



Functional redundancy in Echinocandin B in-cluster transcription factor *ecdB* of *Emericella rugulosa* NRRL 11440

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ABSTRACT

Echinocandin B is a potent antifungal against the majority of fungal pathogens and its biosynthesis occurred by *ecd* and *hty* gene clusters in *Emericella rugulosa* NRRL 11440. We elucidated the functional necessity of in-clustered transcription factor; *ecdB* in the production of echinocandin B. We deleted the *ecdB* gene and found that Δ *ecdB* mutant has no significant effect on echinocandin B production. The expression level of most of the *ecd* and *hty* cluster genes was not significantly altered except few of them up-regulated in knockout strain. The complete abrogation in *ecdB* gene expression was observed in Δ *ecdB* strain. However, the interactions of purified EcdB protein with DNA sequence of *ecda*, *ecdH*, *ecdK* and *ecdI* promoter was confirmed *in-vitro*. Our results conclude that EcdB protein *in-vitro* binds to the *ecda*, *ecdH*, *ecdK* and *ecdI* promoter but *in-vivo*, it could not significantly affect the gene expression and echinocandin B production in *Emericella rugulosa*.

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1. Introduction

Fungal infections cause a life-threatening problem with higher morbidity and mortality that accounts for an over billion people globally. More than 11 million people were affected with serious fungal infections with 1.5 million deaths annually [1]. *Candida* spp. majorly contribute to such invasive and superficial infections in immunocompromised and debilitated patients that undergone to the intensive care unit (ICU) treatments with a 40% mortality [2]. To overcome the disease burden, different classes of antifungal drugs such as azoles, polyenes, pyrimidines, allylamines, morpholines, and echinocandins are used for the treatment of fungal infections [3]. Long course of medication, indiscriminate and unregulated usage of antifungals and the problem of drug

resistance have emerged in most of the fungal pathogens [4]. Majority of these therapeutic drugs are being ineffective for the treatment of fungal infections [4]. Echinocandins class of antifungals still gain an attention due to the occurrence of rare resistance, hence it used as a frontline protection to cure such fungal infections [4,5]. In spite of lower dosage requirements, echinocandins also have less toxicity to host, favorable metabolic kinetics, and quick fungicidal effect against *Candida* spp. [6]. Different forms of echinocandins are now known that capable to synthesize either naturally or semi-synthetically from different strains [7–9]. Echinocandins, a potent first line antifungal is accidentally discovered in the 1970s by the Ciba-Geigy from *Aspergillus nidulans* var. *Echinulatus* [10] that also produced by *Aspergillus nidulans* var. *roseus* NRRL 11440 [11]. A variety of echinocandins such pneumocandin B₀, mulundocandin, aculeacin, FR190293, FR901379 (WF11899 A), and FR209602 also produced from different fungal isolates reported earlier [12–18]. However, in apparent time, various semi-synthetic derivatives of echinocandins were also developed; anidulafungin [8], caspofungin [9], and micafungin [19] are such examples.

Echinocandin B (ECB) is a cyclic hexapeptide linked with a linoleic acid, that acts on 1,3- β -glucan synthase, leading to inhibition of cell wall synthesis [20]. All the amino acid residues of the ECB get hydroxylated during its synthesis [21]. The genes involved in the biosynthesis of ECB have been elucidated as *ecd* and

Abbreviation: Arg, Arginine; bp, base pair; ECB, echinocandin B; CTAB, cetyl trimethylammonium bromide; d, day; GMM, glucose minimal medium; HPLC, high performance liquid chromatography; h, hour; ICU, intensive care unit; kb, kilo base pair; MEGA, molecular evolutionary genetics analysis; MP, maximum parsimony; ORF, open reading frame; PCR, polymerase chain reaction; RT-PCR, reverse transcription polymerase chain reaction; SPR, subtree-pruning-regrafting; TF, transcription factor; UTR, un-translated region; WT, wild type; YG, yeast glucose medium.

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hty clusters in *Emericella rugulosa* NRRL 11440 [21]. The *ecd* cluster has role in its biosynthesis and maturation which comprised with 12 genes (*ecdA* to *ecdL*) among these *ecdA* encoded for a non-ribosomal peptide synthetase (NRPS), *ecdB* for transcription factor, *ecdL*; an ABC transporter, *ecdI*; an acyl-AMP ligase and oxygenases (*ecdG*, *ecdH*, and *ecdK*) [21]. Another, *hty* gene cluster has six genes (*htyA* to *htyF*) which collectively synthesize non-proteinogenic amino acid, L-homotyrosine [21]. This building block amino acid is synthesized by acetyl-CoA and 4-hydroxyphenyl-pyruvate as a precursor molecule [21]. The group of investigators revealed the function and action mechanism of encoded products of *ecdA*, *ecdI*, *htyA* [21], *ecdK*, *ecdG* and *ecdH* genes [22].

The synthesis of secondary metabolites are controlled by a range of regulatory factors but majorly regulated by two ways either by in-clustered transcription factor (TF) or global regulators which trigger in response to environmental nutrient sources (carbon, nitrogen, amino acids) and other physical parameters such as temperature, light, and pH etc. [23]. Similar to pneumocandin B₀ biosynthetic gene cluster of *Glarea lozoyensis*, ECB also contains a single in-clustered TF factor [12,21]. Functions of such in-cluster transcription factors (TFs) and other factors that regulate their biosynthesis have not been characterized so far in echinocandins biosynthesis. For the first time in this manuscript, we targeted our study to elucidate the regulatory behaviour of in-cluster TF *ecdB* on ECB production in *E. rugulosa* NRRL 11440. We found that *ecdB* gene showed functional redundancy in the ECB production which has a very distant relationship with pneumocandin B₀ in-cluster TF.

