Research Article

Colicin E2 Expression in Lactobacillus brevis DT24, A Vaginal Probiotic Isolate, against Uropathogenic Escherichia coli

Disha Trivedi, Prasant Kumar Jena, and Sriram Seshadri

Institute of Science, Nirma University, Sarkhej-Gandhinagar Highway, Chharodi, Ahmedabad, Gujarat 382481, India

Correspondence should be addressed to Sriram Seshadri; sriram.seshadri@nirmauni.ac.in

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Novel therapeutic approaches are needed to combat the urinary tract infection in women. During menstruation elevated protein concentration and increase in oxygen and carbon dioxide concentrations with decrease in vaginal Lactobacilli all together contribute to urinary tract infections. Lactobacillus species are a predominant member of the vaginal microflora and are critical in the prevention of a number of urogenital diseases. In order to increase antimicrobial potential of vaginal Lactobacilli, bacteriocin colicin E2 which has specific activity against uropathogenic Escherichia coli has been overexpressed in vaginal probiotic Lactobacillus brevis DT24. Recombinant Lactobacillus brevis DT24 expressing colicin E2 showed much higher inhibitory activity against uropathogenic Escherichia coli than wild type L. brevis DT24 in vitro. Efficacy of probiotic Lactobacillus brevis DT24 expressing colicin E2 protein is required for further in vivo evaluation.

1. Introduction

Urinary tract infection (UTI) is the most widespread infection in women worldwide after intestinal infection [1]. UTIs affect an estimated 1 out of 3 women before the age of 24 [2, 3]. Up to 40 to 50% of the female population will develop a symptomatic UTI at some time during their lives [2, 3] or develop complicated UTIs [4]. Recurrent UTI (rUTI) is a common syndrome in young healthy women. Previous studies suggest that 27% to 44% of women, who experienced an initial UTI, develop rUTI [5, 6].

UTI has the potential for severe and life-threatening sequelae if left untreated or undertreated. Possible sequelae include pyelonephritis which can lead to renal scarring and sepsis [7]. UTI can be particularly dangerous in pregnant women in whom it has been shown that up to 50% of those with asymptomatic bacteriuria (ABU) leads to develop pyelonephritis. In addition, these women experience higher rates of intrauterine growth restriction and low birth weight infants. The presence of a UTI has also been shown to increase the risk of preterm labor, preterm birth, pregnancy-induced hypertension, preeclampsia, amnionitis, and anemia [8].

Escherichia coli are among the most significant human pathogens, responsible for up to 90% of all community acquired and almost 50% of nosocomial UTIs. E. coli is a ubiquitous human pathogen responsible for both community and hospital-acquired infections [9, 10]. A number of virulence determinants facilitate the ability of uropathogenic E. coli to colonize the urinary tract and exert cytopathic effects, including type 1 fimbriae [11], P fimbriae [12], Dr adhesions [13], hemolysin [14, 15] cytotoxic necrotizing factor 1 [16], flagella [17], capsule polysaccharide [18], lipopolysaccharide O antigen [19], and TonB-dependent iron transport systems [20]. During UTI outer membrane proteins of uropathogenic E. coli like porins (OmpA, OmpC, OmpX, NmpC, and LamB) and outer membrane assembly factors, including YaeT and YeaE, as well as nucleoside and vitamin B12 receptors Txs and BtuB, are overexpressed [21].

Colicin E2 (ColE2) is a proteinaceous bacterial toxin produced by some strains of Escherichia coli that exhibits inhibitory activity against uropathogenic E. coli [22] via binding to an outer membrane receptor—the TonB-dependent vitamin B12 transporter, BtuB [23].
Earlier reports suggest that there is an increased antibiotic resistance in *E. coli*. Initially, resistance was limited to certain specific antibiotics, such as ampicillin or trimethoprim [24], but recently the horizon of resistance has expanded, with the emergence of broad resistance to third generation antibiotics [25–28].

*Lactobacillus* species are an important group of bacteria that inhabit the gastrointestinal tract and represent the predominant microorganism found in the healthy vaginal ecosystem [29–33], producing a variety of compounds [34–38] that inhibit potentially pathogenic microorganisms. It is for this reason that *Lactobacillus* species have been studied as a potential probiotic for the prevention and treatment of urogenital disease in women [39–42]. During menstruation, the vaginal pH becomes neutral, most likely due to the influx of menses blood, which has a pH range of 6.9 to 7.2 [43]. In addition, menses blood in the vagina also results in an elevated protein concentration [43] and increase in oxygen and carbon dioxide concentrations [44], leading to decrease in vaginal Lactobacilli, which all contribute to colonization of uropathogenic *E. coli* which leads to UTIs [45–48].

