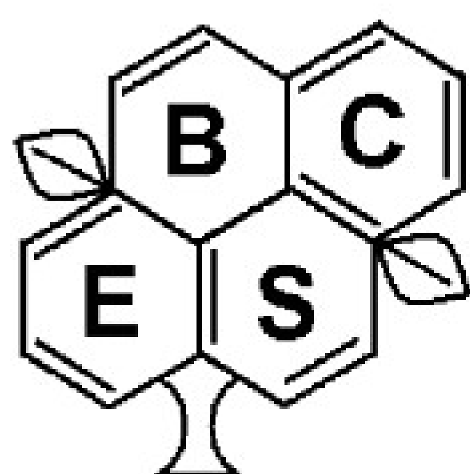

Journal

CLONING AND EXPRESSION OF CHITINASE-B ENCODING GENE (*CHIB*) OF A HIGHLY CHITINOLYTIC SELECTED FROM EGYPTIAN *SERRATIA MARCESCENS*

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ABSTRACT

Serratia marcescens exhibits three unique chitinolytic proteins *ChiA*, *ChiB* and *ChiC* represent bacteria chitinolytic machinery of chitin complete degradation. In this situation, different strategies were adopted to utilize *Serratia* chitinase-encoding gene(s). For acheving of bio-safety issues, the most common procedure is cloning a proper gene in different environmentally accepted organism. In this concern, intermediate steps i.e,isolates evaluation, gene isolation, plasmid construct, bacteria transformation and gene expression are routine work for this purpose. This study describes primer design to amplify chitinase-B encoding gene of highly chitinolytic selected isolate among some Egyptian local isolates of *Serratia marcescens*. Thenafter, cloning this gene in Jm109 *E. coli* strain thorough plasmid constructing (pBK-CMV). The designed primer succeeded to amplify chitinase-B encoding gene of all isolates with variable efficiency indicating different degrees of primer mismatching. This observation suggested differences between standard published sequences and such ones of Egyptian *Serratia marcescens.* A high cloning and transformation efficiency as well as chitinase expression were achieved in Jm109 *E. coli.*

Keywords: *Serratia marcescen,* Chitinas-B, encoding gene, Chitin, transformation, *E.coli,* Plasmid construction,

INTRODUCTION

Chitin, a polymer of N-acetylglucosamine (NAG), represents a major structural component of many agronomically important pests including insects, fungi, and nematodes Bird and McClure (1976). The enzymatic digestion or deformation of the chitin component of these organisms could present an effective method for their control. Microorganisms produce chitinases to digest chitinous nutrient or to partially hydrolyze chitinous cell wall for cell proliferation (Flak et al, 1992). Furthermore, the production and delivery of chitinase to the specific site of infectivity by appropriate rhizoplane- or phyloplane-colonizing bacteria could present a novel method for biological control. Therefore, chitinases may be exploited for the control of fungal and insect pathogens of plants (Aalten *et al*., 2000).

Chitinases are found primarily in two of glycohydrolases families, pfam18 and pfam19. Family 18 contains several runs of conserved amino acids, for the enzymes from *Coccidioides immitis*, *Trichoderma harziamum*, *Aphanocladuim album* and *Serratia marcescens.* Most of chitinases sources like insects, bacteria, human….etc, belong to family 18 and this family has two subfamily, subfamily A (includes both chitinase-A & chitinase-B) and subfamily B (includes chitinase-C). Family 19 contains primarily enzymes from plant sources, although there are some representatives from bacterial sources (Brurberg et al 1994).

This study describes (i) primer design for gene isolation (chitinase-B) based on aligned sequences embedded in different databases, (ii) cloning produced PCR proper fragment in suitable vector and (iii) transformation of *E. coli* with constructed plasmid containing chitinase-B corresponding sequence.

MATERIALS AND METHODS

I. Bacterial strains, media and culture conditions:

Ten local isolates of *Serratia marcescens* collected from different soil sources were characterized, evaluated and used as bacterial donor of chitinase-encoding gene. Jm109 *E. coli* commercial strain was used as transformation host (Promega Corporation). All bacterial strains were routinely maintained on LB media except for bacteria first isolation since nutrient agar was used . For chitinolytic screening a (1/10X) LB media supplemented with 0.5% colloidal (swollen) chitin was used.

