BIOTROPIA No. 20, 2003: 36 - 48

HETEROLOGOUS EXPRESSION OF A CHITINASE GENE FROM *AEROMONAS CAVIAE* IN *PSEUDOMONAS FLUORESCENS*

AMARILA MAHK[U'+], ANTONIUS SUWANTO[1,2,'"], BUDI TJAHJONO[3], ROB HARLING[4]

'Southeast Asian Regional Center for Tropical Biology (SEAMEO BIOTROP), Bogor,
[2] Department of Biology, Faculty of Mathematics and Sciences, [3] Department of Plant Pests and Diseases, Faculty of Agriculture, Bogor Agricultural University, Bogor-16144, Indonesia: '
'Department of Crop Science. Scottish Agricultural College, Edinburgh EH9 3JG, UK.

ABSTRACT

A transcriptional fusion for an *Aeromonas caviae* chitinase gene was constructed under the control of a constitutive promoter of the kanaraycin resistance gene (PKm^R). The construct was inserted into a medium copy number broad host range plasmid vector to yield recombinant plasmid pAM340, which harbored transcriptional fusion PKm^R- *chi.* Another transcriptional fusion, *Ptac-chi,* in a recombinant plasmid pAM630, was conducted as comparison. Triparental mating of *E. coli* carrying the recombinant plasmids with *Pseudomotws fluorescens* 5100, a phyllosphere bacterium, was performed. *Pseudomonas fluorescens* 5100 exconjugants were examined for constitutive expression of chitinase employing a spectrophotometric assay; they showed stronger chitin degradation activity than *Escherichia coli* transformants. Using a fungal antagonism plate assay, this chitinolytic *P. fluorescens,* however, could not inhibit selected phytopathogenic fungi.

Keywords: *Aeromonas caviae/* chitinase gene/transcriptional fusion/PKm'V *Vtac-chilPseudomonas fluorescens*

INTRODUCTION

Chitinase is an enzyme that catalyzes the hydrolysis of chitin to oligomers. Chitin is an insoluble linear p-1, 4-linked unbranched polymer of 7V-acetylgluco-samine, and is a major constituent of fungal cell wall. Chitinase is found widely distributed amongst many organisms, but its physiological role is different among them.

In chitinase-producing plants, this enzyme is used for self-defense against plant pathogens and pests, i.e. to hydrolyse chitin in the cell wall of certain fungal pathogens and cuticle of pests (Boiler *et al.* 1983; Hedrick *et a*\. 1988; Oppenheim and Chet 1992). It has been suggested that chitinase production in plants is induced by microbial infections or other injuries (Hedrick *et al.* 1988). In chitinase-producing bacteria, extracellular chitinases are produced to digest chitin and utilize

* Author to whom all correspondence and reprint requests should be addressed : Tel : +62 (251) 625965; Fax:+62 (251) 315107 ; E-mail : asuwanto@indo.net.id + Present address : Department of Pharmacy, Faculty of Mathematics and Natural Sciences, University ofIndonesia.

it primarily as a carbon and energy source (Gooday 1990). The exploration and utilization of bacterial chitinase in plant protection is centred around its use to protect against pathogenic fungi as a biocontrol mechanism. This potential has been a major concern in constructing biocontrol agents against fungi. Several studies regarding this have been reported (Inbar and Chet 1991; Koby *et al.* 1994).

We have cloned and sequenced a heterogenous chitinase gene *(chi)* from a soilborne *Aeromonas caviae* isolated from a blackpepper plantation in Bangka Island, Indonesia in our previous study (Malik *et al. In press).* This gene has 97% identity with the chitinase gene *chiA* from *A. caviae* (UO9139) (Sitrit *et al.* 1995). In this present study, we intended to utilize this gene under control of a constitutive promoter, i.e. the kanamycin resistance gene from *Tn903,* in a suitable vector for expression in a phyllosphere bacterium, as a means of producing a chitinolytic biocontrol agent. We cloned this *chi* gene into a relatively small size and medium copy number broad host range plasmid vector, pBBRlMCS-2 (Kovach *et al.* 1994), rather than into a high copy number plasmid as reported by Koby *et al.* (1994). This strategy was employed to avoid accumulation of defective *Escherichia coll* mutants during high expression of chitinase (Koby et *al.* 1994). Therefore, the aim of this work was to construct a chitinolytic *P. fluorescens* biocontrol strain through the introduction of a chitinase gene under the control of the Km^R promoter as a transcriptional fusion (PKm^R-c/j/).

