

Inactivation of *pqq* genes of *Enterobacter intermedium* 60-2G reduces antifungal activity and induction of systemic resistance

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Abstract

Enterobacter intermedium 60-2G, a phosphate solubilizing bacterium, has the ability to induce systemic resistance in plants against soft rot pathogen *Erwinia carotovora*. Glucose dehydrogenase, an enzyme that utilizes pyrroloquinoline quinone (PQQ) as a cofactor, is required for the synthesis of gluconic acid by *E. intermedium* 60-2G. Here, we report that the *pqqA* and *pqqB* genes are required for phosphate solubilization and induced systemic resistance against a soft rot pathogen in tobacco. Mutations in either the *pqqA* or *pqqB* gene abolished the production of 2-ketogluconic acid and eliminated the ability of *E. intermedium* to solubilize hydroxyapatite. Addition of gluconic acid to the growth media restored the ability of the *pqqA* mutant to produce 2-ketogluconic acid. Interestingly, both *pqqA* and *pqqB* mutants of *E. intermedium* lost their ability to inhibit the growth of the rice pathogen *Magnaporthe grisea* KI-409. Additionally, induced systemic resistance against the soft rot pathogen was attenuated in the *pqq* mutants. These functions were restored by complementation with the wild-type *pqq* gene cluster. Our findings suggest that PQQ plays an important function in beneficial traits including phosphate solubilization, antifungal activity, and induced systemic resistance of *E. intermedium*, possibly by acting as a cofactor for several enzymes including glucose dehydrogenase.

Introduction

Root colonization by certain soil microorganisms enhances plant growth and induces systemic resistance against plant disease (Bloemberg & Lugtenberg, 2001). Certain strains of *Rhizobium*, *Pseudomonas*, and *Bacillus* species have the ability to solubilize mineral phosphate into forms that can be utilized by the plants for their growth and development (Hilda and Reyanldo, 1999; Cho *et al.*, 2003). Earlier, we isolated a bacterium, *Enterobacter intermedium* 60-2G, from grass rhizosphere that exhibited a strong ability to solubilize phosphate (Kim *et al.*, 2002a). This bacterium was also found to promote plant growth and suppress plant disease due to induction of systemic resistance (Kim *et al.*, 2002b). *Enterobacter intermedium* 60-2G produced an antibiotic compound (3-methylthiopropanoic acid) that is presumably involved in inhibiting the growth of plant pathogenic fungi (Kim *et al.*, 2003b). However, the molecular mechan-

isms and the microbial determinants involved in plant growth, induced systemic resistance, and antifungal activity of *E. intermedium* are largely unknown.

Phosphorus is essential for plant growth and development. However, in natural soils, this essential micronutrient occurs as insoluble complexes or organically bound mainly as phytates (Altomare *et al.*, 1999). Certain soil microorganisms possess the ability to solubilize these insoluble forms (Goldstein, 1995) via the production of organic acids such as citric acid and gluconic acid (Asea *et al.*, 1988). Gluconic acid is generated by the direct oxidation of glucose that is mediated by a membrane bound glucose dehydrogenase. Subsequently, gluconic acid is enzymatically converted to 2-ketogluconic acid and 2, 5-diketogluconic acid (Olijve & Kok, 1979; Goldstein, 1995). The 2-ketogluconic acid (pKa = 2.66) is more effective than gluconic acid (pKa = 3.41) in solubilizing phosphate (Kim *et al.*, 2002a).

The glucose dehydrogenases of *Acinetobacter calcoaceticus* (Goosen *et al.*, 1989) and *Klebsiella pneumoniae* (Meulenberg

et al., 1992) require pyrroloquinoline quinone (PQQ) as a redox cofactor for activity. PQQ is also the cofactor for an alcohol dehydrogenase in *Pseudomonas testosteron* (Duine & Jongejan, 1989). In *K. pneumoniae* and *Rahnella aquatilis* (Meulenbergh *et al.*, 1992; Kim *et al.*, 1998), the genes encoding *pqq* are encoded by an operon consisting of six genes (*pqqA*, *B*, *C*, *D*, *E*, and *F*); the functions of each of these genes have yet to be completely elucidated. Sequence analysis of the *ppq* operon of *E. intermedium* 60-2G revealed a similar organization of six genes (Kim *et al.*, 2003a). The *pqqA* gene product provides precursors for PQQ biosynthesis, and the *pqqB* gene product is thought to facilitate the transport of PQQ across the cytoplasmic membrane into the periplasm, by modification of an existing transport system (Velterop *et al.*, 1995). In *Escherichia coli* and *Rhizobium*, *pqq* mutants produce the apo-glucose dehydrogenase enzyme and do not secrete gluconic acid (Van Schie *et al.*, 1987; Boiardi *et al.*, 1996).

