Copper, Zinc-Superoxide Dismutase from Clinically Isolated *Escherichia coli*: Cloning, Analysis of *sodC* and Its Possible Role in Pathogenicity

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Abstract Superoxide dismutase has been discovered within the periplasm of several Gram-negative pathogens. We studied the Cu,Zn-SOD enzyme in *Escherichia coli* isolated from clinical samples (stool samples) collected from patients suffering from diarrhea. Antibioqram studies of the isolates were carried out to determine the sensitive and resistant strains. The metal co-factor present in the enzyme was confirmed by running samples in native gels and inhibiting with 2 mM potassium cyanide. A 519 bp *sodC* gene was amplified from resistant and sensitive strains of *Escherichia coli*. Cloning and sequencing of the *sodC* gene indicated variation in the protein and amino acid sequences of sensitive and resistant isolates. The presence of *sodC* in highly resistant *Escherichia coli* isolates from diarrheal patients indicates that *sodC* may play role in enhancing the pathogenicity by protecting cells from exogenous sources of superoxide, such as the oxidative burst of phagocytes. The presence of SodC could be one of the factors for bacterial virulence.

Keywords Gram-negative bacteria · *Escherichia coli* · Drug resistance · Superoxide dismutase · *sodC* · Pathogenicity

Introduction

Superoxide dismutases (SODs) are present in most bacteria, catalyzing the conversion of oxygen radical (O_2^-) into hydrogen peroxide and oxygen [1], resulting in the removal of free radicals generated during the reduction of molecular oxygen. Three types of SODs are found in bacteria. Mn-SOD and Fe-SOD encoded by *sodA* and *sodB*, respectively are located in the cytoplasm where they catalyse dismutation of O_2^- during aerobic metabolism. Copper and zinc (Cu, Zn)-SOD encoded by *sodC* has been identified in the periplasm of a wide range of Gram-negative bacteria, including *Escherichia coli* [2], *Salmonella typhimurium*, *Salmonella choleraesuis* and *Salmonella dublin* [3, 4], *Haemophilus* species [5], *Brucella abortus* [6]. Cu, Zn-SOD protect bacteria from toxic oxygen-free radicals generated outside the cell or in the periplasm itself, since superoxide is unable to cross membrane [7]. Therefore, Cu, Zn-SOD has been proposed to be a virulence factor in bacteria potentially exposed to toxic free radicals produced by the host in response to bacterial infection. Experiments have demonstrated the role of bacterial Cu, Zn-SOD in the virulence and pathogenicity of microorganisms [8]. Human pathogens like *Salmonella*, *Haemophilus influenzae*, *H. parainfluenzae* and *Legionella pneumophila* [9, 10] demonstrate the presence of *sodC* gene, while it is hardly detectable in non-invasive bacteria [11, 12]. In the present study we report the presence of *sodC* gene from *Escherichia coli* both pathogenic and nonpathogenic, collected from patients with suspected cases of diarrhea by polymerase chain reaction (PCR) [13], highlighting differences in the protein and nucleotide sequence of isolates and also suggesting a possibility of SodC as a factor for bacterial virulence.

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Materials and Methods

Collection of Samples

Clinical samples (stool samples from patients with suspected cases of diarrhea) were collected using precautionary measures from the patients attending Hospitals and Diagnostic centres in Gulbarga city, Karnataka, India (K.B.N. Hospital, Basaveshwara Hospital, Pooja Diagnostics and Hyderabad–Karnataka Diagnostics and Medical Research Institute Pvt. Ltd.). The collected stool samples were screened for the presence of Gram-negative bacteria using specific media viz. MacConkey and Eosin methylene blue (EMB) agar.

Characterization and AntibioGram of Isolated Bacteria

The isolated bacteria were identified based on the cultural, morphological and biochemical characteristics [14]. The standard strain used was *Escherichia coli* 2622 procured from IMTECH-Chandigarh, India. AntibioGram of the isolated and identified *E. coli* was carried out by Kirby-Bauer disc diffusion method [15]. For all the isolates and reference strain, Muller Hinton agar plates were used. Commercially available antibiotic discs procured from Span Diagnostics Ltd. were used. Organisms were classified as sensitive (S), and resistant (R) according to the Clinical and Laboratory Standards Institute [16] guidelines (formerly NCCLS [17]).