MATERIALS AND METHODS Bacterial

strains, plasmids, and growth conditions

Bacterial strains and plasmids used or constructed during this study are described in Table 1. *E. coli* strains which were used as hosts for cloning experiments throughout this study, and their derivatives also, were routinely grown in Luria Bertani (LB) medium (broth or solidified with 1.5% agar) at 37°C for 18 hours. Ampicillin (100 ng/ml), kanamycin (25 μg/ml), trimethoprim (100 μg/ml) and gentamycin (10 μg/ml) were added to the growth media when needed. *Pseudomonas fluorescens* strains were grown routinely in King's B (KB) 10% agar (King *et al.* 1954) at 25°C for 18-20 hours. Spontaneous rifampicin resistant mutants (Rii) of P. *fluorescens,* which was done according to the method described by Eisenstadt *et al.* (1994), were isolated by plating a 25uL aliquot of a 10-fold concentration of a stationary phase culture onto plates containing a gradient concentration from 10 to 100 ug/ml of rifampicin.

DNA manipulation

Plasmid DNAs were isolated either by alkaline lysis (Sambrook *et al.* 1989), or hv DNA nurification kits (Promeea Wis.. or BioRad, Richmond, Calif.). Restriction

Table 1. Bacterial strains and Piasmids

Bacterial strains and plası	Relevant characteristics	Reference or source
E. coli		
DH5a	F", for a-complementation, general host for cloning	Sambrook *et al.* 1989
TOP10	F", for a -complementation, general host for cloning	Invitrogen, Inc. (Carlsbad, Calif.)
HB101(pRK2013)	mod" Res", general helper strain for bacterial conjugation	Dittae/fl/1980
TOP10(pAM340)	TOP10 carries fusion transcription PKm^R-c/i;	This study
TOP10(pAM630)	TOP10 carries fusion transcription *Ptac-chi*	This study
P.jluorescens		
5100	Phyllosphere biosurfactant* strain, wildtype, derived from *Brassica oleracea* var *italica*	Campbell *et al* 1995
5100 Rif	Spontaneous rifampicin resistance mutant PfSlOO	This study
5100(pAM340)	Pf 5100 (Rif) carries fusion transcription PKm^R-c/i;	This study
5100(pAM630)	Pf 5100 (Rif) carries fusion transcription *Ptac-chi*	This study
Aeromonas caviae WS7l	Chitinolytic, wildtype	Malik *etal In press*
Plasmids		
pUC19	*lacZ* Ap^R cloning vector	Sambrook *et al.* 1989
PAS385	MCS from pSL301(£coRI-.eo/I) cloned into pUC19 *(Smal-EcoRl),* Ap^R	Suwanto & Kaplan 1992
pAS396	Source of trimethoprim resistance gene (Tp^R) in pAS385	Suwanto & Kaplan 1992
pBBRlMCS-2	Broad-host range, medium copy number, Km^R	Kovache/a/. 1994
pUC4K	PKm^R source, Km^R gene from *1n903*	*OknetaL* 1981
p34S-Gm	Source of gentamycin resistance gene (Gm^R)	Dennis & Zylstra 1998
pWS506	pAS385 carrying 2.9 kb *Xho\ + ffmdlU* chitinase gene fragment from *A. caviae* WS7b	Malik *et aL In Press*
pAM201	c/i/-Tp^R DNA fragment as *Sail* cassette in pAS385	This study
pAM330	*PKnf-chi* in pBBRlMCS-2, Tp^R	This study
pAM340	PKm^R-c/i/ in pBBRlMCS-2, Tp", Gm^R	This study
pAM630	*Ptac-chi* in pBBRlMCS-2, Gm^R	This study

fragments were purified from agarose gels with the Gene Clean kit (BiolOl *Inc.,* La Jolla, Calif.). DNA filled-in reaction, utilizing T4 DNA Polymerase (Boehringer Mannheim Biochemicals, Indianapolis, Ind.), DNA fragment ligations, recombinant DNA transformation, and other accessory techniques were carried out as described by Sambrook *et al* (1989). *Escherichia coli* transformants harboring the chitinase recombinant were selected on LB-chitin agar plates, supplemented with the appro-

ing PKm^R, as well as *Ptac,* were selected by a blue-white assay on LB agar plates, containing IPTG (0.5mM), and X-Gal (5-bromo-4-chloro-3-indolyl-b-d-thiogalacto-pyranoside) 40 mg/ml. Plasmids were maintained by selection in the presence of appropriate antibiotics.