II. Isolation of bacteria:

Different sources of soils was used for bacteria isolation. Firstly, variable numbers of isolated bacteria were plated on nutrient agar medium. Secondly, screening of bacteria isolates was carried out on both routine LB and LB-chitin media. Ten single purified colonies were adopted for further identification. The agar plate dilution method was carred out according to Thiery and Francon (1997). Bacterial colonies properties were determined and cells were observed under microscope after proper staining (gram sta.). Essential biochemical tests were carried out according to Collines and Lyne (1984). The bacterial isolates were identified according to Bergy's Manual (Farmer, 1984; Banjo et al, 2006).

III. Substrate preparation:

Swollen chitin was prepared according to the method described by Jeuniaux (1966) with some modifications. Twenty-five grams of chitin (Sigma) was suspended in 250 ml of 85% H_3P0_4 and stored at 4°C for 24 h. Swollen chitin was collided by adding 2 liters of deionized water to the mixture. The colloidal chitin was washed until the pH rose to 5.5. Then, few drops of sodium hydroxide (1N) were added to raise the pH to 7.0. This mixture was centrifuged at 8,000 x g for 10 min, the resulted pellets were resuspended in 1 liter of deionized water and then centrifugation step was repeated and the pelleted chitin was finally stored at 4°C until use. Pelleted swollen chitin was dried (approximately 4 days at 53°C) and then grinded in blender to obtain powdered substrate. LB chitin medium was prepared by adding 5 gram swollen chitin to tenth dilution of common LB concentration and for solid media 20 g bactoagar (DIFCO) were added.

IV. Plasmids and restriction nucleases:

Two plasmids were used throughout of this investigation. pGEMT easy vector (T/A cloning) purchased from Promega corp. was used for PCR product rescue and pBK-CMV phagemid vector purchased from Stratagene corporation was used for plasmid transformation and expression. For enzymatic manipulation of proper sequence and plasmid construction *EcoRI*, *HindIII* and *BamHI* restriction enzymes were used (Fermantas co.). For ligation and plasmid construct T4 DNA ligase (Vivantis co.) was used. To enhance

ligation efficiency a small quantity of ATP was added to the reaction mix and ligation approach was carried out in refrigerator overnight at 4°C (Sambrook et al., 1989).

V. Primer Design:

A set of six DNA sequences of Chitinase-B encoding genes was downloaded from gene bank *(http://www.ncbi.nlm.nih.gov)*. Table (1) summarizes gene sources and its accessions in gene bank. Thenafter, alignments of downloaded sequences were carried out using ClustalW software, public available at *www.ebi.ac.uk/clustalw/..*, (Thompson et al., 1994) to illustrate their identities and extract the consensus sequence of target gene. To design universal primer for gene isolation the six sequences were manipulated using Discovery Studio™ Gene (purchased from Accelrys Inc.). The obtained set of two primers for this purpose is:
5`ATA**GAATTC**-GAATTCAACATCCACTCTGGAGAAATGC3`
serves as forward primer and:
5`ATA**AAGCTT**-CCCGGGGAATAGAGACGAAGGGACGC3` as reverse one. Add-on nucleotides for restriction manipulation and plasmid cloning are bolded underlined characters for *EcoRI* and *HindIII* restriction sites; respectively.

Table1: Accession number of used sequences used in the present study.

Title	Accession no.
Serratia marcescens chitinase (chiB)	EU019912
Serratia marcescens chitinase B (chiB) gene,	DQ868535
Serratia marcescens chiB gene	X15208
S.marcescens (BJL200) chiB gene for chitinase	Z36295
Serratia marcescens gene for chitinase B,	AB015997
Serratia marcescens chitinase (chiB) gene,	AF454463

IV. Recombinant DNA techniques and PCR condition

PCR was performed by Techne 512 Themal Cycler using the followed program one cycle of 95°C for 5 min, 35 cycle of 95°C for 30 sec, 58°C for 30 sec and 72°C for 2 min, and finalization at 72°C for 10 min. PCR total volume was 10 µl containing 5 µl *Pfu* master

mix (2X), 4 µl of primer mixture (1 µM of each Primer-I and Primer-II) 1 µl of template DNA (50 nM f.c.). For large quantity of PCR product 1 µl of amplified gene was used as DNA template in 50 µl reaction total volume. To achieve TA cloning system for PCR product rescue in pGEMT plasmid a Taq 2X master mix (GentBio co.) was used and *Taq* DNA polymerase concentration was raised to 1.5U/50µl by *Pfu* enzyme. Chromosomal DNA isolation and plasmid DNA preparation was carried out by alkaline lyses method. Restriction enzyme digestion, ligation, CIAP treatment, cloning into pBK-CMV vectors and selection of recombinant colonies by IPTG and X-gal were carried out using standard techniques (Sambrook et al., 1989).

