Evaluation of Biofilm Formation in Candida tropicalis Using a Silicone-Based Platform with Synthetic Urine Medium

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Abstract: Molecular mechanisms of biofilm formation in Candida tropicalis and current methods for biofilm analyses in this fungal pathogen are limited. (2) Methods: Biofilm biomass and crystal violet staining of the wild-type and each gene mutant strain of C. tropicalis were evaluated on silicone under synthetic urine culture conditions. (3) Results: Seven media were tested to compare the effects on biofilm growth with or without silicone. Results showed that biofilm cells of C. tropicalis were unable to form firm biofilms on the bottom of 12-well polystyrene plates. However, on a silicone-based platform, Roswell Park Memorial Institute 1640 (RPMI 1640), yeast nitrogen base (YNB) + 1% glucose, and synthetic urine media were able to induce strong biofilm growth. In particular, replacement of Spider medium with synthetic urine in the adherence step and the developmental stage is necessary to gain remarkably increased biofilms. Interestingly, unlike Candida albicans, the C. tropicalis ROB1 deletion strain but not the other five biofilm-associated mutants did not cause a significant reduction in biofilm formation, suggesting that the biofilm regulatory circuits of the two species are divergent. (4) Conclusions: This system for C. tropicalis biofilm analyses will become a useful tool to unveil the biofilm regulatory network in C. tropicalis.

Keywords: Candida tropicalis; biofilms; synthetic urine; ROB1

1. Introduction

Fungal infections caused by Candida species commonly occur in the skin, genitals, or mucous membrane [1]. Lethal candidiasis occurs in immunocompromised patients and is usually in the form of invasive candidiasis [2,3]. In Europe and the United States, the dominant Candida species of candidiasis include Candida albicans (~50%), Candida glabrata (~30%), Candida parapsilosis (~12%), Candida tropicalis (~7%), and Candida krusei (~1%) [4]. However, in tropical and subtropical areas, C. tropicalis is widely found and has become the dominant fungal pathogen in non-albicans Candida species [5–8]. Moreover, C. tropicalis develops fluconazole resistance much more rapidly than C. albicans [9]. Indeed, the increasing rate of fluconazole resistance in C. tropicalis isolates (15%) is greater than that in C. albicans isolates (4%) [10,11]. C. tropicalis isolated from the environment and soil also exhibits reduced susceptibility to fluconazole due to the usage of agricultural azole drugs [11]. However, compared to C. albicans, C. tropicalis has been relatively less investigated.

Biofilms are mixed communities of microbes that adhere to a surface and are embedded within an extracellular matrix comprised of polysaccharides, proteins, and extracellular DNA [12].
can grow on in-dwelling medical devices and therefore are highly associated with virulence and drug resistance, thereby hampering clinical treatment [13,14]. The formation of mature biofilms in C. albicans requires four major distinct steps: early, intermediate, maturation, and dispersal [12]. During the early stage, planktonic cells are present as in the yeast phase and must adhere to a surface. Later, pseudohyphae and hyphae are formed. The increased hyphal growth further promotes extracellular matrix production, leading to biofilm maturation and eventual dispersal [15]. Central to the understanding of the mechanisms underlying C. albicans biofilm formation is the regulation by a complex regulatory network in which several major transcriptional factors are involved in biofilm growth [16–18]. Here, we focus on six major regulators, Bcr1, Brg1, Efg1, Rob1, Ndt80, and Tec1 [16]. Loss of any one of these regulators in C. albicans significantly compromises biofilm formation in vitro and in vivo [16].

In C. tropicalis, a flow model has been used to examine C. tropicalis biofilm formation on latex and silicone catheters using synthetic urine (SU) medium [19]. Bovine serum- and Spider-induced biomass dry weight to determine C. tropicalis biofilms on the bottom of polystyrene plates have also been reported [20]. The former assay requires additional equipment; the latter analysis highly depends on the wash step that may cause variability with different scientists. Hence, to obtain optimal biofilm formation with C. tropicalis, several culturing conditions for biofilm development were evaluated in this study. We found that using SU in the adherence step enables C. tropicalis to form biofilms on silicone. Moreover, we showed that presence of magnesium in SU is necessary for proliferation and biofilm formation by C. tropicalis. Furthermore, deletion of the BCR1, BRG1, TEC1, EFG1, or NDT80 genes but not the ROB1 gene in C. tropicalis reduced biofilm biomass. Reintroduction of the C. tropicalis ROB1 into C. albicans rob1Δ mutant strains could not restore biofilm growth. These results suggest divergent functions of C. tropicalis ROB1 (CIROB1) compared to that of its relative species C. albicans. Overall, we established a method for C. tropicalis biofilm analysis on silicone using SU medium. This method will help the field study the molecular mechanisms of biofilm formation in C. tropicalis.

