A fraction from *Escherichia coli* with anti-*Aspergillus* properties

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The products of various strains of *Escherichia coli* (BL21, DH5α, HB101 and XL Blue) were investigated for antymycotic properties using pathogenic isolates of *Aspergillus*. Co-culture experiments revealed that *E. coli* strains exhibited variable activity against *Aspergillus fumigatus*. The lysates prepared from DH5α, HB101 and XL Blue strains of *E. coli* showed inhibitory activity against *A. fumigatus* in the protein concentration range of 62·50 to 250·00 μg ml⁻¹. The highest activity was seen in the lysate of BL21, which inhibited the growth of *A. fumigatus* and *Aspergillus flavus* completely at a concentration of 31·25 μg protein ml⁻¹. The MIC of BL21 lysate against *Aspergillus niger* was found to be 62·50 μg ml⁻¹. The in vitro toxicity of BL21 lysate was evaluated using a haemolytic assay. A BL21 lysate protein concentration of 1250·00 μg ml⁻¹ was found to be nontoxic to human erythrocytes. The standard drug amphotericin B lysed 100 % of erythrocytes at a concentration of 37·50 μg ml⁻¹. SDS-PAGE showed the presence of at least 15 major proteins in the lysate of BL21. Ion-exchange chromatography resolved the BL21 lysate into five fractions and fraction III was found to be endowed with anti-*Aspergillus* properties. The MIC of this fraction was found to be 3·90 μg ml⁻¹. Further work on the purification of the active molecule and its characterization is in progress.

INTRODUCTION

Infections due to opportunistic fungal pathogens such as *Aspergillus* and *Candida* species are emerging as major causes of morbidity and mortality in immunocompromised patients despite widespread use of several antifungal drugs (Walsh *et al.*, 1996; Richardson & Kokki, 1998). The antifungal agents currently available for treatment of these infections include amphotericin B and its liposomal formulations, 5-fluorocytosine, triazoles and allylamines, which are used singly or in combination (Maertens *et al.*, 2002; Chiu et al., 2000). These drugs not only have limited efficacy, all of them induce severe toxicity or immunosuppression (Georgopapadakou & Walsh, 1996). Further, resistance of fungi to most drugs has emerged, which has triggered considerable interest in identification of novel antifungal molecules (Viscoli & Castagnola, 1998).

The recent past has witnessed the introduction of a few promising second-generation triazoles (voriconazole, ravuconazole and posaconazole) but they are still undergoing clinical trials. These molecules are reported to show broad spectrum *in vitro* activity against clinical isolates of pathogenic fungal species (Graybill *et al.*, 1998). Studies carried out on voriconazole, ravuconazole and posaconazole using immunocompromised animal models (Sheehan *et al.*, 1999) and the published data from clinical trials have demonstrated improved antifungal efficacy (Walsh *et al.*, 2002). However, the serious problem of cross-resistance may still persist since these molecules have structural similarities with first-generation triazole drugs and use the same pathway for exhibiting antifungal activity.

Antifungal proteins such as thaumatin-like proteins (Ye *et al.*, 1999), gluconases (York *et al.*, 2004), chitinases (Choi *et al.*, 2004), ribosome-inactivating proteins (Ng & Parkash, 2002), cyclophilin-like proteins, miraculin-like proteins (Ye *et al.*, 2000), cysteine-rich peptides, like thionins (Epple *et al.*, 1997), and lipid-transfer proteins (Regente & de la Canal, 2000) have been reported from a variety of sources including bacteria, mammals, insects and plants for treating fungal infections (Selitrennikoff, 2001). Although these proteins have been shown to inhibit the growth of pathogenic fungi, many of them were found to be highly toxic (Conlon *et al.*, 2003). However, less-toxic antifungal proteins have been described from bacterial sources (Woo *et al.*, 2002).
Escherichia coli is a common commensal and an important component of gut flora. It serves useful functions in the body by suppressing the growth of harmful bacterial species and by synthesizing appreciable amounts of vitamins. It is reported to be useful in treating severe pseudomembranous colitis (Matricardi et al., 2003) and is also found to be associated with anti-allergic effects (Georg & Schlorer, 1998). However, it is not known if E. coli interacts with mycotic infections to exert any antifungal effect. In view of the importance of E. coli in human health, the present study was undertaken to identify, isolate and characterize the potential antifungal fractions of E. coli.

