Pakistan J. Zool., vol. 48(6), pp. 1769-1774, 2016.

Cloning and Expression of p40 Gene Isolated from Probiotic Bacteria of Dairy Origin

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ABSTRACT

Probiotic bacteria promote gut health by producing beneficial factors. Amongst those factor, *p40* (putative secreted protein) which is a 40 kDa protein; has been found to be effective in inhibiting the cytokine induced apoptosis in intestinal epithelial cells and promote cell survival and proliferation. In this study different probiotic bacteria were isolated from cheese, yoghurt and probiotic sachet. The isolates were characterized, identified and screened for the presence of *p40* gene by using gene specific primers. The isolated strains were identified as *Enterococcus faecium*, *Lactobacillus casei*, *Lactobacillus paracasei* and *Lactobacillus rhamnosus* by biochemical and 16s rRNA sequence analysis. The *p40* gene was found in *Lactobacillus rhamnosus* amongst isolated strains. Successful cloning and expression of *p40* was done in *Escherichia coli* (BL-21 strain). Probiotics are routinely used to boost gut health and due to the safety concerns associated with the use of live probiotic bacterial administration, purified *p40* could be used as an alternative to protect gastro-intestinal (GI) disorders.

Article Information

Received 31 August 2015
Revised 25 February 2016
Accepted 23 April 2016
Available online 25 September 2016

Authors' Contribution

SR, MSA and ZA designed the study and analyzed the data. RM and MA performed the experimental work. AH wrote the article. ZA supervised the work.

Key words

Escherichia coli,
Probiotics**,**
p40 protein
Lactic Acid Bacteria
Lactobacillus rhamnosus

INTRODUCTION

The word "probiotic" originated from Greek words 'pro bios' which means 'for life' opposite to 'antibiotics' which means 'against life'. Probiotics are defined as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (FAO/WHO, 2002). Strains of lactic acid bacteria (LAB) including species of *Lactobacillus, Lactococcus, Enterococcus,* and *Bifidobacterium* are usually used as probiotics (Motta *et al.*, 1991; Klein, 2003).

Probiotics are live bacteria that can resist the rigors of the human digestive system, compete with pathogens, and help to improve the gut flora balance and has been used for long times in food ingredients of human and animals (Alvarez-Olmos and Oberhelman, 2001). The probiotics, colonized in intestine produce a number of beneficial health effects (Bengmark, 2000; Benchimol and Mack, 2004; Brown and Valiere, 2004).

The usage of live bacteria is never completely safe (Wassenaar and Klein, 2003) and secondly bioavailability of bacteria in GI tract is difficult to determine (Yan *et al.*, 2007). Live probiotic strains administration to the patients of acute pancreatitis is associated with the high mortality rate in those patients (Besselink *et al.*, 2008). One alternative to avoid the safety concerns associated with the administration of live probiotic bacteria is to identify and purify therapeutically useful proteins of probiotics. Therapeutic use of the purified proteins can avoid the risks associated with the usage of live probiotic strains.

Bacterial *p40* is a soluble protein normally secreted by *Lactobacillus rhamnosus*. It is involved in the activation of Akt, inhibition of epithelial cell apoptosis, cellular growth promotion of epithelial cells and prevention of cytokine-mediated gastrointestinal injury and disease through specific signaling pathway (Yan *et al.*, 2007). In this study we isolated probiotic bacteria from different dairy products of Pakistan and their probiotic properties have been characterized. The isolates were identified and screened for *p40* gene followed by the expression of *p40* gene in *E. coli*. Purified *p40* could be used as an alternative to probiotic bacteria to protect GI tract and disorders related to it.

MATERIALS AND METHODS

Isolation and preliminary identification

Probiotic bacteria were isolated form cheese and yoghurt using lactobacillus MRS agar (Oxoid) under anaerobic conditions. Selected colonies were streaked on MRS agar to obtain a pure culture. Isolated strains were initially examined by Gram staining and catalase test (Aslam and Qazi, 2010).

