A Non-Pathogenic Environmental Isolate of *Pseudomonas aeruginosa* MCCB 123 with Biotechnological Potential

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A B S T R A C T

Pseudomonas aeruginosa MCCB 123 is a potent producer of enzymes such as β-1,3 glucanase, LasA and LasB proteases, which have been identified to have greater significance in the biotechnology sector. However, pathogenicity assessment of bacteria is essential for its industrial survival. Pathogenicity was assessed by a panel of virulence assays such as the presence of type III secretion toxin genes, motility assays, biofilm formation, adhesion and invasion assays on Hep-2 and HeLa monolayers and antibiogram profiling. Analysis of the major cytotoxic *exoU* gene revealed the absence of the gene, thus confirms the non-cytotoxic phenotype of the bacterium. *P. aeruginosa* exhibited three types of motilities viz., swimming, swarming and twitching motilities and proved to be a moderate biofilm producer with a 3.10±0.52-fold increase in the optical density at Abs_{570} when compared to control. Antibiogram suggested the possibility of antibiotic treatment as an effective method for eradication of its biofilm. The non-pathogenic nature of the bacterium suggests industrially viability of this organism for the production of biotechnological relevant enzymes.

Introduction

Pseudomonas aeruginosa is bacterium that is ubiquitously distributed in aquatic habitats and soil and is a normal bacterial flora of intestine, mouth and skin. The colonization is normally harmless and infection occurs only when general or local defence mechanism is reduced (Kiewitz and Tummler, 2000). i.e., it is an opportunistic human pathogen (Lyczak, 2000 and Ortiz-Herrera, 2004). It is frequently isolated from hospital environments, clinical specimens and soil and water environments (Palleroni, 1992). As several members of *P. aeruginosa* are known human pathogens, it is pertinent to differentiate between pathogenic and non-pathogenic strains.

Environmental isolates of *P. aeruginosa* can be found in soils, surface and ground water and the number of living cells in the soil does not reach the level of infection risk (Atzel *et al.,* 2008). Clinical bacterial isolates are believed to be pathogenic when compared to environmental counterparts. The major reason for its emergence as a pathogen is due to its intrinsic resistance to antibiotics and disinfectants (Senthil *et al.,* 2011).

The bacterium *Pseudomonas aeruginosa* MCCB 123 used in the present study was an environmental isolate isolated from coir retting ground of Chellanum, kerala, India. The organism was found to be potent producer of relevant industrial enzymes such as β-1,3 glucanase (Jose *et al.,* 2014) LasA protease (Jose *et al.,* 2017) and LasB proteases (Jose *et al.,* 2017). However, assessment of pathogenicity of this enzyme producer strain is essential for its industrial survival. Therefore, an evaluation has been made on the pathogenicity of this bacterium for its industrial acceptance.

Materials and Methods

Identification of bacterial isolate

The bacterial isolate was identified by phenotypic characterization followed by molecular characterization by way of 16S rRNA gene sequencing. Amplification of 16S rRNA gene was performed using universal primers 16 S1 (GAG TTT GAT CCT GGC TCA) and 16 S2 (ACG GCT ACC TTG TTA CGA CTT). The amplified PCR product of 16S rRNA was purified using QIAEX II gel purification kit (Qiagen) and was used for cloning into pGEM-T Easy vector (Promega, USA) and sequenced using ABI PRISM 3700 Big Dye Sequencer at Microsynth AG, Switzerland.

Assessment of pathogenicity

Detection of type III toxin genes (*exoU* and *exoS*) by PCR

Genomic DNA of the bacterium was extracted by phenol-chlorofom method (Sambrook and Russell, 2001) as described above. PCR amplification of *exoU* and *exoS* gene was carried out using the primers as described by Zhu *et al.,* (2006). A 428-bp fragment of the *exoU* gene was amplified using primers *exoU*

(F) (5´-GGG AAT ACT TTC CGG GAA GTT-3´) and *exoU* (R) (5´ -CGA TCT CGC TGC TAA TGT GTT-3´). A 1352-bp fragment of the *exoS* gene was amplified using primers *exoS* (F) (5´-ATC GCT TCA GCA GAG TCC GTC-3´) and *exoS* (R) (5´ -CAG GCC AGA TCA AGG CCG CGC-3´).