2. Materials and methods

2.1. Strains and media

The echinocandin B (ECB) producing strain *Emericella rugulosa* NRRL 11440 was purchased from Fungal Genetics Stock Centre (FGSC), Kansas City, USA [21], formally known as *A. nidulans* var. *roseus* ATCC 58397 [24]. The culture of *E. rugulosa* and *Candida albicans* were maintained at 30 °C in YEPD medium (HiMedia). The minimal medium (GMM) used for ECB production opted from the Cramer lab protocol with slight modification; Sodium nitrate (NaNO₃) replaced with 10 mM Arginine (Arg) in our study and named as Arg medium. The spores were produced in YG medium (2% Dextrose, 1% Yeast extract supplemented with 40 µl trace elements) [25]. Cell susceptibility analysis of ECB was performed against *C. albicans* DSY 294 strain.

2.2. Echinocandin B production and measurement

The 2×10^6 *E. rugulosa* spores were grown in 50 ml Arg medium for 10 d. Only Arg medium was used for comparative ECB production and transcriptional studied of *ecdB* knockout background. ECB production was initially tested by confrontation assay (susceptibility) against *C. albicans* followed its detection by HPLC. Briefly, confrontation assay was done by placing filter disc containing extract on pre-inoculated *C. albicans* cells in YEPD agar plates. Zone of inhibition formed by *E. rugulosa* extract against *C. albicans* was measured. Samples for HPLC analysis were performed as method opted by Hu et al., with minor modifications [26]. The harvested culture was lyophilized followed by methanol extraction at 30 °C for overnight. The filtered sample was run on analytical HPLC system (Waters) using an RP-C18 column (4.5 × 250 mm, I.D. WAT054375) using 20 µl sample injection. The standard of ECB (Santa Cruz Biotechnology: SC-362020) was run and monitored at 222 nm. The ECB concentration was calculated by peak(s) area and statistical analysis was applied using GraphPad Prism v5.01 (GraphPad Software, Inc.).

2.3. mRNA isolation

The mRNA was isolated using Magnetight Oligo(dT) particles (Merck) for transcriptional studies. The *E. rugulosa* culture was grown on Arg medium for 5 d. The total RNA (aqueous phase) was isolated by using 100 mg tissue with the TRIzol reagent as per the manufacturer instructions (Invitrogen). The aqueous phase, containing the RNA was mixed with equal volume of binding buffer (100 mM Tris-Cl, pH 7.5; 150 mM NaCl; 10 mM EDTA, pH 8.0) and applied on to pre-equilibrated Magnetight Oligo(dT) particles (0.25 mg) incubated for 10 min then particles were captured by the magnet. The bounded mRNA was washed (100 mM Tris-Cl, pH 7.5; 150 mM NaCl; 10 mM EDTA, pH 8.0) and recovered by elution buffer (100 mM Tris-Cl, pH 7.5; 25 mM EDTA, pH 8.0) at 60 °C temperature and quantified by Nano-drop Spectrophotometer and stored at -80 °C for further use.

2.4. cDNA preparation and transcriptional studies

The cDNA was prepared by using 200 ng of mRNA sample with M-MLV reverse transcriptase enzyme (Invitrogen) according to manufacturer's instructions. For transcriptional studies, specific primers were designed from the exonic regions of all 18 genes of the *ecd* and *hty* gene cluster along with β-actin as an experimental control (Table IS). The transcriptional study was conducted by semi-quantitative RT-PCR using 1.5 µl cDNA reaction mixtures in 20 µl PCR reaction along with all necessary ingredients. The PCR was set up using a thermal cycler (Multiplex PCR, Applied Biosystems) with amplification for 42 cycles and 10 µl PCR products was subjected to the agarose gel electrophoresis and the image was captured in ChemiDoc Gel Imaging System (Bio-Rad). The relative gene expression was measured by band intensity which was evaluated using Image Lab v4.1 (Bio-Rad) and statistical analysis was applied by GraphPad Prism v5.01 (GraphPad Software, Inc.).

2.5. Genomic DNA isolation

The *E. rugulosa*, *ecdB* knockout colonies were screened by PCR analysis using genomic DNA as a template for PCR reaction. The genomic DNA was isolated by the CTAB method as described earlier [27]. The concentration of genomic DNA was measured by Nano-drop Spectrophotometer.

2.6. Disruption of *ecdB* gene (Δ *ecdB*)

To prepare the *ecdB* deletion mutant, pCSN44 vector was selected, it contains hygromycin phosphotransferase selection marker gene (*hph*) against hygromycin B. The disruption cassette was prepared by cloning of 1 kb both upstream (*ecdB* promoter flanked with 77 bp of *ecdB*-ORF) and downstream (3'UTR flanked with 273 bp of *ecdB*-ORF) DNA sequence of *ecdB* gene in the pCSN44 vector (Fig. 2) using list of primers as mentioned elsewhere (Table IIS). These fragments were cloned in such a way the *hph* gene was sandwiched between both fragments in a pCSN44 vector as described elsewhere [28]. The linearized deletion cassette (digested with *KpnI*) was transformed in *E. rugulosa* NRRL 11440 protoplast and plated under the hygromycin B selection for 48–72 h, as described elsewhere [25]. The protoplast was prepared using method reported by Szewczyk et al., with slight modifications, we used an enzyme NS35072 (Gifted from Novozymes) for cell wall digestion [25]. The colonies appeared under hygromycin B selection were sub-cultured to get a pure line for further studies. These colonies were screened by PCR using *ecdB*-ORF specific primers (Table IIIS) followed by *EcoRI* digestion (an *EcoRI* site present in *hph* gene). The specific *ecdB* knockout

(Δ ecdB) clones were further confirmed by Southern blot analysis by using listed primers (Table IIIS).