We speculate that the phosphate-solubilizing activity of *E. intermedium* 60-2G is correlated with the production of organic acids, involving processes requiring PQQ as an essential cofactor for the initial enzyme, glucose dehydrogenase. In this paper, we report on the functional role of *pqqA* and *B* genes in production of 2-ketogluconic acid from glucose, phosphate solubilization, inhibition of fungal phytopathogens, and induction of systemic resistance. These results indicated that PQQ is essential for the beneficial plant effects of *E. intermedium* 60-2G.

Materials and methods

Bacterial strains, plasmids, and growth conditions

Escherichia coli and *E. intermedium* 60-2G (Kim *et al.*, 2002a) were grown on Luria-Bertani (LB) agar medium or LB broth in a culture with shaking (Sambrook *et al.*, 1989) at 37 °C and 30 °C, respectively. Then appropriate filter-sterilized antibiotics were added at the following concentrations: ampicillin 100 µg mL⁻¹, kanamycin 50 µg mL⁻¹, spectinomycin 50 µg mL⁻¹, and tetracycline 25 µg mL⁻¹. Hydroxyapatite agar or broth medium (HY medium) contained the following: glucose 10 g, MgSO₄ 0.2 g, NaCl 1 g, CaCl₂ 2H₂O 0.2 g, NH₄NO₃ 1.5 g, yeast extract 0.5 g, and hydroxyapatite 4 g per 1 L of distilled water. Potato dextrose agar (PDA) (Difco, Detroit, MI) was used for growth of the rice blast fungus, *Magnaporthe grisea*, obtained from Korean Agricultural Culture Collection (KACC) (Suwon, RDA, Korea).

Construction of the *E. intermedium pqqA* and *pqqB* mutants

DNA manipulations for cloning and subcloning were carried out as described by Ausubel *et al.* (1989) and Sambrook

et al. (1989). The genomic DNA of *E. intermedium* was isolated by the CTAB/NaCl method (Ausubel *et al.*, 1989). The plasmid DNA was isolated with CTAB (Del Sal *et al.*, 1989) and the alkaline lysis method (Sambrook *et al.*, 1989). The *pqqA* and *pqqB* genes cloned into the p7HB5 plasmid (Kim *et al.*, 2003a) were disrupted by the insertion of a 0.9-kb SmaI fragment into unique PshAI and HpaI sites within the *pqqA* and *pqqB* genes. The 0.9-kb SmaI fragment harbored a kanamycin-resistance gene from the pRL648 plasmid (Elhai & Wolk, 1988). The chromosomal *pqq* genes in *E. intermedium* 60-2G were exchanged for the disrupted versions, as described previously (Miller *et al.*, 1997) using the pCPP54 exchange vector (Tc^R). Three putative mutants were selected on the basis of their resistance to kanamycin and sensitivity to tetracycline, on plate medium containing 5% sucrose. Southern blot analysis was performed by fusing a digoxigenin-labeled *pqqDE* gene probe to confirm the disruption of the *pqqA* or *pqqB* genes. Two oligonucleotides for use as PCR primers [forward primer (5'-GGC TGC TGG CCG AAC TGA CTT-3') and the reverse primer (5'-GGC CGC AAG AAG CAT TAT TAG-3')] were designed as exact matches to published sequence of *pqqD* and *pqqE* in *R. aquatilis*. (GenBank AF007584). PCR amplification conditions were as described previously (Kim *et al.*, 2003a). The PCR products were randomly-labeled with digoxigenin and used as a probe to detect *pqq* genes from *E. intermedium*. Hybridization analysis was performed using the non-radioactive Genius system (Roche Molecular Biochemicals, Mannheim, Germany) with the digoxigenin-labeled probes.

To complement the *pqq* mutants, the *pqq* genes of *E. intermedium* were cloned into the broad host range vectors, pCPP45 and pCPP46, both of which harbor tetracycline resistance genes. The 13-kb EcoRI fragment containing the complete *pqq* operon (*pqqA* to *pqqF*) in pCSW7 (Kim *et al.*, 2003a) was inserted into pCPP45 (p45E13), and the 5.0-kb HindIII/BamHI fragment containing *pqqA* to *pqqE* was inserted into pCPP46 (p46HB5). The resulting broad host range vectors containing the *pqq* operon from *E. intermedium* were transferred to the *pqqA* or *pqqB* mutants by tri-parental mating using pRK2073 as a helper plasmid. Tetracycline-resistant transconjugants were evaluated for the presence of the plasmid and solubilization of insoluble phosphate.