Biochemical characterization

Gram positive and catalase negative strains were

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selected. The isolates were characterized by biochemical tests of gas production, temperature tolerance (10°C, 40°C and 45°C), NaCl tolerance (2%, 4% and 6.5% NaCl), acid tolerance (2, 3 and 7 Ph), bile salt tolerance (0%, 0.2%, and 0.4% bile salt), antibiotics resistance (Ampicillin, Chloramphenicol, Tetracycline, Streptomycin and Gentamycin), gelatinase activity, arginine hydrolysis test (L-arginine hydrochloride), haemolysis activity, antimicrobial activity and fermentation of sugars (glucose, ribose, sucrose, lactose, galactose, and maltose). This biochemical characterization was performed as described by Yavuz Durmaz (2007).

Amplification and sequencing of 16SrRNA gene

Genomic DNA was isolated from selected probiotic strains using DNA isolation kit (Invitrogen, USA). Polymerase Chain Reaction (PCR) for amplification of *16SrRNA* gene was performed using primers RS1 (forward)
5'AAACTCAAATGAATTGACGG 3'and RS3 (reverse)
5'-ACGGGCGGTGTGTAC-3'
with PCR thermo-cycler (BIOER XP Cycler) programmed as initial denaturation of DNA for 5 min at 94°C, 35 cycles of 1 min at 94°C, 45sec at 55°C, and 1 min at 72°C and final extension for 5 min at 72°C. *16S rRNA* PCR product was visualized on 1% agarose gel in 0.5X Tri acetate buffer. PCR products obtained were purified using PCR purification kit. DNA sequencing was done with the help of Centre for Applied Molecular Biology (CAMB), Lahore, Pakistan on DNA sequencing system by Dideoxy chain terminaton DNA sequencing method. Identification of the sequenced strains was checked by NCBI BLAST (Basic Local Alignment Search Tool).

Cloning and expression of p40 gene

DNA from isolated strains was used as template for amplification of *p40* gene using *p40* specific forward primer
(5'-GCATATGAAATTCAATAAAGCAATGATGAC- 3') and reverse primer
(5'-CTCGAGTTACCGGTGGATGTAAAC-3').
PCR thermocycler was programmed as initial denaturation of DNA for 3 min. at 94°C, 35 cycles of 45 sec at 94°C, 50 sec at 58.2°C, and 1.5 min at 72°C and final extension for 5 min at 72°C. The *p40* gene PCR product was visualized on 1% agarose gel. The PCR product was purified by invitrogen gel extraction kit method. The 3'-dA overhang portion of PCR fragment of *p40* gene was ligated in pTZ57R/T vector (Fermentas) for making the recombinant DNA vector. The gene fragment was ligated into pTZ57R/T cloning vector. The recombinant vector was transformed into DH5α competent cells by following Sambrook and Russell (2001). The transformed white colonies were further characterized by colony PCR, plasmid PCR and restriction analysis using Nde 1 and Xho 1 restriction enzymes. For expression of *p40* gene, it was subcloned in pET-28a (+) (Novagen, Germany) expression vector (5,369 bp) containing the kanamycin resistant gene as a marker. The chemical competent cells of expression host *E. coli* strain BL21-codon plus were transformed independently with pET-28a-*p40* expression vectors. The BL21-codon plus strains were maintained on Lauri-Bertani agar plates at 37°C. The BL21-codon plus transformants containing the pET-28a-*p40* expression vectors were confirmed by colony PCR. The *p40* gene was expressed in pET vectors under T7 lac promoter by using isopropyl β-D-thiogalactoside (IPTG) as inducer. Various concentrations of IPTG were used to optimize the expression of protein. Moreover, incubation time was varied with different IPTG and lactose concentrations to get the optimum expression. The expression level of recombinant protein at different IPTG concentrations and time interval was checked by electrophoretic pattern on 10% SDS-PAGE.

RESULTS

Biochemical characteristics of probiotic strains

Eight probiotic bacteria were isolated from cheese, yoghurt and probiotic sachets. Primary screening of probiotic isolates was done by selecting gram positive and catalase negative strains (Table I). RM-3 was ruled out as it was catalase positive. Biochemical analysis revealed that the probiotic isolates belongs to *Lactobacillus* and *Enterococcus* genera (Table II).