2.7. Expression of GST::EcdB protein and DNA protein interaction

The truncated 162 bp *ecdB* ORF was cloned in pGEX-2TK vector at *Bam*HI/*Eco*RI sites, briefly described in our personal communication. The cloned product was expressed in *E. coli* -BL21(DE3)pLysS expression host and GST-fused EcdB₁₋₅₄ (GST::EcdB) protein purification was performed using Glutathione Sepharose beads (Sigma) as described by manufacturer instructions. The interaction of GST::EcdB protein with *ecdB* promoter was checked by non-labelled Electrophoretic mobility assay (EMSA) using varying concentration of GST::EcdB protein (500, 750, 1000, and 1500 ng) and a fixed 100 ng concentration of *ecdB* promoter DNA (1 kb). We also tested interactions of EcdB protein with some other important genes of ECB biosynthetic gene cluster such as *ecdB*, *ecdC*, *ecdH*, *ecdK*, *ecdI*, *htyA* and with T7 promoter (pTZ57 r/t) used as a negative control. Binding of protein with the promoter was carried out in binding buffer (50 mM Tris-HCl, pH 7.4; 50 mM NaCl; 1 mM DTT; 5 mM MgCl₂; 6% Glycerol). The reaction mixture was incubated 30 min at 4 °C and the DNA-protein complex was resolved on 1% agarose gel at 120 V for 3 h at 4 °C using 0.5 X TBE as reported earlier [29]. The DNA control was kept in order to compare the shift of the complex so formed, the BSA and purified GST protein was also taken as negative control. The gel was visualized through ChemiDoc imaging system after ethidium bromide (0.5–1 µg/mL) staining.

2.8. Phylogenetic tree construction

The amino acid sequences of various fungal transcription factors (TFs) were retrieved from Aspdg (<http://www.aspdg.org>), NCBI (<https://www.ncbi.nlm.nih.gov>) and UniProt (<http://www.uniprot.org/>) databases. The sequences were randomly selected from known in-clustered TFs of secondary metabolite (SM)

biosynthetic gene cluster of various fungi. We also took a TF (GLAREA10050) of *Glarea lozoyensis* ATCC 20868, present within pneumocandin B₀ biosynthetic gene cluster. The phylogenetic tree was constructed by multiple sequence alignment of all protein sequences performed using Molecular Evolutionary Genetics Analysis version 7.0 (MEGA7) software [30]. The evolutionary history was inferred using the Maximum Parsimony (MP) method with 1000 bootstrap test value. This phylogenetic analysis was done using EcdB TFs amino acid sequences of *E. rugulosa* NRRL 11440 as a reference. All positions containing gaps and missing data were eliminated. There were a total of 267 positions in the final dataset.

3. Results

3.1. Phylogenetic relation of *ecdB* transcription factor (TF)

In order to reveal the relationships of EcdB protein with other in-clustered TF which involved in fungal secondary metabolites synthesis including pneumocandin (belongs to echinocandin group). The phylogenetic tree of protein sequences was constructed using Molecular Evolutionary Genetics Analysis version 7.0 (MEGA7) software [30]. The most parsimonious tree with length was 4313 with 0.647428, 0.311677 and 0.202196 of consistency index, retention index and composite index respectively, for all sites and parsimony. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches [31]. The MP tree was obtained using the Subtree-Pruning-Regrafting (SPR) algorithm [32] with search level 1 in which the initial trees were obtained by the random addition of sequences (10 replicates). The phylogenetic analysis of *ecdB* gene present within the gene cluster of ECB of *E. rugulosa* NRRL 11440 is close proximity to ApdR of *A. nidulans* FGSC A4, which acts as a local regulator of the aspyridone biosynthetic gene cluster (Fig. 1). Furthermore, the EcdB TF also

