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Curcumin Epigenetically Represses Histone Acetylation of Echinocandin B Producing *Emericella rugulosa*

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Abstract: Echinocandin B is a natural product that possesses potent antifungal property against a wide array of fungi. This antifungal agent is produced by *Emericella rugulosa*. The biosynthetic genes of echinocandin B are physically organized in two gene clusters (*ecd* and *hty*). The *ecd* gene cluster comprises 12 genes (*ecd*A–L). On the other hand, *hty* contains six genes (*hty*A–F). These gene clusters regulate Echinocandin B expression and play an essential role in chromatin modifications. The present study elucidates the epigenetic regulatory network of echinocandin B production using Histone acetyltransferase (HAT) inhibitor; curcumin using transcriptional gene expression analysis. The High-performance liquid chromatography (HPLC) analysis revealed suppression of the echinocandin B levels in the cells treated with curcumin. Curcumin was also found to repress the expression of different *ecd* genes by several folds. Taken together, we conclude that curcumin targets echinocandin B production by inhibiting histone acetylation as well as disrupting interspecies consortium communication, which eventually leads to a decrease in the echinocandin B synthesis.

Keywords: echinocandin B; curcumin; histone modifier; histone acetyltransferase (HAT) inhibitor; epigenetic regulation

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1. Introduction

The emergence of drug resistance against most of the antifungal agents has triggered a prevalence of superficial and invasive Candidiasis and Aspergillosis ailments with a high rate of fatalities. In this light, Echinocandins are considered the first-line antifungals that exhibit both fungistatic and fungicidal activity against most of the fungal pathogens including azole-resistant strains. Echinocandin B is a non-competitive inhibitor of β-1, 3-Glucan synthetase, which restricts the synthesis of Glucan, a key component of the cell wall [1–3]. Echinocandin is known to have minimal toxic effects on the host than other counterparts like Amphotericin B. Echinocandin B is reported to be naturally synthesized in *Emericella rugulosa* NRRL11440 under specific conditions [4,5]. Due to the therapeutic and economical importance of echinocandin B, the genetic and physiological studies dealing with this secondary metabolite production have been the subject of intense research. The biosynthetic genes of echinocandin B are physically organized in two gene clusters (*ecd* and *hty*). The *ecd* gene cluster comprises 12 genes (*ecd*A-L) and *hty* contains six genes (*hty*A-F). For instance, the biosynthetic gene cluster of echinocandin (*ecd*) contains a six-module nonribosomal peptide synthetase (NRPS) encoded by e*cdA*, transcription factor *ecdB*, an acyl-AMP ligase *ecdI* and oxygenases *ecdG*, *ecdH*, and e*cdK*. *ecdI* activates the linoleate as linoleyl-AMP and installs it onto the first thiolation domain of *ecdA* for the maturation of the echinocandin B molecule [4,6,7].

It has been well established that the synthesis of the fungal secondary metabolite is strongly influenced by environmental factors and nutrients. These factors include carbon

and nitrogen source, pH, light, redox, iron starvation, interspecies communication, etc. [8,9]. Regulation of secondary metabolite gene clusters is largely based on the modulation of a chromatin structure. This chromatin status restricts access to the underlying genetic material and thus results in reversible gene repression [10,11]. Accessibility to chromatin is mainly regulated at the level of post-translational modifications (PTMs) of histone proteins which define the degree of compactness ranging from a loose (euchromatin) to a very dense (heterochromatin). Protein modifications by acetylation, methylation, ubiquitination, and phosphorylation mainly represent on to different amino acids of N-terminal tails and globular domains of histone H2B, H3, and H4 proteins [11]. These post-translational modifications (PTMs) on histone proteins play an important role in chromatin-mediated regulation and various other biological processes [12,13]. The post-translational modifications of chromatin through acetylation and methylation of histone proteins serve as the signals for the recruitment or dismissal of silencing or activating factors for modulation of secondary metabolites biosynthesis. During a regulatory cycle, these markers can be altered again by deacetylases, demethylases, and phosphatases, or one modification can be replaced by another on the same residue. This is the case in lysine modification, which can either be acetylated or methylated on the same nitrogen atom. The PTM is a vital epigenetic mechanism that controls cell growth and other biological phenotypes without alteration of DNA sequence. Usually, the catalytic enzymes performing the specific addition or removal of the post-transcriptional modifications are part of specialized histone-modifying complexes. For instance, histone acetylation mainly linked to the activation of secondary metabolites synthesis is controlled by the action of histone acetyltransferases (HATs) and deacetylases (HDACs) [11]. However, histone methylation rendered by histone methyltransferases (HMT) opposes the activation of the secondary metabolites production [10]. These modifications are usually induced under nutrient-limiting conditions, humidity, temperature, UV irradiation, salt, or unfavorable pH [8]. In recent years, fungi have gained high attention to study epigenetic regulation as they are often involved in the synthesis of therapeutic-relevant secondary metabolites. However, histone modifiers have also been reported to have promising results in clinical practices, particularly in the regression of tumor growth cells with lower cytotoxicity [14–16]. Curcumin is an important compound found in turmeric that has been used for a long time in Asian foods [17]. This yellow color substance showed a variety of medicinal properties [18–22]. Earlier studies also reported that curcumin modifies the histones proteins to restrict acetylation and hence acts as the HAT inhibitor [18–24]. The present study is focused on investigating the role of curcumin in the production of echinocandin B antifungal in *Emericella rugulosa* NRRL11440.