METHODS

Bacterial strains. E. coli BL21 (MTCCB1678), DH5α (MTCCB1652) and HB101 (MTCCB82) were obtained from the Institute of Microbial Technology, Chandigarh, India, whereas the XL Blue strain of E. coli was purchased from Banglore Genei.

Pathogens. Aspergillus fumigatus isolates ITCC4517 (IARI, Delhi), ITCC1634 (IARI, Delhi) and 190/96 (VPCCI, Delhi), Aspergillus flavus isolates ITCC5192 (IARI, Delhi), ITCC5076 (IARI, Delhi) and 223/96 (VPCCI, Delhi), and Aspergillus niger isolates ITCC5405 (IARI, Delhi) and 56/96 (VPCCI, Delhi) were employed in the current study. These pathogenic species of Aspergillus were cultured in the laboratory on Sabouraud dextrose agar plates.

Preparation of lysate of E. coli strains. The 72 h exponential phase cultures of E. coli strains were centrifuged at 5000 rpm for 30 min. The pellet was suspended in sonication buffer (50 mM Tris/HCl, 50 mM EDTA, 5 mM DTT, 1 mM PMSF) and sonicated for 20 s bursts at 200 W and 10 s cool period using a sonicator (Misonix, Sonicator 3000). The sonicate was centrifuged at 15 000 rpm for 30 min using a Sorvall RC 5C centrifuge. The supernatant was used as lysate. The lysate was dialysed against water at 4°C for 24 h and lyophilized. The protein concentration of bacterial lysate was determined by the bicinchoninic acid method of Smith et al. (1985).

Antimycotic activity. The antifungal activity of bacterial components was analysed by microbroth-dilution, disc-diffusion and spore-germination-inhibition assays (Rajesh & Sharma, 2002). These assays were repeated at least three times.

Microbroth-dilution assay. The spores (1 × 10⁶) of Aspergillus were harvested from 96 h cultures and treated with different concentrations of bacterial products in a 96-well culture plate. The plates were incubated at 37°C and examined macroscopically after 48 h for the growth of Aspergillus mycelia.

Disc-diffusion assay. This test was performed in radiation-sterilized Petri plates of 10-cm diameter (Tarsons). Sterilized discs (5-mm diameter of Whatman paper) impregnated with different concentrations of the bacterial products were placed on the surface of agar plates already inoculated with Aspergillus spores (1 × 10⁶). The plates were incubated at 37°C and examined at 48 h for the zone of inhibition, if any, around the discs.

Spore-germination-inhibition assay. Various concentrations of the test samples in 90 μl of culture medium were prepared in 96-well flat-bottom micro-plate cultures (Nunc Nunclon) by double-dilution method. The wells were prepared in duplicate for each concentration. The wells of the culture plates were inoculated with 10 μl of spore suspension containing 100 ± 5 spores. The plates were incubated at 37°C for 16 h and then examined for spore germination under an inverted microscope. The number of germinated and non-germinated spores was counted.

Based on the results of co-culture antimycotic activity experiments, the BL21 strain of E. coli was further investigated to identify its active fraction(s).

Fractionation of E. coli (BL21) lysate. The lysate prepared from the BL21 strain of E. coli was subjected to fractionation by ion-exchange chromatography. A 1500 mg amount of lysate protein was dissolved in Tris/HCl, pH 7.4, and loaded onto a DEAE-cellulose column (5.000×8.00 cm) pre-equilibrated with the same buffer. The non-adsorbed proteins were eluted with Tris/HCl, pH 7.4. The elution of adsorbed proteins was carried out using a 200-ml linear gradient from 0 to 1.00 M of NaCl in Tris/HCl pH 7.4. The flow rate was adjusted to 1.00 ml min⁻¹ and 2.00 ml fractions were collected. The OD₂₈₀ of the fractions was measured. The OD₂₈₀ values of various fractions were plotted against the fraction number. The peaks were pooled and analysed for antifungal activity. The proteins recovered in peaks showing antifungal activity were subjected to SDS-PAGE using 12.5% gel. SDS-PAGE was carried out according to the method of Laemmli & Favre (1973).