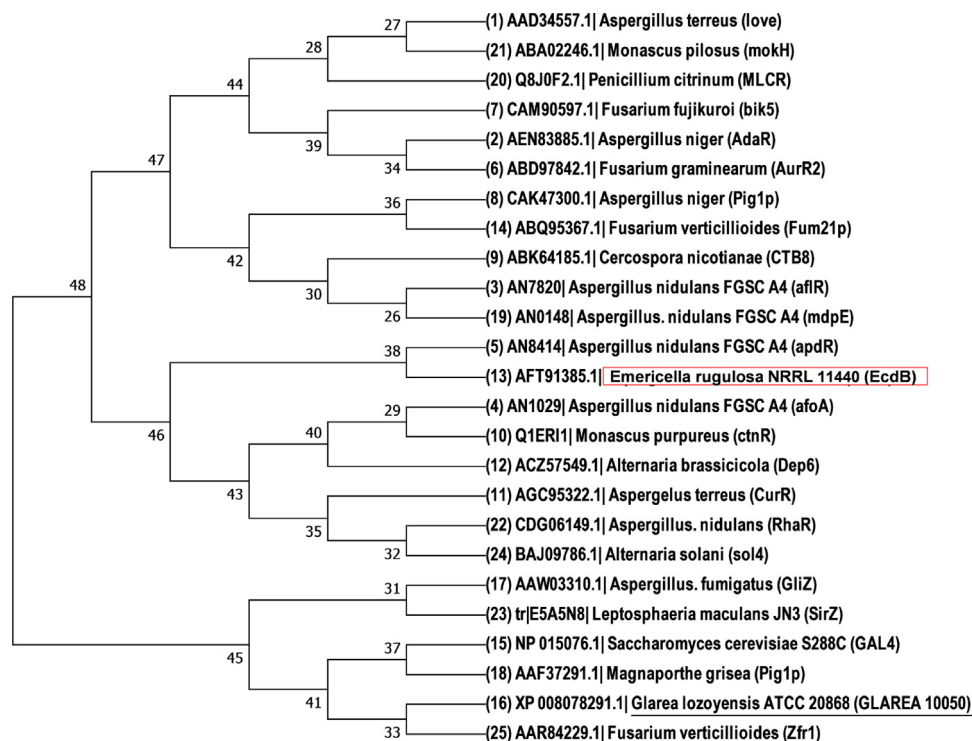


Fig. 1. Phylogenetic tree of EcdB transcription factor. The evolutionary history of EcdB (highlighted in square) transcription factor was evaluated along with 25 other fungal secondary metabolites regulatory transcription factors, including Pneumocandin B₀ (member of echinocandin family) in-cluster TF GLAREA 100050 (underlined). The tree was constructed using Maximum Parsimony method in MEGA7 with 1000 bootstrap value.

showed similarity with other in-cluster TF of fungal secondary metabolite such as AfoA, CtnR, Dep6, CurR, RhaR and Sol4 which regulate asperfuranone, citrinin, depudecin, dehydrocurvularin, L-rhamnose, and solanopyrone biosynthetic gene cluster respectively. The TF, GLAREA 100050 of *Glarea lozoyensis* (in-cluster TF of pneumocandin B₀ biosynthetic gene cluster) was present in another clade (distant) and are closer to Fum21 of *Fusarium verticillioides* ATCC 20868 (Fig. 1).

3.2. Disruption of *ecdB* in *E. rugulosa*

The TF of pneumocandin biosynthetic gene cluster shows a distant sequence similarity with the *ecdB* sequence which appeals to further characterize the regulatory behaviour of the *ecdB*. We targeted to disrupt the *ecdB* gene by preparing a disruption vector. The disruption vector was constructed by introducing 1 kb promoter and 3'UTR region of *ecdB* flanked with *ecdB*-ORF on both sides of the *hph* gene as described in materials and methods (Fig. 2). This disruption vector was termed as pPBU which was linearized and transformed in the host strain *E. rugulosa* NRRL 11440 under hygromycin selection. Approximately, 30 distinct colonies appeared after 2–3 d of incubation. These transformants were first screened by PCR analysis followed by restriction digestion using *EcoRI* enzyme (present in *hph* gene).

A PCR product with the expected size of 1.9 kb was obtained using *ecdB*-ORF primers (Table IIIS). Upon digestion of PCR product with the *EcoRI*, obtained two distinct DNA fragments with complete digestion of expected molecular size of 850 bp and 1050 bp whereas a partial digestion was observed when disruption cassette was non-specifically integrated (ectopically) in the host genome (Fig. 2). No such partial or complete fragments were obtained in case of the PCR product of wild type (WT) DNA digested with the same enzyme. Out of 30, 4 transformed colonies (13%) were found to be positive with successful disruption of *ecdB* gene and rest of them were ectopically integrated. These knockout

strains were termed as $\Delta ecdB111$, $\Delta ecdB112$, $\Delta ecdB114$, $\Delta ecdB117$ whereas $\Delta ecdB111$ was selected to use in this study. The positive clones were further confirmed by Southern blotting using *KpnI* restriction digestion. A single 7.5 kb band appeared in *ecdB* knockout strains whereas WT strain fails to produce such band (Fig. 2). These results clearly demonstrate that *ecdB* gene was specifically disrupted under the *hph* selectable gene.

3.3. ECB production level in *ecdB* knockout strain

To confirm the role of *ecdB* TF in the ECB production, the positive clone $\Delta ecdB111$ was grown in Arg medium along with WT for 10 d; extraction was performed as mentioned in the methods and tested for ECB susceptibility against *C. albicans*. The sensitivity profile of the *ecdB* knockout was found to be similar to WT and no significant difference was observed in the zone of inhibition (Fig. 3). Further to test the accurate ECB production, quantitative analysis was performed using analytical HPLC. The ECB standard was used as a reference which was detected at 16.75 min of retention time at 222 nm. Peak with similar retention time was also observed in case of WT and knockout strains. There is no significant difference in the ECB production level was observed in both WT and knockout strain with $p > 0.05$ in a paired *t*-test (Fig. 3). Our results suggest that *ecdB*, a TF located in-cluster of ECB biosynthetic genes not directly involved in the biosynthetic regulation of ECB production.