2. Materials and Methods

2.1. Media and Reagents

Yeast extract-peptone-dextrose (YPD), Roswell Park Memorial Institute 1640 (RPMI 1640), Lee’s glucose, synthetic defined (SD), and synthetic complete dextrose (SCD) media used in experiments were prepared as described previously [19,21–25]. Notably, mannitol in Spider medium was replaced with 1% glucose for C. tropicalis biofilm analyses. SU (pH 5.8) was prepared according to previous reports [19,24]. All chemicals were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, U. S. A.) unless otherwise stated.

2.2. Plasmid and Strain Construction

The yeast strains and primers used in this study are listed in Table 1 and Table S1. All C. tropicalis mutants were derived from the C. tropicalis MYA3404 strain [26]. To generate the C. tropicalis tec1Δ, bcr1Δ, brg1Δ, rob1Δ, efg1Δ, and ndt80Δ strains, the 5’ and 3’ flanking regions of TEC1 (primers 505/506 and 507/508), BCR1 (primers 549/550 and 551/552), BRG1 (primers 609/610 and 611/612), ROB1 (primers 625/626 and 627/628), EFG1 (primers 675/676 and 677/678), and NDT80 (primers 601/602 and 603/604) were PCR amplified using the indicated primers. The respective 5’ and 3’ PCR products were digested with ApaI/XhoI and SacII/SacI and cloned into the plasmid pSFS2A [27] to generate the plasmids pSFS-ctEcg1 KO, pSFS-ctBcr1 KO, pSFS-ctBrg1 KO, pSFS-ctRob1 KO, pSFS-ctEfg1 KO, and pSFS-ctNd80 KO. Each plasmid was digested with ApaI/SacI and transformed into YL477 (MYA3404) [26] to generate heterozygous Δtec1Δ/TEC1, Δbcr1Δ/BCR1, Δbrg1Δ/BRG1, Δrob1Δ/ROB1, Δefg1Δ/EFG1, and Δndt80Δ/NDT80 strains. The SAT1 marker was recycled under treatment with 2% maltose. The heterozygous strains were retransformed with the respective deletion construct to generate the tec1Δ (YL1380 and YL1381), bcr1Δ (YL1404 and YL1406), brg1Δ (YL1402 and YL1403), rob1Δ (YL1413 and YL1414), efg1Δ
(YL1410 and YL1411), and ndt80Δ (YL1382 and YL1384) mutant strains. Primers 509/6, 7/510, and 511/512 were used to verify the tec1Δ genotype. Primers 553/6, 7/554, and 555/556 were used to verify the bcr1Δ genotype. Primers 613/6, 7/614, and 615/616 were used to verify the brg1Δ genotype. Primers 629/6, 7/630, and 631/632 were used to verify the rob1Δ genotype. Primers 679/6, 7/680, and 681/682 were used to verify the efg1Δ genotype. Primers 605/6, 7/606, and 607/608 were used to verify the ndt80Δ genotype.

Table 1. Strains used in this study.