Haemolytic assay. The basic method of Latoud et al. (1986) with slight modifications was employed to determine the haemolytic effect of bacterial proteins with antifungal activity. Human erythrocytes, collected from apparently healthy individuals, were washed three times with PBS by centrifugation at 1500 r.p.m. for 10 min. A 2% erythrocyte suspension was incubated at 37°C for 1 h with different concentrations of lysate ranging from 5000-0 to 1.22 μg protein ml⁻¹. After incubation, cells were pelleted at 5000 r.p.m. for 10 min. The supernatant was collected and the A₅₅₀ was determined using a spectrophotometer (UV Vis Spect Lambda Bio 20, Perkin Elmer). In negative control sets, only buffer was used for background lysis, whereas in positive controls, lysis buffer was used for completely lysing the erythrocytes. For each sample the percentage of maximum haemolytic activity was determined.

RESULTS AND DISCUSSION

Antifungal activity of E. coli lysates

The antifungal activity of lysates prepared from BL21, DH5α, HB101 and XL Blue strains of E. coli was examined by microbroth-dilution assay. These strains exhibited mild to moderate activity (MICs in μg ml⁻¹: DH5α, 62.50; HB101, 125.00; XL Blue, 250.00). The lysate of the BL21 strain of E. coli showed potent and broad-spectrum antifungal activity against Aspergillus species. It was observed that a protein concentration of 31.25 μg ml⁻¹ of BL21 lysate inhibited the growth of A. fumigatus and A. flavus in microbroth-dilution and spore-germination-inhibition assays (Fig. 1). A higher concentration of bacterial lysate of BL21 was required to inhibit the growth of A. niger. In the disc-diffusion assay, the MIC of BL21 lysate against A. fumigatus and A. flavus was found to be 6.25 μg disc⁻¹ (Table 1). Magnusson et al. (2003) screened 1200 isolates of lactic acid bacteria for anti-Aspergillus activity and observed strong inhibitory activity against A. fumigatus but several isolates showed reduced antifungal activity after storage and handling. Lactobacillus coryniformis subsp. coryniformis strain Si5 was also found to be a producer of broad-spectrum proteinaceous antifungal compound (Magnusson & Schnurer, 2001).
Fractionation of *E. coli* BL21 lysate

The results of SDS-PAGE showed various protein bands in the lysate of *E. coli* BL21 (Fig. 2). It was, therefore, pertinent to fractionate these proteins and identify the active antifungal fraction(s). The lysate of *E. coli* BL21 was fractionated by DEAE cellulose column. The proteins were resolved into five fractions (FI to FV). The elution profile of proteins of *E. coli* (BL21) obtained after ion-exchange chromatography is shown in Fig. 3. Different fractions were collected and examined for antifungal activity as described.

Antifungal activity of fractions of *E. coli* BL21 lysate

It was observed that activity resided mainly in FIII as its MIC was found to be 3.90 μg ml⁻¹ against *A. fumigatus* using microbroth-dilution and spore-germination-inhibition assays. In disc-diffusion assay, the MIC of FIII was found to be 1.25 μg disc⁻¹ (Fig. 4). The SDS-PAGE of FIII on 12.5 % gel demonstrated two major bands in the molecular mass range of 29 to 43 kDa (Fig. 2).

Dahot (1998) studied the antifungal properties of protein fractions of *Moringa oleifera* leaves and found that one of the chromatographic fractions inhibited the growth of *A. niger* at a minimum concentration of 75.0 μg ml⁻¹. This fraction was not found to be effective against *A. flavus* and *A. fumigatus*. Dahot (1999) also fractionated the leaf extract of *Indigofera oblongifolia* using Sephadex G-25 column to obtain four fractions. Their chromatographic fraction AP4 was active against *A. fumigatus, A. flavus* and *A. niger* at the concentration of 50.0, 15.0 and 25.0 μg ml⁻¹, respectively. But AP3 inhibited the growth of *A. fumigatus* and *A. flavus* at the concentration of 50.0 and 10.0 μg ml⁻¹, respectively. Bottone & Peluso (2003) identified the *Bacillus pumilus* (MSH) which had antifungal activity against Mucoraceae and *Aspergillus* species, but it was not tested against *A. niger*. No data were given by Bottone & Peluso (2003) regarding the toxicity of the antifungal compound. The results of the present investigation showed that lysate of *E. coli* (BL21) inhibited the

