indicated (LZM0 contains no zinc; LZM25 and LZM2000 contain 25 μM and 2,000 μM ZnSO₄, respectively), and 25 μM FeSO₄ 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 (dH₂O), and transferred into filament-inducing medium at an optical density at 600 nm (OD₆₀₀) of 0.2. To stimulate filamentation, we used spider medium (1% mannitol, 1% nutrient broth, 0.2% K₂HPO₄ [pH 7.2]), liquid YPD, or H₂O 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 Apal-Xhol 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* Wu284 as the template. A SacII-Sacl 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 Apal/BgIII-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-BgIII fragment was amplified via PCR with the primers PRA1-1 and PRA1-2. Genomic DNA from *C. dubliniensis* Wu284 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 pcdZRT2E1 were constructed in a similar way by amplifying a XhoI-BgIII 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 electroperoration 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.

### TABLE 1 Strains in this study

<table>
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<th>Strain</th>
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<td>This study</td>
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⁴ SAT1-FLIP denotes the SAT1 flipper cassette (33).
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 LYM0 without additional zinc. After starvation in LYM0 for 24 h at 30°C, cells were diluted to an OD_600 of 0.01 in LYM 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 x 10^6 cells/ml were inoculated into 200 ml LYM 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 LYM0 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 RNaseasy 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* CAGL030960g (CgCsr1), *S. cerevisiae* YJL056C (ScZap1), *Aspergillus fumigatus* Afu1g10080 (ZafA), and *Cryptococcus neoformans* serotype A CNAG_05392 (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://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 10^6 cells/ml. An inoculum of 10^6 yeast cells/egg was applied to the chorionic-allantoic 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.

**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* (CbCsr1), 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 Cys-His-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 CdcSR1, called CSR1 here, in *C. dubliniensis*. To this end, we created both a csr1ΔΔ 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 Wü284 and in the csr1ΔΔ 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 or without zinc (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 ZnSO₄), 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 csr1ΔΔ, csr1ΔΔ+ZRT1OE, and csr1ΔΔ+PRA1OE strains) failed to adapt and grow in the low-zinc medium (Fig. 2B). Only artificial upregulation of ZRT2 in the csr1ΔΔ mutant could largely phenocopy wild-type growth under low-zinc conditions (Fig. 2B).

Addition of 2,000 μM ZnSO₄ to the LZM rescued the growth defect of all csr1ΔΔ mutant strains (Fig. 2C). We concluded that Csr1 is a key regulator of *C. dubliniensis* for growth in environments with low zinc. While upregulation of neither ZRT1 nor PRA1 in the csr1ΔΔ background improved growth under conditions of low zinc, the ZRT2 expression of CSR1 mutant displayed intermediate growth in LZM plus 25 μM ZnSO₄, 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 *HWPI* 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 *HWPI* were analyzed by quantitative real-time PCR (qRT-PCR). Cells were precultured for 24 h in LZM plus 2,000 μM ZnSO₄ 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 csr1ΔΔ 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 *CSR1* 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 *HWPI* at all time points (compared to *TEF3*) in the wild type. As expected, no CSR1 gene expression was measured in the csr1ΔΔ 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 csr1ΔΔ 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* csr1ΔΔ mutant in serum-containing medium (11). To test the possible relevance of *C. dubliniensis* Csr1 for initiation of germ tubes, the wild type, the csr1ΔΔ mutant, and the revertant were tested for germ tube induction in water with 10% serum or in lipic acid 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 *ZRT1OE, ZRT2OE, PRA1OE, csr1ΔΔ+ZRT1OE, csr1ΔΔ+ZRT2OE, and csr1ΔΔ+PRA1OE*. 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 *HWPI*, and together they demonstrate that in *C.
C. 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 csr1ΔΔ* 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* csr1ΔΔ 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 csr1ΔΔ mutant (csr1ΔΔB) similarly exhibited a clear, but not

**FIG 2** Growth of wild-type and mutant strains depends on extracellular zinc levels. *C. dubliniensis* Wu284 and the csr1ΔΔ, csr1ΔΔ+CSR1, ZRT1Δ, csr1ΔΔ+ZRT1ΔE, ZRT2Δ, csr1ΔΔ+ZRT2ΔE, PRA1Δ, and csr1ΔΔ+PRA1ΔE 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) ZnSO4. 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.
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 C2H2 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/H9004/H9004 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/H9004/H9004 knockout strain.

In C. albicans, the csr1Δ/Δ mutant shows impaired growth under zinc limitation (11, 18). We observed a similar growth defect of the C. dubliniensis csr1Δ/Δ mutant. However, in C. albicans csr1Δ/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 csr1Δ/Δ did not lead to any phenotypic rescue. The artificial expression of this zinc transporter or the zinc scavenger pro-
proteins in several Candida species (43% identities and 59% positives) than with Zrt1 to transport zinc into the fungal cell (25). Based on their close relationship, we expect a similar mechanism to be present in 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 Saccharomyces. 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 Saccharomyces, 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 csr1Δ/Δ mutant and noticed a strong dependency of ZRT1 on CSR1.

Overall, data on ZRT2 gene expression in Saccharomyces 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 Saccharomyces 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 Saccharomyces. 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 Saccharomyces (50). We observed the same phenomenon in C. dubliniensis with a delayed and reduced but measurable upregulation of ZRT2 even in the csr1Δ/Δ background. Interestingly, in addition to ZRT2, ZRT3 and PRA1 also remained responsive to zinc starvation in a csr1Δ/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 up-regulation of ZRT3 in C. dubliniensis within 4 h of zinc starvation. Likely, the cells had filled their vacuolar storage during the adaptation 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 csr1ΔΔ 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-intrastragically 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 csr1ΔΔ deletion mutant to produce hyphae in vitro.

A filamentation defect has been observed for the C. albicans csr1ΔΔ 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 csr1ΔΔ 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 phenomenon, 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 second liver target 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 csr1ΔΔΔ background. Interestingly, a C. albicans csr1ΔΔΔ 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 csr1ΔΔΔ 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 Wu284 (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 csr1ΔΔΔ strains is of special interest, as hyphal 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 csr1ΔΔΔ 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 csr1ΔΔΔ 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 to 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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