Reaction mixture (final volume 25 µl) contained 2.5 µl 10 X buffer, 1 µl 10 pmol each of oligonucleotide primer, 1µl DNA template, 2.5 µl 2.5 mM each deoxynucleoside triphosphate, 1 µl Taq polymerase, and the remaining volume made up with sterile Milli Q water. The amplification profile consisted of initial denaturation at 94°C for 2 min followed by 30 cycles of annealing of primers for 30s at 59°C for *exoU* and 68°C for *exoS* and primer extension at 72°C for 1.5 mi. The PCR product was separated on 1 % agarose gel.

Swimming motility

Swimming motility was done according to the method of Deligianni *et al.,* (2010). Swim plates were prepared by using 1% tryptone, 0.5 NaCl, 0.3 % (w/v) agar. The plates were inoculated with the bacterium using a sterile tooth pick and incubated overnight at 37°C. The ability to swim was assessed by the radius of the colony. The swimming zones were measured after 48 h incubation at 37°C. The experiments were conducted in triplicates.

Swarming motility

Swarming motility was done according to Deligianni *et al.,* (2010). The medium used consisted of 0.5% nutrient broth, 0.5 % glucose and 0.5 % agar.

Plates were inoculated with a 5 µl aliquot from an overnight culture of the bacterium in LB broth on the top of the agar and incubated at 37°C for 48 h.

Twitching motility

Twitching motility was done according to Head and Yu (2004). Freshly prepared and briefly dried twitch plates (Tryptic soy broth solidified with 1% (w/v) Difco granulated agar) were stab inoculated with a sharp toothpick into the bottom of the Petri dish.

After incubation at 37°C for 24 h, the agar was removed from the twitching activity plate and the plate was stained with 0.25% (w/v) Commassie blue for 30 minutes. The stain was removed and the twitching activity was measured in centimetres.

Ability to form Biofilm

Biofilm assay was performed according to Head and Yu (2004). Overnight cultures of the bacterium was diluted 1:100 in fresh LB medium, dispensed 125 µl to the wells of a 96-well micro titre plate and grown for 15 h at 37°C without aeration.

After incubation, the wells were stained with 100 µl of 0.25 % crystal violet for 30 min at 25ºC. Stain was discarded and the plate was rinsed three to five times in standing water and allowed to dry. Stained biofilm was solubilised with 200 µl of 95 % ethanol for 10 min and the optical density was read at 570 nm. Assays were done triplicates. A suitable control (LB medium without inoculation) was also kept.

Biofilms were classified according to the method of Stepanovic et al., (2000). When there was no increase of optical density over control, it was considered as a non-biofilm producer. Meanwhile, up to a 2-fold increase in optical density was considered as a weak producer, up to 4-fold increase in optical density as moderate producer and greater than 4-fold increase in optical density as strong producer.

Adhesion and invasion assay on Hep-2 and HeLa monolayers

Adherence and invasion assays were carried out by the modified method of Prasad et al., (1996). An 18 h old culture of the bacterium was centrifuged at 10,000g for 15 min at 4°C. The bacterial pellet was re-suspended in MEM with 10% FBS and the inoculum was adjusted to 10^7 CFU/ml.

Hep-2 and HeLa cells were grown to confluence in 24-well plate using Minimal Essential Medium (MEM) containing 10% FBS at 37ºC in 95% air and 5% CO_2. The monolayers were inoculated with 200µl bacterial suspension in triplicate wells. In to the other set of triplicate wells, gentamicin (200µg ml^{-1}) was added. The plate was incubated for 3 h at 37ºC in 95% air and 5% CO_2. In to the first set of triplicate wells antibiotic was not added in order to enumerate the number of bacteria that have adhered and invaded the cell lines. All the wells were washed with sterile MEM with 10% FBS to remove un-adhered bacterial cells. The monolayers were lysed with 0.01% Triton X-100.