**Table 1.** Activity of BL21 lysate against *Aspergillus* species by microbroth-dilution and disc-diffusion assays

<table>
<thead>
<tr>
<th>Conc of BL21 lysate (μg ml⁻¹)</th>
<th>Microbroth-dilution assay</th>
<th>Disc-diffusion assay zone of inhibition (mean diameter in mm)</th>
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<tr>
<td></td>
<td><em>A. fumigatus</em></td>
<td><em>A. flavus</em></td>
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<tr>
<td>125.00</td>
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<td>+</td>
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<td>62.50</td>
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<td>31.25</td>
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<td>15.60</td>
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<td>7.825</td>
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+, Activity, –, no activity.
growth of \( A. \) \textit{fumigatus} at a concentration of 31.25 \( \mu \text{g ml}^{-1} \) and the activity mainly resided in ion-exchange chromatographic fraction FIII, the MIC being 3.90 \( \mu \text{g ml}^{-1} \).

**Cytotoxicity**

The results of toxicity experiments revealed that the total protein lysate of \( E. \) \textit{coli} investigated in the present study was nontoxic up to 1250 \( \mu \text{g ml}^{-1} \) to human erythrocytes. The higher doses exerted insignificant toxicity and only marginal haemolysis (5.8 \%) was detected at the concentrations up to 5000 \( \mu \text{g ml}^{-1} \) (Fig. 5). The amphotericin B lysed all erythrocytes at a concentration of 37-5 \( \mu \text{g ml}^{-1} \). Similarly, Hong \textit{et al}. (1999) found that a novel antimicrobial peptide did not show haemolytic activity up to the concentration of 500 \( \mu \text{g ml}^{-1} \). Woo \textit{et al}. (2002) also found antifungal SAP protein, isolated from \textit{Streptomyces}, nontoxic up to 250 \( \mu \text{g ml}^{-1} \) to human dermal fibroblasts, but it was found to be toxic at all higher doses. However, Sorensen \textit{et al}. (1996) found 100 \% haemolysis by syringomycins at the concentration of 20 \( \mu \text{g ml}^{-1} \) and found it to be more toxic than amphotericin B to erythrocytes. Thionins and defensins have been reported to be effective antifungal proteins against human pathogens but they exerted nonspecific cytotoxic activity against a wide range of normal and malignant targets, including cells resistant to TNF (tumour necrosis factor)-alpha and NK (natural killer)-cytolytic factor. They appear to kill mammalian target cells and microorganisms by a common mechanism (Lehrer \textit{et al}. , 1993). The antifungal peptides purified from \textit{Bacillus cereus} were also reported to be highly toxic to human erythrocytes (Latoud \textit{et al}. , 1986).

The toxicity of most reported antifungal proteins to mammalian cells, therefore, has become a major limitation in using them as lead molecules for developing new antifungal drugs and using them for further formulations of better drugs. The much lower toxicity of the potent antifungal fraction (FIII) identified in the current study thus emphasizes its usefulness in new therapeutics. It may also be possible to develop specialized probiotics using \( E. \) \textit{coli} strains for treating \textit{Aspergillus}-induced disorders.

**Conclusion**

The observations of the present study indicated that the BL21 strain of \( E. \) \textit{coli} synthesizes potent anti-\textit{Aspergillus} proteins with extremely low toxicity to human cells. Such preparations may be important leads for developing new therapies for treating fungal infections. Further, the rehabilitation of gut flora with BL21 strain of \( E. \) \textit{coli} may therefore be useful in providing the protection against pathogenic fungi. The treatment of pseudomembranous colitis was indeed achieved by using non-specified strain of \( E. \) \textit{coli}.  

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**REFERENCES**


Antifungal fraction from *E. coli*


