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Expression of *pqq* Genes from *Serratia marcescens* W1 in *Escherichia coli* Inhibits the Growth of Phytopathogenic Fungi

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Serratia marcescens* W1, isolated from cucumber-cultivated soil in Suwon, Korea, evidenced profound antifungal activity and produced the extracellular hydrolytic enzymes, chitinase and protease. In order to isolate the antifungal genes from *S. marcescens* W1, a cosmid genomic library was constructed and expressed in *Escherichia coli*. Transformants exhibiting chitinase and protease expression were selected, as well as those transformants evidencing antifungal effects against the rice blast fungus, *Magnaporthe grisea*, and the cucumber leaf spot fungus, *Cercospora citrullina*. Cosmid clones expressing chitinase or protease exerted no inhibitory effects against the growth of fungal pathogens. However, two cosmid clones evidencing profound antifungal activities were selected for further characterization. An 8.2 kb *Hin*dIII fragment from these clones conditioned the expression of antagonistic activity, and harbored seven predicted complete open reading frames (ORFs) and two incomplete ORFs. The deduced amino acid sequences indicated that six ORFs were highly homologous with genes from *S. marcescens* generating pyrroloquinoline quinone (PQQ). Only subclones harboring the full set of *pqq* genes were shown to solubilize insoluble phosphate and inhibit fungal pathogen growth. The results of this study indicate that the functional expression of the *pqq* genes of *S. marcescens* W1 in *E. coli* may be involved in antifungal activity, via as-yet unknown mechanisms.

Keywords : antifungal activity, biocontrol, pyrroloquinoline quinine, solubilization of phosphate

Several biocontrol rhizobacteria have been utilized as microbial pesticides for the control of plant pathogens (Bloemberg and Lugtenberg, 2001). Antimicrobial products from beneficial bacteria include exoenzymes, including proteases, lipases, chitinases, and glucanases (Buchenauer, 1998; Dunlap et al., 1998; Park et al., 2005) and metabolites, including hydrogen cyanide (HCN) (Voisard et al., 1989), siderophores (Leong, 1986), antimicrobial compounds (Haas and Keel, 2003), and biosurfactants (Stanghellini and Miller, 1997). Competitive root colonization is another trait relevant to biocontrol (Chia-A-Woeng et al., 2000; Lugtenberg et al., 2001). Certain rhizobacteria have been also shown to augment plant growth via a variety of mechanisms, including the generation of indole-3-acetic acid (Lambrecht et al., 2000) and the solubilization of insoluble nutrients, such as phosphates, in soil (Goldstein, 1995), and to enhance plant growth and induce systemic resistance by production of butanediol (Han et al., 2006; Ryu et al., 2004).

Phosphorus is one of the most important macro-nutrients with regard to plant growth and development. However, phosphorus available in soil is readily converted into insoluble complexes, including tri-calcium-phosphate (Altomare et al., 1999). Insoluble phosphate can be solubilized by the organic acids generated by rhizobacteria. The conversion of glucose to gluconic acid by glucose dehydrogenases (GDH) is involved in this process, as is its oxidation to 2-ketogluconic acid via the activity of gluconate dehydrogenase (Goldstein, 1995). Glucose dehydrogenase requires pyrroloquinoline quinone (PQQ) as a redox cofactor (Meulenberg et al., 1992). Genes encoding for the enzymes inherent to PQQ biosynthesis were cloned from *Klebsiella pneumoniae* and consisted of six open reading frames (*pqqA* to *pqqF*) (Meulenberg et al., 1992). The D-gluconic acid generated by the rhizobacterium *Pseudomonas* strain, AN5, was shown to inhibit the growth of *Gaeumannomyces graminis* var. *tritici*. However, the genes involved in the production of D-gluconic acid and the mechanisms of D-gluconic acid in antifungal activity have yet to be thoroughly characterized (Kaur et al., 2006). In addition, there have been no studies conducted, in our knowledge, regarding the functions of *pqq* genes in antifungal activity.