Screening of *Escherichia coli* for Cu, Zn-Superoxide Dismutase

Preparation of Periplasmic Extracts

Escherichia coli isolates were grown overnight in Luria–Bertani (LB) broth (10 mg/ml tryptone, 5 mg/ml yeast extract, 10 mg/ml NaCl, pH 7.2). Periplasmic extracts were prepared from bacterial cultures by the lysozyme-EDTA method described by Battistoni and Rotilio [12]. 250 ml of 30 h culture was centrifuged (Sigma) at 8000 rpm for 15 min. The dry pellet obtained was re-suspended in 5 ml of the isotonic solution comprising Tris–HCl (30 mM; pH 8.0), Sucrose (20% wt./vol.), EDTA (1 mM). To this, 2 ml of lysozyme solution (10 mg/ml) was added. The tube was mixed by inversion, incubated for 10 min on ice and centrifuged at 12000 rpm for 10 min. The supernatant was decanted and stored at –20°C for later analysis.

Visualization of SODs in PAGE

Superoxide dismutase was detected by non-denaturing polyacrylamide gel electrophoresis (PAGE), (7.5%) according to Laemmli [18]. The stacking gel used was 5%

pH 6.8, 7.5% separating gel pH 8.8 using the buffer system of Davis [19]. SOD activity in PAGE gels was visualized by modification of the Beauchamp and Fridovich [20] method as described by Steinman [21]. In a two-step staining procedure, the gels were incubated in 2.45 mM Nitroblue tetrazolium chloride [(NBT) (in dark)] for 20 min and in a solution containing 28 µM riboflavin and 28 mM *N,N,N',N'*-tetramethylethylenediamine (TEMED) in a 36 mM potassium phosphate buffer (pH 7.8) for 10 min (in dark). When the gels were pre-treated with 2 mM potassium cyanide, Cu, Zn-SOD was inactivated. Fe-SOD was inactivated by hydrogen peroxide, but Mn-SOD was resistant to both [22, 23]. The achromatic band indicating the zone of SOD activity was developed by illumination (Fig. 1).

Amplification of *sodC* from *E. coli*

In vitro amplification of *sodC* from isolated and reference strains of *E. coli* was carried out by polymerase chain reaction (PCR) using genomic DNA as template and oligonucleotides, designed based on the existing *sodC* sequence from database (NCBI). The oligonucleotide primers (Sigma–Aldrich) used were *Ecoli*_{for} 5'-ATGAAACGT TTAGTCTGGC-3'(Forward Primer) and *Ecoli*_{rev} 5'-CT TAATTACACCACAGGCATAGC -3'(Reverse Primer). PCR (PTC-150 Minicycler-MJ Research Inc., USA.) was carried out using 50–80 ng of genomic DNA as template. The reaction mixture comprised 200 µM dNTP, 200 nM primers (Sigma–Aldrich), 2% Dimethylsulfoxide (DMSO) and 1.5 U XT-5 polymerase (Bangalore Genie) in 1x reaction buffer. The XT-5 polymerase is a mixture of Taq DNA polymerase and proof reading polymerase ensuring no mutation in the PCR amplicon. In the final extension step 0.5 U of Taq DNA polymerase was added to ensure addition of 3' overhang dA, so that the amplicon could be cloned into T/A based cloning vector pTZ57R/T. Samples were processed through 30 cycles of initial denaturation at 94°C for 3 min, denaturation at 94°C for 30 s, annealing at 50°C for 30 s, elongation at 72°C for 30 s, final elongation at 72°C for 10 min. *sodC* amplicons were purified by using the GenElute Gel Extraction Kit (Sigma–Aldrich) as per manufacturer's protocol.

Cloning of *sodC*

Ligation of *sodC* into Cloning Vector pTZ57R/T

The purified *sodC* was ligated into cloning vector pTZ57R/T (InsTAclone PCR Cloning kit, MBI Fermentas) as per manufacturer's protocol. The ligation reaction mixture consisting of Insert DNA, vector DNA, 2x ligation buffer, T4 DNA ligase and nuclease free water were vortexed

Fig. 1 Achromatic bands against the dark background indicate the presence of SOD enzymes in periplasmic extracts from isolates: Lane 1 *E. coli* 130, Lane 2 *E. coli* 131, Lane 3 *E. coli* 140, Lane 4 *E. coli* 2622, Lanes 5,6,7,8 enzyme extracts of above strains treated with 2 mM KCN indicating the inhibition of Cu, Zn-SOD activity

briefly and centrifuged for 3–5 s, incubated at 22°C for 16 h.