| Strain | Species | Genotype | Source     |
|--------|---------|----------|------------|
| SC314  | C. albicans | Wild-Type | [26]       |
| RBY717 | C. albicans | ura3::imm434/URA3 ino1::imm434/IRO1 | [28]       |
| YL477  | C. tropicalis | wild-type sequence strain (MYA3404) | [26]       |
| YL1344 | C. albicans | bcr1Δ/bcr1Δ | [21]       |
| YL1346 | C. albicans | brc1Δ/brc1Δ | [21]       |
| YL1348 | C. albicans | efg1Δ/efg1Δ | [21]       |
| YL1350 | C. albicans | ndt80Δ/ndt80Δ | [21]       |
| YL1354 | C. albicans | rob1Δ/rob1Δ | [21]       |
| YL1356 | C. albicans | tec1Δ/tec1Δ | [21]       |
| YL1380 | C. tropicalis | tec1Δ/tec1Δ/-SAT1 | This study |
| YL1381 | C. tropicalis | tec1Δ/tec1Δ/-SAT1 | This study |
| YL1382 | C. tropicalis | ndt80Δ/ndt80Δ/-SAT1 | This study |
| YL1384 | C. tropicalis | ndt80Δ/ndt80Δ/-SAT1 | This study |
| YL1402 | C. tropicalis | brg1Δ/brg1Δ/-SAT1 | This study |
| YL1403 | C. tropicalis | brg1Δ/brg1Δ/-SAT1 | This study |
| YL1404 | C. tropicalis | bcr1Δ/bcr1Δ/-SAT1 | This study |
| YL1406 | C. tropicalis | bcr1Δ/bcr1Δ/-SAT1 | This study |
| YL1410 | C. tropicalis | efg1Δ/efg1Δ/-SAT1 | This study |
| YL1411 | C. tropicalis | efg1Δ/efg1Δ/-SAT1 | This study |
| YL1413 | C. tropicalis | rob1Δ/rob1Δ/-SAT1 | This study |
| YL1414 | C. tropicalis | rob1Δ/rob1Δ/-SAT1 | This study |
| YL1417 | C. albicans | efg1Δ/efg1Δ/::CtEFG1-SAT1 | This study |
| YL1419 | C. albicans | efg1Δ/efg1Δ/::CtEFG1-SAT1 | This study |
| YL1450 | C. albicans | bcr1Δ/bcr1Δ/::CtBCR1-SAT1 | This study |
| YL1451 | C. albicans | bcr1Δ/bcr1Δ/::CtBCR1-SAT1 | This study |
| YL1456 | C. albicans | rob1Δ/rob1Δ/::CtROB1-SAT1 | This study |
| YL1457 | C. albicans | rob1Δ/rob1Δ/::CtROB1-SAT1 | This study |
| YL1493 | C. tropicalis | bcr1Δ/bcr1Δ/::BCR1-SAT1 | This study |
| YL1494 | C. tropicalis | bcr1Δ/bcr1Δ/::BCR1-SAT1 | This study |
| YL1495 | C. tropicalis | brc1Δ/brc1Δ/::BRG1-SAT1 | This study |
| YL1496 | C. tropicalis | brc1Δ/brc1Δ/::BRG1-SAT1 | This study |
| YL1497 | C. tropicalis | rob1Δ/rob1Δ/::ROB1-SAT1 | This study |
| YL1498 | C. tropicalis | tec1Δ/tec1Δ/::TEC1-SAT1 | This study |
| YL1499 | C. tropicalis | tec1Δ/tec1Δ/::TEC1-SAT1 | This study |
| YL1504 | C. tropicalis | rob1Δ/rob1Δ/::ROB1-SAT1 | This study |
| YL1570 | C. tropicalis | efg1Δ/efg1Δ/::EFG1-SAT1 | This study |
| YL1571 | C. tropicalis | efg1Δ/efg1Δ/::EFG1-SAT1 | This study |
| YL1572 | C. tropicalis | ndt80Δ/ndt80Δ/::NDT80-SAT1 | This study |
| YL1573 | C. tropicalis | ndt80Δ/ndt80Δ/::NDT80-SAT1 | This study |

The TEC1, BCR1, BRG1, ROB1, EFG1, and NDT80 complementation constructs were made by amplification of their endogenous promoters and ORFs using primers 937/938, primers 929/930, primers 931/932, primers 935/936, primers 805/806, and primers 933/934, respectively. The TEC1, BCR1, ROB1, EFG1, and NDT80 PCR products were digested with Xho1/Xho1 and cloned into
pSFS2A to generate pSFS-ctTEC1 AB, pSFS-ctBCR1 AB, pSFS-ctROB1 AB, pSFS-ctEFG1 AB, and pSFS-ctNDT80 AB, respectively, whereas the BCR1 PCR product was digested with Apal/BamHI and cloned into pSFS2A to generate pSFS-ctBCR1 AB. To generate the complementary strains, plasmid pSFS-ctTEC1 was linearized with Pmel and transformed into mutants to create Δtec1/Δtec1::TEC1, YL1498, and YL1499. Plasmid pSFS-ctBCR1 AB was digested with BglII and transformed into mutants to create Δbcr1/Δbcr1::BCR1, YL1493, and YL1494. Plasmid pSFS-ctBRG1 AB was partially digested with BsaI and transformed into mutants to create Δbrg1/Δbrg1::BRG1, YL1495, and YL1496. Plasmid pSFS-ctROB1 AB was digested with Apal/Xhol and transformed into mutants to create Δrob1/Δrob1::ROB1, YL1497, and YL1504. Plasmid pSFS-ctEFG1 AB was digested with Hpal and transformed into mutants to create Δefg1/Δefg1::EFG1, YL1570, and YL1571. Plasmid pSFS-ctNDT80 AB was partially digested with ApalI and transformed into mutants to create Δndt80/Δndt80::NDT80, YL1572, and YL1573. To avoid ectopic integration, primers 1795/1796, 1797/1798, 1799/1800, 1801/1802, 1803/1804, and 1805/1806 were used to verify the C. tropicalis CITEC1, CtBCR1, CtBRG1, CtROB1, CtEFG1, and CINDT80 complementary strains, respectively.