Previously, we isolated a rhizobacterium, *Serratia marcescens* W1, which evidenced profound antifungal activities against a variety of plant pathogenic fungi, and

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also produced extracellular hydrolytic enzymes including chitinase and protease (Kim, 1996). In this paper, we address studies targeted toward the identification of the genes involved in the antifungal activity of this strain. We identified genes encoding for *S. marcescens* W1 chitinase and protease, as well as antifungal activity, via expression in *E. coli*. Neither chitinase nor protease was associated with antifungal activity. The expression of the *S. marcescens pqq* biosynthetic genes in *E. coli* evidenced substantial antifungal effects.

Materials and Methods

Bacterial strains, plasmid and culture conditions. Bacteria were stored in 15% glycerol at -70°C. The rhizobacterium, *S. marcescens* W1, was isolated from cucumber fields in Suwon, Korea (Kim, 1996). The fungal pathogens, *Magnaporthe grisea* strain K101 and *Cercospora citrullina* were acquired from the NAIB collection (Suwon, South Korea). The fungal isolates were grown on potato dextrose agar (PDA, Difco Inc. Deteroit, MI) at 26°C, and *E. coli* and *S. marcescens* W1 were grown on Luria Bertani (LB, Difco Inc., Deteroit, MI) agar medium, or in LB broth at 37°C and 30°C, respectively. Antibiotics, when employed, were added at the following concentrations: 100 μg/ml of ampicillin for the growth of *E. coli* clones. Hydroxyapatite agar or broth medium (HY) containing glucose 10 g, $MgSO_4$ 0.2 g, NaCl 1 g, $CaCl_2{\cdot}2H_2O$ 0.2 g, NH_4NO_3 1.5 g, yeast extract 0.5 g, and hydroxyapatite 4 g per liter of distilled water was employed to characterize the solubilization of the insoluble phosphate (Kim et al., 2002).

Construction of a genomic library and screening for biocontrol traits. A genomic library of *S. marcescens* W1 was constructed into a *Bam*HI/*Xba*I digested Supercos vector (Stratagene cat. #251301, La Jolla, CA, USA), using the partially *Sau*3AI-digested genomic DNA of *S. marcescens* W1, in accordance with the protocols established by Stratagene (La Jolla, CA, USA). The detection of antifungal genes in the cosmid was conducted via the screening of 3,000 clones for activity against *M. grisea* K101 or *C. citrullina*. The fungus was grown via the transfer of agar plugs into the centers of PDA agar plates, and fungal growth was facilitated by 5 days of incubation at room temperature, until the fungal mycelium covered 30% of the plate. Ten μl of 14 h LB broth cultures of *E. coli* carrying cosmid clones (10^9 cfu/ml) were applied to the fungal mycelia, and the cultures were incubated for an additional 3 days. *E. coli* clones generating growth inhibition zones were retained for the purposes of characterization.

In order to isolate the chitinase or protease-producing cosmid clones, 3,000 *E. coli* transformants were inoculated onto M9 minimal medium agar containing 2% colloidal chitin (Yamura and John, 1961), or LB agar containing 1% skim milk (Dahler et al., 1990). After 2 days of incubation, the *E. coli* strains evidencing clear zones around the inocula were selected for characterization.

DNA manipulation. Cloning and subcloning were conducted as previously described by Ausubel et al. (1989) and Sambrook and Russel (2001). The DNA fragments were isolated and purified from the agarose gels using a Zymoclean gel DNA recovery kit (Zymo Research, Orange, CA). Southern or colony hybridization was conducted using the nonradioactive Genius system (Roche Biochemicals, Indianapolis, IN). The plasmids were isolated with a miniplasmid purification system (Bioneer Inc., Daejeon, Republic of Korea). The chromosomal DNA was prepared via the hexadecyltrimethylammonium bromide (CTAB) method (Ausubel et al., 1989). Nucleotide sequence analyses were acquired using an ABI1301 DNA sequencer (Applied Biosystems, Foster City, CA) at the Korea Basic Science Institute (KBSI), Gwangju Branch. The nucleotide and deduced amino acid sequences were determined with NCBI Blast (http://www.ncbi.nlm.nih.gov) and Expasy (http://www.expaxy.hcuge.ch/) software.

