CHAPTER IV

DISCUSSION

Chitinase production of *Burkholderia cepacia*

Burkholderia cepacia was isolated from soil in Bangkok, Thailand. Shells of prawns were buried underground to concentrate bacteria which can use chitin as carbon source, by producing chitin degrading enzyme. After one week, the decomposed prawn shell was resuspended in sterile distilled water. The suspension, in ten fold dilutions, was spread on colloidal chitin minimum medium plate and incubated at room temperature. Bacteria colonies, which can produce clear zone on the screening plate, were isolated and chitinase activity was determined. The turbidity reduction of a colloidal chitin suspension method was used for the assay.

Chitinase production profile (Figure 9) of *Burkholderia cepacia* shows the same pattern of chitinase production in both colloidal chitin and flake chitin minimum medium. The highest chitinase activity is on the second day of cultivation. This suggests that *Burkholderia cepacia* was able to be induced to produce chitinase when grown in the presence of either colloidal chitin or flake chitin with the same induction pattern.

Characterization of crude chitinase

A. Optimum pH

Crude chitinase was incubated with colloidal chitin in buffer at pH 2-10. The highest chitinase activity was detected when crude chitinase was incubated with colloidal chitin in 0.1 M citrate buffer, pH 7.0. Further experiments were conducted at this condition. Optimum pH of crude chitinase from *Burkholderia cepacia* was compared with purified chitinase from other organisms as shown in Table 6. Optimum pH of crude chitinase from *Burkholderia cepacia*, 7.0, was observed closely to

Table 6 Comparison of the Characteristics of Chitinase from several Microorganisms.

Species	Molecular weight (MW)*	Optimum pH	Optimum temp(°C)	Reference
Burkholderia cepacia				
Crude chitinase	at least 1 species (47,500)	7.0	40	
Aeromonas sp. 10S-24				
Chitinase I	112,000	4.0	50	Ueda, M. *et al.*, 1995
Chitinase II	115,000	4.0	50	
Alteromonas sp. strain O-7	70,000	8.0	50	Tsujibo, H. *et al.*, 1992
Enterobacter sp. G-1:				
Chitinase A	60,000	7.0	40	Park, J. *et al.*, 1996
Streptomyces sp. J13-3	31,000	6.0	45	Okazaki, K. *et al.*, 1995
Streptomyces olivaeoviridis	47,000	7.3	45-55	Blaak, H., *et al.*, 1993
Streptomyces thermoviolaceus OPC-520				
Chi 30	30,000	4.0	60	Tsujibo, H., *et al.*, 2000

* determined by SDS-PAGE

chitinase A from *Enterobacter* sp. G-1 and chitinase from *Streptomyces olivaeoviridis*.

B. Optimum Temperature

The effect of temperature on chitinase activity was determined at temperature 10-65 °C in 0.1 M citrate buffer, pH 7.0. The results shown in Figure 11 could be inferred that the optimum temperature of crude chitinase from *Burkholderia cepacia* is 40°C. Table 6 shows the comparison of optimum temperature of chitinase from many organisms. Crude chitinase from *Burkholderia cepacia* shows optimum temperature, 40 °C, similar to chitinase A from *Enterobacter* sp. G-1.

C. Substrate specificity

Soluble and insoluble substrate, in the form of glycol chitin and colloid or solid flakes was used in chitinase assay to understand chitinase substrate preference and specificity. Soluble substrates are hydrolyzed easily, however, substrates that are insoluble are less accessible to the enzyme, only the surface of the substrate can be hydrolyzed. Insoluble substrates are also different in their degree of crystallinity. Chitinase can hydrolyze substrates with low crystallinity, which offers high enzyme accessible surface, easier than substrates with high crystallinity. Since, the high degree of packaging if the polymer strands is resistant to chitinase binding and hydrolysis. Therefore, insoluble substrates with high crystallinity such as flake chitin or crab shell chitin are more difficult to hydrolyze than substrate that is amorphous in structure such as colloidal chitin. However, there are chitinases that possess activity towards highly structured chitin.

In our experiment, chitinase hydrolyzed colloidal chitin and glycol chitin approximately 13 folds better than flake chitin and crab shells chitin. Flake chitin and crab shells have higher crystallinity than colloidal chitin so that they have lower enzyme accessible surface than colloidal chitin. This indicates that our enzyme has low preference towards chitin with high degree of crystallinity.