RESULTS AND DISCUSSION

Bacteria isolation and identification:

In the present study different sources of soils were used for isolation of genus *Serratia*. Depending on colonies characteristics, morphological specification and physiological properties a nine bacterial isolates were identified according to Bergey's Manual (1984). As summarized in Table (2), the obtained results demonstrate that the examined nine isolates were assumed to be *Serratia marcescens* (Farmer 1984 and Banjo et al., 2006).

Chitinase activity analysis of *S. marcescens*:

The well identified isolates were tested on LB-chitin agar plates. All isolates of *Serratia marcescens* were grown on LB chitin plate and incubated at 37 degree for 48 hour and chitinase positive colonies were selected according the diameters of clearing zone around colony (Gohel et al., 2005). Although the nine isolates hydrolyzed chitin (as clear zone), a variable lyses degrees was observed. Figure (1) shows comparable clear zones resulted in different diameters of chitin lyses. The resulted characteristics chitinolytic activities are given in Table (3) where four isolates out of the tested nine ones orderly gave the highest chitinase profile (isolates; S1, S3, S2 and S7). All chitinolytic isolates showed their abilities of lyses on plates within short period ranged from two to three days.

Table 2. Differential characteristics of the isolated (*Serratia*).

Characteristics &Biochemical tests		Isolates
Morphology		Short rods
Spores		-
Gram staining		-
Capsule		-
Motility		Motile
Pigments		Red undifusible
Oxygen requirements		Aerobic & facultatively anaerobic
Growth temp. (°C):	4	-
	30	+
	37	+
	41	-
	50	-
Growth pH:	4	±
	5.7	-
	6.8	±
	9.0	-
Growth in NaCl (%):	5	-
	7	-
	10	-
Gelatin hydrolysis		+
Starch hydrolysis		-
Glucose		+
Maltose		+
Manitol		+
Sucrose		+
Citrate		+
Voges Proskauers (VP)		+
Indol		-
Nitrate reduction		-

+: Positive reaction or Good growth, ±: Moderate growth, -: No growth and ND: Not detected.