Expression of *S. marcescens* W1 *pqq* genes in *E. coli*. Plasmids harboring various subclones of *S. marcescens* W1 *pqq* genes were transformed into *E. coli* DH5α. The transformants were grown for 24 hours in HY broth with agitation at 200 rpm at 30°C from an inoculum of 10^6 cfu/100 ml medium. The pH of the culture filtrate was then measured with a pH meter, and the organic acids production were determined via HPLC analysis, as previously described (Kim et al., 2002). The soluble phosphorus concentrations of the culture filtrates were determined using a spectrophotometer at 660 nm (Kim et al., 2002).

GenBank accession number. The nucleotide and amino acid sequences of the *S. marcescens* W1 extracellular chitinase and *pqq* genes were deposited into the GenBank database under the accession numbers DQ868535 and DQ868536, respectively.

Result and Discussion

Cloning of chitinase and protease genes from *S. marcescens* W1. The screening of 3,000 *E. coli* cosmid clones with genomic DNA fragments from *S. marcescens* W1 revealed seven clones that generated extracellular chitinase, and five clones that generated extracellular protease. The subcloning of one (C1) of the cosmid clones

Table 1. Fungal, bacterial strains and plasmids used in this study

Organism, strain or plasmid	Relevant genotype or description	Source or reference
Fungal strains		
Magnaporthe grisea	Rice blast	NASTI collection[a]
Cercospora citrullina	Cucumber leaf spot	NASTI collection
Bacterial strains		
Serratia marcescens		
W1	Antagonist to plant-fungal pathogen	Isolated from soil
Escherichia coli		
TG1	*supE hsdΔ5 thi(lac- proAB)* F'[*traD36 pro AB*$^+$ *lacI*^{q} *lacZΔM15*]	NASTI collection
NM554	*recA13,araD139, Δ(ara,leu) 7696,Δ(lac)17A,galU,galK, hsrR,rpsl(str^r),mcrA,mcrB*	Stratagene
AG1	*recA1,endA1,gyrA96,thi-1, hsdR17(r_K-,m_K+),supE44,relA1*	Stratagene
DH5α	*supE44 Δlac U169(φ80 lacZΔ M15) hsdR17 recA1 endA1 gyrA9 thi-1 relA1*	Stratagene
Plasmids		
SuperCos	Cosmid Vector, Amp^r Stratagene	
pUC18	Cloning vector, Amp^r	Promega
pUC19	Cloning vector, Amp^r	Promega
pSUPC1	35 kb fragment in SuperCos, chitinase(+)	This study(C1)
pCHI-I	11 kb *Eco*RI fragment from pSUPC1 cloned in pUC19, chitinase(+)	This study(C38)
pCHI-II	2.4 kb *Eco*RI/*Kpn*I fragment from pCHI-I cloned in pUC18, chitinase(+)	This study(C94)
pSUPC6	35 kb fragment in SuperCos, protease(+)	This study(C6)
pPRO-I	4.7 kb *Eco*RI fragment from pSUPC6 cloned in pUC19, protease(+)	This study(C27)
pPRO-II	3 kb *Hinc*II fragment from pPRO-I cloned in pUC19, protease(+)	This study(C46)
pSUPC5	35 kb fragment in SuperCos, antifungal activity(+)	This study(C5)
pSUPC32	27 kb fragment in SuperCos, antifungal activity(+)	This study(C32)
pANT-I	8.2 kb *Hind*III fragment from pSUPC32 cloned in pUC19, antifungal activity(+)	This study(C52)
pANT-II	6.9 kb *Hind*III/*Eco*RI fragment from pANT-I cloned in pUC19, antifungal activity(+)	This study(C78)
pANT-III	6.9 kb *Hind*III/*Eco*RI fragment from pANT-I cloned in pUC18, antifungal activity(+)	This study(C81)
pANT-IV	4.6 kb *Sal*I/*Hind*III fragment from pANT-I cloned in pUC18, antifungal activity(−)	This study(C74)
pANT-VI	3.6 kb *Hind*III/*Sal*I fragment from pANT-I cloned in pUC19, antifungal activity(−)	This study(C76)
pPRO-III	1.3 kb *Eco*RI/*Hind*III fragment from pANT-I cloned in pUC18, antifungal activity(−)	This study(C79)