The lysed monolayer suspensions were then serially diluted (10^{-1} to 10^{-6}) and 100 µl of each suspension were plated on LB agar and incubated at 37°C for 24 h. The viable count was determined as colony forming units (CFU) on the plates multiplied by its dilution factor. Viable bacteria recovered from the wells with gentamicin were considered intracellular (those which invaded the cells) and bacteria recovered from the wells without gentamicin were considered as the extracellular + intracellular. The adherence was calculated by the formulae: (CFU/ml from well without gentamicin at particular dilution - CFU/ml from the well with gentamicin at the same dilution) × dilution factor). The assays were carried out in triplicates.

Antibiotic susceptibility testing

Antimicrobial susceptibility of the bacterium was determined according to the modified disk diffusion method of Zhu *et al.,* (2006). The bacterial culture was adjusted to 10^7 CFU/ml (equivalent to a 0.5 McFarland standard) and was plated onto Muller-Hinton agar. The plates were allowed to dry for 5 min and not more than 6 antibiotic discs (Himedia) were applied on each agar plate and were then incubated at 37°C for 18 h. The zone of bacterial inhibition was examined by measuring the annular radius after incubation.

The organism was considered as susceptible, reduced susceptible or resistant to a particular antibiotic on the basis of the diameters of the inhibition zones that matched the criteria of the manufacturer's interpretive table, which followed the recommendations of the Clinical and Laboratory Standards Institute (CLSI, 2007).

Results and Discussion

Identification of the bacterial isolate

Phenotypic identification

The cells appeared as Gram negative short rods. The isolate was motile, oxidase positive, produced diffusible greenish pyocyanin pigment, have denitrification activity and capable of growing at 41°C. Moreover, *Pseudomonas* MCCB 123 produced acid from xylose and mannose and was positive for the utilization of glucose and sucrose as a sole carbon source for its growth.

It was positive for gelatin, starch, casein hydrolysis. The characteristics considered have been detailed in Table 1. The phenotypic characteristics were suggestive of the bacterium as *Pseudomonas aeruginosa,* according to Baumann and Schubert (1984).

Molecular identification by 16S rRNA gene sequencing

DNA amplified using universal primers of 16S rRNA gene primers, cloned into pGEM–T easy Vector partially sequenced using T7 and SP6 vector primers. When the sequence was compared with the GenBank data base using available from NCBI (www.ncbi.nlm. nih.gov), it showed 100% query coverage with 16SrRNA gene sequence of *Pseudomonas aeruginosa*. The sequence is deposited in the GenBank with the accession number FJ665510.

Assessment of pathogenicity of *P.aeruginosa* MCCB 123

Analysis of type III secretion toxin genes

Analysis of the presence of type III secretion toxin encoding genes revealed that *P. aeruginosa* MCCB 123 did not harbour *exoU* gene, while a positive amplification with an amplicon of 1352 bp was obtained for *exoS* gene (Fig. 1).

Motility assays

P. aeruginosa MCCB 123 exhibited swimming and swarming motilities with distance of the migration of 1.46 ±0.05 cm and 1.53 ±0.11 cm radius, respectively, from the inoculation point. The twitching motility was distinguished by the presence of twitch zone formed by colony expansion with a zone diameter of 3.4 ±0.17cm. The results are shown in Table 2.

Biofilm formation

P. aeruginosa MCCB 123 is considered as a moderate biofilm producer with 3.10±0.52-fold increase in the optical density when compared to control (LB medium without inoculation) at Abs_{570} (Table 3).

Table.1 Phenotypic characterisation of *Pseudomonas aeruginosa* MCCB 123

Tests	Results
Motility	motile
Grams stain	gram negative rods
Oxidase	positive
Citrate utilization	positive
Agrinine dihydrolase	positive
Denitrification	negative
Starch hydrolysis	positive
Gelatin hydrolysis	Positive
Casein hydrolysis	positive
Lecithinase	positive
Production of pyocyanin	positive
Growth at 41°C	positive
Utilisation of:	
Glucose	positive
Trehalose	negative
Sucrose	positive
Acid production from:	
D(-) Ribose	negative
D(-) Xylose	positive
L(+) Arabinose	negative
D(-)Sorbitol	negative
Mannose	positive
Rhamnose	negative
Lactose	negative
Sucrose	negative
Trehalose	negative
Adonitol	negative