2. Results

2.1. Effect of Curcumin on E. rugulosa Growth

In order to assess the effect of curcumin on *E. rugulosa* NRRL11440 growth, 10 days aged spores were grown in GMM-arginine medium in the absence and presence of 100 and 200 µM curcumin (dissolved in DMSO) at the static condition for further 10 days. Results showed that the growth of mycelial spores of *E. rugulosa* NRRL11440 was induced when it was treated with curcumin as compared to the control cells (Figure 1).

2.2. Effect of Curcumin on Echinocandin B Production

To check the effect of curcumin which is a known histone methyltransferase (HAT) inhibitor on echinocandin B antifungal production in *E. rugulosa* NRRL11440 strain.

The cell culture grown in echinocandin B producing medium (GMM-arginine) was treated with a fixed 100 µM concentration of curcumin for 10 days in static conditions. The HPLC analysis was performed with both the curcumin-treated and non-treated (Control) methanol extract samples using RP-C18 column (4.5 × 250 mm) for quantitative estimation of echinocandin B synthesis. Results show that the pure echinocandin B compound was used as a standard. It was detected at almost 18 min of retention time as shown in Figure 2A. The comparative analysis of echinocandin B production was also observed both

in the control and curcumin-treated sample in particular to the observed peak with similar retention time both in the control and curcumin-treated sample (Figure 2B,C).

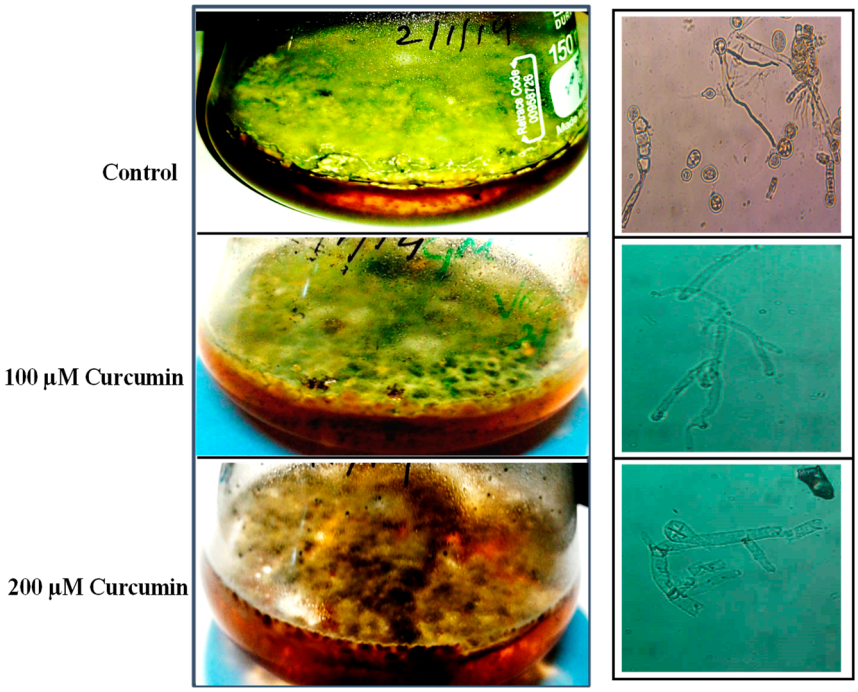

Figure 1. Effect of curcumin on *Emericella rugulosa* growth: Ten-days aged *Emericella rugulosa* NRRL11440 mycelium grown in GMM-Arg medium. The mycelial growth was observed under a microscope in the absence and presence of 100 µM and 200 µM curcumin.