To generate the C. albicans ROB1 (CtROB1) open-reading frame fused with the C. albicans ROB1 promoter (CaROB1p) construct, CtROB1 and CaROB1p were amplified with primer pair 753 and 754 and primer pair 755 and 756, respectively. Similarly, CtBCR1 (primers 763/764) and CaBCR1p (primers 765/766) as well as CtEFG1 (primers 745/746) and CaEFG1p (primers 747/748) were amplified with indicated primers, respectively. The PCR products were mixed and amplified again with primers 754/755, 764/765, and 746/747 to create CaROB1p-CtROB1, CaBCR1p-CtBCR1, and CaEFG1p-CtEFG1 gene fragments, respectively. The fused CaROB1p-CtROB1, CaBCR1p-CtBCR1, and CaEFG1p-CtEFG1 fragments were digested with Apal/Xhol, Apal/BamHI, and Apal/Xhol, respectively, and cloned into pSFS2A to construct pSFS-CaROB1p-CtROB1, pSFS-CaBCR1p-CtBCR1, and pSFS-CaEFG1p-CtEFG1, respectively. The pSFS-CaROB1p-CtROB1 construct was digested with Pmel and transformed into the C. albicans rob1Δ to generate CtROB1 expression strains YL1456 and YL1457 in C. albicans. The pSFS-CaBCR1p-CtBCR1 construct was partially digested with EcoRI to generate CtBCR1 expression strains YL1450 and YL1451 in C. albicans. pSFS-CaEFG1p-CtEFG1 construct was digested with Hpal to generate CtEFG1 expression strains YL1417 and YL1419 in C. albicans. These two independent knockout and knock-in strains exhibited similar phenotypes in our pretest experiments. Only one strain was therefore selected for further analysis in this article. To confirm that each construct was integrated into the right position, primers 1807/766, 1808/756, and 1809/748 were used to verify the CaBCR1p-CtBCR1, CaROB1p-CtROB1, and CaEFG1p-CtEFG1, respectively, in C. albicans.

2.3. Biofilm Assay and Biofilm Staining

To develop the biofilms on the bottom of polystyrene plates, overnight YPD cultures grown at 30°C were used to inoculate polystyrene plates pretreated with bovine serum at an OD600 of 0.5. Cells were left to adhere to the plates in Spider at 37°C and shaking at 100 rpm for 2 h. Plates were then washed with Phosphate-buffered saline (PBS) three times by pipetting after removed supernatants. Fresh Spider medium was added to each well, and plates were further incubated for 48 h at 37°C at 100 rpm [20]. Supernatants were removed, and samples were then photographed. The adhered cells were scratched out of the plastic well and quantified by measuring the OD600. Determination of slime index (SI) was based on the previous report with slight modifications [29]. SI was calculated from the biofilm formation values obtained by OD method in relation to the growth culture values measured by OD600 prior to washing the wells. SI establishes a relation between biofilm formed and culture growth (SI = (biofilm/growth culture) × 100) [29].

Established protocols to measure the dry weight of biofilms in a silicone model of biofilms were performed, with slight modifications [21]. Preweighed 1.5 cm × 1.5 cm sterile silicone squares (Bentec Medical, PR72034-06N, Woodland, CA, USA) were preincubated in bovine serum (Gibco, 1861237; ThermoFisher Scientific Inc., Waltham, MA, USA) for 12 h at 37°C and 100 rpm in a polystyrene 12-well plastic plate. Serum-treated silicone was washed with 2 mL of PBS. The washed silicone was placed in 2 mL of Spider, Lee’s glucose, RPMI, yeast nitrogen base (YNB) + 1% glucose, SD, SCD +
50% serum, or SU medium for adhesion. Notably, mannitol in Spider medium was replaced with 1% glucose for *C. tropicalis* biofilm analyses. *C. tropicalis* and *C. albicans* cells were grown overnight in YPD medium, and approximately $1 \times 10^7$ cells were added on top of each silicone square. The inoculated plate was incubated with gentle agitation (100 rpm) at 37 °C for 4 h for adhesion. Silicone squares were washed with 2 mL of PBS, and incubation continued in 2 mL of fresh Spider medium for 24 h at 37 °C with gentle shaking (100 rpm). Supernatants were removed, and silicone squares were allowed to dry overnight before weighing to determine biofilm mass. Four to five replicate biofilms grown in separate wells were used for each strain.