Analysis of solubilization of insoluble phosphate and production of organic acids

Phosphate-solubilizing bacteria were selected based on their ability to produce clear zones around the bacterial colonies after 3 days of incubation on standard agar plates containing 0.4% hydroxyapatite (HY medium) (Kim *et al.*, 2002a). *Enterobacter intermedium pqq* mutants, the complemented *E. intermedium pqq* mutants and the *E. intermedium* wild-type strains were grown in HY broth medium. Bacterial

growth, pH of the medium, phosphorus concentrations, and levels of organic acid were measured after 0, 12, 24, and 48 h growth on a reciprocal shaker (200 r.p.m.) at 30 °C. One hundred milliliters of hydroxyapatite broth was dispensed into each of the 250-mL bottles, followed by inoculation with 10 µL of each bacterial culture (10^8 CFU mL⁻¹) grown in LB broth for 24 h at 30 °C. Changes in medium pH were measured with a pH meter equipped with a glass electrode. The soluble phosphate concentration of the culture filtrates was determined using a spectrophotometric method at 660 nm (Olsen & Sommers, 1982). Organic acids were identified and quantified by HPLC analysis as described previously (Kim *et al.*, 2002a) using a Shimadzu SPD-10A HPLC system (Shimadzu, Tokyo, Japan) with a UV-Vis detector at 210 nm and a RS pak KC-811 column manufactured by Shodex (Shoko Co., Tokyo, Japan).

Growth determination

Bacterial growth and pH change of the medium were measured in potato dextrose broth (PDB) medium (Difco Inc., Detroit, MI). Cultures of *E. intermedium* 60-2G strains, previously grown on LB for 48 h, were incubated at 28 °C on PDB for 24 h. Culturable cell density was determined by plating serial dilutions of the culture on LB agar plates and assessment of CFU after 3 days growth at 28 °C.

Induction of systemic resistance and fungal inhibition assay

Induction of systemic resistance was examined in tobacco with *Erwinia carotovora* ssp. *carotovora* SCCI as the challenging pathogen, as described previously (Han *et al.*, 2006). Tobacco cv. Xanthi seeds were surface-sterilized with 3% (w/v) sodium hypochlorite and washed thoroughly with sterile distilled water. Individual seeds were placed in 12-well microtiter plates (SPL Inc., Republic of Korea) containing 1 mL of 0.5% (w/v) Murashige and Skoog salt (MS) agar supplemented with 3% sucrose in each well. Two weeks after seeding, 10 µL of bacterial suspensions (1×10^8 CFU mL⁻¹) of the wild-type, the *pqq* mutants, and the complemented *pqq* mutants were applied onto the roots. One week after application of bacterial treatments, the tobacco plants were challenged with *E. carotovora* ssp. *carotovora* SCCI by pipetting 2 µL of the pathogen (1×10^8 CFU mL⁻¹) onto a leaf. One to two days after pathogen challenge, soft-rot disease was rated by counting the number of symptomatic leaves (leaves that showed wilting and water soaking) and expressed as a percentage of all examined leaves. The control plants were treated with 2 µL sterile water. Three independent experiments were performed and each treatment included at least 21 plants.

Fungal antagonism of the *E. intermedium* strains was assessed with the rice blast fungus *M. grisea*. The fungus was

inoculated in the centers of PDA agar plates followed by 5 days of incubation at room temperature, until the fungal mycelium covered 30% of the plate. Aliquots (10 µL) of *E. intermedium* strains (10^9 CFU mL⁻¹) were then spotted around the fungal mycelia. After incubation for an additional 2–3 days, the inhibition zone surrounding the bacterial spots was measured.

Statistical analysis

Data were analyzed by ANOVA using SPSS 12.0K for WINDOWS software (SPSS Institute, Korea). The significance of the effects of bacterial treatment was determined by Duncan's multiple range test ($P=0.05$).