Table (3). Diameter of clearing zone for *S. marcescens*

Strain	S1	S2	S3	S4	S5	S6	S7	S8	S9
Clearing zone mm	7	5.5	6.5	4	3.5	1	4.5	4	4

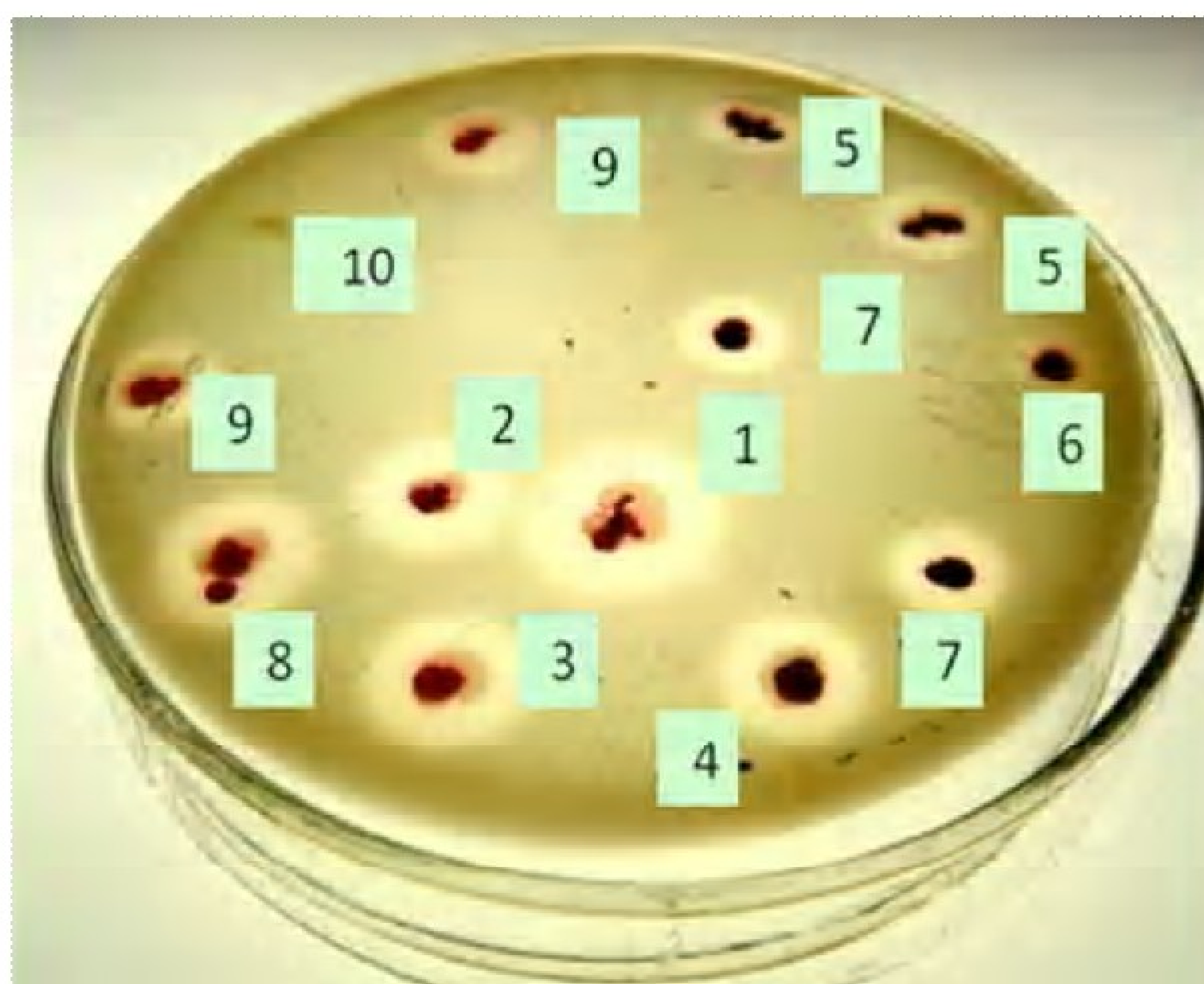

Figure (1) : The efficiency of the tested *S. marcescense* on hydrolysis chitin (express as clean zone m.m.)

Chitinase-B sequence analysis and primer design:

Sequences of chitinase-B encoding genes were collected to analyze the homology among published data to choose conserved domain which can be a site for primer design. The forward primer was ATAGAATTC-GAATTCAACATCCACTCTGGAGAAATGC and under lined bases are add-on *EcoRI* restriction site for cloning in pBK-CMV. The reverse primer was ATAAAGCTT-CCCGGGAATAGAGACGAAGGGACGC and *HindIII* restriction site for cloning is add-on underlined characters. The alignment of used sequences indicates a highly conserved sequence among downloaded sequences showing universality of target gene. As shown in Figure (2) the aligning result shows a conserved domain inside the open reading frame and few bases upstream the ORF (enough for primer binding). Figure (2, below) presented primer design resulted in common sequence except base number 2 from 3′ end (*A*, in the case of Z36295 sequence) that showed a site of mismatching might explain the result obtained of variable intensities of PCR products. This characteristic is considered a basis for demonstrating probable differences between universal and local isolates. Tantawi et al., (2004) illustrated 96.92 identity between isolated local strains and accessed databases embedded sequences.

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X15208.1       GAGACCGATACGTCTGTCGTGCCGTTCCCGGTTTCCAACATCACGCCGGCCAAAGCCAAA 243
AF454463.1     GAGACCGATACGTCTGTCGTGCCGTTCCCGGTTTCCAACATCACGCCGGCCAAAGCCAAA 126
AB015997.1     GAGACCGATACGTCTGTCGTGCCGTTCCCGGTTTCCAACATCACGCCGGCCAAAGCCAAA 271
EU019912.1     GAGACCGATACGTCTGTCGTACCGTTCCCGGTTTCCAACATCACGCCGGCCAAAGCCAAA 117
Z36295.1       GAGACCGATACGTCCGTCGTGCCATTCCCGGTTTCCAACATTACGCCGGCCAAAGCCAAA 300
DQ868535.1     GAGACCGATACGTCTGTCGTGCCGTTCCCGGTTTCCAACATCACTCCGGCCAAAGCCAAA 240
               ************** ***** ** **************** ** ***************