3.4. Transcriptional behaviour of ECB biosynthetic gene cluster

To analyze the transcriptional response of different genes present in both *ecd* cluster (*ecdA-ecdL*) and *hty* cluster (*htyA-htyF*) of ECB the *E. rugulosa* culture was grown on Arg medium for 5 d and isolate the mRNA pool. For transcriptional studies, semi-quantitative RT-PCR analysis of WT and the *ecdB* knockout strain was performed using the different set of gene primers (Table IS). Our results imply that all

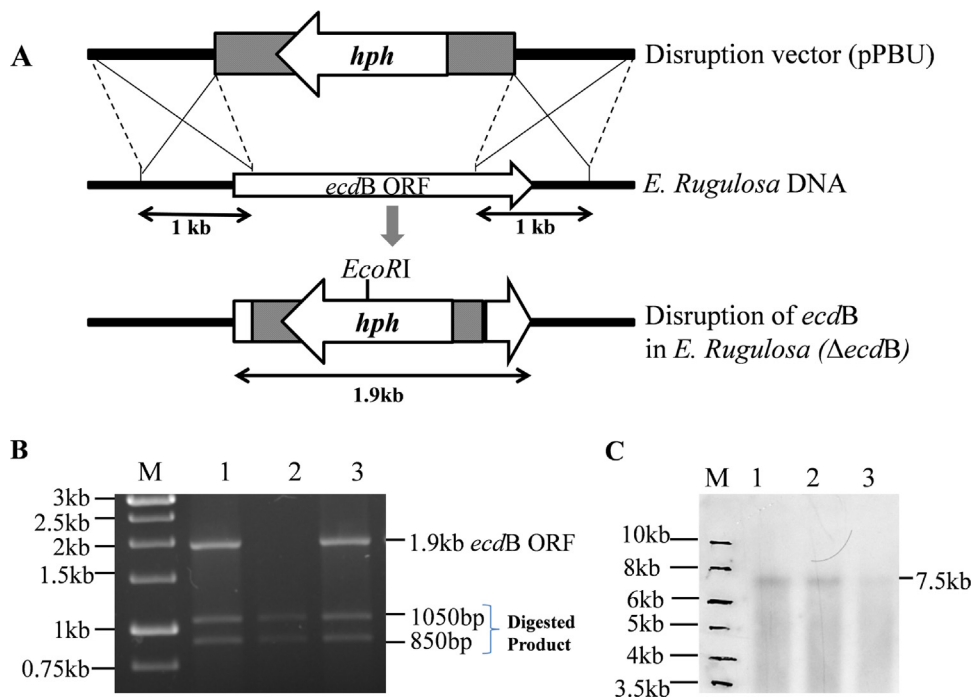


Fig. 2. Disruption of the *ecdB* gene in *E. rugulosa*. (a) Block diagram representation of *ecdB* gene replacement with hygromycin phosphotransferase (*hph*) gene via homologous recombination. The dotted line depicts the flanking regions used for homologous recombination. (b) Confirmation of *ecdB* knockout mutant by *EcoRI* digestion (*hph* gene contain an *EcoRI* restriction site) of *ecdB* PCR product (1.9 kb). Lane M: 1 kb DNA ladder, Lane 1 and 3: partial digestion occurred with *EcoRI* that depicted the ectopic clones. Lane 2: The expected size 1050 bp and 850 bp DNA fragments appeared on complete digestion. (c) Southern analysis of *ecdB* knockout clones. Lane 1, 2 and 3 showed $\Delta ecdB111$, $\Delta ecdB112$ and $\Delta ecdB114$ knockout clones respectively.