Enzymes hydrolyze substrate in homogeneous reaction better than heterogeneous reaction. Since in heterogeneous reaction, the substrate is less accessible to the enzyme, only the surface of the substrate can be hydrolyzed. Therefore, higher amount of products should be obtained from glycol chitin, a soluble substrate, than colloidal chitin, an insoluble substrate. However, glycol chitin, which was in homogeneous reaction, was hydrolyzed only 87% compared to colloidal chitin. This result suggested that the substituted group at C'6 of glycol chitin may interfere with ability of the enzyme to bind with the substrate, which resulted in lower chitinase activity when glycol chitin was used as a substrate.

Chitosanase activity was also determined in this experiment. Substrates containing both N-acetylglucosamine and glucosamine residues, 78% deacetylated chitosan and 90% deacetylated chitosan, were used. High degree of deacetylation indicates high glucosamine units in the molecule of the polymer. Chitinase can only hydrolyze β-1,4 linkage at C1 of N-acetyl glucosamine residues, but not at glucosamine residues. Consequently, substrates with higher degree of deacetylation, more glucosamine units, is a poorer substrate for chitinase than a substrate with lower degree of deacetylation. In contrast, percent of deacetylation affects chitosanase activity in an adverse way. Chitosanase hydrolyze substrate with higher percent of deacetylation better than substrate with lower percent of deacetylation. In our study, 78% deacetylated chitosan gave higher amount of reducing sugar compared with 90% deacetylated chitosan when both of them were used as a substrate. The activity dropped as the percent of deacetylation increases. This phenomena indicates that the reducing sugar released resulted from the activity of chitinase. No chitosanase activity was detected in cultured medium.

D. Estimation of molecular weight

On SDS-PAGE stained for chitinase activities, only one major band was presented on SDS-PAGE after activity staining. This result suggested that there is at least one major form of chitinase produced from *Burkholderia cepacia* and secreted into the culture medium. The estimated molecular weight of this chitinase species was considered to be 47.5 kDa. As show on Table 6, this species of chitinase from

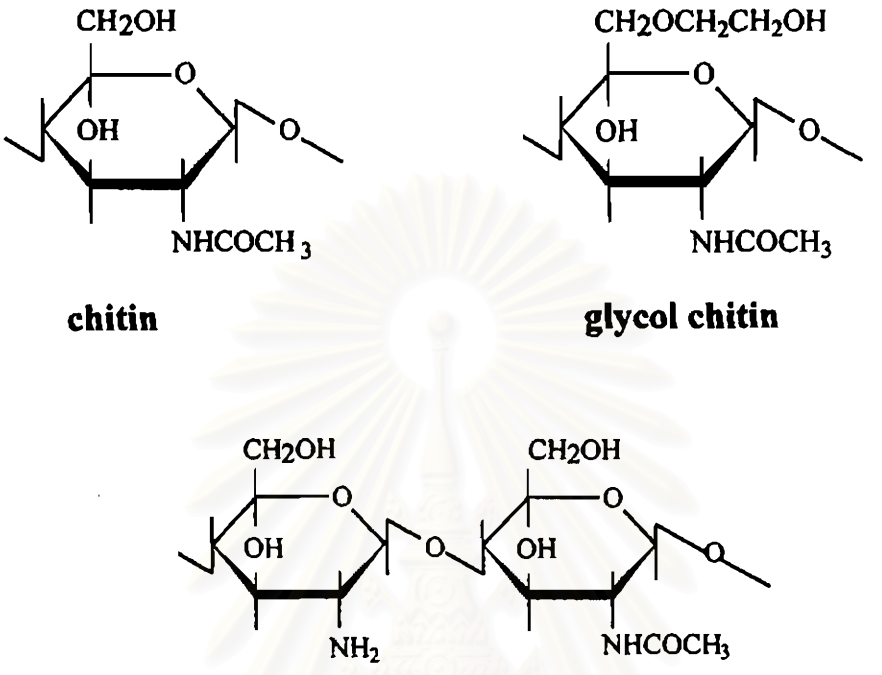

Figure 21 Structure of chitin and chitin related compounds.

Burkholderia cepacia, 47.5 kDa, has a molecular weight closed to chitinase from *Streptomyces olivaeoviridis.*

Detection of Chitinase gene by Southern blot Analysis

To detect the DNA fragment containing chitinase gene, Southern blot analysis was performed. Degenerate probe specific for chitinase gene, CB1, was designed by the conserved amino acid sequence in chitinase gene, FDGLDLDWEYP.