X15208.1       CAGCTGACGCACATTAACTTCTCGTTCCTGGATATCAACAGCAACCTGGAATGCGCCTGG 303
AF454463.1     CAGCTGACGCACATTAACTTCTCGTTCCTGGATATCAACAGCAACCTGGAATGCGCCTGG 186
AB015997.1     CAGCTGACGCACATTAACTTCTCGTTCCTGGATATCAACAGCAACCTGGAATGCGCCTGG 331
EU019912.1     CAGCTGACGCACATTAACTTCTCGTTCCTGGATATCAACAGCAACCTGGAATGCGCCTGG 177
Z36295.1       CAGCTGACGCACATCAACTTCTCGTTCCTGGATATCAACAGCAATCTGGAATGCGCCTGG 360
DQ868535.1     CAGGTGACGCACATCAACTTCTCGTTCCTGGATATCAACAGCAATCTGGAATGCGCCTGG 300
               *** ********** ***************************** ***************

X15208.1       GATCCGGCCACCAACGACGCCAAGGCGCGCGATGTGGTCAACCGTTTAACCGCGCTCAAA 363
AF454463.1     GATCCGGCCACCAACGACGCCAAGGCGCGCGATGTGGTCAACCGTTTAACCGCGCTCAAA 246
AB015997.1     GATCCGGCCACCAACGACGCCAAGGCGCGCGATGTGGTCAACCGTTTAACCGCGCTCAAA 391
EU019912.1     GATCCGGCCACCAACGACGCCAAGGCGCGCGATGTGGTCAACCGTTTAACCGCGCTCAAA 237
Z36295.1       GATCCGGCCACCAACGACGCCAAGGCGCGCGATGTGGTCAACCGTCTGACCGCGCTCAAA 420
DQ868535.1     GATCCGGCCACCAACGACGCCAAGGCGCGCGATGTGGTCAATCGTCTGACCGCGCTCAAA 360
               **************************************** *** * ************
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            150       160       170       180
DQ868535    TAAAAACATCCACTCTGGAGAAATGCCATGTCCACACGTAA
AB015997    GAAAAACATCCACTCTGGAGAAATGCCATGTCCACACGCAA
Z36295      TAAAAACATCCACTCTGGAGAAATACCATGTCCACACGCAA
X15208      GAAAAACATCCACTCTGGAGAAATGCCATGTCCACACGCAA