Results and discussion

Pqq mutants are impaired in phosphate solubilization

In order to examine the role of *pqq* genes in phosphate solubilization, we first constructed mutants in which *pqqA* or *pqqB* genes were individually inactivated. Southern blot analysis of genomic DNA isolated from the *pqq* mutants revealed that the sizes of the hybridizing fragments were about 1 kb bigger than the wild type due to insertion of the kanamycin gene in the *pqqA* and *pqqB* coding regions (data not shown). The ability of the *pqqA* mutant to solubilize mineral phosphate was evaluated using the hydroxyapatite plate assay. After a 3-day incubation at 30 °C, a clear zone around the wild-type *E. intermedium* was seen (Fig. 1). The *pqqA* mutant did not solubilize hydroxyapatite as evidenced by the lack of clear zones around the colonies (Fig. 1).

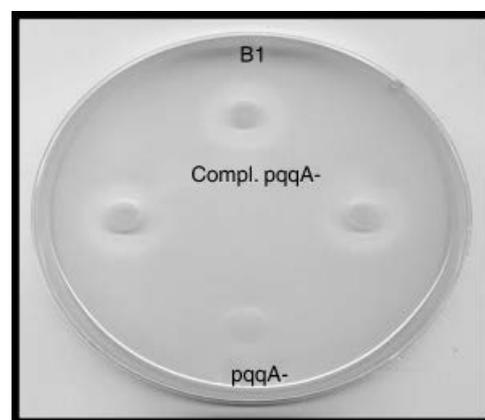


Fig. 1. Effect of *pqq* mutations on phosphate solubilization. B1, *Enterobacter intermedium* 60-2G wild-type; *pqqA* –, *pqqA* mutant; Compl. *pqqA* –, *pqqA* containing the *pqq* operon of *E. intermedium*. Phosphate solubilization activity with the *pqqB* mutant and the complemented mutant gave results similar to those observed with the *pqqA* mutant (data not shown).

Complementation with the wild-type operon restored mineralized phosphate solubilization ability to the *pqqA* mutant (Fig. 1), confirming that *pqqA* was required for the solubilization of phosphate by *E. intermedium*. Similarly, the *pqqB* mutant was unable to solubilize hydroxyapatite (results not shown).

Previous studies have demonstrated the existence of a direct correlation between the phosphate-solubilizing ability of bacteria and the acidification of growth media due to release of organic acids, including gluconic acid (Van Schie *et al.*, 1987; Illmer *et al.*, 1995). Therefore, we assessed the changes in pH when *E. intermedium* 60-2G, the *pqq* mutants, and the complemented mutants were grown in liquid medium containing hydroxyapatite. After 36 h, the pH values of culture media in which *E. intermedium* 60-2G and the complemented *E. intermedium pqq* mutants were grown dropped to 4.0. The reduction in the pH of the culture medium was correlated with increased levels of soluble phosphate (Fig. 2b). In contrast, the pH of the culture media in which the *pqqA* mutant or the *pqqA* mutant carrying the empty vector was grown remained at 5.0 even after 48 h growth (Fig. 2a). The amount of soluble phosphate measured from the culture media in which wild-type *E. intermedium* and the complemented mutants were grown was *c.* 1600 p.p.m. The soluble phosphate levels in the culture media in which *pqq* mutants or the mutants harboring empty vectors were grown remained at < 200 p.p.m. (Fig. 1b).

The acidification of culture media by *E. intermedium* 60-2G was correlated with the accumulation of gluconic acid and 2-ketogluconic acid (Kim *et al.*, 2002a). In the case of wild-type *E. intermedium*, the concentration of 2-ketogluconic acid reached a concentration of 12 000 p.p.m. after 48 h growth, whereas no 2-ketogluconic acid was detected in culture filtrates of the *pqqA* mutant (Fig. 3a). The amount of 2-ketogluconic acid in the complemented *pqqA* mutant was similar to that of the wild-type strain (data not shown). Interestingly, the addition of gluconic acid to the growth media enabled the *pqqA* mutant to produce 2-ketogluconic acid (Fig. 3b), suggesting the presence of a functional gluconate dehydrogenase in *pqqA* mutant. The production of 2-ketogluconic acid reached a maximal level at 24 h postinoculation in both the wild-type and *pqqA* mutant strain when gluconic acid was supplied as a sole carbon source (Fig. 3b). These data indicate that the *pqq* genes of *E. intermedium* 60-2G are involved in the generation of organic acids which mediates the acidification of the culture media and solubilization of insoluble phosphate (Hilda & Reyando, 1999).