Transformation of *sodC* Cloned pTZ57R into *E. coli* DH5 α

The TransformAid Bacterial Transformation Kit (MBI, Fermentas) was used for transforming 10 μ l of ligation mixture into competent *E. coli* DH5 α cells, spread plated on LB solid medium containing 100 μ g/ml ampicillin and incubated at 37°C for 12 h. The recombinant clones were analysed for the presence and orientation of the DNA insert using restriction digestion analysis. The plasmid DNA was isolated from an overnight bacterial culture using a convenient plasmid Miniprep method. The GeneElute Plasmid Miniprep kit (Sigma–Aldrich) was used as per manufacturer's protocol.

Sequencing of the Clones

After confirming the *sodC* clones of *E. coli* (pTZ57R 2622, pTZ57R 130, pTZ57R 131, pTZ57R 140) by restriction digestion analysis, they were submitted for sequencing (MWG Biotech Pvt. Ltd., Bangalore, India). Multiple alignments of nucleotide and protein sequences of *E. coli* 2622, *E. coli* 130, *E. coli* 131, *E. coli* 140 were carried out with CLUSTAL W program.

Results

The antibiogram of isolated *E. coli* using 10 antibiotics was carried out and the results showed that reference strain

E. coli 2622 and isolate *E. coli* 140 is resistant to 10 antibiotics compared to isolate *E. coli* 130 which is resistant to 7 antibiotics and *E. coli* 131 which is resistant to only 1 antibiotic. Multi-drug resistant and sensitive strains from the antibiogram studies were screened for Cu, Zn-superoxide dismutase by PAGE. Amplification of *sodC* gene of *E. coli* isolates and reference strain all resulted in 519 bp (Fig. 2). The *sodC* gene cloned into pTZ57R/T vector was transformed into *E. coli* DH5 α . The transformants were selected on LB agar medium containing 100 μ g/ml ampicillin. Positive transformants were selected by isolating plasmids and restriction enzyme analysis. The positive transformants of *E. coli* 2622 reference strain was labeled as pTZ57R2622 and similarly for isolates as pTZ57R130, pTZ57R131 and pTZ57R140. Sequencing of the *sodC* cloned pTZ57R2622 resulted in 519 nucleotide base pairs, which corresponded to the *sodC* gene sequence of *E. coli* from NCBI GenBank database on nucleotide-nucleotide BLAST analysis. Putative protein translation of

Fig. 2 Ethidium bromide stained 1% agarose gel showing ~520 bp PCR product of *sodC* gene. Lane 1 Negative control: without template, Lane 2 *sodC* amplicon of *E. coli* 2622, Lane 3 *sodC* amplicon of *E. coli* 130, Lane 4 *sodC* amplicon of *E. coli* 131, Lane 5 *sodC* amplicon of *E. coli* 140, Lane 6 DNA ladder

Fig. 3 Multiple alignment of *sodC* nucleotide sequences among *E. coli* isolates and *E. coli* reference strain

the sequenced fragments was carried out with EXPASY. The nucleotide sequences of all the four *E. coli* viz. *E. coli* 2622 (reference strain), *E. coli* 130, 131 and 140 are deposited in NCBI GenBank and the accession numbers are EU447295, EU447292, EU447293 and EU447294, respectively. The isolated *E. coli* strain numbers 130, 131 and 140 yielded nucleotide sequence of 519 bp. The putative translated protein had a length of 171 amino acids for all three strains. The multiple alignment of amino acid sequences of SodCs of *E. coli* 130, *E. coli* 131, *E. coli* 140 revealed 100% sequence identity with the *E. coli* SodC protein sequence available in the NCBI GenBank database (Figs. 3, 4). The nucleotide sequence homology of the isolated strains with that of the reference strain *E. coli* 2622 showed variation at position 327, cytosine (C) is replaced by thymine (T), was observed in all the three isolates when compared to the reference strain. Similarly at position 171, adenine (A) is replaced by guanine (G) in *E. coli* 130 strain. In *E. coli* strain 140 SodC sequence showed 2 variations at positions 365 and 474, in former guanine (G) is replaced by adenine (A) and at 474 position, adenine (A) is replaced by cytosine (C). Both the isolated *E. coli* strain numbers 130, 131 showed no mutations at protein level, however, few base differences at nucleotide sequence level (due to degeneracy of codon). On the other hand isolate *E. coli* 140 showed mutations in amino acid sequence too, at position 122, glycine (G) is replaced by aspartate (D), and at position 158 lysine (K) is replaced by N (asparagine). The total number of metal binding amino acid residues (histidine; H and aspartate; D) in *E. coli* 2622 reference strain and isolates *E. coli* 130 and 131 were found to be 18 but in