Crystal violet staining was performed by decanting the silicone, with slight modifications [30]. Cells were stained for 30 min with 2 mL of 0.2% aqueous crystal violet per silicone. Silicone was washed with 2 mL of PBS and destained with 1 mL of 95% ethanol for 30 min. Two hundred microliters of the destain solution was placed in a 96-well plate, and absorbance at 520 nm was read using an ELISA reader (Waltham; Thermo Fisher Scientific Inc., Waltham, MA, U. S. A.). The experiment was performed in three experimental replicates.

### 2.4. Statistical Analyses

All statistical analyses were performed with Excel software. Differences were analyzed using a one-tailed or two-tailed Student’s *t*-test with a 95% confidence interval.

### 3. Results

#### 3.1. Effects of Different Culture Conditions on Biofilm Formation in the *C. tropicalis* Wild-Type Strain (MYA3404)

We compared the effects of different media during the adhesion step on biofilm growth. These media included Spider, Lee’s glucose, RPMI, YNB + 1% glucose, SD, SCD + 50% serum, and SU media [19,21–25]. We first tested the biofilm cells of *C. tropicalis* on the bottom of 12-well polystyrene plates. However, the biofilm growth of *C. tropicalis* in different culture media was easily washed out, although cells incubated with SCD supplemented with 50% serum for adhesion showed some biofilm formation around the well and a minor amount on the bottom (Figure 1a). We further calculated the slime index (SI), where growth constitutes a correction factor in the determination of biofilm formation. Figure 1b showed that *C. tropicalis* exhibited significant increase in the slime index in SU medium compared to that of the Spider medium. Nevertheless, the final outcome of low quantifiable biofilms by measuring cell density in Figure 1a still caused difficulty in determination. We therefore evaluated whether these culture conditions for the adhesion stage could profoundly affect *C. tropicalis* biofilm formation on silicone-based materials. As shown in Figure 2, the use of RPMI 1640, YNB + 1% glucose, or synthetic urine media as the adherence medium induced the most biofilm growth, particularly with the SU medium. Thus, SU medium was primarily used to analyze *C. tropicalis* biofilm formation on silicone in this work.
Figure 1. Effects of different culture conditions on *C. tropicalis* biofilm formation on the bottom of 12-well polystyrene plates: (a) Representative images of biofilm formation of the *C. tropicalis* wild-type strain (MYA3404) on the bottom of 12-well polystyrene plates. Quantitative results showed that no medium induced cell adhesion and biofilm formation significantly compared to that of the Spider medium on the bottom of 12-well polystyrene plates. Values are the mean ± SD from six replicates. (b) *C. tropicalis* exhibited a significant increase in the slime index in synthetic urine medium. Values are the mean ± SD from six replicates. **p < 0.01.
Figure 2. RPMI, YNB + 1% glucose, and synthetic urine (SU) media as adhesion conditions exhibited increased biofilm dry weights. The C. tropicalis wild-type strain MYA3404 was cultured under different culture conditions for biofilm analysis in 12-well tissue culture plates with silicone. (a) Representative images of biofilm formation of the C. tropicalis wild-type strain on silicone. (b) Quantitative analysis of the C. tropicalis wild-type strain in a biofilm assay on silicone squares: Values are the mean ± SD from five replicates. * p < 0.05; ** p < 0.01, and *** p < 0.001.

3.2. Replacement of Spider Medium with Synthetic Urine in the Adherence Step Profoundly Affected C. tropicalis Biofilm Growth but not that of C. albicans