Consensus    AAAAACATCCACTCTGGAGAAATgCCATGTCCACACGcAA
```

Figure (2): Clustal W alignment of downloaded sequences of CHIB gene demonstrate high homology among them. Below a DS GENE analysis of designing primers (only forward primer presented) showing ORF start and ribosomal binding site. The base 2 from 3` end represent mismatching site (G/A) derived from Z36295 sequence (row 3).

PCR analysis:

PCR was success with all samples and all products are at the predicted size (~ 1.6 Kb). Figure (3) presents PCR products obtained from reaction with the isolates used. The traced distinct bands might be divided into two groups according to the band intensity. The results obtained suggest that isolates with low band intensity reflects mismatching in forward primer. On the basis of mismatching different

sequence might be explained as low intensities bands. Although all isolates produced amplified proper product, the differences in clearing zones observed might be due to differences in contribution of other genes in chitinolytic machinery (like, ChiA and/or ChiC). Other reason might be referred to variable promoter, regularity elements and/or such signals capabilities.

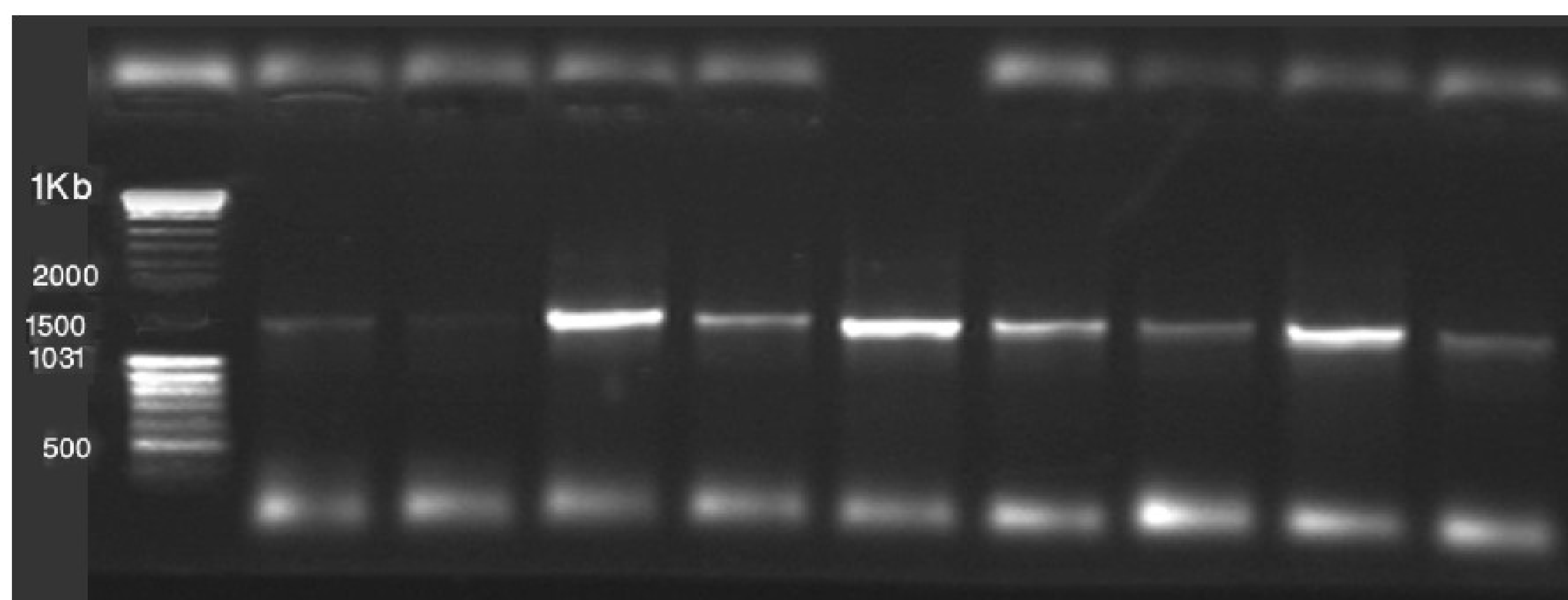

Figure (3): PCR products of Chitinase-B encoding gene from nine bacterial isolates

Cloning of S. *marcescens* gene encoding chitinase-B:

The PCR product of a highly chitinolytic isolate is directly rescued in pGEMT easy cloning system for further analysis. Then, *S. marcescens* PCR product of Chitinase-B gene was cloned in *E. coli* Jm109 with pBK-CMV vector, screened on IPTG/X-gal and chitin overlay agar plate approach to identify chitinase-producing clones. Figure (4) represents original plasmid (pBK-CMV) and produced constructed plasmids. White colonies