[a] National Agricultural Science and Technology Institute

into pUC19, C94 (pCHI-II), generated a 2.4 kb *Eco*R1/*Kpn*I fragment which encoded for chitinase activity. The chitinase gene (*chiB*) of *S. marcescens* W1 harbored a predicted 1,693 bp ORF with deduced amino acids of 487 residues, corresponding to a subunit molecular weight of approximately 55.6 kD and pI 6.26. The deduced amino acid sequence of *S. marcescens* W1 *chiB* gene evidenced the conserved catalytic site, YGFDGVDIDIWEYP, and exhibited an identity of 92% with the deduced amino acid sequence of the *chiB* gene of *S. marcescens* QMB1466, but evidenced no homology with the *chiA* gene of this strain. A putative signal peptide was detected in the deduced amino acid sequence of the *chiB* gene of *S. marcescens* W1 (Watanabe et al., 1997).

Another cosmid, clone pSUPC6, which conditioned the highest levels of protease activity, was subcloned to generate C46 (pPRO-II) harboring a 3.0 kb *Hinc*II fragment that encoded for protease activity (Table 1). The protease gene in this fragment had a predicted 1,044 bp open reading frame, and a deduced amino acid of 347 residues, corresponding to a subunit molecular weight of approximately 38.7 kD and pI 12.16. The *S. marcescens* W1 protease gene evidenced an identity of 73.4% with the deduced amino acid sequence of the protease gene of *S. marcescens* ATCC21074, and 53.5% with that of *Erwinia carotovora*. The putative zinc binding sites, HELTHG and QSGALNESLSDVFGS, were located downstream of the start codon (data not shown).

Neither the chitinase nor protease subclones (C94 and C46) evidenced antifungal activity against *M. grisea* (data not shown). Chitin and protein are primary cell wall components of plant pathogenic fungi. Therefore, chitinase

and protease production are believed to be involved in the biological control of phytopathogenic fungi by biocontrol microorganisms, including bacteria and fungi (Buchenauer, 1998; Dunlap et al., 1998; Park et al., 2005). In addition, the metalloprotease of *S. marcescens* has been demonstrated to be involved in pathogenicity in insects (Flyg and Xanthopoulos, 1983). However, our results indicated that the expression of chitinase or protease from *S. marcescens* W1 in *E. coli* did not inhibit the growth of *M. grisea* and *C. citrullina*. We have no direct evidence which might explain this observation, but we theorize that the overexpression of single chitinase or protease in *E. coli* may be insufficient with regard to the inhibition of fungal pathogenic growth. Investigations are currently underway to determine the roles of chitinase or protease in antifungal activity against plant pathogenic fungi,, via the generation of single or double knockout mutants of the extracellular enzymes.

Cloning of antifungal genes of *S. marcescens* W1. The screening of the 3,000 cosmid library clones for growth inhibitory effects against *M. grisea* K101 demonstrated activity from cosmid clones C5 (35 kb) and C32 (27 kb), which evidenced some common restriction fragments (Fig. 1). A 1.3 kb *Eco*RI/*Hin*dIII fragment, when employed as a probe, detected an 8.2 kb *Hin*dIII fragment from C32 and a 6.9 kb *Hin*dIII/*Eco*RI fragment from C32. The sequencing of the 8.2 kb fragment evidenced seven putative ORFs, six of which evidenced similarity to other *ppq* genes (Kim et al., 2003; Meulengerg et al., 1992). A putative ORF exhibiting homology with a membrane dipeptidase was also located upstream of the proposed *pqqA* gene. Similar dipeptidases are zinc-dependent metalloenzymes, which hydrolyze a wide variety of dipeptides (Adachi and Tsujimoto, 1995). Our BLAST search analysis of the dipeptidase gene showed that the dipeptidase homologues were conserved upstream of the *pqq* operons of *Acinetobacter calcoaceticus* and *K. pneumoniae*.