Previous studies have shown that C. albicans biofilms were formed in vitro using SU medium [24], and Spider medium has frequently been used for the adhesion step in biofilm development by C. albicans [16,21,22]. We therefore evaluated whether changes in the adhesion condition with SU medium affected biofilm development in both C. albicans and C. tropicalis. The results showed that both Spider and SU media were able to induce biofilm development (~12.1 and ~11.3 mg, respectively) in C. albicans (Figure 3a), whereas biomass weights with SU medium significantly increased (~7.7 mg) compared to that with Spider medium (~2.3 mg) in C. tropicalis (Figure 3a). Consistent with a previous report, EFG1 is required for biofilm growth in C. tropicalis (Figure 3a), and a mutant was used as a negative control [20]. Biofilm staining using crystal violet also showed similar conclusions, in which more biofilms were stained in SU medium than in Spider medium in the C. tropicalis wild-type strain (Figure 3b).
Figure 3. Quantitation of biofilms of *C. albicans* and *C. tropicalis* wild-type (WT) and *efg1Δ* strains in the Spider and SU biofilm formation conditions: (a) The use of SU medium resulted in increased *C. tropicalis* biofilm formation, whereas *C. albicans* showed a similar biomass when cultured with Spider medium and SU. Representative images of biofilm formation were shown below. (b) The extent of *C. albicans* and *C. tropicalis* biofilms were measured using crystal violet and resulted in similar conclusions in Figure 3a. Values are the mean ± SD from four replicates. ** *p* < 0.01. The *C. albicans efg1Δ* and *C. tropicalis efg1Δ* strains were used as negative controls.

3.3. Effects of Depletion of Each Ingredient in SU on *C. tropicalis* Biofilm Growth

To understand why SU is able to induce *C. tropicalis* biofilm formation, we depleted each ingredient and examined its effect on biofilm growth. The results showed that depletion of MgCl2 or KH2PO4 caused a significant reduction in biofilm formation, especially that of the former (Figure 4a). Lack of CaCl2, Na2SO4, Na2C6H5O7, urea, or creatinine resulted in reduced biofilms but without significant effects (Figure 4a). Given that the magnesium chloride concentration in Spider medium (0.25 g/L) is much lower than that in SU medium (0.65 g/L), we further tested whether increases in the magnesium concentration level in Spider medium to the same level as that in SU were able to promote biofilm growth. As shown in Figure 4b, adding exogenous magnesium did not significantly induce biofilm development, suggesting that it could be a combinational effect of SU in biofilm induction. Furthermore, in order to determine whether growth defects occur in SU medium without any magnesium, thereby causing a severe deficiency in biofilm formation, growth curves were
analyzed. The results showed that magnesium depletion caused a striking effect on cell growth, indicating that the presence of magnesium is necessary in proliferation, leading to an impact on biofilm formation (Figure 4c).

Figure 4. Effects of the ingredients of SU on biofilm formation in C. tropicalis: (a) Depletion of MgCl2 or KH2PO4 in SU significantly inhibited biofilm formation. (b) The addition of exogenous MgCl2 in Spider medium caused very only slight effects on biofilm dry weights in both C. albicans and C. tropicalis. Values are the mean ± SD from five replicates. * p < 0.05; *** p < 0.001. (c) Growth curves of C. tropicalis strains at 30 °C in SU with or without additional MgCl2 showed the requirement of magnesium for C. tropicalis proliferation. Growth rates were monitored every 2 h using a Biowave density meter.

3.4. ROB1 is not Required for Biofilm Development in C. tropicalis

In C. albicans, six transcription factors, Bcr1, Brg1, Efg1, Tec1, Rob1, and Ndt80, form a complex regulatory circuit controlling biofilm formation. Loss of each gene causes dramatic biofilm defects [16,21]. Genomic sequence data have shown that the six biofilm-associated genes are also present in C. tropicalis [20,26,31]. Six transcription factor amino acid sequences of both C. albicans and C. tropicalis obtained from the Candida Genome Database and UniProt websites were aligned, and alignments were carried out using EMBOSS Pairwise Sequence Alignment. Results showed that C. tropicalis Bcr1, Brg1, Efg1, Tec1, Rob1, and Ndt80 share 53.4%, 44%, 55.4%, 61.4%, 35.9%, and 72.1% identities with each respective homolog in C. albicans (Table 2). CtBrg1 and CtRob1 exhibited much less identity with CaBrg1 and CaRob1 than the others. To determine whether the homologs of major transcriptional factors play crucial roles in biofilm development in C. tropicalis, each C. tropicalis homologous transcriptional gene was deleted. Compared with the wild-type strain (MYA3404), five mutant strains of each gene exhibited a significant reduction in biofilm biomasses (Figure 5a). rob1Δ showed no effect and no statistically significant difference compared with the wild-type strain. Crystal violet assays also revealed results similar to the previous results on silicone (Figure 5b). Furthermore, reintroduction of a functional gene in each mutant but not rob1Δ restored or partially restored biofilm formation in C. tropicalis (Figure 5c).
Table 2. Sequence alignment of *C. tropicalis* Bcr1, Brg1, Efg1, Tec1, Rob1, and Ndt80 with the respective homolog in *C. albicans*.