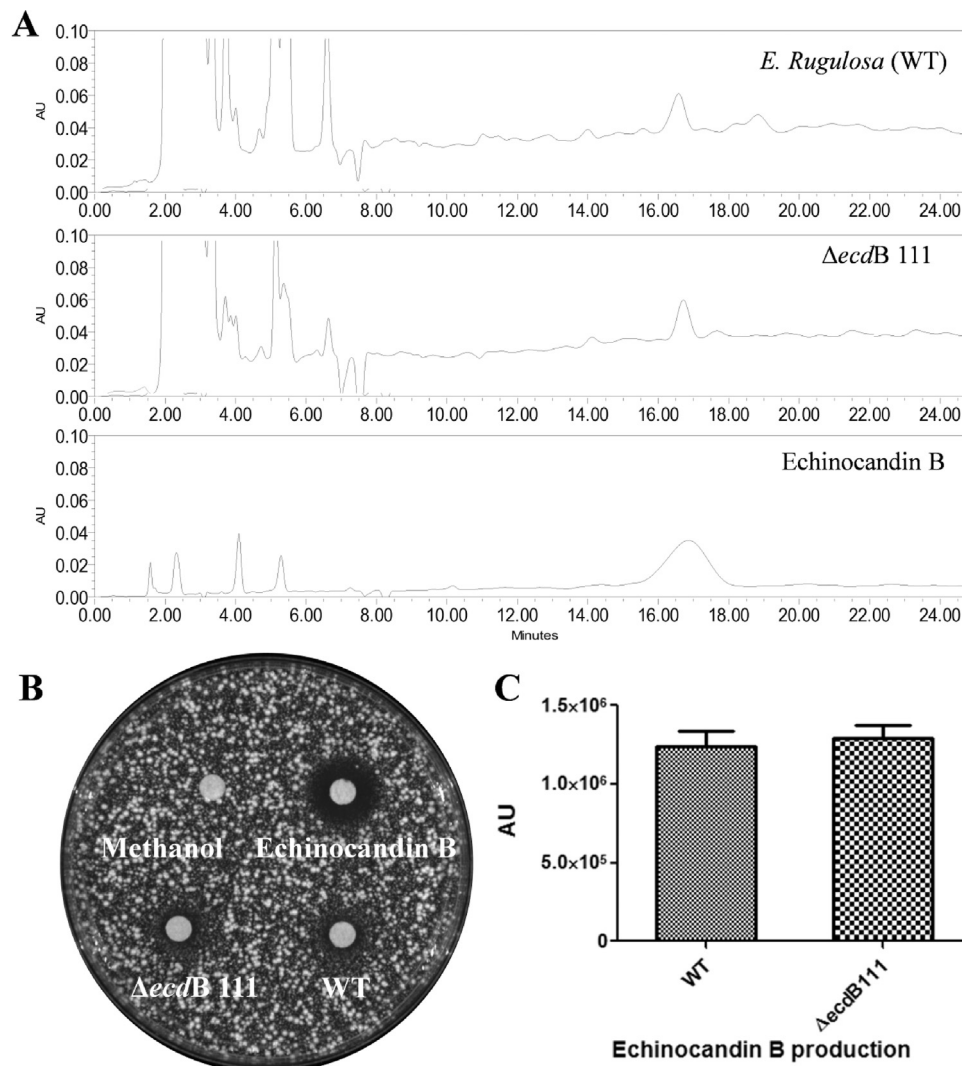


Fig. 3. Quantitative estimation of echinocandins B production in *ecdB* knockout strain. (a) Chromatogram of methanol extracts of both wild-type (WT) and a Δ *ecdB*111 mutant of *E. rugulosa* grown on Arg medium at 30 °C for 10 d. The samples were run using RP-C18 column, echinocandins B standard was taken as a reference which was detected in 222 nm at 16.75 min. (b) Antifungal activity of methanol extracts of WT and Δ *ecdB*111 strain of *E. rugulosa* along with echinocandins B standard (1 mg/ml) and absolute Methanol (M) were used as positive and negative controls, respectively. (c) Comparative analysis of echinocandins B production based on peak area calculation in arbitrary unit (AU) of three independent replicates.

genes of the cluster spanning the region from *ecdA-ecdL* and *htyA-htyF* (GeneBank accession no. JX421684 and JX421685) were transcribed with varying expression level. The transcriptional expression of *ecdA*, *ecdG*, *ecdI*, *ecdK*, *htyA*, *htyB*, *htyC* and *htyD* genes was found to be abundantly expressed, *ecdC*, *ecdH*, *ecdJ*, *htyE* and *htyF* are moderately expressed whereas low expression was found in case of *ecdB*, *ecdD*, *ecdE*, *ecdF* and *ecdL* genes in WT strain (Fig. 4). The higher and moderately expressing genes majorly belong to oxygenases (*ecdG*, *ecdH*, *ecdK* and *htyE* except *htyF*), ligase (*ecdI*) and aconitase (*htyD*) whereas low expressing genes are majorly encoded for transporters (*ecdC*, *ecdD* and *ecdL*), glycosyl hydrolase (*ecdE*) and glycosidase (*ecdF*) proteins (Fig. 4).

Gene deletion analysis revealed that *ecdB* is dispensable for ECB production in *E. rugulosa* because *ecdB* deletion has not altered the ECB production. To further test the effect of deletion of an *ecdB* gene on the expression pattern of other genes of the ECB genes clusters, a comparative expression profiling of *ecdB* knockout versus WT was performed. We noticed that expression level of an *ecdB* gene was found to be completely lost in case of *ecdB* knockout strain. In contrast, such gene deletion did not significantly ($p > 0.05$) affect the expression of other genes present in gene

clusters including *ecdA* (NRPS) whereas few genes are significantly up-regulated *ecdC*, *ecdF*, *htyA*, *htyB* and *htyC* at $p < 0.05$ by applying paired *t*-test (Fig. 4). These results suggest that deletion of *ecdB* TF has no direct effect at transcription level for regulation of ECB production.

3.5. Interaction of EcdB protein with the *ecdA* promoter

To further evaluate the role of *ecdB* TF in the ECB biosynthesis, we purified GST fused DNA binding domain (GST::*EcdB*) using bacterial expression system. A single band with 33 kDa of GST::*EcdB* protein (Fig. 5b) was obtained which facilitates us to explore the interaction of GST::*EcdB* protein with the DNA sequence of promoter region of NRPS encoded *ecdA* gene, responsible for ECB production. The EMSA study revealed a successful interaction of GST::*EcdB* protein with the promoter region of *ecdA* as observed by shifting in the DNA mobility at subsequent increasing protein concentration. The highest interaction was observed at 1000–1500 ng protein with 100 ng *ecdA* promoter DNA (Fig. 5c & d). Moreover, *EcdB* interaction was also obtained with the promoter sequences of *ecdH*, *ecdI* and *ecdK* genes (Fig. 5d). In contrast, no