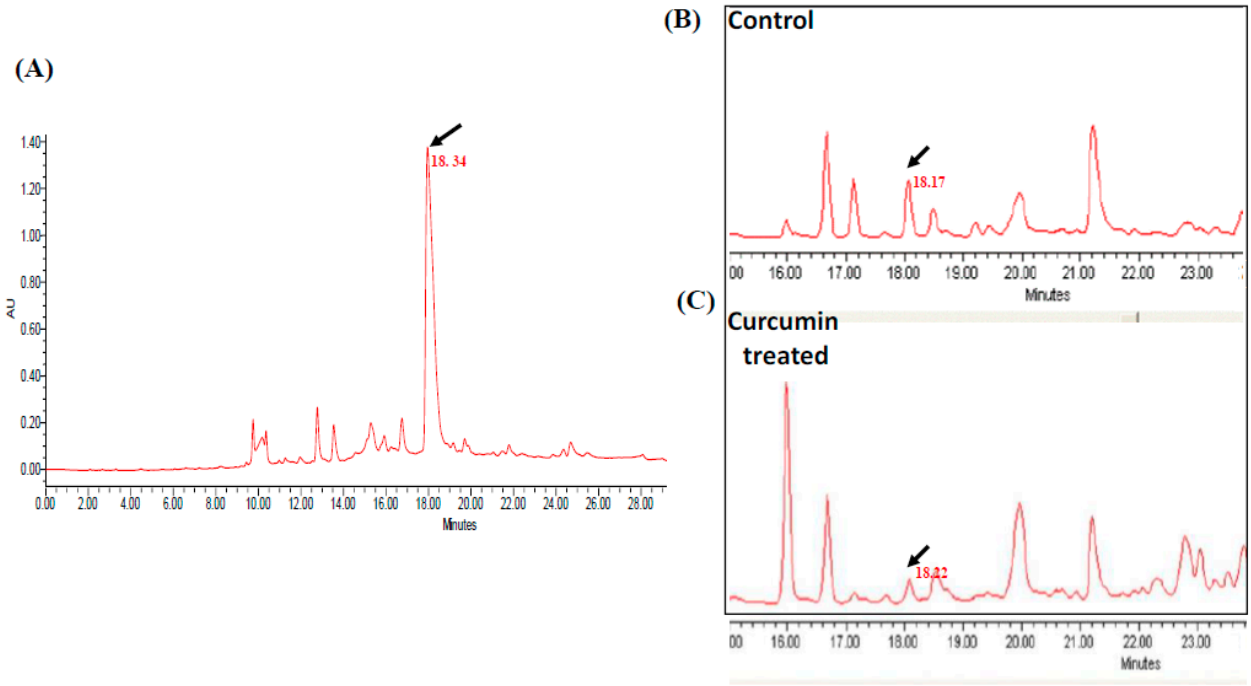

Figure 2. Estimation of echinocandin B biosynthesis: The methanolic extract sample recovered from the ten-days aged cell mat of *E. rugulosa* NRRL11440 was loaded the on RP-C18 column for assessment of Echinocandin B production. Arrow indicates the Echinocadin B peak on chromatogram. (**A**) Pure Echinocandin B sample as standard (1 mg/mL); (**B**). Methanolic extract of without curcumin treated (control) sample. (**C**). Methanolic extract of 100 µM curcumin treated sample.

However, the echinocandin production in the curcumin-treated sample was sharply reduced, revealed by the peak intensity corresponding to ~18 min of retention time. These results indicate that the HAT inhibitor; curcumin inhibits echinocandin B production.

2.3. Cell Susceptibility Profiling of Curcumin-Treated Sample

To further evaluate the effect of curcumin on echinocandin B production, a cell susceptibility test was performed by confrontation assay using a mycelial mat of *E. rugulosa* NRRL11440, treated with and without curcumin (100 µM) sample for 10 days at static conditions. As expected that *E. rugulosa* NRRL11440 cells treated with curcumin severely suppressed the echinocandin production, resulting to show a very low cell susceptibility against *C. albicans* DSY294 as shown in Figure 3. These results indicate that curcumin limits the synthesis of echinocandin B in *E. rugulosa* NRRL11440.