The putative 77-bp gene, *pqqA*, encoded a 25-amino acid peptide, with a subunit size of 2.98 kD and a pI of 5.94. The presence of glutamic acid and tyrosine residues at positions 8, 17, and 21 was consistent with other deduced PQQA proteins. A frame shift in *K. pmeumoniae pqqA* resulted in an abrogation of PQQ biosynthesis (Meulenberg et al., 1992). In another study, it was demonstrated that conserved glutamic acid and tyrosine in PQQA are the principal precursors for PQQ biosynthesis (Goosen et al., 1992). Consequently, the primary function of the PQQA is to generate PQQ biosynthesis precursors.

The 855-bp *pqqB* encodes a 284-amino acid peptide, with subunits of approximately 30.5 kD and pI 9.39. Although PQQB did not harbor hydrophobic stretches, mutant analysis in *K. pneumoniae* indicated that PQQB may be involved in the transport of PQQ across the cytoplasmic membrane into the periplasm, via the modification of an existing transport system (Velterop et al., 1995). The 749-bp *pqqC* encoded a predicted 249 amino acids, with subunits of 28.76 kD and pI 6.45. The PQQC protein in *K. pneumoniae* has been hypothesized to catalyze the last step in PQQ biosynthesis (Velterop et al., 1995). The 278-bp *pqqD* encoded a predicted peptide of 92 amino acids, with an approximately 10.49 kD and a pI of 5.84. The 1,112-bp *pqqE* encoded a peptide of 370 amino acids, with an approximately 41.49 kD and a pI of 6.31. The 1,841-bp *pqqF* gene encoded a predicted 66.7 kD, 614 amino acid peptide, with a pI of 10.66. The deduced amino acid sequences of the *pqqF* genes from *S. marcescens* W1 evidenced a high degree of similarity to *E. coli* protease III and other proteases (Meulenberg et al., 1992). The roles in PQQ biosynthesis played by the proteins encoded by *pqqD*,

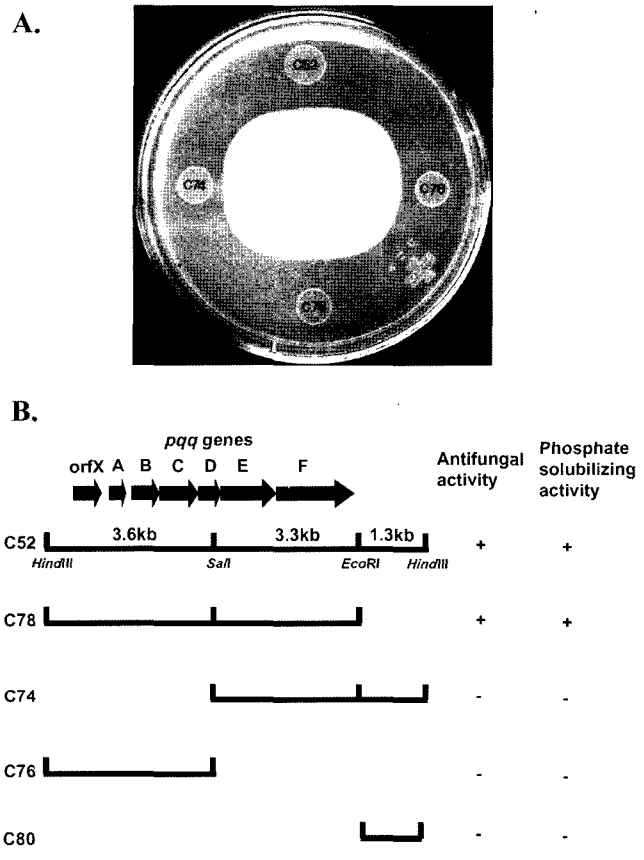

Fig. 1. Antifungal activities, solubilizing phosphate, and restriction map of the sequence in the transformants with the full sequence of *ppq* genes (C52) and its restricted fragments. A: Antifungal activity of constructs with complete *pqq* genes, 8.2 kb C52 and 6.9 kb C78, and deleted genes. B: Restriction map of subclones obtained from 8.2 kb C52 and antifungal activities against *M. grisea* and the phosphate-solubilizing activities of the subclones.