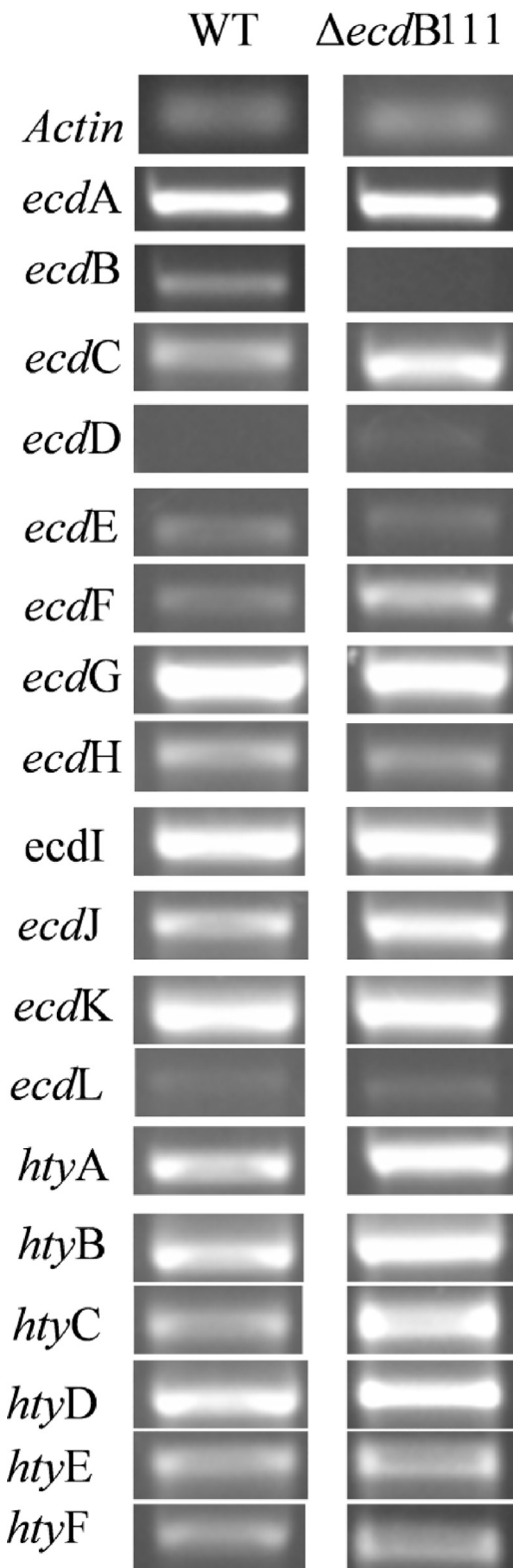


Fig. 4. Transcriptional response of echinocandin B biosynthetic genes in *ecdB* deletion. The expression level of different *ecd* and *hty* genes of echinocandin B biosynthetic gene cluster were tested in *ecdB* knockout strain (Δ *ecdB111*) of *E. rugulosa*. The culture was grown on Arg medium for 5 d at 30 °C, shaking at 180 rpm. Results were obtained from three independent experiments.

such interaction was found with the *ecdB*, *ecdC* and *htyA* promoter sequence (Fig. 5d). These interactions were found to be specific as no DNA mobility shift were observed in GST and BSA (Fig. 5c) whereas EcdB also not interact with T7 promoter (Fig. 5d) used as a negative control for DNA-protein interaction. These results indicate that the *ecdB* TF present in the cluster of ECB biosynthetic gene interacts with NRPS for regulation and synthesis of ECB production.

4. Discussion

It is well known that fungal secondary metabolite biosynthetic genes are present in the clustered form which generally coordinately regulated by a TF present within the gene cluster [18,23,33–35]. Such in-cluster TF leads to transcribe other genes of the cluster that required for complete synthesis of secondary metabolite [23]. The ECB synthesis occurred by two gene clusters termed as *ecd* and *hty* in *E. rugulosa* NRRL 11440 [21]. These *ecd* and *hty* gene cluster are mainly biosynthesize ECB and L-homotyrosin, respectively which is supposed to regulate by its own in-cluster TF *ecdB*. Therefore we first conducted the phylogenetic analysis, phylogenetic tree revealed that EcdB TF protein of *E. rugulosa* NRRL 11440 was found closer to the ApdR TF of *A. nidulans* FGSC A4 (Fig. 1) that has the regulatory role in the aspyridone production [36]. Based on these facts, we assume that both TF genes have been evolved from a common gene ancestor. In contrast, pneumocandin in-cluster TF, GLAREA 100050 of *Glarea lozoyensis* (both are in-cluster TF of echinocandins family) were found to be distantly related to EcdB which suggests that these genes were not originated from the same ancestor. The distant relationship between EcdB and GLAREA 100050 TF showed an interest to characterize the EcdB TF. Therefore we disrupted more than 80% of the *ecdB* gene including >50% DNA binding domain sequence which disrupts the functional architecture of protein and diminishes its probability of interaction with DNA molecule (Fig. 2a). The similar strategy was also applied by Proctor et al., for disruption of fumonisin biosynthetic genes *fum17* and *fum18* in maize pathogen *Gibberella moniliformis* by replacing over 75% of the coding regions of both genes by *HygB* [37]. The *ecdB* knockout strain of *E. rugulosa* (Δ *ecdB*) still produced ECB, as resulted by confrontation assay and HPLC chromatogram (Fig. 3). Opposite to our expectations, Δ *ecdB* strain did not show any significant difference in ECB production as compared to WT (Fig. 3). In response to ECB production results in *ecdB* knockout strain, the transcription of ECB biosynthetic gene cluster was also analysed. No significant difference at expression level was found in *ecdA* (NRPS) and most of the ECB biosynthetic genes whereas *ecdC*, *ecdF*, *htyA*, *htyB* and *htyC* genes were up-regulated it may be due to ambiguities because it does not affect the ECB production. In contrast, a complete loss of *ecdB* gene expression was found in *ecdB* knockout strain (Fig. 4). Similar cases also reported earlier, deletion of some in-cluster TFs such as *hypG*, *ctfR1*, and *cpaR* of *A. flavus*, *A. flavus*, and *A. oryzae* respectively, also did not alter the cyclopiazonic acid (CPA) production as well as transcription level [38–40]. Remarkably, a global regulator, *LaeA* has a regulatory role in CPA production as well as their biosynthetic gene regulation both in *A. flavus* and *A. oryzae* [39,40]. Proctor et al., commented that disruption of regulatory gene *znf1* of fumonisin gene cluster of *Fusarium graminearum* exhibited fumonisin production similar to WT [41]. To further validate the role of this transcription factor in interaction with the promoter sequence, EMSA study of EcdB protein with the promoter region of *ecdA* gene was performed. Interestingly, GST::EcdB protein specifically interacts with *ecdA*, *ecdH*, *ecdI* and *ecdK* promoter revealed by shifting DNA mobility whereas such interaction was not observed in case of *ecdB*, *ecdC*, *htyA* and T7 promoter (Fig. 5c & d). Similar interaction