As a means of increasing the antimicrobial capabilities of *Lactobacillus* species against uropathogenic *E. coli* (UPEC), probiotic *L. brevis* DT24, isolated from vagina of healthy women [49], was used for overexpression of colicin E2.

The objective of this study was to express ColE2 structural gene *ceAB* and immunity gene *ceiB* from *E. coli* into probiotic *Lactobacillus brevis* DT24, secreting colicin E2 to exclude a uropathogenic *E. coli* competitively and completely. In this study genetically engineered probiotic *Lactobacillus brevis* DT24 would exert its antimicrobial effect against the target pathogen directly through the expression of ColE2 gene and indirectly through beneficial properties inherent in probiotics.

### 2. Materials and Methods

#### 2.1. Bacterial Strains, Plasmids, and Growth Conditions.

Bacterial strains used in this study are listed in Table 1. *Lactobacillus brevis* DT24 (NCBI Accession no. JX163909) was routinely cultured in MRS broth (HiMedia, Mumbai) at 37°C for 48 h. For the analysis of expression of colicin E2, recombinant strains were grown in basal MRS medium supplemented with 2% xylose. *Escherichia coli DH5α*, *Escherichia coli BL21 D3*, and *Escherichia coli NCTC 50133* were routinely cultured in Luria Bertani (LB) broth and agar at 37°C for 16 to 24 h and uropathogenic *Escherichia coli* MTCC 729 (Microbial Type Culture Collection, Chandigarh) was routinely cultured in nutrient broth and agar at 37°C for 16 to 24 h. Chloramphenicol (Cm) and streptomycin (Strp) were used for the selection of plasmids.

#### 2.2. DNA Manipulation, *E. coli* Competent Cell Preparation, Transformation, and PCR.

Plasmid DNA was isolated using GeneJet Plasmid Miniprep Kit as per instruction (Fermentas). DNA cloning and transformation procedures followed as previously described [51]. Restriction enzymes were purchased from New England Biolabs. Ligation was carried out by using Rapid Ligation Kit (Fermentas).

#### 2.3. Construction of Plasmids and Transformation of *Lactobacillus*.

The expression plasmid pSLPIII.3, a type of secretion expression vector containing *StpA* as secretion signal and having a cell wall anchor domain, was kindly gifted by Prof. Jos Seegers (Falco Biotherapeutics, The Netherlands). Nucleic acid manipulation and cloning procedures was performed according to standard procedures [51].

Colicin E2 gene fragment of about 2.01 kb encoding the colicin E2 structural gene (*ceAB*) and colicin E2 immunity gene (*ceiB*) was obtained from the plasmid pColE2-P9 (*E. coli* genetic resource centre, Yale University, USA) by polymerase chain reaction (PCR) amplification with the primers 5′-GGATCCATGAGCGGTGGCGAT-3′ (forward) containing a BamHI site (underlined) and 5′-CTCGAGTCAGCC-CTTTTAAAATCTCGTA-3′ (reverse) containing an Xhol site (underlined). PCR conditions were as follows: 30 cycles of 30 s at 94°C, 30 s at 58°C, and 2 min 20 s at 72°C after denaturing for 4 min at 94°C.

The PCR product of colicin E2 gene was cleaved with BamHI and Xhol restriction endonuclease and inserted into the corresponding sites of pPSLIII.3 digested by BamHI and Xhol, respectively, giving rise to pSL-ColE2 (Figure 1).

Electroporation of *L. brevis* DT24 was carried out as previously described [52] with some modifications. In brief, a 2% inoculum of an overnight culture was grown in MRS medium supplemented with 1% glycerine at 37°C until the OD660 of culture was 0.2 to 0.3. The cells were harvested and washed twice with cold washing buffer (5 mM sodium phosphate pH 7.4 and 1 mM MgCl₂). The cells were then resuspended to 1% of the original culture volume in a cold electroporation buffer (1 M sucrose, 3 mM MgCl₂). For electroporation, 45 μL of the cell suspension was mixed with 50 to 500 ng of plasmid DNA and subjected to 2.5 kV, 200 μF, and 25 μF electric pulse in a 0.2 cm cuvette by using a Genepulsar II electroporation system (Bio-Rad Lab). After the pulse, 450 μL of cold MRS was immediately added to the cell suspension, kept on ice