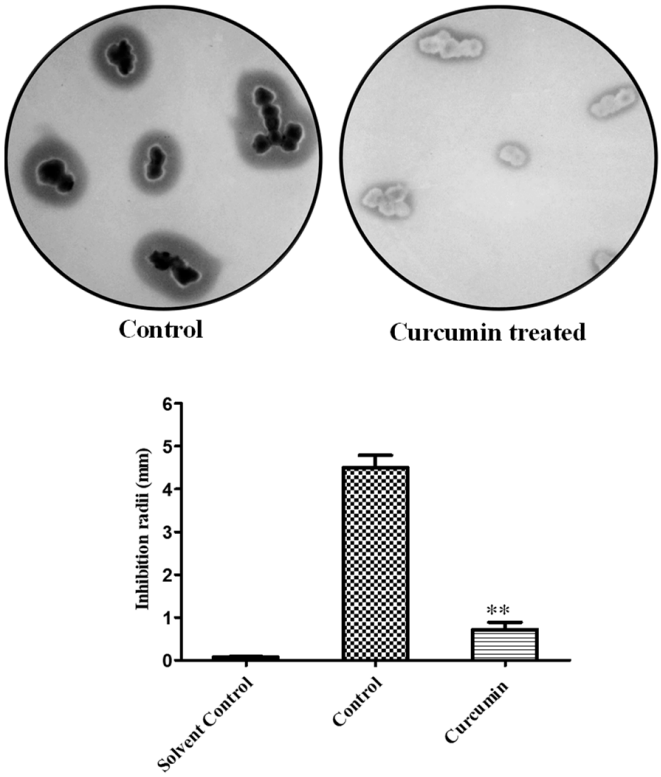

Figure 3. Susceptibility test of curcumin-treated *E. rugulosa* NRRL1144: The cell extract was recovered from the curcumin-treated 10 days aged *E. rugulosa* NRRL11440 cells and the mycelial mat was put on the mid-log aged *Candida albicans* cells spread YEPD agar plate. The zone of inhibition was observed. Two-tailed Student *t*-test was performed for statistical analysis. *p* values of (** $p \leq 0.001$) are considered significant. *p* values of control vs. curcumin: 0.0038.

2.4. Curcumin Transcriptionally Represses Genes Present in the Echinocandin B Gene Cluster

To further evaluate the repression in cell susceptibility in the curcumin-treated sample was the result of post-translational modifications on histone proteins. For this, we analyzed the acetylation incurred at histone protein (H3) in the absence and presence of curcumin (100 and 200 µM) via Western blotting analysis using Anti-acetlyH3 Ab. Figure 4 illustrates that the acetylation on H3 histone proteins was found to be repressed in a dose-dependent manner when the sample was treated with curcumin. It was observed that the expression level of acetylation on histone (3 H) was repressed by 43% and 68% when samples were treated with 100 and 200 µM curcumin, respectively.

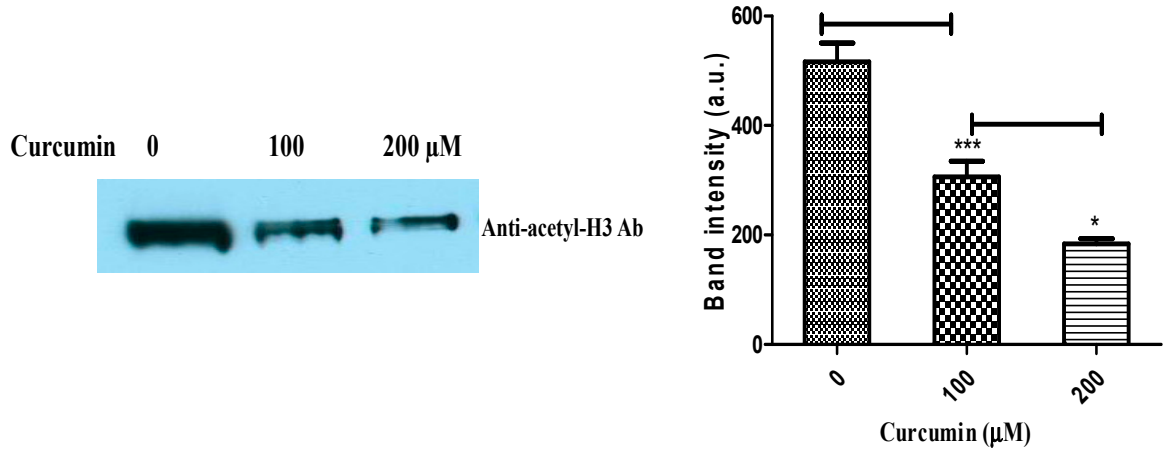

Figure 4. Curcumin epigenetically represses the acetylation of histone protein: The crude membrane was prepared from *Emericella rugulosa* NRRL11440 grown in the absence and presence of 100 and 200 µM curcumin-treated cell cultures as described in Materials and Methods. The Western blot analysis was carried out using Anti-acetyl H3 antibody (1:5000 dilution). The histone acetylation was assessed by band intensity using Image J software. Two-tailed Student *t*-test was performed for statistical analysis. p values of (* $p \leq 0.05$) (*** $p \leq 0.0001$) are considered as significant. Detailed p values of control vs. 100 µM curcumin: 0.0008, 100 µM curcumin vs. 200 µM curcumin: 0.024.

These results suggest that curcumin, a histone acetyltransferase (HAT) inhibitor is able to post-translationally suppress the acetylation process on a histone protein, resulting to repress the echinocandin B synthesis as revealed by western blot.

2.5. Curcumin Transcriptionally Represses Genes Present in the Echinocandin B Gene Cluster

To confirm the role of histone modifier, curcumin in echinocandin B production, we analyzed the gene expression pattern of different genes of the *ecd* gene cluster of echinocandin B. For this, gene expression profiling of *ecd* gene cluster genes, i.e., "*ecdB* which encodes for a transcription factor, *ecdA* encodes for an NRPS (Non-ribosomal Peptide synthetase), *ecdL*; for an ABC transporter, and *ecdK* encodes for a dioxygenase" were analyzed in the absence or 100 and 200 µM curcumin treatment. The expression of the actin gene was used as an internal reference.