As shown on figure 14, 7.0 kb band of *Eco*R I digested chromosomal DNA, 7.8 kb and 10 kb bands of *Bam*H I digested chromosomal DNA, an approximately 23 kb band of *Hin*d III digested chromosomal DNA and 1.8 kb and 1.2 kb bands of *Pst* I digested chromosomal DNA were detected. Detection of two positive bands on *Bam*H I and *Pst* I digested chromosomal DNA suggested that there may be two chitinase genes in *Burkholderia cepacia*'s genome. Moreover, the fact that there was only one fragment from *Eco*R I digested chromosomal DNA also suggested that the chitinase genes might be clustered on the same region in the genome of *Burkhoderia cepacia.*

From the estimated molecular weight of the major chitinase, 47.5 kDa, we predicted that this chitinase comprise approximately 400 amino acid. This gene should be encoded from an approximately 1,200 bp DNA fragment. Therefore, the 7.0 kb fragment from the *Eco*R I digested genomic DNA and the 7.8 and 10 kb fragment from *Bam*H I digested genomic DNA was chosen for further cloning in our work. These fragments had appropriate size for cloning and were also large enough to contain the entire coding region of the gene. Therefore, 6-9 kb fragments and 6-12 kb fragments of *Eco*R I and *Bam*H I completely digested chromosomal DNA were subjected for cloning.

Detection of transformants containing chitinase gene

Recombinant plasmids from approximately 500 colonies of transformants were extracted and screened for chitinase gene by dot blot analysis with DNA probe specific for chitinase, CB1. Plasmids from eighteen different clones which gave positive signal on dot blot analysis

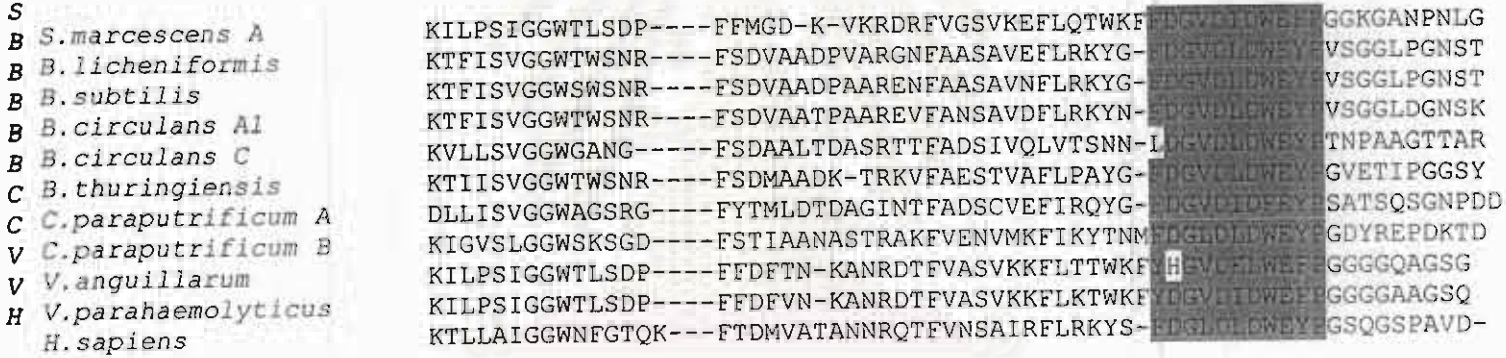

Figure 22 Conserve amino acid sequence, FDGLDLDWEYP, was found in various organisms. Homologous sequences were used to design the degenerate probe.

were digested with either *Eco*R I or *Bam*H I and *Pst* I then hybridized with CB1 probe. pKK243B gave positive results both on dot blot analysis and Southern blot analysis. Southern blot analysis of pKK243B showed 7.8 kb band and 1.8 kb band when pKK243B was digested with *Bam*H I and *Pst* I, respectively. These two positive bands of pKK243B were located on the same position of positive bands in Southern blot analysis of chromosomal DNA of *Burkholderia cepacia* digested with the same enzymes (shown on Figure 15). This result demonstrated that we have successfully clone the 7.8 kb *Bam*H I fragment, and the CB1 probe hybridize with sequences within the *Pst* I 1.8 kb fragment. These results suggested that pKK243B should contain chitinase gene.