Bacterial Conjugation

Recombinant plasmids PKm^R-c/» and *Ptac-chi* were introduced into *P. fluorescens* 5100 (Campbell *et al.* 1995) by bacterial conjugation as described by Ditta *et al* (1980). Triparental mating was performed, employing *E. coli* HB101 (pRK2013) (Ditta *et al.* 1980) as helper, *E. coli* TOP10 (pAM340), as well as *E. coli* TOP10 (pAM630), as donor, and *P. fluorescens* 5100 Rif as recipient. The cells of all bacteria employed in this mating were collected from mid-exponential phase cultures, harvested by centrifiiging recipient, donor, and helper cultures in a ratio of 8:1:1, washing the pellet twice in sterile saline solution, and resuspending in a small volume of LB broth (30 ul). All cell suspensions were mixed, spotted onto LB agar plates without any antibiotic supplements, and incubated at 25°C.

Polymerase Chain Reactions

Standard PCR reactions were performed to verify the presence of cloned *chi* in *P. fluorescens* exconjugants, followed by electrophoretic analysis. An approximately 800-bp fragment *of chi* (accession number AJ431785) was amplified in a GeneAmp 2400 thermal cycler (Perkin Elmer). Single colonies of exconjugants were resus-pended into a reaction mixture prepared with *TaqDNA* polymerase (FINNZYME). The thermocycling program used in this study consisted of denaturation at 94 °C for 60 s, annealing at 54°°C for 60 s, and elongation at 72 °°C for 90 s, for 25 cycles, as a modification of the method described in Chernin *et al.* (1997). A set *of chi* internal primers, which were designed based on *A. caviae* chitinase DNA sequence (accession number AJ431785), were used, i.e. forward primer 5 ' - G T G A A G A A C T A C C A G G C -3', and reverse primer 5 ' - G G C A G A T C A G T T G C A G C T C G -3' (GENSET Biotech, Singapore).

Chitinase Plate Assay **and Semimicro-quantitative** Assay

An assay for chitinase was carried out on LB agar supplemented with colloidal chitin 1% (Hsu and Lockwood 1975) for *E. coli* derivatives, whilst for *P. fluorescens* derivatives this was carried out on 10% KB agar supplemented with colloidal chitin at the same amount. *P. fluorescens* exconjugants were selected on 10% KB agar supplemented with rifampicin (50 ug/ml) and gentamycin after introducing transcriptional fusion recombinant plasmids by bacterial conjugation.

Semimicro-quantitative assay was performed as described previously (Malik *et* a/. *In press).* Cultures of the recombinant bacteria were collected after growing for 18-20 hours both for *E. coli* and *P. fluorescens,* whilst cultures of *A. caviae* were

collected from 15 hours. Enzyme activity was measured in both extracellular and intracellular fractions, after sonicated the cell. The protein concentration was measured according to the method as described by Bradford (1976). Chitinase activity was assayed by a modification of methods as described using colloidal chitin azure as substrate (Wirth and Wolf 1990; Evrall *et al.* 1990; Hood 1990). The amount of dye released from this chromogenic substrate, remazol brilliant blue, was measured at 590 nm. A standard curve was constructed using a chitinase standard from *Streptomyces griseus* (Sigma Chemical, St. Louis, Mo) against colloidal chitin azure as substrate, in citrate-phosphate buffer pH 6.0 as described by Hood (1990), and incubation temperature 37°C for 2 hours. The enzymatic activity was expressed as units of chitinase/mg of protein.

Assay **for Antagonism to Fungi**

An antagonism assay was performed as described by Chernin *et al.* (1995): Test bacteria were grown for 24 and 48 h for *A. caviae* WS7b (Malik *et al. In press)* and *P. fluorescens* strains, respectively, in Luria or nutrient broth at 30°C with aeration. The suspension of cells was streaked in a line at the centre of a PDA plate and incubated at 30 °C for 24 and 48 h for WS7b and *P. fluorescens* strains, respectively. After placing 3-mm diameter agar disks of an actively growing fungal culture of either *Botrytis cinerea* TOM98LD and *Fusarium solani f.* sp. *pisi,* at 3 to 4 cm away from each side of the bacterial growth area, the plates were incubated for 3 to 25 days, until mycelium growing from the two sides on a control plate came into contact.