were selected and grown on LB media (Figure 5), then, plasmid purification was carried out and double digestion with *ECORI* and *HindIII* was done. To verify the insert length, one clone out of four chitinase-positive *E.coli* colonies were introduced to plasmid alkaline purification and double restriction digestions. Thenafter, digestion product was examined on agarose gel (2%) against both undigested and negative (original insert-free plasmid). Figure (6) showed an proper insert after digestion at the predicted fragment size.

The pBK-CMV phagemid vector is a cloning vector (high copy number) derived from prokaryotic systems. Gene expression is driven by the cytomegalovirus (CMV) immediate early promoter in the pBK-CMV phagemid vector. Prokaryotic expression is driven by the lac

promoter, which is repressed in the presence of the LacI protein and is inducible by IPTG/x-gal. Therefore, pBK-CMV was selected as binary vector for obtaining ChitinaseB clones.

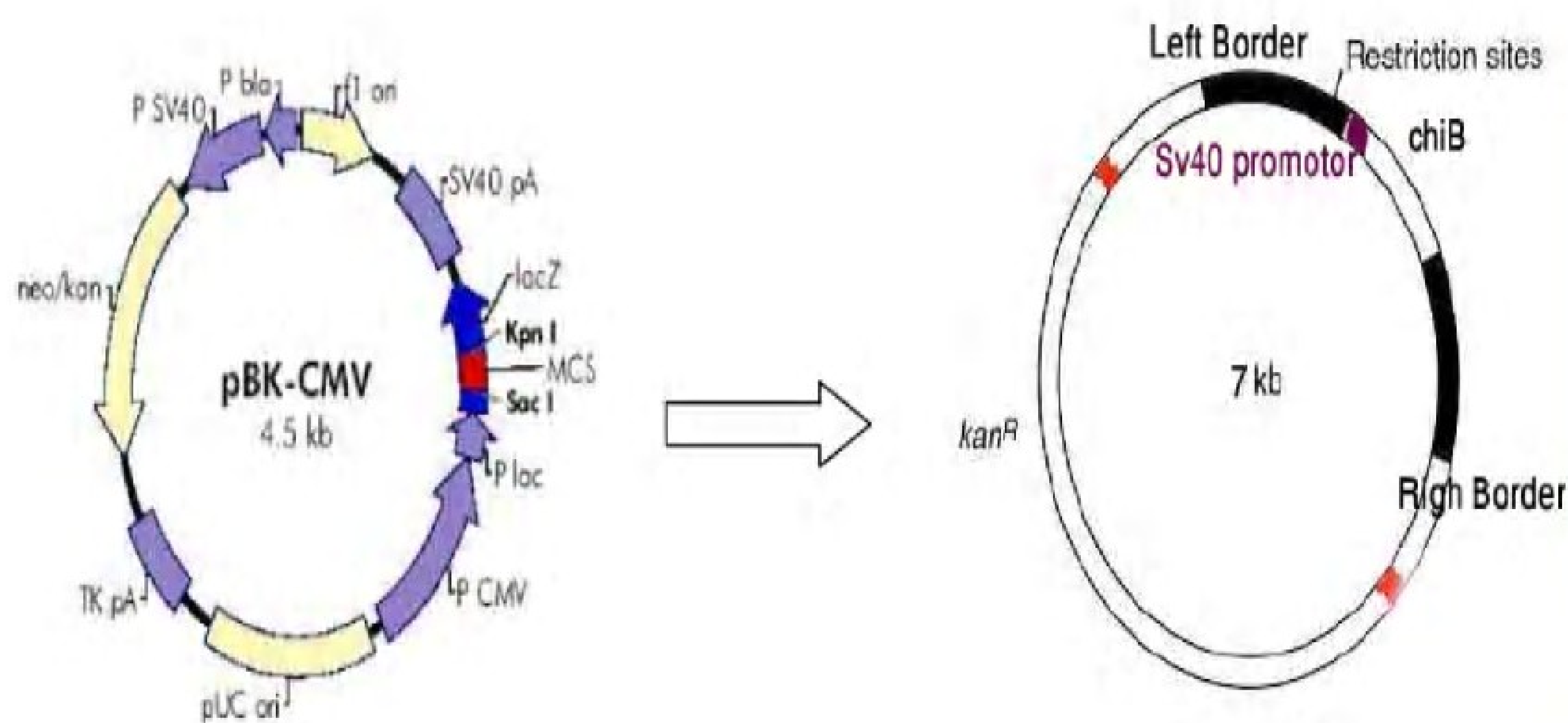

Figure (4): pBK-CMV cloning dual expression system plasmid shows MCS site and restriction map and resulted modified plasmid with proper insert of CHIB gene.

Figure (5): LB-chitin plate test of resulted four clones proved successful cloning and expression of chitinase-B gene in *E. coli.*

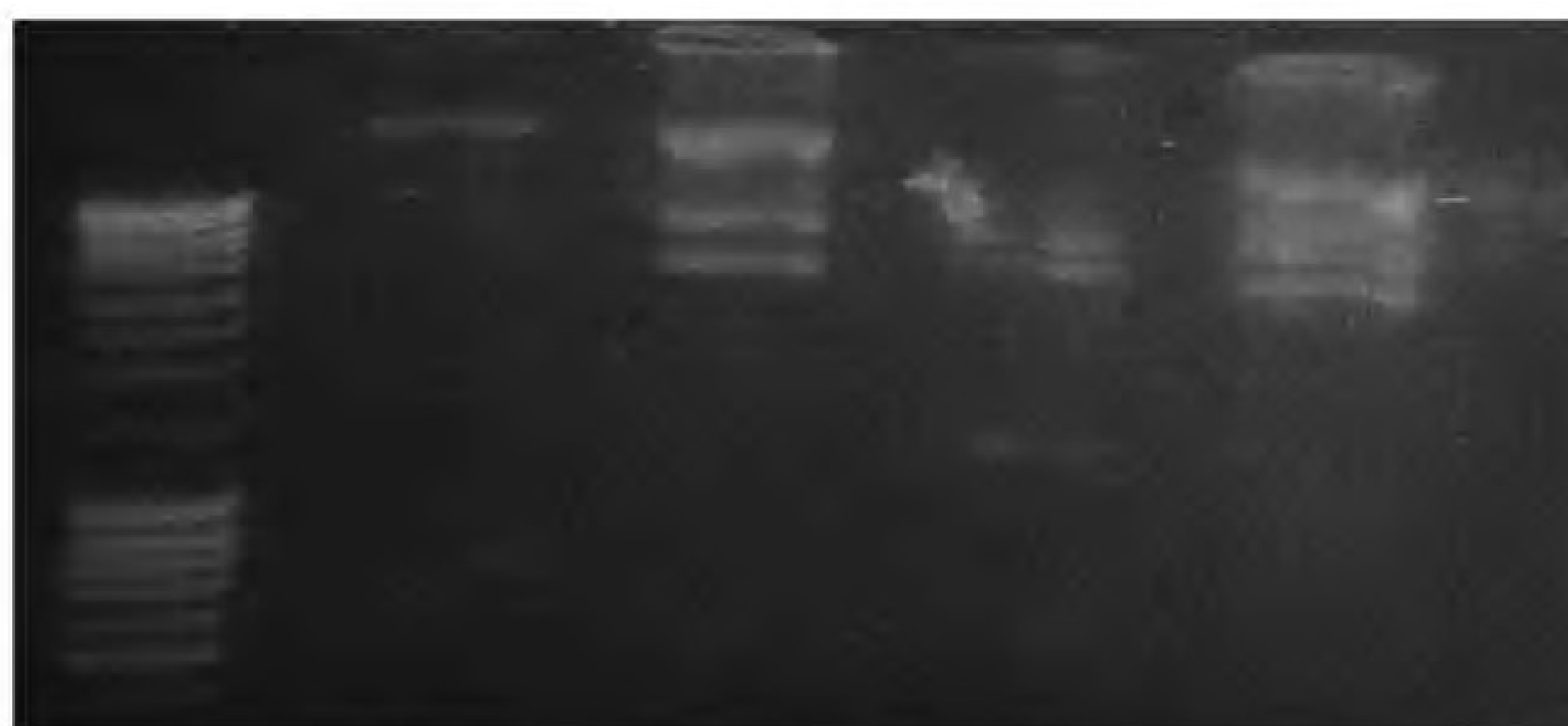

Figure (6): Agarose (2%) sizing examination of double digested plasmid isolated and purified from such cloned *E. coli* evidence proper insert at predicted fragment size.