Table 2. Changes in pH, gluconic acid production, and soluble phosphate concentration of culture filtrate of *E. coli* DH5α containing *pqq* genes[a]

Bacterial isolate	pH	Gluconic acid (μg/ml)	Soluble phosphate (μg/ml)
E. coli DH5α	4.99[b]	5,239[b]	213[b]
S. marcescens W1	4.25	65,469	1,102
E. coli C52	4.53	112,227	921
E. coli C78	4.13	107,247	954

[a]Cells were grown in culture medium containing 0.4% hydroxyapatite at 30°C for 24 hours. Culture filtrates were assayed for pH, gluconic acid concentration, and soluble phosphate as described in Materials and methods.
[b]Values are means of three independent experiments.

pqqE, and *pqqF* remain to be further elucidated.

Expression of *S. marcescens* W1 *pqq* genes in *E. coli*. The 6.9 kb *Hin*dIII/*Eco*RI subclone in pUC19 harbored the full set of *pqq* genes from *S. marcescens* W1 that conditioned antifungal activity, whereas the smaller subclones, the 4.6 kb *Sal*I/*Hin*dIII fragment, 3.6 kb *Hin*dIII/*Sal*I fragment, and the 1.3 kb *Eco*RI/*Hin*dIII fragment, lacked this property and also lacked the full complement of *pqq* genes (Fig. 1).

When *E. coli* DH5α harbored the vector plasmid with the full set of *S. marcescens* W1 *pqq* genes, the solubilization of phosphate was observed, whereas the smaller subclones, harboring incomplete *ppq* genes, did not exhibit such activity (Table 2 and Fig. 1). In another study, phosphate-solubilizing ability was correlated with acidification mediated by the generation of gluconic acid (Van Schie et al., 1987). Therefore, we evaluated the changes in pH occurring when *E. coli* constructs harboring different subclones of the *S. marcescens pqq* genes were grown in liquid medium containing hydroxyapatite. *E. coli* DH5α cultures harboring the vector with the partial *pqq* genes remained at pH 5.5 for up to 48 hours of growth (Table 2), whereas those with the full set of *pqq* genes acidified the culture filtrate to pH 4.0 within 24 hours (Table 2).

We determined (Table 2) that reductions in pH value corresponded to higher levels of gluconic acid generation. Reductions in pH corresponded with an increase in the levels of soluble phosphate in the culture medium; soluble phosphate levels remained at 950 ppm for the strain expressing the complete complement of the *ppq* genes, compared with less than 210 ppm for the strain harboring only the vector with the partial *pqq* genes (Fig. 4).

In summary, we have demonstrated in this study that the genetic manipulation of *E. coli* to express *pqq* genes endows the strains with an antifungal activity against *M. grisea* and *C. citrullina*. We speculate that the *ppq* genes increased the generation of gluconic acid, thereby resulting in a drop in pH values that may prove stressful, thereby inhibiting the growth of the tested fungal pathogens. Another possibility is that PQQ generation in *E. coli* functioned as a cofactor for the production of novel antimicrobial compounds. We are currently investigating this possibility, via the fractionation of antifungal compounds from culture filtrates of *pqq* gene-expressing *E. coli*. The results of our recent study showed that *pqq* mutants of *Enterobacter intermedium* lost their phosphate solubilizing activity and antifungal activity against *M. grisea* (unpublished data). How the function of the *ppq* gene in the rhizosphere-competent *Serratia* isolate affects antifungal activity awaits clarification.

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