Table.2 Motility assays of *Pseudomonas aeruginosa* MCCB 123

Type of motility	Distance migrated (cm)
Swimming motility	1.46 ± 0.05
Swarming motility	1.53 ± 0.11
Twitching motility	3.4 ± 0.17

Table.3 Biofilm assay of *Pseudomonas aeruginosa* MCCB 123

| Test Abs $_{570}$	Control Abs$_{570}$	Test Abs $_{570}$/ Control Abs$_{570}$
1.72 ± 0.28	0.55 ± 0.003	3.10 ± 0.52

Table.4 Adherence and Invasion assay of *Pseudomonas aeruginosa* MCCB 123 on Hep-2 and HeLa cell lines

Adherence assay		Invasion assay	
Hep-2	HeLa	Hep-2	HeLa
0 (CFU/ml)	0 (CFU/ml)	0 (CFU/ml)	0(CFU/ml)

Table.5 Antibiogram of *Pseudomonas aeruginosa* MCCB 123

Antibiotics	Disk content	Zone diameter	Inference
Pencillins			
Carbenicillin	100µg	23	S
Piperacillin	100µg	15	R
Ticarcillin	75 µg	16	S
Cephalosporins			
Ceftazidime	30 µg	21	S
Cefoperazone	75 µg	21	S
Cefotaxime	30 µg	21	I
Ceftizoxime	30 µg	15	I
Carbapenems			
Imipenem	10 µg	32	S
Polymyxin			
Colistin	10 µg	11	S
Aminoglycosides			
Gentamicin	10 µg	21	S
Amikacin	10 µg	17	S
Tobramycin	10µg	22	S
Netilmicin	30 µg	16	S
Fluroquinolones			
Lomefloxacin	10µg	28	S
Ofloxacin	5µg	28	S
Norfloxacin	10 µg	24	S
S:Sensitive	R: Resistant	I:Intermediate	

Fig.1 Analysis of type III secretory toxin genes of *P. aeruginosa* MCCB 123. Lane1, 1 kb DNA ladder; lane 2, shows the absence of exoU gene of the *P. aeruginosa* MCCB 123, lane 3, amplification of 1352 bp of exoS gene of *P. aeruginosa* MCCB 123
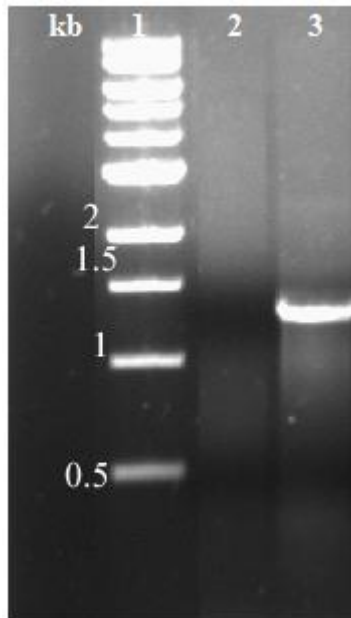
Adhesion and Invasion assay on epithelial cell lines

The capability of *P. aeruginosa* MCCB 123 to invade and adhere human epithelial cell lines (Hep-2 and HeLa) was assessed by gentamicin survival assays and the strain *P. aeruginosa* MCCB 123 was found to be non-adherent and also is not capable of invading into the human epithelial cell lines (Table 4).

Antibiogram

The susceptibility of *P. aeruginosa* MCCB 123 to 16 antibiotics belonging to six categories such as pencillins, cephems, carbapenems, lipopeptides, aminoglycosides and fluroquinolones was examined and listed in Table 5. Out of the 16 antibiotics belonging to six classes, the strain was sensitive to 13 antibiotics, resistant to one antibiotic (piperacillin) and intermediately sensitive to two antibiotics (cefotaxime and ceftizoxime). Confirmation of the pathogenicity of the

producer isolate is important for its industrial acceptance. Pathogenicity of *P. aeruginosa* MCCB 123 was assessed by a panel of virulence assays like the presence of type III secretion toxin genes, motility assays, ability to form biofilm, adhesion and invasion on human epithelial cell lines and antibiotic resistance profiles.