RESULTS Construction

of *Sail-cassette ofchi*

Constitutive promoter of kanamycin resistance gene (PKm^R) from *Tn903* used in this study possesses a unique restriction site, *Xhol* (Oka *et al.* 1981). This *Xhol* site was employed to construct a transcriptional fusion of *A. caviae* WS7b chitinase gene *chi* (accession number AJ431785) under the control of PKm^R (Fig.l). The *chi* gene in pWS506 was cloned without its indigenous promoter (Malik *et al. In press).*

Plasmid pAM201-A carrying a *Sail* cassette *of chi* was constructed through several intermediate-cloning steps (Fig. 1). The first step was the insertion of trimethoprim resistance gene (Tp^R) isolated from pASS 96 (Suwanto and Kaplan 1992), as a marker downstream *ofchi* fragment at *Hpal* site in pWS506. The *chi*-Tp^R fragment was then isolated subsequently employing *HindHl+EcoRV* double digestion. The protruding end of *Hinalll* was made blunt by means of T4 DNA polymerase to obtain blunt end *chi-*Tp^R fragment, which was then ligated with blunt-end *Hpal* of vector pAS385 (Suwanto and Kaplan 1992), generated recombinant plasmid pAM201 (Fig 1). Plasmid pAM201-A carries c/z/-To^R

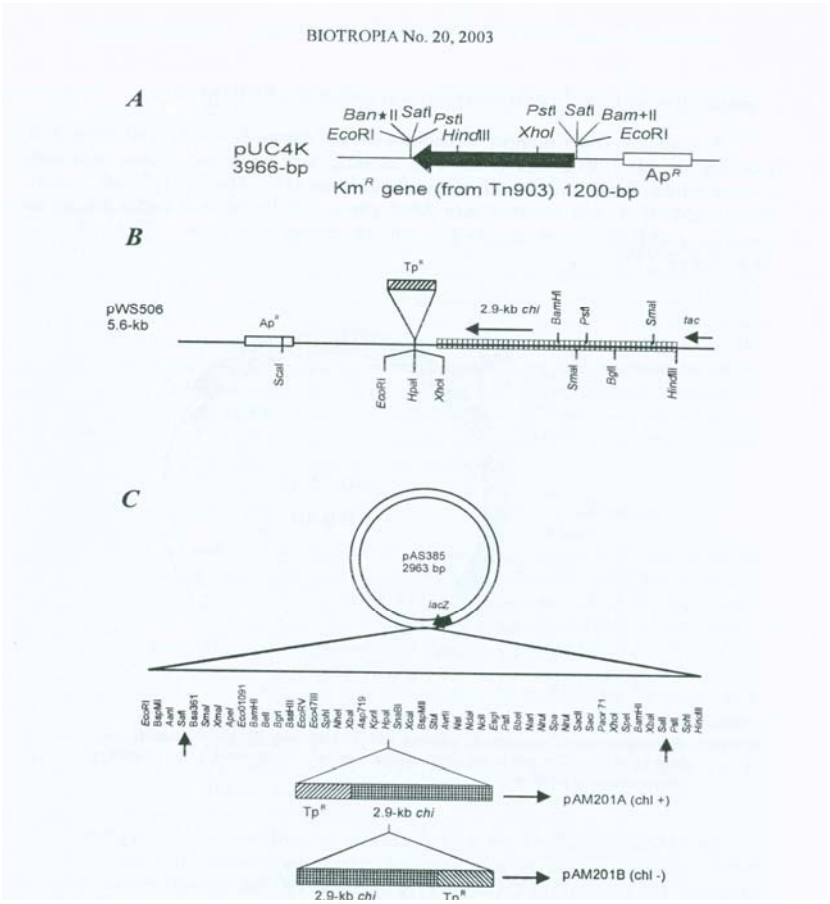


Figure 1. Schematic structure *of Sail* cassette construction *of chi.* The promoter of kanamycin resistance gene (PKm^R) from Tn903 in pUC4K is located upstream of the *Xhol* site (Oka *et* a/,1981) *(A).* A trimethoprim resistance gene (Tp^R) fragment, isolated from pAS396 (Suwanto & Kaplan 1992), was inserted downstream of the *chi* fragment in pWS506 (Malik *et al. In press)* as marker at *Hpal* site in polyl inker site *(B).* The c/i/-Tp^R fragment was isolated employing *Hindlll+ EcoRV* double digestion. The protruding end of *IfiruH.ll* was made blunt, which was then ligated with blunt-end *Hpal* end of linearized vector pAS385 (Suwanto & Kaplon 1992), resulting in recombinant plasmids pAM201-A (chi+) and pAM201-B (chi-) that carry *Sail* cassette of c/i;-Tp^R (C).