Table I.- Preliminary screening of isolated probiotic strains

Sample source	Strain isolated	Gram nature	Catalase test
Cheese	RM-1	Gram positive cocci	Negative
Cheese	RM-2	Gram positive cocci	Negative
Probiotic sachet	RM-4	Gram positive cocci	Negative
Probiotic sachet	RM-5	Gram positive cocci	Negative
Probiotic sachet	RM-6	Gram positive rods	Negative
Yoghurt	RM-7	Gram positive rods	Negative
Probiotic sachet	RM-8	Gram positive rods	Negative

Molecular identification of the probiotic strains

16s rRNA PCR product of about 500 bp was obtained from all isolates and sequenced (Fig. 1). Sequencing analysis revealed the 16srRNA homology of RM-1, RM-2, RM-4 and RM-5 (Gene Bank Accession numbers KJ865849, KJ865850, KJ865851, KJ865852) with

Enterococcus faecium, RM-6 (KJ865853) *Lactobacillus casei*, RM-7 (KJ865854) *Lactobacillus paracasei* and RM-8 (KJ865853) *Lactobacillus rhamnosus.*

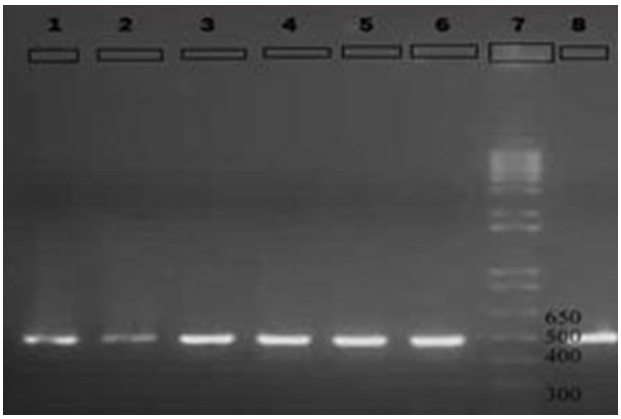

Fig. 1. Gel purified 16SrRNA gene of probiotic isolates. Well no. 7 is loaded with 1kb plus DNA ladder on 1% agarose gel. RM-1, RM-2, RM-4, RM-5, RM-6, RM-7 and RM8 were loaded into well no 1, 2, 3, 3, 5, 6 and 7, respectively.

Antimicrobial activity of probiotic isolates

RM-1 showed antimicrobial activity against *E. coli*, *S. aureus* and *Klebsiella.* RM-2 and RM-5 did not show inhibition against any pathogen while RM-4 showed inhibition of *E. coil.* RM-7 and RM-8 have high activity against *E. coli* while RM-8 also causes inhibition of *Klebsiella* growth. Only RM-6 produced zone of inhibition around MRSA as shown in Table III.

Table III.- Antimicrobial activity of probiotic isolates against Gram positive and Gram negative bacteria.

	E. coli	*S. aureus*	*Klebsiella*	*Pseudomonas*	*MRSA*
RM-1	8mm	9mm	10mm	--	--
RM-2	--	--	--	--	--
RM-4	9mm	--	--	--	--
RM-5	--	--	--	--	--
RM-6	--	--	--	--	7mm
RM-7	7mm	--	--	--	--
RM-8	10mm	--	11mm	--	--

*Antimicrobial activity is shown as zones of inhibition in millimeter (mm)

Tumor suppressor gene (p40) in isolated probiotics

Probiotic strains were screened for the presence of the tumor suppressor gene *p40* using gene specific primers. *P40* was not present in any of the strains except RM-8 *(Lactobacillus rhamnosus).* PCR product of about 1250bp was visualized as a band appearing between 1650 bp band and 1000bp band of 1kb plus DNA ladder (Fig. 2).

p40 gene was cloned into pTZ 57R/T and screened by colony PCR of white colonies (Fig. 3). Recombinant pTZ 57R/T-*p40* was restricted with *Nde*1 and *Xho*1and ligated into pET-28a and directly transformed to BL-21 (DE) with that ligation as shown in Figure 4. Induction was done using varying concentration of IPTG starting from 0.5 to 4.0 mM. Protein of 40 kDa is expressed and analyzed using SDS-PAGE as shown in Figure 5.