To confirm our result from the Southern blot assay, colonies containing pKK243B and pKK1.8PP, generated from ligation of *Pst* I digested pBluescript II KS+ and 1.8 kb *Pst* I digested pKK243B fragment which gave a positive result on Southern blot analysis, were streaked onto LB-glycol chitin agar. The colonies containing pKK243B gave a positive result by forming a visible clearing zone around the colony when stained with Congo red, which demonstrated that pKK243B contains chitinase gene.

To further confirm the results found in glycol chitin plate, colonies harboring pKK243B and pKK1.8PP were cultivated in LB-colloidal chitin and measure chitinase activity. If indeed pKK243B contains chitinase gene, we should be able to detect chitinase activity in the culture medium of *E. coli* harboring these plasmids. The results in Table 5 show that *E. coli* containing pKK243B showed 33% activity compared with chitinase activity from *Burkholderia cepacia.* When the plasmid was extracted and re-transformed, the new transformants also contain comparable chitinase activity. This demonstrated that chitinase activity confers with the presence of pKK243B. When the *E. coli* containing pKK1.8PP was assayed for activity, a much lower activity, 10% compared to chitinase activity from *Burkholderia cepacia*, was observed. This may indicate deletions in the regulatory sequences or parts of the coding sequence of the protein. These results strengthen our previous conclusion from the Southern blot analysis that we have successfully cloned chitinase gene from *Burkholderia cepacia.*

Analysis of Chitinase Gene

The 1.8 kb *Pst* I fragment within pKK1.8PP, which binds to CB1 probe, was sequenced by method of Sanger *et al* (1977). When nucleotide sequence of the DNA fragment, 1706 bp, was translated 6-phase into amino acid sequence, a partial open reading frame of 206 amino acid and open reading frame of 244 amino acid were discovered. Comparing these deduced amino acid sequences with protein sequences in the Genbank database revealed that the partial open reading frame of 206 amino acid has similarity with the amino acid sequence of putative sensor proteins, and the open reading frame of 244 amino acid sequence has similarity with the amino acid sequence of putative two component transcriptional regulator. No open reading frame which have similarity with chitinase gene was found in pKK1.8PP.

However, both results from Southern blot analysis and phenotypic screening demonstrated that colonies harboring pKK243B contains chitinase gene. Chitinase activity was detected in the medium of bacterial culture containing pKK243B. From these results we reasoned that; 1) chitinase gene is located within the *Bam*H I 7.8 kb fragment, but does not bind to the CB1 probe or 2) this DNA fragment from *Burkholderia cepacia* can induce *E. coli* to produce chitinase like activity, but it does not contain chitinase gene.

Recent work on regulation of chitinase gene from *Streptomyces thermoviolaceus* OPC-520 was reported by Tsujibo *et al.* (1999). *Streptomyces thermoviolaceus* OPC-520 produces both family 18 chitinase, Chi 40 and Chi 30, and Family19 chitinase, Chi 35 and Chi 25. Tsujibo *et al.* found that *chi* 40 is regulated by a two-component sensor-regulator system, *chi* S and *chi* R. The pair of regulatory genes, *chi* S and *chi* R, is located directly upstream flanking the *chi* 40 gene (Figure 25). The *chi* S and *chi* R genes are found to encode novel proteins of the two-component transduction system. Activation of the signal transduction cascade in response to chitin or chitobiose leads to induction of the *chi* 40 gene. Interestingly, the gene construct of this two-component sensor-regulator system was similar to what we have found in the sequence obtain from the 1.8 *Pst* I fragment.

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B.cepacia          ---LQSKINPHFLFNALNAISSSIRLNPDTARQLIINLSRYLRYNLELN--DELIDIRKE
E.coli             LRALQSKINPHFLFNALNAISSSIRLNPDTARQLIFNLSRYLRYNIELKD-DEQIDIKKE
B.subtilis         MAFLQSQIKPHFLYNVLNTIISLTHLDIEKAREVTEEFTNYLRMSFDFQNTSAISSFRHE
P.aeruginosa       S--LQARIRPHFLFNS-NSIASLIELDPLKAEHAVLDLSDLFRASLAKP--GTLVSWEEE
                      **::*.****:*  *:* *  .*:  .*..   :::  :* .:     .   . ..*