Figure 5A,B shows that gene expression of transcription factor encoded "*ecdB*, and *ecdA*; an NRPS encoded gene" was completely repressed when cells were treated with curcumin which indicates that transcriptional factor encoded gene ecdB and NRPS genes present within the *ecd* gene cluster have a major role in the echinocandin B production. However, gene expression of e*cdL* and *ecdK* genes showed a dose-dependent expression which drastically repressed expression at 100 µM compared to 200 µM curcumin concentration as shown in Figure 5.

2.6. Interspecies Interactions Suppress the Inhibitory Effect of Curcumin on Echinocandin B Production

It is well established that interspecies interactions tend to induce the biosynthetic gene clusters (BGCs) of secondary metabolites which led to increased production of secondary metabolites. To assess the inhibitory effect of curcumin on echinocandin B production under interspecies interactions established between *E. rugulosa* NRRL11440 and *C. albicans*. For this, *Emericella rugulosa* NRRL11440 cell mat was grown in the absence or presence of 200 µM curcumin. On the 5th day, *C. albicans* cells were cocultured. to observe the interspecies effects on echinocandin B production.

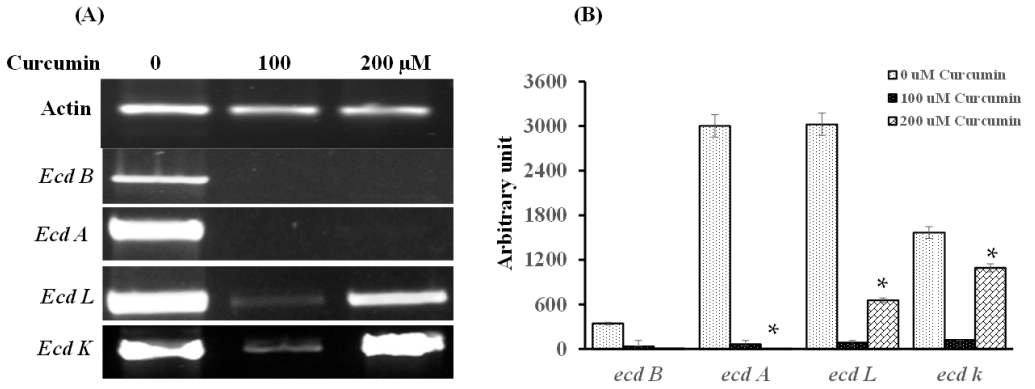

Figure 5. Curcumin transcriptionally represses echinocandin B genes: (**A**).Transcriptional expression of *ecdA*, *ecdB*, *ecdL* and *ecdK* genes of echinocandin B biosynthetic pathway was tested in the absence or presence of curcumin treatment. The expression pattern of the actin gene was measured as a reference. (**B**). Bar diagram representation of gene expression of the tested genes. Statistical analyses were done using the two-way ANOVA test. *p* values of (* $p \leq 0.05$) are considered as significant in comparison to control groups.

Our results showed that the expression level of all the tested genes such as *ecd B*, transcription factor NPRS encoded; *ecd* A, ABC transporter encoded *ecd L* and *ecd K* genes were increased at different extents (Figure 6) when a consortium established with echinocandin B producing *Emericella rugulosa* NRRL11440 strain and *C. albicans*, suggesting to induce echinocandin B production. The induction of these gene expressions was repressed upon curcumin (200 µM) treatment to the consortium, leading to disturb interspecies interactions. These results suggest that curcumin is able to disrupt interspecies communication, leading to a decrease in the echinocandin B synthesis.

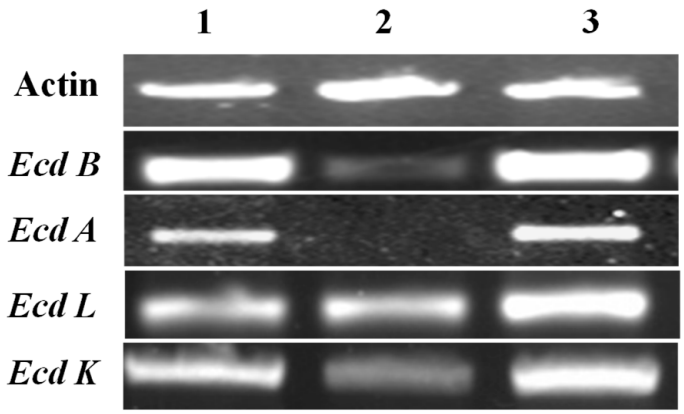

Figure 6. Interspecies interactions suppress curcumin effect on echinocandin B production: Transcriptional expression of *ecdA*, *ecdB*, *ecdL*, and *ecdK* genes of the echinocandin B biosynthetic gene cluster was tested in *E. rugulosa* the absence or presence of *C. albicans*. (1). Gene expression of different genes of echinocandin B biosynthesis gene cluster (BGC) in *E. rugulosa* without coculture *C. albicans*, as control. (2). Gene expression pattern of different genes of echinocandin B BSC in *E. rugulosa–C. albicans* cocultured treated with curcumin (200 µM). (3). Gene expression pattern of different genes of echinocandin B BSCs in *E. rugulosa–C. albicans* cocultured treated without curcumin (200 µM), as a reference.