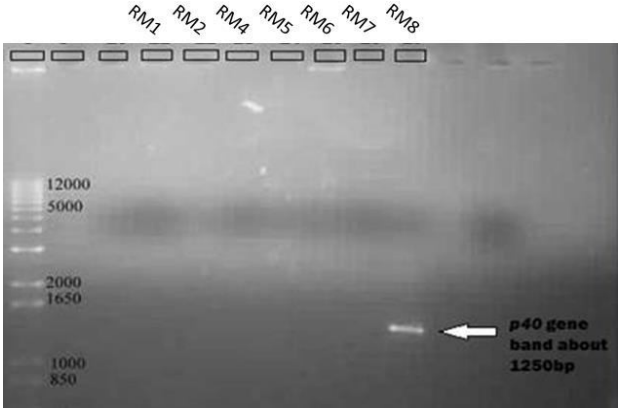

Fig. 2. Screening of probiotic strains for tumor suppressor *p40* gene. Well no. 1 is loaded with 1kb plus DNA ladder.

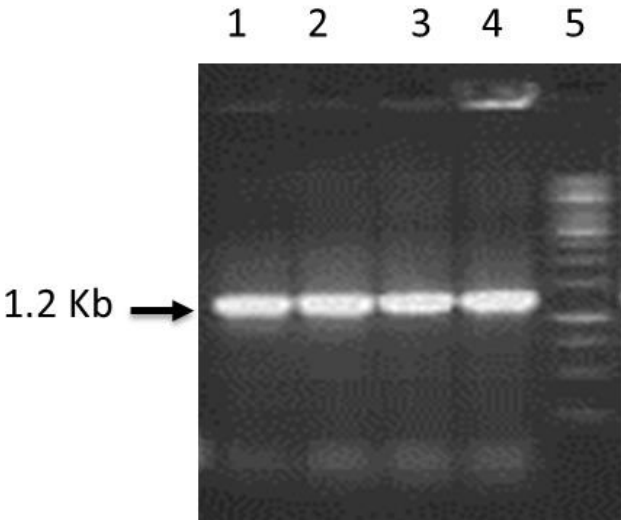

Fig. 3. Colony PCR of ptz-*p40* transformed DH5α cells. Colony PCR of white colonies of transformed DH5α was performed and PCR product was resolved on 1% agarose gel, Lane 1-4 shows PCR bands of white colonies:, Lane 5: marker.

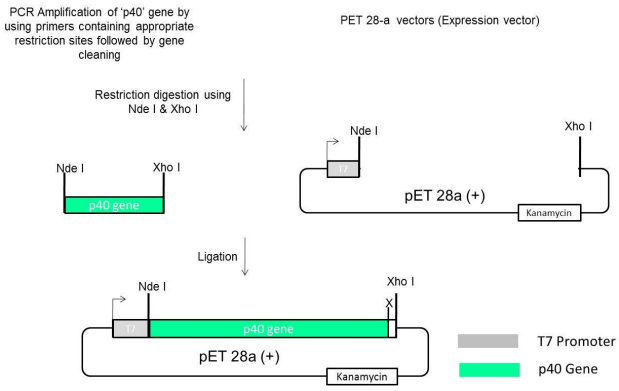

Fig. 4. Schematic Diagram to describe Subcloning of '*p40*' Gene: *p40* gene amplified by using primers containing restriction sites for NcoI in forward primer and XhoI in reverse primer. It was cloned into TA vector to form pTZ-*p40*. The recombinant vector and pET22 were treated with both restriction enzymes. Linearized products were extracted from gel and ligated. T7 shows the promoter and 'X' shows the stop codon.