B.cepacia          LHQIQDYIAIEQARFGAKLTVIYDID-DDVSVRIPSLLIQPLVENAIVHGIQPCKGKGVV
E.coli             LYQIKDYIAIEQARFGDKLTVIYDID-EEVNCCIPSLLIQPLVENAIVHGIQPCKGKGVV
B.subtilis         LSIINSYLSIEKTRFSNRLEVLFDID-EDIDFILPPLMIQPLVENAVLHGVSKKRGGGWI
P.aeruginosa       LALARRYLSIEQYRLGDRLQLDWQVHGVPANLPIPQLTLQPLLENALIYGIQPRVEGGLV
                   *   . *::**: *:. :* : :::.    .  :* * :***:***:::*:.     * :

B.cepacia          VIAVKDQG-DRVKISVKDTGHGINQETIDRVARNEMPGHNIGLLNVHHRVSLLYGEGLHI
E.coli             TISVAECG-NRVRIAVRDTGHGIDPKVIERVEANEMPGNKIGLLNVHHRVKLLYGEGLHI
B.subtilis         KLTAKKQSKNEYHIKVEDNGPGITPEKQIDLLSTDFDR-SVGLKNINQRLKHFCGSELMI
P.aeruginosa       QVEAVYRE-GVFQLCVSN--------PYDEALESPPSKGTRQALHNIDARLGALFGPKASL
                    : .     .  :: * :          :          .* *:. *:  : *    :

B.cepacia          RR-LEPGTEIAFYISKNGGKLHQEPSAPPVGEAS
E.coli             RR-LEPGTEIAFYIPN-----QRTPVASQATLLL
B.subtilis         SSTPDAGTSVSMLIHLAET--TGSPKELKDTERT
P.aeruginosa       SV---ERRDGRHYTCL-----R-YPCARLMQEA-
                             .          *
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Figure 23 Partial amino acid sequence alignment of putative sensor protein in various microorganism.

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B.cepacia          -MKAIIVEDEFLAQEELSYLIKKHSNIDIIA-TFEDGLDVLKYLQTHQVDAIFLDINIPS
E.coli             -MKVIIVEDEFLAQQELSWLIKEHSQMEIVG-TFDDGLDVLKFLQHNRVDAIFLDINIPS
B.subtilis         MLRVLIVDDEMLARDELAYLLKRTNDEMEIN-EAENIESAFDQMMDQKPDLLFLDVDLSG
P.aeruginosa       -MNVLIVDDEPLARERLARLVGQLDGYRVLEPSASNGEEALTLIDSLKPDIVLLDIRMPG
                    :..:**:** **::.*: *: . .    :    .:  ..:  :   : * ::**: :..

B.cepacia          LDGVLLAQNISKFAHRPSIVFITAYKEHAVEAFEIEAFDYILKPYHEARIVTMLQKLEAL
E.coli             LDGVLLAQNISQFAHKPFIVFITAWKEHAVEAFELEAFDYILKPYQESRITGMLQKLEAA
B.subtilis         ENGFDIAKRLKKMKHPPAIVFATAYDQYALKAFEVDALDYLTKPFDEERIQQTLKKYKKV
P.aeruginosa       LDGLQVAARLCEREAPPAVIFCTAHDEFALEAFQVSAVGYLVKPVRSEDLAEALKKASRP
                    :*. :* .: :    * ::* ** .:.*::**::.*..*: **  .  :   *:* .

B.cepacia          HH--RPAGAAEPTSAPSRGSHSINLIKDERIIVTDINDIYYAAADERVTRVYTRREEFVM
E.coli             WQ--QQQTSSTPAATVTRENDTINLVKDERIIVTPINDIYYAEAHEKMTFVYTRRESYVM
B.subtilis         N---RDIVETEQNSHA--GQMKLALSVGESIVIVDTKDIIYAGTEDGHVNVKTFDHSYTV
P.aeruginosa       NRVQLAAALTKPPASGGSGPRSHISARTRKGIELIPLEEVIFFIADHKYVTLRHAQGEVLL
                               :       :    : * :   ::: :  :..  . :     .  :

B.cepacia          PMNLTEFYGRLPEEHFFRCHRSYCVNLAKIREIVPWFNNTYILRLSDLEF-EVPVSRSKV
E.coli             PMNITEFCSKLPPSHFFRCHRSFCVNLNKIREIEPWFNNTYILRLKDLDF-EVPVSRSKV
B.subtilis         SDTLVVIEKKLPDSDFIRVHRSFVVNTEYIKEIQPWFNSTYNLIMKDGS--KIPVSRTYA
P.aeruginosa       DEPLKALEDEFG-ERFVRIHRNALVARERIERLQRTPLGHFQLYLKGLDGDALTVSRRHV
                      :  :  .:  . *.* **.  *    *..:     . : * :.. .   :.***  .