The obtained construct will be used for further studies in prokaryotic and eukaryotic gene transfer for binary expression phase in both two systems. In the present study, *Serratia marcescens* was selected as the source of chitinase-encoding gene(s) for the following reasons; (i) crude preparations of chitinases from *S. marcescens* are commercially available, (ii) an effective affinity chromatographic purification procedure for the *S. marcescens* chitinases has been reported (Molano et al 1977) and (iii) the gene(s) encoding these chitinase(s) and their associated regulatory signals is likely to be recognized and expressed directly in *E. coli*. The last reason is refer to taxonomic base since both bacterial systems belong to the same group, Enterobacteriaceae.

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كلونة و التعبيرعن جين الكيتينيز B فى ال *Serratia marcescens* المصرية

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تنتج بكتريا *Serratia marcescens* ثلاثة انواع من البروتينات المحللة للكيتين *ChinA*, *ChinB* و *ChinC*. في هذه الدراسة تم استخدام عدد من الأستراتيجيات لدراسة تلك الجينات. نظرا لاعتبارات الأمن و السلامة على الصحة Biosafety تم استخدام نظام معترف به بيئيا لكلونة تلك الجينات. تم عمل عزل للجينات هدف الدراسة و دمجها في البلازميد المناسب و اجراء التحول الوراثي للبكتريا المناسبة و من ثم دراسة تعبير تلك الجينات عن نفسها. خلال هذه الدراسة تم اختيار سلالات من البكتريا هدف الدراسة المعزولة من مصر و التي تتميز بالكفاءة في تحليل الكيتين و تصميم بادئات لعزل جين *ChinB* من هذه البكتريا. تم دمج الجين في البلازميد pBK-CMV و اجراء التحول الوراثي لبكتريا *E. coli* jm109 باستخدام البلازميدات الجديدة constructs. نجحت البادئات المصممة في عزل و مضاعفة الجين هدف الدراسة و لكن بدرجات متفاوتة من الكفاءة مما يثبت حدوث خطأ في الأرتباط mismatching في بعض الاحيان مع بعض العزلات. اثبتت هذه النتيجة وجود اختلافات وراثية طفيفة بين تلك العزلات المحلية من *Serratia* ذات درجات التحليل المختلفة للكيتين. ثبت نجاح الطريقة المستخدمة في كلونة و التحول الوراثي و التعبير الجيني للجين هدف الدراسة من خلال بكتريا *E. coli* jm109.