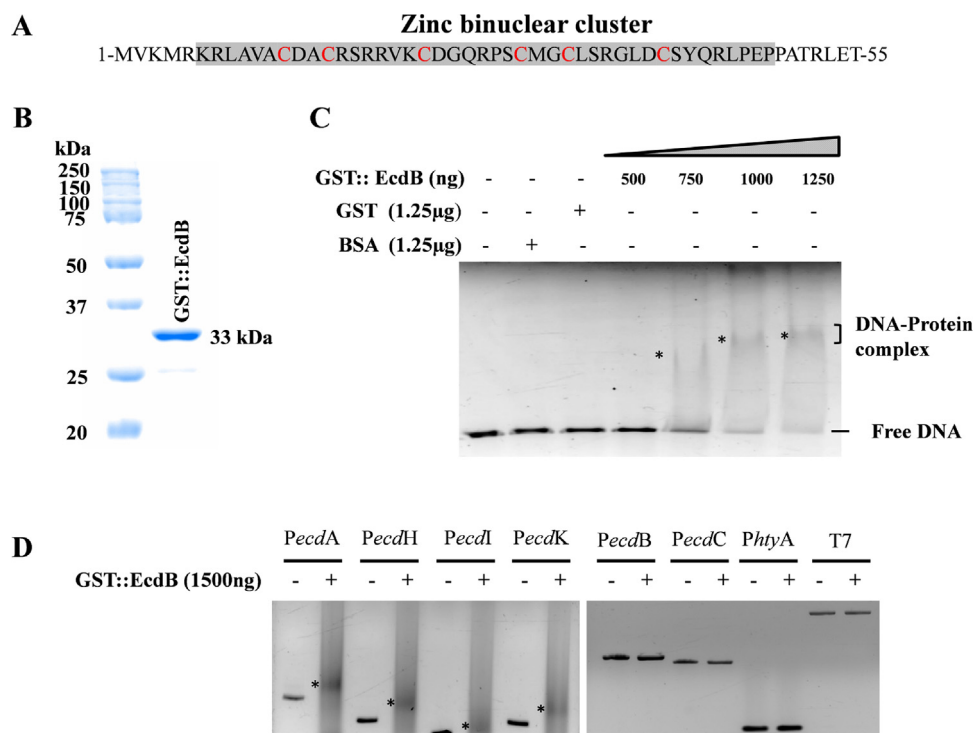


Fig. 5. DNA-Protein interaction of EcdB protein with *ecdA* and other promoters. (a) Sequence analysis of DNA binding domain (highlighted) of EcdB protein determined by *in-silico* analysis, Zn(II)2cys6 residues (highlighted by red). (b) Purified GST tagged EcdB protein (GST::EcdB) with 33 kDa size. (c) Interaction of *ecdA* promoter with different concentration of GST::EcdB protein (Lane 4–7) along with BSA (Lane 2) and GST (Lane 3) as negative control, star (-) indicates the DNA band shift. (d) Interaction of EcdB protein with different promoters of other echinocandin B biosynthetic genes along with T7 promoter as negative control.

of in-clustered TF investigated earlier, with DNA-binding domain of TF *terR* of terrein gene cluster [42]. All together our results imply that, *ecdB* knockout strain did not significantly alter the ECB production and transcription of associated genes and exhibits a un-regulatory behavior. In contrast, interaction of EcdB protein with the *ecdA* promoter confirms the functional redundancy of *ecdB* gene. Wu et al., well reported the functional redundancy of yeast TFs and concluded that only 3% of binding targets of TFs were severely affected in knocked out strain [43]. Similar redundancy was also observed that *ecdB* transcription factor could not interact to activate transcription of ECB biosynthetic genes *in-vivo* but the truncated EcdB protein efficiently binds with the *ecdA* promoter *in-vitro*.

5. Conclusion

Taken together our results revealed that deletion of the *ecdB* gene elicited an effect similar to WT without affecting both ECB production and transcriptional behaviour of ECB biosynthetic gene cluster except few genes whereas EcdB protein interact with *ecdA* promoter *in-vitro*. This analysis concluded that EcdB protein binds to the promoter region of *ecdA*, *ecdH*, *ecdI* and *ecdK* but such interaction *in-vivo* are transient that could not activate transcription of the concerned genes.

Author contributions

Arvind Kumar- Performed all the experiments and manuscript written.

Varun Jaiswal- Phylogenetic analysis was done.

Vinay Kumar- Helped in designing the Southern analysis.

Amitava Dey- Helped in HPLC analysis.

Antresh Kumar- Study designed, supervised and manuscript edited.

Conflict of interest

Authors declares there is no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.btre.2018.e00264>.

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