Phenotypes of *E. intermedium pqq* mutants

In our previous study, we demonstrated that *E. intermedium* inhibited the growth of *M. grisea* (Kim *et al.*, 2002b). In

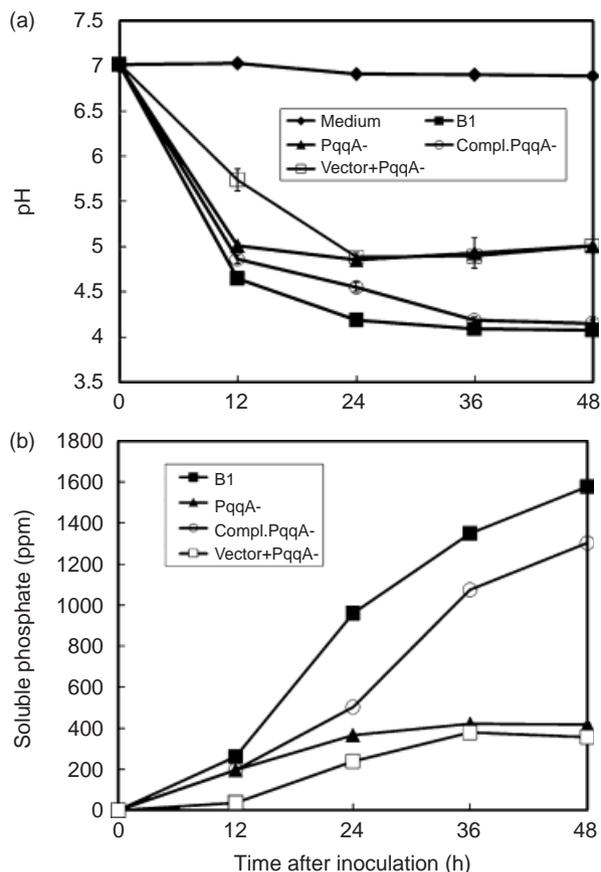


Fig. 2. Changes in the pH of culture filtrates and solubilization of insoluble phosphate of the wild type and *pqqA* mutant of *Enterobacter intermedium*. Cells were grown on glucose minimal medium supplemented with 0.4% hydroxyapatite at 30 °C. The pH of the culture filtrate and soluble phosphate concentration were determined at 12 h intervals. Medium, noninoculated HY medium; B1, *E. intermedium* 60-2G; *pqqA* –, *pqqA* mutant; Compl. *pqqA* –, *pqqA* mutant containing a full length of *pqq* gene cluster of *E. intermedium*. Vector+*pqqA* –, *pqqA* mutant containing a broad host range vector pCPP45. Vertical bars represent the SEs of three independent experiments. Treatment with the *pqqB* mutant gave results similar to those observed with the *pqqA* mutant (data not shown).

order to examine if *pqq* genes play any role in antifungal activity, we examined the ability of *pqq* mutants to inhibit the growth of *M. grisea* in a plate assay (Fig. 4). The wild-type and complemented *pqq* mutants inhibited the mycelial growth of the blast fungus, whereas no antifungal activity was evidenced by the *pqq* mutants (Fig. 4). Cucumber plants grown in presence of wild-type *E. intermedium* 60-2G in a soil-less media containing rock phosphate as the sole phosphate source showed enhanced growth when compared with plants grown in the absence of rock phosphate. Interestingly, this growth promotion was not observed when *pqqA* or *pqqB* mutants of *E. intermedium* 60-2G were used (data not shown). A possible explanation of this loss in the