3. Discussion

Among the many remarkable discoveries in the genomics, the revelation of the fungal isolates possessing greater numbers of gene clusters encoded for the production of secondary metabolites is arguably the most terrific one. Echinocandin B is a secondary metabolite which is synthesized in *E. rugulosa* NRRL11440 strains under specialized condi-

tions. This important antifungal entity is often used for the treatment of Candida infections, particularly for the treatment of drug-resistant fungal infections. The biosynthetic genes of echinocandin B are arranged into two (*ecd* and *hty*) gene clusters [4]. It has been established that the secondary metabolites' clusters located distal to the chromosome ends are also regulated by a heterochromatin-dependent mechanism. Regulation by chromatin structure is a proficient mechanism for post-translational regulation of secondary metabolite production. Chromatin regulation largely depends on DNA and histone modifications such as acetylation, methylation, or phosphorylation which inhibit or induce the transcription of certain genomic regions under specific conditions. Many researchers have proved that fungal secondary metabolite biosynthesis clusters are controlled by chromatin-based mechanisms, histone modifiers, and proteins involved in heterochromatin formation. The acetylation of histones coordinated by histone acetyltransferase (HATs) is involved in the activation of gene expression, cellular metabolisms, and protein stability [25]. The HATs positively regulate the biosynthetic gene clusters by transferring the acetyl group to the N-terminal of histone lysine. Earlier studies revealed that GcnE and MYST-type histones acetyltransferases reported in A. nidulans are involved in regulating the activation of secondary metabolites synthesis [26]. However, disruption of such genes led to loss of secondary metabolites production [26,27]. To the best of our knowledge, this is the first evidence showing the effect of curcumin on the biosynthesis of echinocandin B. The curcumin treatment to *E. rugulosa* NRRL11440 showed severe impairment of the echinocandin B production as supported by the HPLC analysis (Figure 2). It is suggested that curcumin which acts as a HAT inhibitor restricts histone acetylation, resulting to affect the echinocandin B production. The suppression of echinocandin B production was further corroborated by the confrontation assay. The cell susceptibility effect was completely eliminated with no significant inhibition zone observed when *E. rugulosa* NRRL11440 cells were treated with curcumin. In order to investigate the effect of curcumin on the expression of genes present in the echinocandin B genes cluster, it was observed that expression of transcription factor encoded *ecdB* gene, and *ecdA*; NRPS encoded gene was repressed in curcumin-treated cells which indicates that *ecdB* and *ecd* A gene present within the *ecd* gene cluster of echinocandin B has an important role in the echinocandin B production. However, the expression of multi-drug ABC transporter encoded e*cdL* and α-ketoglutarate-dependent nonheme iron oxygenases encoded *ecdK* gene repressed more at 100 μM curcumin than 200 μM curcumin concentration (Figure 5). An earlier study described the role of *ecdK* in the maturation of echinocandin B synthesis [28]. It is expected that higher curcumin (200 μM) treatment which repressed echinocandin B production, is able to tailor the echinocandin B molecule in *E. rugulosa* NRRL11440. However, the role of *ecdL* is still to be characterized. Therefore, Curcumin transcriptionally regulates echinocandin B biosynthetic genes by modulating them epigenetically especially restricting the recruitment of the acetylation on histone protein as revealed by the western blot analysis shown in Figure 4. A different group of studies has revealed the effect of histone modifiers on the modulation of secondary metabolite production [10,29,30]. Epigenetic histone modifiers such as Histone deacetylase (HDAC) and DNA Methyltransferase (DNMT) inhibitors induced the expression of a secondary metabolite pathway in endophytic *Nigrospora sphaerica* [27,31]. Similar findings were also obtained when azacytidine; a DNA methyltransferase (DNMT) inhibitor epigenetically modulates the synthesis of antibacterial secondary metabolites endophytic *Streptomyces coelicolor* [29,32]. It is well described that interspecies communication led to stimulate the secondary metabolites production. The perseverance of such cell-cell communications for the cell defense against the surroundings or inhibition of the growth of competitors for their survival which led to regulating the activation of biosynthetic gene clusters for secondary metabolites production. We also found that the expression level of all the tested genes of echinocandin B biosynthetic gene clusters (BSCs) such as *ecd B* encoded for a transcription factor, NPRS encoded; *ecd* A, ABC transporter encoded *ecd L* and *ecd K* genes were increased at different extents when echinocandin B producing *Emericella rugulosa* NRRL11440 was grown with *C. albicans*, suggesting to induce echinocandin B production.