B.cepacia          KEFRKLMRL-
E.coli             KEFRQLMHL-
B.subtilis         KELKKLLHI-
P.aeruginosa       AGVRRLMHQL
                     .::*::
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Figure 24 Amino acid sequence alignment of putative 2-component transcriptional regulator in various microorganism

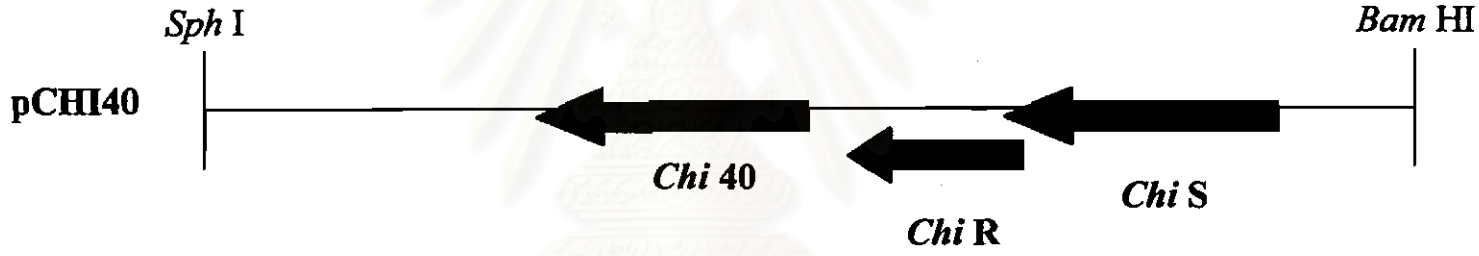

Figure 25 Restriction map of pCHI40.
Arrows indicate the ORF and the direction of transcription

From recent findings by Tsujibo *et al.* (1999), we predict that there should be a chitinase gene in pKK243B following the two-component regulatory system we have sequenced. This chitinase gene should belong to family 19, since it does not bind with CB1 probe, specific for family 18 chitinases. Moreover, both family 18 and 19 chitinase were reported in *Burkholderia gladioli.* It is possible that *Burkholderia cepacia* also contains both family 18 and 19 chitinase. The proposed location of chitinase gene in pKK244B is shown in Figure 26.

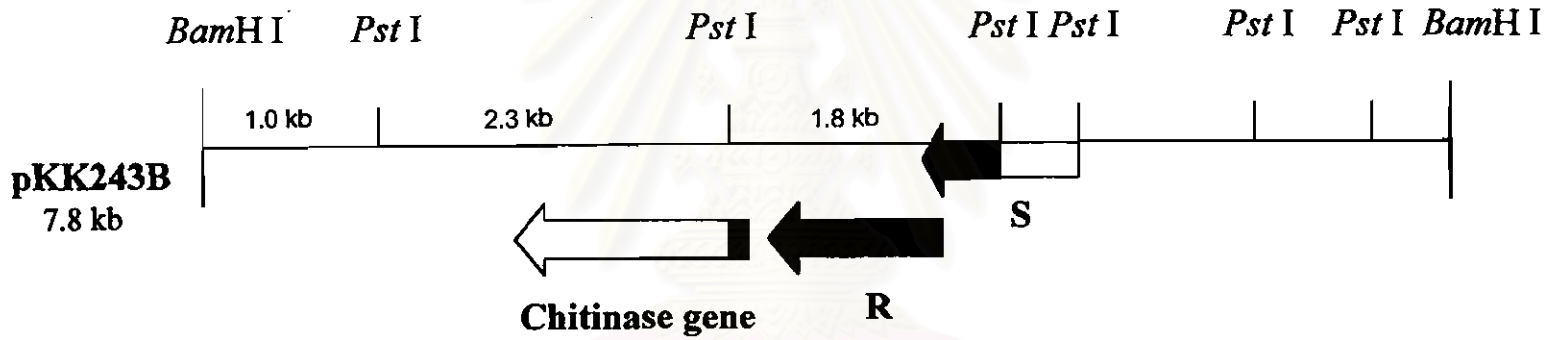

Figure 26 Proposed location of chitinase gene in 7.8 kb insert fragment of pKK243B.
Arrows indicate the ORF and the direction of transcription. S and R are representative of putative sensor protein and putative 2-component transcription regulator