Construction of *PKm^R-chi* transcriptional fusion in pBBRlMCS-2

A medium copy number broadhost-range plasmid vector, pBBRlMCS-2 (Kovach *et al.* 1994), was utilized as cloning vehicle to introduce *PKm^R-chi* transcriptional fusion into *Pseudomonas fluorescens* 5100. The c/»/-Tp^R *Sail* cassette from pAM201-A was inserted into *Xhol* site of pBBRlMCS-2 under PKm^R to generate pAM330, in which c/»'-Tp^R will be transcribed under control of Km^R promoter (Fig 2).

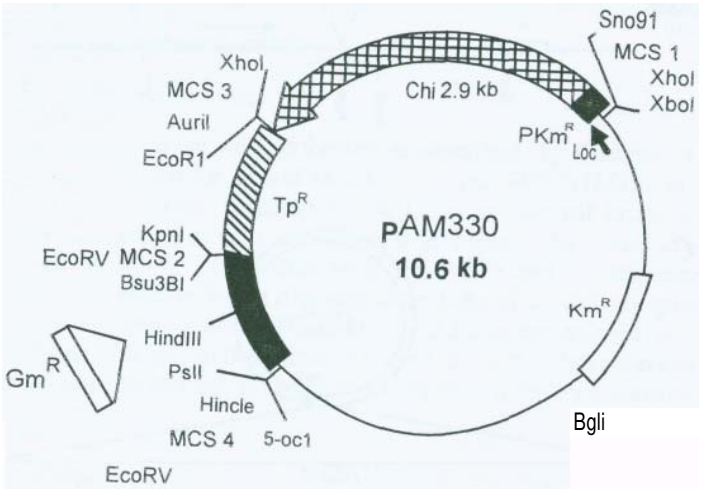
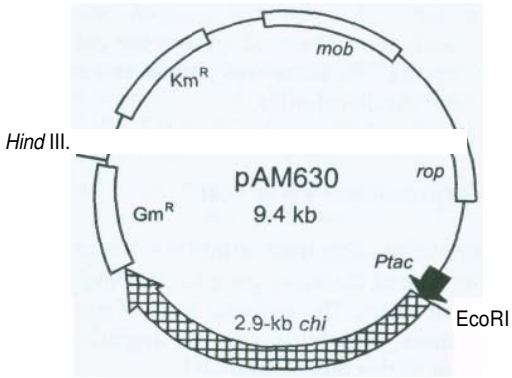


Figure 2. Restriction map of recombinant plasmid pAM330 carrying PKm^R-c/i; transcriptional fusion in pBBRlMCS-2. The additional Gm^R marker was inserted at ///«dIII site downstream of Tp^R, which generated pAM340

In addition to Tp^R marker, a Gm^R cassette isolated from p34S-Gm (Dennis and Zylstra 1998) was used as an alternative marker by inserting the cassette downstream of Tp^R resulting in pAM340 (Fig 2), since Tp^R marker was not sufficient to select for Pf exconjugants after mobilizing pAM330 from the *E. coli* transformant.

Construction of *Ptac-chi* transcriptional fusion in pBBRlMCS-2

A *BamHl* fragment of *Ptac* isolated from pKK223-3 was inserted into *BamHl* site in pBBRlMCS-2, generating plasmid pAM601. Chitinase gene *chi,* which was isolated from pAM202 as *Hindlll-EcoRl* c/i/-Gm^R fragment, was inserted subsequently into this plasmid vector, resulting in recombinant transcriptional fusion plasmid harboring *Ptac-chi,* designated as pAM630 (Fig 3). Plasmid pAM202 was generated from pAM201-B by replacing Tp^R with Gm^R.



Abbreviations : Km" = Kanamycin resistance gene; PKm^R = Promotor of Kanamycin resistance gene; Ap^R = Ampicillin resistance gene; Tp" = Trimethoprim resistance gene; Gm^R = Gentamycin resistance gene; *chi* = chitinase gene

Figure 3. Restriction map of recombinant plasmid pAM630 carrying *ftac—chi* transcriptional fusion in $_P$BBRlMCS-2

Chitinase expression of transcriptional fusions in *E. coli* and *P. fluorescens.*

Screening of *E. coli* transformants harboring pAM340, or pAM630 on chitin agar plate showed only weak clearing zones observed after 7 and 11 days of incubation, respectively (Table 2). On the other hand, *E. coli* harboring pWS506 showed strong clearing zones after only 3 days of incubation.