One of the virulence determinants of *P. aeruginosa* is the presence of type III secretion toxin genes such as *exoU, exoS, exo T* and exoY (Zhu *et al.,* 2006). Analysis of the important type III toxin encoding genes, *exoU* and *exoS* can confirm the cytotoxic and invasiveness of *P. aeruginosa* (Choy *et al.,* 2008). Those strains that harbour *exoU* gene are considered as cytotoxic phenotype and those that harbour *exoS* are considered as invasive phenotype, and strains that neither harbour *exoU* and *exo*S genes are considered as neither cytotoxic nor invasive (Zhu *et al.,* 2006). The cytotoxicity of the non-invasive strains of *P. aeruginosa* are mainly due to the

action of *exoU* gene (Hauser *et al.,* 2002). Secretion of *exoU* has been regarded as a marker for high virulent strains of *P. aeruginosa* isolated from the infection, but *exoS* was not consistently associated with increased virulence (Schulert *et al.,* 2003). The reason for *exoS* positive strains being associated with lower virulence may be attributed to the poor expression of *exoS* phenotypes in these isolates and such isolates are phenotypically classified as "neither invasive or nor cytotoxic" (Zhu *et al.,* 2002). Analysis of type III toxin genes in *P. aeruginosa* MCCB 123 showed the presence of *exoS* gene, while *exoU* gene, a major contributor to the potential pathogenesis of *P. aeruginosa* (Lin H-H *et al.,* 2006) was not detected. *exoU* has been previously shown to play a major role in mediating a cytotoxic phenotype of *P. aeruginosa* against lung epithelial cells and HeLa cells (Zaborina *et al.,* 2006). The absence of *exoU* gene in *P. aeruginosa* MCCB 123 confirms the non-cytotoxic phenotype on the strain, while the presence of *exoS* gene shows the invasive phenotype. Even though, *P. aeruginosa* MCCB 123 carry the gene for invasive phenotype (*exoS* gene), it is neither capable of adhesion nor invasion on both the epithelial cell lines tested. This may be due to the absence of effector protein (*exoS*) responsible for invasion. The presence of the *exoS* gene and the absence of *exoU* gene in *P. aeruginosa* MCCB 123 suggest the genetic differences between the environmental isolate and the other clinical isolates (Kaszab *et al.,* 2011). Zaborina *et al.,* (2006) demonstrated that most of the multi-drug resistant clinical isolates of *P. aeruginosa* with barrier disruptive phenotypes harboured *exoU* gene and displayed cytotoxicity against Caco-2 monolayers. However, clinical isolates that harboured *exoS* gene were not cytotoxic to Caco-2 cells. Fleiszig *et al.,* (1997), screened clinical isolates of *P. aeruginosa* for their ability to invade into corneal epithelial cells

of mice. After 1 h of invasion assay, there were no significant differences in the invasion among the isolates, but following a 3 h of infection, *P. aeruginosa* could be differentiated into invasive and cytotoxic strains and suggested that invasion was inversely correlated with cytotoxicity. Fleiszig *et al.,* (1997), tested the invasion of *P. aeruginosa* strains on polarized MDCK cells and found low levels of invasion for both cytotoxic and invasive strains at 1h. However, at 3 h of infection, the percentage of associated bacteria invaded had increased approximately 4 to 9-fold for the invasive isolates but decreased 13- to 15-fold for the cytotoxic isolates. In addition, the total number of associated bacteria for the cytotoxic isolates increased 6 to 8-fold, while there was little to no increase for invasive isolates. However, in case of *P. aeruginosa* MCCB 123 used in the present study, even after 3 hrs of incubation on both the monolayers (Hep-2 and HeLa), the strain was unable to adhere to or invade the cell line and thus the organism could be considered as a phenotype which was neither adhesive (cytotoxic) nor invasive agreeing with Zhu *et al.,* (2006), who stated that there were phenotypes of *P. aeruginosa* that could be considered as neither cytotoxic nor invasive.