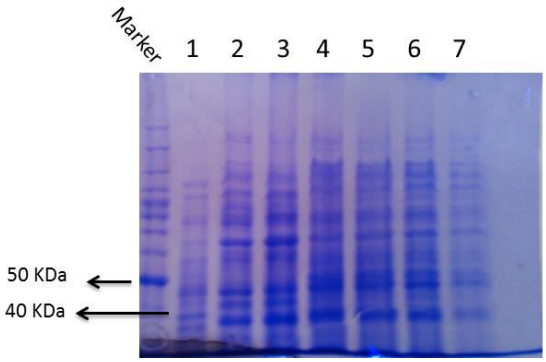

Fig. 5. Induction of *p40* using different concentrations of IPTG. *p40* gene was cloned into pET 28, expressed in BL21 and resolved on 10% SDS-PAGE, 1 un-induced, 2-7 shows *p40* expression induced at 0.5, 1.0, 1.5, 2.0, 3.0 and 4.0 mM IPTG concentrations, respectively.

DISCUSSION

Probiotics mainly include *Lactobacillus, Lactococcus, Enterococcus* and *Bifidobacteria.* Since ancients times probiotics are providing health benefits to humans by being functional part of fermented foods such as milk, cheese, kefir, sauerkraut, pickle, butter milk and other fermented products as well as of supplements containing probiotics in lyophilized or solid form. Due to this reason cheese, yoghurt and probiotic sachets were used as samples for isolation of probiotics.

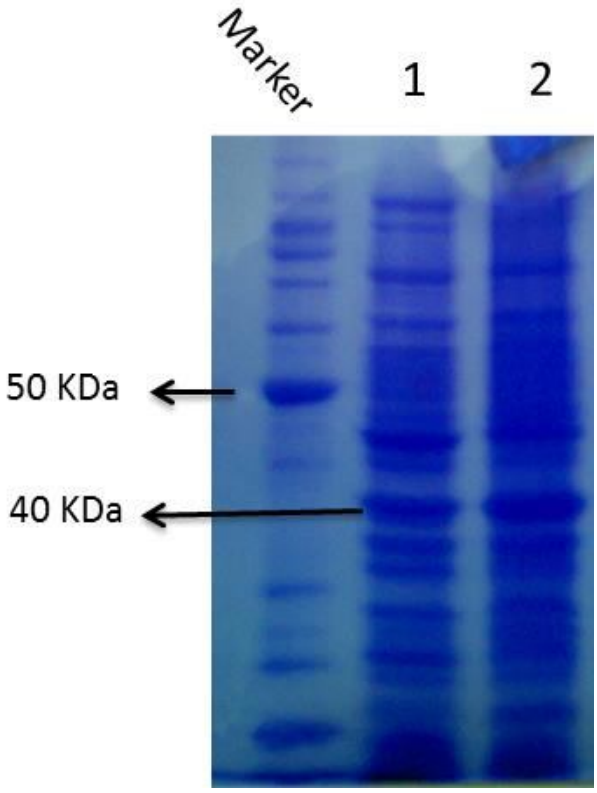

Fig. 6. Induction of *p40* using IPTG and lactose. *P40* gene was cloned into pET 28, expressed in BL21 and resolved on 10% SDS-PAGE, 1 1mM IPTG induced sample, 2 lactose induced.

Initially, eight strains were isolated and preliminary screening of isolated strains was done by Gram staining and catalase test. LAB are known to be Gram positive and catalase negative (Bhatt *et al.*, 2012). RM-3 was ruled out because it showed catalase positive test and catalase positive enzyme is an attribute of *Staphylococcus* genera (Cappuccino and Sherman, 2013).

Gastrointestinal tract (GIT) is associated with low pH, high bile salts and NaCl concentrations and these are effective barriers to the bacteria for their entry into GIT (Huang and Adams, 2004). As Probiotic bacteria are normal flora of GIT so the probiotic isolates were assessed for their potential to survive in low pH, high bile salts and NaCl. Isolated strains showed good survival at acidic conditions of pH 2 and pH 3 which indicates that their potential to survive in harsh acidic conditions. On passage through the intestinal tract, the next gastric barrier is the bile salts (Nueno-Palop and Narbad, 2011).

Table II.- Biochemical characterization and carbohydrate fermentation pattern of isolated probiotic strains.