Table 2. Chitinolytic expression of *chi* on chitin agar plates.

Strains	Expression control	Chitinolytic activity as clearance zone*	Incubation time (days)
E. co//TOP10(pWS506)	*Plac*	+++	3
E. co//TOP10(pAM340)	PKm^R	+	7
E. co//TOP10(pAM630)	Ptoc	+	11
P. fluorescens 5100 (pAM340)	PKm^R	++	7
P. fluorescens 5100 (pAM630)	Ptoc	++	11

* Relative chitin degradation ability was indicated as +, ++ and +++ for slightly, moderate, and very clear zone, respectively.

Plasmid pAM340 and pAM630 were mobilized into spontaneous rifampicin resistance (Rif) mutants of strain Pf5100. Screen ino fnr pf phitmr.Krf:/-

selection, in conjunction with PCR techniques using *chi* primers as described above. Approximately 800 bp bands were observed on agarose gel after electrophoresis of exconjugants (data not shown). The latter was a rapid and easy method to verify the exconjugants after isolating single colonies.

Constitutive chitinase expression of *PKm^R-chi*

The modified chitin azure assay used in this study could demonstrate the constitutive expression of the cloned chitinase gene by growing the *E. coli* recombinant and Pf exconjugant without chitin. The colloidal form of chitin azure, which consists of oligomers and monomers, was fairly easy to degrade by chitinolytic activity compared to the flake form of this compound.

We also constructed a transcriptional fusion of the same chitinase gene under the *tac* promoter (Fig. 3), in order to compare the promoter activity. Expression assays of both transcriptional fusions were carried out under uninduced growing conditions. The result showed that constitutive expression of *chi* under PKm^R was slightly stronger (Tables 2 and 3). By comparing chitinolytic activity of these two *P. fluorescens* 5100 exconjugants to the wild type bacterium *A. caviae* WS7b (the origin of *chi* gene in this study), it could be demonstrated that constitutive chitinolytic activity of these two strains of *P. fluorescens* were less active. This is consistent with the result of our previous study on *E. coli* recombinant harboring *chi* of WS7b in a pUC vector that WS7b presumably carries more than one chitinase gene, and has a complex regulation of chitinase gene expression.

Table 3. Chitinase activity assay

Strains	Chitinase assay (unit/mg protein)	
	Extracellular	Intracellular
A. caviae WS7b	7.70"	ND
E. CO//TOP10	0.29	0.41
E. co//TOP10(pWS506)	2.31	4.17
Eco/iTOP10(pAM340)	0.93	1.12
E. co/;TOP10(pAM630)	0.91	1.05
P. fluorescens 5100 Rif"	0.77	0.84
P. fluorescens (pBBRlMCS-2)	0.78	0.83
P. fluorescens (pAM340)	1.91	3.27
P. fluorescens (pAM630)	0.97	2.53

#as positive control,collected from90 hrs of growth incubation time ND = not determined

Antifungal Plate Assay

The antagonism assay of *P. fluorescens* strains harboring pAM340, and pAM630, was conducted with *A. caviae* WS7b as positive control for chitinolytic activity. *P. fluorescens* 5100 Rif and *P. fluorescens* (pBBR!MCS2) were used as negative control strains. The inhibition zones between the pathogenic fungus *Botrytis cinerea* TOM98LD and the tested strains were observed after 3 to 24 days of incubation. The positive control, WS7b, showed an inhibition zone up to 17 mm. However, all bacteria strains tested did not show any fungal growth inhibition against *Fusarium solani f.* sp. *pisi.*

DISCUSSION

A transcriptional fusion of *A. caviae* chitinase gene *(chi)* under Kanamycin resistance gene promoter (PKm^R) has been constructed. Km^R gene is known to be expressed constitutively. By inserting *chi* under this promoter, we assume this will cause simple constitutive regulation *of chi.* The expression of other chitinase genes has been reported under nonindigenous promoter, *Ptac* (Koby *et al.* 1994; Downing and Thomson 2000). However, the expression of chitinase gene under the control of PKm^R has previously not been reported. In this study, we demonstrated the constitutive expression of chitinase under this promoter by a simple semi micro-quantitative chromogenic assay using colloidal chitin azure as substrate. The enzyme activity was determined by measuring the amount of remazol brilliant blue dye released employing spectrophotometer.