| Protein in *C. tropicalis* | Bcr1 | Brg1 | Efg1 | Tec1 | Rob1 | Ndt80 |
|---------------------------|------|------|------|------|------|-------|
| Identity to respective homolog in *C. albicans* | 53.4% | 44.0% | 55.4% | 61.4% | 35.9% | 72.1% |
| Similarity to respective homolog in *C. albicans* | 62.1% | 50.5% | 60.2% | 73.7% | 50% | 75.2% |

Figure 5. Except the rob1Δ mutant strain, the lack of each gene in *C. tropicalis* caused a significant reduction in biofilms. Biofilms were determined by (a) biomass dry weight and (b) the OD600 at the wavelength for crystal violet absorbance. Each experiment was repeated independently at least three times. (c) Reintroduction of each functional gene in each respective mutant strain except the ROB1 complementary strain was able to recover biofilm formation. Values are the mean ± SD from five replicates. *p < 0.05, **p < 0.01, and ***p < 0.001.

3.5. Introduction of the *C. tropicalis* ROB1 into *C. albicans* Rob1Δ Was Not Able to Restore Biofilm Growth in *C. albicans*

To further elucidate the role of *C. tropicalis* ROB1 (CtROB1) in biofilm formation, we transformed CtROB1 into the *C. albicans* rob1Δ mutant strains. As shown in Figure 6, CtROB1 in the *C. albicans* rob1Δ could not promote biofilm development. CtBCR1 and CtEFG1 were selected as the parallel control groups, as deletion of any one gene caused severe impairment of biofilm growth in *C. tropicalis* (Figure 5). Results showed that reintroduction of CtBCR1 and CtEFG1 into *C. albicans* bcr1Δ and efg1Δ strains, respectively, restored biofilm development in *C. albicans* (Figure 6). These findings demonstrated that CtROB1 does not play an important role in biofilm formation in *C. tropicalis*. Furthermore, the results also suggest that, although *C. tropicalis* and *C. albicans* are evolutionarily closely related, characteristics of biofilm regulatory circuits of the two species are somehow different.
Figure 6. Heterogeneous expression of *C. tropicalis* ROB1 in the *C. albicans* rob1Δ could not recover biofilm growth. Values are the mean ± SD from three replicates. Reintroduction of *CtBCR1* and *CtEFG1* into *C. albicans* bcr1Δ and efg1Δ strains, respectively, restored biofilm development in *C. albicans*, whereas *Carob1Δ::CtROB1* exhibited few biofilms. ***p < 0.001.

4. Discussion

The pathogenicity of chronic and recurrent infections has been associated with the production of biofilms for *C. albicans* and other non-albicans *Candida* species [12,15,22]. It is known that different culture conditions affect biofilm growth in several *Candida* species [19,22,25]. In this study, we established a reliable method for *C. tropicalis* biofilm development with a silicone-based platform. Three media, RPMI 1640, YNB supplemented with 1% glucose, and SU, as an adhesion medium were able to induce strong biofilm growth of *C. tropicalis*. Four media, Spider, Lee’s glucose, SD, and SCD supplemented with 50% serum, produced no or less biofilms. Spider medium contains nutrient broth (beef extract and peptone) to supply amino acids, peptides, and proteins [32]. Similarly, Lee’s glucose and SCD serum media contain a variety of amino acids [22,25,32]. SD medium contains fewer amino acids [22,25,32]. Interestingly, vitamins and inorganic salts are not the major constituents in these media. By contrast, both YNB and RPMI 1640 are rich in vitamins and inorganic salts, although RPMI 1640 also contains a variety of amino acids [22,25,32]. SU medium contains urea, creatinine, and many inorganic salts [19,24]. This fact implies that vitamins and inorganic salts are important for *C. tropicalis* adhesion and biofilm growth on silicone. Moreover, the glucose contents of RPMI 1640 (2 g/L), YNB (9 g/L), and SU (3 g/L) media are lower than those of Spider (10 g/L), Lee’s glucose (12.5 g/L), SD (20 g/L), and SCD + serum (20 g/L) media. High glucose content in a medium may support and promote planktonic growth rather than biofilm development in *C. tropicalis*. Interestingly, the compositions of SU are relatively simple, but this medium induced *C. tropicalis* biofilm formation the most. This result might explain why *Candida* species favor urinary tract infection [33].