<table>
<thead>
<tr>
<th>Strains and plasmids</th>
<th>Description</th>
<th>Source</th>
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</thead>
<tbody>
<tr>
<td><em>Lactobacillus brevis</em> DT24</td>
<td>Probiotic vaginal isolate</td>
<td>In this study</td>
</tr>
<tr>
<td><em>Escherichia coli</em> NCTC 50133</td>
<td>Contains pColE2-P9, produces colicin E2</td>
<td>[50]</td>
</tr>
<tr>
<td><em>Escherichia coli</em> DH5α</td>
<td>Transformation host</td>
<td>MTCC</td>
</tr>
<tr>
<td><em>Escherichia coli</em> BL21 D3</td>
<td>Expression Host</td>
<td>MTCC</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>Uropathogenic strain</td>
<td>MTCC</td>
</tr>
<tr>
<td>Plasmids</td>
<td>Description</td>
<td>Source</td>
</tr>
<tr>
<td>pSLPIII.3</td>
<td>Cm', <em>E. coli/LAB</em> shuttle vector</td>
<td>Prof. Jos Seeger, Lactrys, The Netherlands</td>
</tr>
<tr>
<td>pColE2-P9</td>
<td>Stp', vector for colicin E2</td>
<td><em>E. coli</em> Genetic Stock Centre, Yale University</td>
</tr>
<tr>
<td>pSL-ColE2</td>
<td>Cm', <em>SlpA</em>, and ColE2</td>
<td>In this study</td>
</tr>
</tbody>
</table>
The construct of recombinant vectors expressing colicin E2. The ColE2 gene (*ceaB*) and its immunity gene (*ceiB*) on plasmid DNA (pColE2-P9) were amplified using the polymerase chain reaction (PCR) with the primers. The PCR product was cleaved with BamHI and XhoI restriction endonuclease and inserted into the corresponding sites of pSLP111.3, giving rise to pSLP111.3-pColE2-P9.

for 10 min, and incubated for 3 h at 37°C. The transformants were plated onto MRS agar plates and incubated for 48 to 72 h. The transformation efficiency was calculated as the number of transformants per microgram of plasmid DNA.

2.4. Molecular Weight Determination. Transformed ColE2 gene having *Lactobacillus brevis* DT24 was grown in MRS medium at 37°C for 24 hr and centrifuged (10,000 rpm, 30 min, and 4°C) to collect supernatants. The collected supernatants were filter sterilized (0.20 μm; Axiva). Ammonium sulfate was slowly added to the cell-free supernatants to 60% saturation and stirred for 4 h at 4°C and centrifuged (10,000 rpm, 30 min, and 4°C). The precipitate was resuspended in 10 mL of 25 mM ammonium acetate buffer (pH 6.5) and desalted by dialysis using a 1,000 Da cutoff dialysis membrane (Sigma) against the same buffer. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was used for further separation, as described by Laemmli [53].

2.5. Antimicrobial Activity of Recombinant *L. brevis* DT24 Expressing Colicin E2. Antimicrobial activity of cell-free supernatant (filter sterilized) was measured by well diffusion assay. Filtrates were neutralized (set to pH 6.5) with 5 N NaOH. Nutrient agar plates were flooded with pathogenic bacteria (0.1% of overnight grown uropathogenic *E. coli* strain), air-dried, and then 6 mm diameter wells were punctured in each plate. The prepared supernatants were poured into respective wells (25 μL) and incubated for 24 h at 37°C. *L. brevis* DT24 was used as negative control and *E. coli* NCTC 50133 was used as positive control.

3. Results


Plasmid pSL-ColE2 was constructed by inserting a 2.01 kb PCR amplicon obtained from pColE2-P9 DNA containing *ceaB* and *ceiB* into pSLP111.1 (Figure 1). Transformants containing pSL-ColE2 demonstrated inhibitory activity against the uropathogenic *E. coli* (Figure 1). The presence and orientation of the *ceaB* and *ceiB* insertion in pSL-ColE2 was verified by PCR (Figure 2).

3.2. Extracellular Expression of Colicin E2. *L. brevis* DT24 transformants harboring recombinant pSL-ColE2 were tested for extracellular expression in 500 mL of MRS broth. Transformant and control strains containing vector only were
incubated with vigorous shaking (200 rpm), and cell growth was monitored by checking optical density at 600 nm. After 24 hrs of growth 52kDa protein was observed in SDS-PAGE analysis (Figure 3).

3.3. Antimicrobial Activity of Recombinant L. brevis DT24 Expressing Colicin E2
Antimicrobial properties of transformed L. brevis DT24-ColE2 showed higher zone of inhibition (56 mm) compared to Wild Type L. brevis DT24 (23 mm) were shown in Figure 4. But there is no difference in the inhibition zone showed by L. brevis DT24 ColE2 and E. coli NCTC 50133.

4. Discussion
Various techniques have identified Lactobacillus species as the predominant microorganism found in the vaginas of most healthy and fertile women [40, 41]. Lactobacillus species have been studied as a potential probiotic for the prevention and treatment of urogenital disease in women [39–42]. During menstruation, the vaginal pH becomes neutral, most likely due to the influx of menses blood, which has a pH range of 6.9 to 7.2 which leads to lower Lactobacilli number in vagina and chances of infections like urinary tract infections and bacterial vaginosis.