The results from the expression of chitinase transcriptional fusion assay demonstrated that both transcriptional fusions were expressed in *E. coli,* and were able to degrade chitin in the medium. Longer incubation time for chitinolytic activity of pAM340 and pAM630 compared to pWS506, might be due to the influence of plasmid copy number in this expression (Table 1). Plasmid pAM340 and pAM630 were constructed on a medium copy number plasmid vector pBBRlMCS-2, while pWS506 was constructed on a high copy number plasmid vector pUCl 9.

The strategy to isolate and insert the promoters into plasmid vector pBBRlMCS-2 before fusion with *chi,* rather then subcloned *chi* under PKm^R directly in pUC4K, as well as under *Ptac* in pKK223-3, was carried out to yield a broad host-range plasmid vector that carry a constitutive promoter PKm^R, as well as the strong promoter *Ptac,* which will be useful in future studies of other heterologous gene expressions.

The result of the semimicro-quantitative of chitinase activity assay indicated that *chi* was expressed constitutively both in *E. coli* or *P. fluorescens* 5100 recombinants (Table 3). However, chitinase activity of intracellular fractions were higher than the extracellular fractions which can be assumed that the chitinase product might not be well secreted in *E. coli,* as we have discussed in our previous study, as well as in *P. fluorescens* (Malik *et al. In press).*

The inability of transformed *P. fluorescens* strains to slow fungal growth is more likely caused by secretion problem, as well as low expression of chitinase product in *P. fluorescens* based on the results obtained from chitinolytic expression on agar plates and semiquantitative chitinase assays as shown in Tables 2 and 3. The gene was isolated from *A. caviae,* and was cloned and expressed in *E. coli* and *P. fluorescens,* which are not indigenous hosts. Nevertheless, the fact that the protein expression machinery between these three microorganisms are not the same, could explain this low chitinolytic activity. It could be also suggested that this chitinase gene *(chi)* in its source bacteria *A. caviae* WS7b, did not act alone to slow fungal growth. It might also require other antifungal mechanisms as reported previously in Jones *et al.* (1986) and Sundheim *et al.* (1988).

CONCLUSIONS

Transcriptional fusion of the *chi* gene under the control of kanamycin resistance gene promoter generated constitutive expression of chitinase in both *E. coli* and *P. fluorescens* 5100, which has previously not been reported. This chitinase gene expression might be useful to be developed further for construction of a biocontrol strain to prevent the growth of phytopathogenic fungi.

ACKNOWLEDGMENT

This work was supported by Graduate Team Research Grant (URGE) Grant 029/HTPP/II/ URGE/96 to B.T., and a Higher Education LINK Award from the Department for International Development (DFID)/British Council to R.H. and A.S.

REFERENCES

Boiler, T., Gehri, A., Mauch, F. and U. Vogeli. 1983. Chitinase in bean leaves: induction by ethylene, purification, properties, and possible function. Planta 157,22-31.

Bradford, M.M. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72,248-254.

Campbell, L.J., Marling, R., Chard, J. and M. Sutton. 1995. Calabrese: factors controlling symptom development in bacterial spear rot. Final report, project FV104b, Horticultural Development Council, Bradbourne House, East Mailing, Kent ME 19 6DZ, UK.

Cherain, L., Z. Ismailov, S.Haran, and I. Chet. 1995. Chitinolytic *Enterobacter agglomerans* antagonistic to fungal plant pathogens. Appl. Environ. Microbiol. **61,**1720-1726.

Chernin, L.S., de La Fuente, L., Sobolev, V., Haran, S., Vorgias, C.E., Oppenheim, A.B. and I. Chet. 1997. Molecular cloning, structural analysis, and expression in *Escherichia coli* of a chitinase gene from *Enterobacter agglomerans.* Appl. Environ. Microbiol. 63, 834 -839.

Dennis, J.J. and G.J. Zylstra. 1998. Plasposon: Modular self-cloning minitransposon derivatives for rapid genetic analysis of Gram-negative bacterial genomes. Appl. Environ. Microbiol. 64,2710-2715.