This inducive effect was repressed after supplementation of curcumin (200 μM), a HAT inhibitor in the coculture of *Emericella rugulosa* NRRL11440 and *C. albicans* consortium, leading to upset interspecies interactions through chromatin modifications, resulting to decrease gene expression of echinocandin B biosynthetic genes.

In the present work, curcumin treatment to *E. rugulosa* NRRL11440 was found to suppress the echinocandin B antifungal biosynthesis by repression of gene expression of a transcriptional factor *ecdB* and an NRPS-coded *ecdA* which shows reduced cell susceptibility against *C. albicans*. Curcumin may turn out to be an effective histone acetyltransferase inhibitor for limiting acetylation on the histone proteins, that restrict echinocandin B production in *E. rugulosa* NRRL11440. This study signifies that the echinocandin B synthesis in *E. rugulosa* is regulated at an epigenetic level which may pave the way to further explore epigenetic regulation for the induction of echinocandin B production.

4. Materials and Methods

4.1. Chemicals, Strains, and Media

The echinocandin B producing *Emericella rugulosa* NRRL11440 strain was purchased from Fungal Genetics Stock Cente (FGSC), Manhattan, KS, USA, and it was maintained in YG medium at 30 °C. For echinocandin B production, *Emericella rugulosa* NRRL11440 strain was grown in GMM (Glucose minimal medium) supplemented with 10 mM of Arginine for 10 days in static conditions at 30 °C. For assessment of drug susceptibility analysis. *Candida albicans* DSY 294 strain was obtained as a kind gift from Prof. Dominique Sanglard, Centre Hospitalier Universitaire Vaudois (CHUV), Lausanne, Switzerland. *Candida albicans* DSY 294 strain culture and grown in the YEPD medium. The YG, YEPD, and GMM medium were purchased from the Hi-Media (Mumbai, India).

4.2. Production of Echinocandin B

Emericella rugulosa NRRL11440 strain was grown in YG medium at 30 °C at 220 rpm for three days. Spores formed were spread on YG plates and incubated at 30 °C for 10 days. Spores were harvested by drenching the plate with 5 mL of 0.1% Tween 20 and scraping off the mycelium with a sterile spreader without damaging the agar. The spore solution was agitated for 1 min. Spores were counted and inoculated 10^6 spores/mL in GMM (Glucose minimal medium) supplemented with 10 mM of Arginine as the sole nitrogenous source [4,5]. Further, to check the role of histone modification in echinocandin production, 100 and 200 μM Curcumin, a HAT (Histone acetyltransferase) inhibitor of histone modifiers was added to GMM-arginine medium at the time of adding spores. The cell culture was grown at 30 °C for 10 days in static conditions. The harvested culture was lyophilized and extracted with methanol at 30 °C for overnight for further use.

4.3. Measurement of Echinocandin B Production

The echinocandin B biosynthesis in *E. rugulosa* NRRL11440 in the absence and presence of curcumin was assessed by HPLC analysis. Samples for HPLC analysis were performed as per the method described earlier with minor modifications [5]. The methanol-extracted harvested sample of *E. rugulosa* NRRL11440 was filtered with a 0.22 μm filter. A 25 μL sample was injected into the analytical HPLC system (Waters, Milford, MA, USA) to run using an RP-C18 column (4.5 × 250 mm). The standard of echinocandin B (Santa Cruz Biotechnology, Dallas, TX, USA: SC-362020) was run and monitored at 222 nm. The echinocandin B concentration was calculated by peak(s) area and statistical analysis was applied using GraphPad Prism v5.01 (GraphPad Software, Inc., Boston, MA, USA).

4.4. Confrontation Assay

The *E. rugulosa* spores grown in 50 mL GMM-Arg medium were used for comparative analysis of echinocandin production with and without treatment of curcumin as per cell susceptibility methods described earlier [33–37]. The production of echinocandin B in the absence or presence of curcumin histone modifier was tested by confrontation assay

(susceptibility) against the *C. albicans* DSY 204 strain. Briefly, a confrontation assay was done by placing the *E. rugulosa* mycelial mats on pre-inoculated *C. albicans* DSY 294 cells in the YEPD agar plate. The cell mat was recovered after 10 days of culture and washed with autoclaved water and cut into small pieces with a sterile blade. These small pieces were put on pre-inoculated *C. albicans* DSY 294 cells in YEPD agar plates and incubated at 30 °C for 24 h. The zone of inhibition formed by *E. rugulosa* NRRL11440 mat was measured.