E. coli 140, 19 amino acid residues are present, though there is mutation at two amino acid residues of SodC sequence of *E. coli* 140 isolate, indicating there is no change in the SOD activity and in the functional group of the enzymes.

Discussion

It has been shown that Cu, Zn-SOD enzyme plays a protective role against superoxide generated by macrophages. *sodC* genes are present in most of the pathogenic Gram-negative bacteria. Here in our study we have reported the difference in sequence pattern of sensitive and resistant *Escherichia coli*. The antibiotic sensitivity of strains *E. coli* 2622, *E. coli* 130, *E. coli* 131, *E. coli* 140 indicated that *E. coli* 2622, *E. coli* 130 and *E. coli* 140 are highly resistant to different antibiotics when compared to *E. coli* 131 which is sensitive to antibiotics. The *sodC* sequence of *Escherichia coli* was amplified using specific *sodC* primers. With these primers we amplified the *sodC* gene (519 bp) from the genomic DNA of reference strain *E. coli* 2622 and clinical isolates *E. coli* 130, 131, 140. The *sodC* sequence from all three isolates revealed that there is no variation in the sequence length of *sodC*; it supports the earlier report by Imlay and Imlay [2]. The *E. coli* Cu, Zn-SOD enzyme like other bacterial Cu, Zn-SODs is secreted into the periplasm. Because superoxide cannot cross membranes, this localization indicates that the enzyme has evolved to defend periplasmic biomolecules against an extracytoplasmic superoxide source. Sequence homology among the

Fig. 4 Multiple alignment of amino acid sequences of SodC (translated) among *E. coli* isolates and *E. coli* reference strain

nucleotide sequences of three *E. coli* isolates and one reference *E. coli* 2622 strain revealed that all the three *E. coli* isolate *sodC* sequences showed the nucleotide variation at four places with that of reference *E. coli* 2622 *sodC* gene sequence. Highest changes in the nucleotide sequences were observed in *E. coli* 140 when compared to the *sodC* sequence of *E. coli* reference strain 2622. Overall 7 histidines and 11 aspartate were present in the SodC of all *E. coli* isolates except in *E. coli* 140 isolate. The above data suggests that isolate *E. coli* 140 differs from other isolates and the reference strain *E. coli* 2622 in their nucleotide and amino acid sequences. One of the reasons for this phenotypic change in the SodC of *E. coli* 140 could be the resistance to multi drugs. Such studies have been reported earlier by Zhang et al. [24], who observed the changes in SOD gene sequence of isoniazid resistant *M. tuberculosis* strain when compared with that of SodC of susceptible strain. This suggests that SodC may act as virulence factors of pathogenic bacteria. *sodC* genes had previously been reported in few other bacteria [2, 3, 25], among them *B. abortus* was not only a pathogen but also a facultative intracellular parasite which survives and multiplies in an intracellular environment within host phagocytic cells. Similar to other pathogenic microorganisms, *E. coli* having the *sodC* gene is found to have significant intracellular survival which suggests that bacteria encounter an oxidative stress upon invasion of epithelial cells and hence overproduction of Cu, Zn-SOD provides a defense mechanism [26]. Within phagocytes, organisms must protect from the respiratory burst host defense reaction, either by inhibiting its initiation or by resisting the bactericidal action of superoxide and other free radicals produced. A periplasmic SOD, appropriately located to dismutate superoxide generated exogenously in this way, can be considered as an enhancer of bacterial virulence. In our study the genotypic variation in protein sequences at the active sites

of resistant *E. coli* strain 140 containing *sodC* when compared with the sensitive strains indicates that the presence of SodC in multidrug resistant isolates may enhance the pathogenicity of the organism due to the ability of SodC in the periplasm to neutralize free radicals generated by the respiratory burst of host phagocytes hence can be considered as one of the factors leading to virulence.

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