Ditta, G., Stanfield, S., Corbin, D. and D.R. Helsinski . 1980. Broad-host range DNA cloning system for Gram-negative bacteria: construction of gene bank of *Rhizobium melihti.* Proc. Natl. Acad. Sci. 77,7347-7451.

Downing, K.J. and J.A. Thomson. 2000. Introduction of the *Serralia marcescensa chiA* gene into an endophytic *Pseudomonas Jluorescens* for the biocontrol of phytopathogenic fungi. Can J Microbiol 46, 363-369.

Eisenstadt, E., B. C. Carlton and B. J. Brown. 1994. Gene mutation. *In:* Gerhardt, P., R. G. E. Murray, W. A. Wood, N.R. Krieg (Eds). Methods for General and Molecular Bacteriology. American Society for Microbiology, Washington, D.C.: 297-315.

Evrall, C.C., Atwell, R.W., and C.A. Smith. 1990. A semi-micro quantitative assay for determination of chitinolytic activity in microorganisms. J. Microbiol. Methods 12,183-187.

Gooday, G.W. 1990. Physiology of microbial degradation of chitin and chitosan. Biodegradation 1, 177-190.

Hedrick, S.A., Bell, J.N.., Boiler, T. and C.J. Lamb. 1988. Chitinase cDNA cloning and mRNA induction by fungal elicitor, wounding, and infection. Plant Physiol. 86,182-186.

Hood, M.A. 1990. Comparison of four methods for measuring chitinase activity and the application of the 4-MUF assay in aquatic environments. J. Microbiol. Methods 13, 51-160.

Hsu, S.C. and J.L. Lockwood. .1975. Powdered chitin agar as a selective medium for enumeration of Actinomycetes in water and soil. Appl. Microbiol. 29, 422-426

Inbar, J and I. Chet. 1991. Evidence that chitinase produced by *Aeromonas caviae* is involved in the biological control of soil-borne plant pathogens by this bacterium. Soil Biol. Biochem. 23, 973-978.

Jones, J.D.G., K.L.Grady, T.V.Suslow, and J.R.Bedbrook. 1986. Isolation and characterization of genes encoding two chitinase enzymes from *Serratia marcescens.* EMBO J. 5: 467-473.

King, E.O., Ward, M.K. and D.E. Raney. 1954. Two simple media for the demonstration of pyocyanin and fluoreescin. J. Labor. Clin. Med. 44, 301-307.

Koby, S., Schickler, H., Chet, I. and A.B. Oppenheim. 1994. The chitinase encoding Tn7-based chiA gene endows *Pseudomonas Jluorescens* with the capacity to control plant pathogens in soil. Gene 147,81-83.

Kovach, M.E., Phillips, R.W., Elzer, P.H., Roop II, R.M. and K.M. Peterson. 1994. pBBRlMCS: A broad-host range cloning vector. Biotechniques 16, 800-802.

Malik, A., Wenuganen, S., Suwanto, A. and B. Tjahjono. Cloning, DNA sequence and expression of *Aeromonas caviae* chitinase gene. Molec. Biotech. (in press)

Oka, A., Sugisaki, H. and M. Takanami. 1981. Nucleotide sequence of the Kanamycin resistance transposon Tn905. Mol. Biol 147: 217-226.

Oppenheim, A. B. and I. Chet. 1992. Cloned chitinases in fungal plant pathogen control strategies. Trends Biotechnol. 10,392-394.

Sambrook, J., Fritsch, E.F. and T. Maniatis. 1989. Molecular cloning. Cold Spring Harbor Laboratory Press. Cold Spring Harbor. New York. USA

Sitrit, Y., Vorgias, C.E., Chet, I. and A.B. Oppenherm. 1995. Cloning and primary structure of the *chiA* gene from *Aeromonas caviae.* J. Bacteriol. 177, 4187-4189

Sundheim, L., A.R. Poplawsky and A.H. Ellingboe. 1988. Molecular cloning of two chitinase genes from *Serratia marcescens* and their expression in *Pseudomonas* species. Physiol. Mol. Plant. Pathol. 33: 483-491.

Suwanto, A. and S. Kaplan. 1992. A self-transmissable narrow-host range endogenous plasmid of *Rhodobacter sphaeroides* 2.4.1: Physical structure, incompatibility determinants, origin of replication, and transfer function. J. Bacteriol. 174, 1124-113

Wirth, S.J. and Wolf, G.A. (1990. Dye-labelled substrates for the assay and detection of chitinase and lysozyme activity. J Microbiol. Methods 12,197-205.