4.5. RNA Isolation

For RNA isolation, a 100 mg fungal mycelial mat was ground up to a fine powder with liquid nitrogen. The RNA extraction was performed using TRIzol® (Life Technologies, Carlsbad, CA, USA). The ground tissue was homogenized with 1 mL Trizol buffer and incubated at room temperature for 5 min. 0.2 mL of chloroform was added to the sample and shaken vigorously for 15 s and incubated further at room temperature for 5 min. The sample was centrifuged at 12,000 rpm for 15 min at 4 °C. The top aqueous phase was taken out carefully and 0.5 mL of isopropanol was added and allowed to precipitate at room temperature for 1 h. The sample was centrifuged at 12,000 rpm for 15 min at 25 °C [38–42]. The precipitant pellet was washed twice with 1 mL of 75% ethanol at 12,000 rpm for 5 min. The concentration and purity were measured on a Nanodrop (Thermo Scientific, Waltham, MA, USA). RNA integrity was analyzed on 0.8% formaldehyde agarose gel prepared in MOPS buffer.

4.6. cDNA Synthesis

The cDNA was synthesized using 1 µg RNA from each sample which was reverse transcribed using ProtoScript II Reverse Transcriptase (NEB, Ipswich, MA, USA). Firstly, the RNA sample was mixed with 2 µL of oligo dT (50 µM) and 1 µL dNTPs (10 mM) and the volume was adjusted to 12 µL with nuclease-free water. This mix was heated at 65 °C for 5 min. It was spun briefly and placed promptly on ice. Then 4 µL of 5X ProtoScript II RT Reaction buffer, 2 µL DTT (0.1 M), 1 µL murine RNAse inhibitor (40 U/µL), and 1 µL ProtoScript II Reverse Transcriptase (200 U/ul). This was incubated at 42 °C for 1 h. The enzyme was inactivated at 80 °C for 5 min [5].

4.7. Transcription Gene Expression Profiling

To analyze the relative gene expression of the echinocandin genes RT-PCR was performed using 1.5 µL cDNA reaction mixtures in 25 µL PCR reaction mixture using gene-specific primers. The PCR was set up in a thermal cycler (Multiplex PCR, Applied Biosystems) with amplification for 35 cycles [5]. PCR amplification from different cDNA samples was used to determine the relative expression of the genes of the echinocandin B biosynthetic gene cluster in the presence of curcumin, a HAT inhibitor. Quickly, Genes *ecdA*, *ecdB*, *ecdL*, *ecdK*, and actin (internal control) were amplified from cDNA prepared from different samples. The 10 µL PCR products were run on 1% agarose gel and the image was captured in ChemiDoc Gel Imaging System (BioRad, Hercules, CA, USA). The relative gene expression was measured by band intensity which was evaluated using Image Lab v4.1 (BioRad, Hercules, CA, USA).

4.8. E. rugulosa and C. albicans Interspecies Interactions

To establish an interspecies consortium, the three days aged spores of *E. rugulosa* were harvested by drenching them with 0.1% Tween 20 (5 mL), and 10^6 spores/mL spores were inoculated in GMM-Arg (Glucose minimal medium-10 mM Arginine) medium [5]. Further in this, 200 µM Curcumin has supplemented at the time of spores inoculum. The cell culture was grown at 30 °C initially for 8 days in static conditions. On the 9th day, 1×10^4 *C. albicans* cells were cultured with the existing culture and further grew for the next 2 days. The harvested culture mat was washed well (three times) with distilled water and further used for RNA isolation for gene expression analysis.

4.9. Crude Membrane Preparation

To prepare the crude membrane, *E. rugulosa* (10^6 spores/mL) spores were grown in GMM-Arg medium (50 mL) with and without treatment of different curcumin concentrations for 10 days, at 30 °C under static conditions. This assay was performed according to the method described earlier with some modifications [34]. The harvested culture mat was washed with PMSF (100 µM) containing distilled water. The cell mat was transferred into the prechilled mortar and homogenization buffer (10 mM Tris-Cl pH 7.5, 0.5 mM EDTA, 1 µg/mL TPCK, TLCK, and 100 µM PMSF) was added to crush the cell mat with pestle using liquid nitrogen. In order to remove cell debris, it was centrifuged at 2500 rpm for 5 min, 4 °C. The supernatants were then centrifuged for 60 min at 25,000 rpm, 4 °C to recover the pellet to dissolve in 3 mL, resuspension buffer (10 mM Tris-Cl pH 7.5, 150 mM NaCl, 10% Glycerol, 1 µg/mL TPCK, TLCK and 100 µM PMSF). The resuspension samples were quantified and aliquoted (100 µL) in tubes for further use.

4.10. Immunodetection of Histone Acetylation

The Western blot analysis was carried out using an anti-acetyl H3 antibody (1:5000 dilution) as described in the previous studies [34].

4.11. Statistical Analyses

Statistical analyses were assessed using GraphPad prism v5.01 (Graph Pad Software, La Jolla, CA, USA). p values were calculated via Student t-test and ANOVA test. $p < 0.05$ was significant.

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