After analysis of the effect of each ingredient in SU on biofilm growth, we found that depletion of magnesium exhibited the greatest impact on cell proliferation, thereby affecting biofilm formation. Metal ions are important elements for prokaryotic and eukaryotic cells and play crucial roles in numerous enzymatic reactions to maintain physiological function, including DNA replication, transcription, and biosynthesis of precursors [34,35], but they can also be toxic to living organisms if present in excess [36]. Furthermore, several reports have shown that cation concentrations influence extracellular product formation and biofilm-associated growth in microorganisms. For example, calcium and magnesium cations can enhance *Pseudomonas aeruginosa* adherence ability [37]. Increases in Mg²⁺ concentration promote cell attachment and biofilm formation in both *Staphylococcus aureus* and *Pseudomonas fluorescens* [38,39]. Calcium increases the amount of extracellular matrix composition associated with *Pseudoalteromonas* sp. biofilms [40].
However, 50 mM and higher Mg\textsuperscript{2+} concentrations significantly inhibit biofilm formation in Bacillus species [41]. The adherence ability of *Staphylococcus epidermidis* is enhanced in low concentrations of magnesium [42]. In *C. parapsilosis*, an increase in Mn\textsuperscript{2+}, with a maximum at 2 mM, significantly induced biofilm formation [43]. Moreover, different metal ions can suppress or enhance *C. albicans* and *C. tropicalis* biofilm formation. For example, Co\textsuperscript{2+}, Cu\textsuperscript{2+}, Ag\textsuperscript{+}, Cd\textsuperscript{2+}, Hg\textsuperscript{2+}, Pb\textsuperscript{2+}, AsO\textsubscript{2}\textsuperscript{−}, and SeO\textsubscript{3}\textsuperscript{2−} inhibit hyphal formation in biofilms of both *Candida* species, whereas CrO\textsubscript{4}\textsuperscript{2−} triggers a transition to the hyphal cell morphotype in *C. tropicalis* biofilms [44]. These data indicate that the effects of metal ions on microorganisms are highly dependent on the different types of added metal ions and microbial species.

Spider medium, containing fewer magnesium ions than SU medium, is often used for *C. albicans* biofilm assays, but it fails to induce *C. tropicalis* adhesion and biofilm formation. The addition of exogenous magnesium to the Spider medium resulted in only very slight, nonsignificant increases in biomass dry weights. These results indicated that *C. albicans* and *C. tropicalis* displayed strikingly variable heterogeneous growth, adhesion, and biofilm-forming potential in different growth media. However, the mechanisms of why SU is able to induce *C. albicans* and *C. tropicalis* biofilm formation remain unknown. It is possible that the outcome results from the fact that a number of metal ions, urea, and creatinine have minor effects on biofilm formation, contributing to the impact.

Mechanisms underlying biofilm formation in *C. albicans* are controlled by a central regulatory network with six transcriptional factors: Bcr1, Brg1, Efg1, Tec1, Rob1, and Ndt80 [16]. However, studies regarding biofilm formation mechanisms are very limited in non-albicans *Candida* species, and biofilm formation regulated by the circuit of six transcription factors may vary in *Candida* species. For example, in *C. parapsilosis*, only EFG1 and BRG1 deletion strains exhibited similar effects on biofilm formation as those observed in *C. albicans*, whereas BRG1 and TEC1 do not play critical roles in biofilm growth in *C. parapsilosis* [45]. Furthermore, in contrast to in *C. albicans*, four transcription factor genes, CPH2, UME6, CZF1, and GZF3, were characterized for their roles in biofilm formation and are unique to *C. parapsilosis* [45]. These six homologous genes have also been identified in *C. tropicalis* [20,26]. Interestingly, the degree of sequence identity of two species corresponds to biofilm results, in which the lower identity causes less effect in biofilm formation in *C. tropicalis* mutant strains. Both *C. tropicalis* brg1\textsuperscript{Δ} and rob1\textsuperscript{Δ} strains formed more biofilms compared to the other mutant strains, although brg1\textsuperscript{Δ} produced reduced biofilm significantly. Furthermore, expression of *CtROB1* in the *C. albicans* rob1\textsuperscript{Δ} was not able to recover biofilms. These findings suggest that, although the functions of five biofilm-associated regulators in both *C. albicans* and *C. tropicalis* are relatively conserved, mechanisms regarding biofilm formation in *C. tropicalis* are distinct from those of its relative *C. albicans*. Furthermore, it will be worthwhile to explore the transcriptional expression profile between planktonic cells and biofilm cells using an SU-induced biofilm platform to understand the *C. tropicalis* biofilm regulatory network.

**Supplementary Materials:** The following are available online at www.mdpi.com/2076-2607/8/5/660/s1, Table S1: Oligonucleotides used in this study.

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