Biochemical Test	RM-1	RM-2	RM-4	RM-5	RM-6	RM-7	RM-8
Production of gas from glucose	+	+	+	+	+	-	-
Acid Tolerance **	Very good	Very good	Very good	Good	Good	Very good	Good
Bile salt tolerance**	Excellent	Excellent	Excellent	Good	Poor	Poor	Very good
Gelatinase activity	-	-	+	-	-	+	+
Arginine hydrolysis	-	+	+	+	-	-	-
Haemolysis activity	-	-	-	-	-	-	-
Temperature tolerance							
Growth at 10°C	++*	++	++	++	--*	--	++
Growth at 40°C	++	++	++	++	++	++	++
Growth at 45°C	++	++	++	++	--	--	++
Salt Tolerance							
Growth in 2% NaCl	++	++	++	++	++	++	++
Growth in 4% NaCl	++	++	++	++	++	++	++
Growth in 6.5% NaCl	++	++	++	++	--	--	++

* shows experiment was done in duplicates

** Classification criteria included four arbitrary level of acid condition tolerance: excellent if the isolate survived at pH 2/ 0.4% after 24h; very good if the isolate survived at pH 2/ 0.405 after 6h but not after 24h; good if the isolate survived at pH 3/ 0.2% after 24h but not at pH2; poor if the isolate did not survive in any experimental condition. An isolate survived if it demonstrated a surviving percentage equal or greater than 50%.

+ shows Positive results

++ shows strongly positive results

-- Negative results

Isolated strains showed a good degree of survival at 0.2% and 0.4% bile salt concentration which demonstrates their ability to withstand intestinal conditions.

One of the important characteristic of probiotic bacteria is the production of antimicrobial compounds such as bacteriocin, pediocin, lactacin, enterocin and acids. These antimicrobial compounds help in colonization of probiotic bacteria and adherence to epithelium by washing off pathogenic bacteria in gut and intestinal tract (Giraffa, 2003). Antimicrobial activity of the probiotic isolates was assessed against *E. coli, MRSA, Klebsiella* and *S.* RM-2 and RM-4 did not show inhibitory activity against pathogens. *E. coli* growth was inhibited significantly by RM-1, RM-4, RM-7 and RM-8 while RM-1 and RM-8 are effective against *Enterobactericeae* and *S. aureus* indicating their potential to inhibit enteric infections. None of the strain showed inhibition of *Peudomonas.* The importance of RM-7 is that it shows significant antimicrobial infection against *MRSA* which are leading cause of nosocomial infections. Our results are in line with previously reported data that lactic acid bacteria are more effective against Gram negative bacteria than Gram positive bacteria (De Kwaadsteniet *et al.*, 2005; Drider *et al.*, 2006). Tested strains neither showed gelatinase nor hemolysis activity which indicates that isolates strains are not virulent and can be used as probiotics (Barbosa *et al.*, 2010).

Sugar fermentation profiling indicated that RM-1, RM-2, RM-4 and RM-5 belong to the genus *Enterococcus* and RM-6, RM-7 and RM-8 species belong to the genus *Lactobacillus.* This was also confirmed by 16S rRNA gene sequences. RM-1, RM-2, RM-4 and RM-5 were identified as *Enterococcus faecium*, RM-6 as *Lactobacillus casei* and RM-7 as *Lactobacillus paracasei*. RM-8 was identified as *Lactobacillus rhamnosus*.

Lactobacillus rhamnosus confirmed by 16s rRNA sequencing was proceeded for *p40* gene amplification and its subsequent cloning and expression using pET-28a+ expression vector. The transformed bacteria were induced with various concentrations of IPTG to get the protein expression. It was noted that at higher concentration of IPTG the protein expression was significantly lower than that obtained using 1 mM concentration. Protein expression was optimized by varying the induction time and temperature and maximum expression was observed at 35^{o}C when incubated for 6 hours with 1 mM IPTG induction. In parallel experiments, *p40* recombinant clones were induced with lactose for different time periods and almost same amount of expression upon 8 hour incubation was found. In future purified *p40* could be assessed in-vitro and *in vivo* for its potential to suppress tumor and protect GI tract.

ACKNOWLEDGEMENT

This work was supported by a grant from the University of the Punjab, Lahore, Pakistan.

Statement of conflict of interest

Authors have declared no conflict of interest.

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