Common features of *P. aeruginosa* with high destructive capability on intestinal cell lines include high swimming motility, increased adhesiveness and the presence of *exoU* gene (Zaborina *et al.,* 2006). The non-adhesive nature and the absence of *exoU* gene indicated the non-pathogenic nature of *P. aeruginosa* MCCB 123. However, the strain exhibited three types of motilities such as swimming, swarming and twitching motilities and proved to be a moderate biofilm producer with a 3.10 ± 0.52-fold increase in the optical density when compared to control (medium without inoculation) at Abs_{570}. Biofilms exhibits increased resistance to antimicrobial agents

due to the production of extracellular polymeric substances, presence of high concentration of β-lactamases, slower metabolic rates of the cells due to nutrient limitation and the presence of persistent cells (Deligianni *et al.,* 2010). The characteristic property of bacterial biofilm is their remarkable resistance to antibiotics. The overall resistance depends upon the entire population of cells and therapy needs to be directed against a multicellular community (Stewart and Costerton, 2001).

Environmental isolates of *P.aeruginosa* are more susceptible to antibiotics when compared to their clinical counterparts (Ruiz *et al.,* 2004). The antibiogram of *P. aeruginosa* MCCB 123 shows that the strain is sensitive to most of the antibiotics tested belonging to various classes, pencillins (carbenicillin, ticarcillin), cephems (ceftazidime, cefoperazone), carbapenems (imipenem), lipopeptides (colistin), aminoglycosides (gentamicin, amikacin, tobramycin, netilmicin), fluroquinolones (lomefloxacin, ofloxacin, norfloxacin), showed intermediate sensitivity to cefotaxime and ceftizoxime which belong to the class of cephems and is resistant to piperacillin which belongs to the class of penicillin. Antibiotics commonly used in the treatment of *P.aeruginosa* infection belong to the classes such as pencillins, cephalosporins, aminoglycoside, fluroquinolones, polymixin and carbapenems (Hancock and Speert, 2000).

The results of antibiogram of *P. aeruginosa* MCCB 123 suggested the possibility of antibiotic treatment as an effective method for eradication of its biofilm. The sensitivity of *P.aeruginosa* MCCB 123 to both ceftazidime and cefoperazone suggests that these can be used as a single agent against *P. aeruginosa* (Hancock and Speert, 2000). Gentamicin is usually effective against non-clinical

environmental isolates of *P. aeruginosa* (Tripathy *et al.,* 2007), while the pathogenic strains from clinical environment from cystic fibrosis patients were found to be resistant to this antibiotic (Deredjian *et al.,* 2011). *P. aeruginosa* MCCB 123 showed sensitivity to gentamicin. Broad spectrum fluoroquinolones are used for the treatment of *Pseudomonas* keratitis (O'Brien *et al.,* 1995 and Bower *et al.,* 1996), fluoroquinolones and aminoglycosides for the treatment of endophthalmitis (Elder and Morlet, 2002). Fluroquinolones were also reported to be effective drugs for non-clinical (environmental isolates) of *P. aeruginosa* (Kaszab *et al.,* 2011 and Silva *et al.,* 2008). *P. aeruginosa* MCCB 123, being an environmental isolate, was sensitive to all the fluroquinolones tested. The organism also showed intermediate sensitivity to third generation cephalosporins such as cefotaxime and ceftazidime.

Environmental isolates of *P. aeruginosa* from compost is also reported to be resistant to third generation cephalosporins such as cefotaxime and ceftazidime (Kaszab *et al.,* 2011). Further, the pathogenic clinical strains of *P. aeruginosa* were reported to be resistant to various classes of antibiotics belonging to the classes such as aminoglycosides, carbapenems, cephalosporins (Brown and Izundu, 2004). However, *P. aeruginosa* MCCB 123 is sensitive to the tested antibiotics belonging to the above said classes indicating that the organism is controllable by antibiotics.

The isolate was found to exhibit non-cytotoxic characteristics based on invasion and adhesion assays on human epithelial monolayers. Its sensitivity to antibiotics indicated that it could be controllable by antibiotic therapy. The high level of proteolytic activity of the bacterium suggested its suitability in industrial applications.

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