Colicin E2 production by a nonpathogenic organism may have clinical applicability as a means to prevent catheter-associated urinary tract infection [22]. Evans et al. [54] demonstrated the possibility of developing oral whole-cell vaccines against diarrhea caused by enterotoxigenic E.coli by modifying this E. coli by the in situ destruction of chromosomal and plasmid DNAs by ColE2. The colicin operon is carried on a plasmid and includes a structural gene (ceaB) encoding for the bacteriocin, an immunity gene (ceiB) that protects the producer cell from the toxin, and a lysis gene (celB) that leads to death of the producer cell and release of ColE2 into the surrounding medium [23]. Sensitivity of gram-negative microorganisms to ColE2 is conferred by the binding of the bacteriocin to an outer membrane receptor, the TonB-dependent vitamin B12 transporter, BtuB [23]. After transport across the membrane, ColE2 acts as an endonuclease, degrading the DNA of the sensitive cell. During UTIs uropathogenic E. coli overexpresses surface protein BtuB which can act as receptor for binding colicin E2.

The colicin E2 gene (ColE2) from E. coli cloned in shuttle secretion vector (pSLP111.3) and successfully expressed in our lab probiotic isolate Lactobacillus brevis DT24 and its impact on the inhibitory activity of host organism were examined. The expression of ColE2 in Lactobacillus brevis DT24 was studied by isolating proteins extracellularly.

One of the challenges of transforming ColE2 in Lactobacillus is the differences in the transport mechanisms of bacteriocins in gram-negative and gram-positive microorganisms [55]. In gram-negative microorganisms, ColE2 is thought to be released into the surrounding medium after CelB-mediated lysis of the producer cell. Expression of celB leads to changes in the cell envelope and results in activation of Omp LA, an outer membrane phospholipase A [23]. Mutation or deletion of the lysis protein has been shown to interfere with release, and in such cases, colicin remains in the cytoplasm [23]. In gram-positive microorganisms, secretion does not occur through cell lysis and is not a lethal event for the cell. Instead, secretion is dependent on a signal peptide,
which typically contains conserved double-glycine regions and is mediated by a bacteriocin-specific transport system or the sec-dependent export pathway [56]. Although ColE2 does not contain a signal peptide to direct the secretion of the protein, it is predicted to contain 6 double-glycine regions at the N terminus, which may function in a manner analogous to a signal peptide. Thus, ColE2 may be secreted by a gram-positive host without lysis of the producer cell. This feature is important if the genes encoding for colicin production are to be transferred to and expressed by a Lactobacillus [57].

This study demonstrated that genes associated with bacteriocin production from a gram-negative microorganism could be cloned, expressed, and secreted by a gram-positive microorganism in the absence of a lysis protein (CelB) and with addition of a signal peptide. In the present work, genes associated with ColE2 production (ceaB and cell) were transferred to L. brevis DT24, probiotic isolate from vagina. The level of ColE2 production by the colicin-producing transformants of L. brevis DT24 was similar to that of E. coli NCTC 50133, from which the ColE2-encoding genes (pColE2-P9) were derived.

Secretion of ColE2 proteins into the surrounding medium by E. coli NCTC 50133 and the pSLPII.3-ColE2 transformants occurred before cell leakage was observed. The mechanism proposed for secretion of ColE2 from E. coli involves release of the colicin caused by the lysis protein CelB [58–63]. Braun et al. [64] found that inactivation of celB resulted in decreased release of colicin from the cells, compared with cells containing intact celB.

Cloning and expression of ColE2 in L. brevis DT24 allowed evaluation of the transformant as a bioactive compound for use in treatment of UTI. Similar strategies were used for treatment of Staphylococcus aureus infection by expressing antimicrobial protein lysostaphin in vaginal probiotic Lactobacillus plantarum WCFS1 [65] and inhibition of HIV by expressing anti-HIV proteins which were capable of blocking the HIV entry into human peripheral blood mononuclear cells in probiotic organism Lactobacillus reuteri RC-14 [66].

5. Conclusion

This study has demonstrated that the expression of E. coli colicin E2 (ColE2) into Lactobacillus showed increased expression of colicin E2 in extracellular level to inhibit the infectious disease occurred by uropathogenic E. coli. The probiotic properties of host Lactobacillus brevis DT24 were increased in the terms of antimicrobial activity against pathogenic E. coli. Oral administration of probiotics has clear effects on the numbers and activities of intestinal and fecal bacteria. The administration oral probiotics help to reduce the transfer of yeast and urogenital pathogenic bacteria from the rectum to vagina [67]. It may be possible to use these L. brevis DT24-ColE2 probiotics in biotherapy (i.e., as vehicles for the secretion of colicin E2 in the gastrointestinal tracts as well as uro vaginal tract for the treatment of UTI as well as other gastrointestinal infectious diseases).

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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