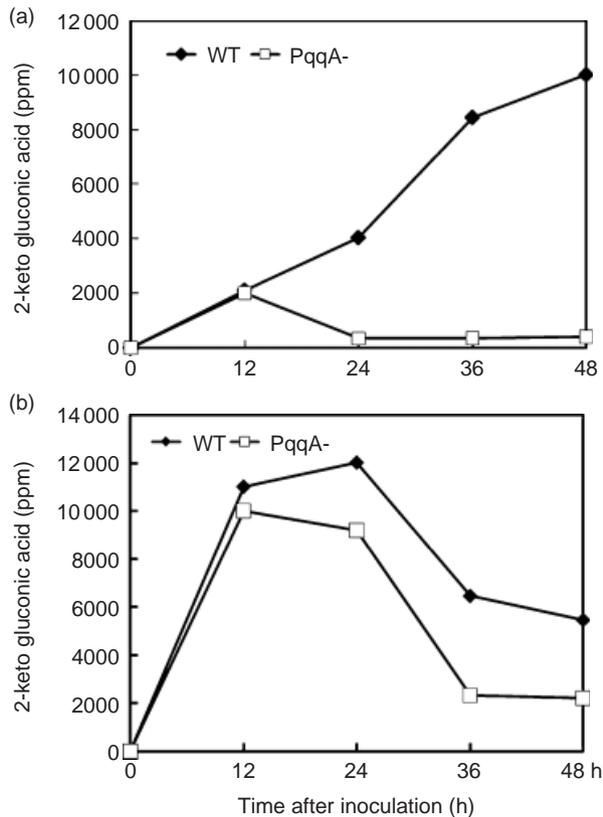


Fig. 3. Production of 2-ketogluconic acid by *Enterobacter intermedium* strains with glucose (a) or gluconic acid (b) used as a sole carbon source in minimal medium. Cells were grown in culture liquid medium with different carbon sources containing 0.4% hydroxyapatite at 30 °C. Culture filtrates were assayed by HPLC for 2-ketogluconic acid at 12 h intervals. WT, *E. intermedium* 60-2G; pqqA⁻, pqqA mutant. The complemented pqqA mutant produced the organic acid with time profiles identical to those of the wild-type strain. The data are from one of three independent experiments evidencing similar results. The pqqB mutant showed results similar to those of the pqqA mutant (data not shown).

inhibition of fungal growth and plant growth promotion might involve less acid production by the pqq mutants. Another possible explanation is that the organic acid itself may have antifungal activity as has been suggested by Kaur *et al.* (2006). However, gluconic acid itself did not have any antifungal activity in our study (data not shown). We have shown previously that expression of the pqq genes of *Serratia marcescens* W1 in *E. coli* resulted in antifungal activity against a variety of phytopathogenic fungi (Kim *et al.*, 2006). We have also demonstrated that *E. intermedium* 60-2G produces 3-methylpropanoic acid, an antibiotic that has antifungal activity. Therefore, it is possible that PQQ may be required for the production of the antifungal 3-methylpropanoic acid (Kim *et al.*, 2003b). However, the production of 3-methylpropanoic acid was not altered by the pqq mutations (data not shown) indicating that pqqA

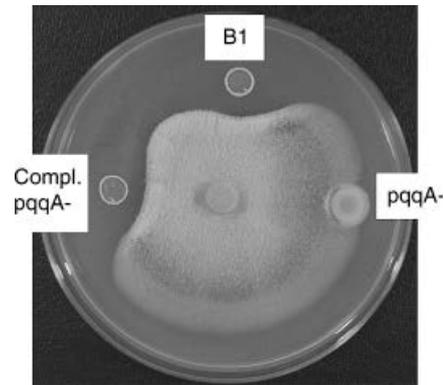


Fig. 4. Effect of pqq mutations on inhibition of the phytopathogenic fungus, *Magnaporthe grisea* KI-409 grown on potato dextrose agar plate. B1, *Enterobacter intermedium* 60-2G wild-type; pqqA⁻, pqqA mutant; Compl. pqqA⁻, pqqA containing the pqq operon of *E. intermedium*. Fungal growth changes caused by the pqqB mutant and its complemented mutant were similar (data not shown). The picture is from one of three independent experiments evidencing similar results.

and pqqB genes are not directly involved in the synthesis of 3-methylpropanoic acid.

Colony morphology of the pqq mutant on PDA plates without fungal pathogens was clearly different from that of the wild type (Fig. 4). Comparing the growth in PDB liquid medium, the pqq mutants grew continuously during 48 h cultivation whereas that of the wild type and the complemented pqq mutants declined. We observed that the decline correlated with acidification of the medium (Fig. 5). After 12 h, bacterial cultures of the wild type and the complemented pqq mutants were at 4.0 pH, declining to pH 3.5 by 24 h (Fig. 5). We correlate the decreased fungal growth with these strains with increased acidification. These results indicate that a decrease in pH caused by the wild type and the complemented pqq mutants might be involved in antifungal activity.

Induced systemic resistance

Induced systemic resistance was observed when the tobacco roots were colonized with the wild-type and the complemented pqq mutants (Fig. 6). *Enterobacter intermedium* pqqA mutant, however, was unable to induce systemic resistance as evidenced by high disease incidence (Fig. 6). However, gluconic acid itself did not show induction of systemic resistance against soft-rot disease (data not shown). Our results suggest that functional pqq genes are required for induced systemic resistance against soft rot in tobacco. Studies with another plant growth-promoting rhizobacterium, *Paenibacillus polymyxa* E681, have demonstrated that an enzyme in PQQ synthesis is involved the plant-rhizobacterium interaction, particularly under conditions in

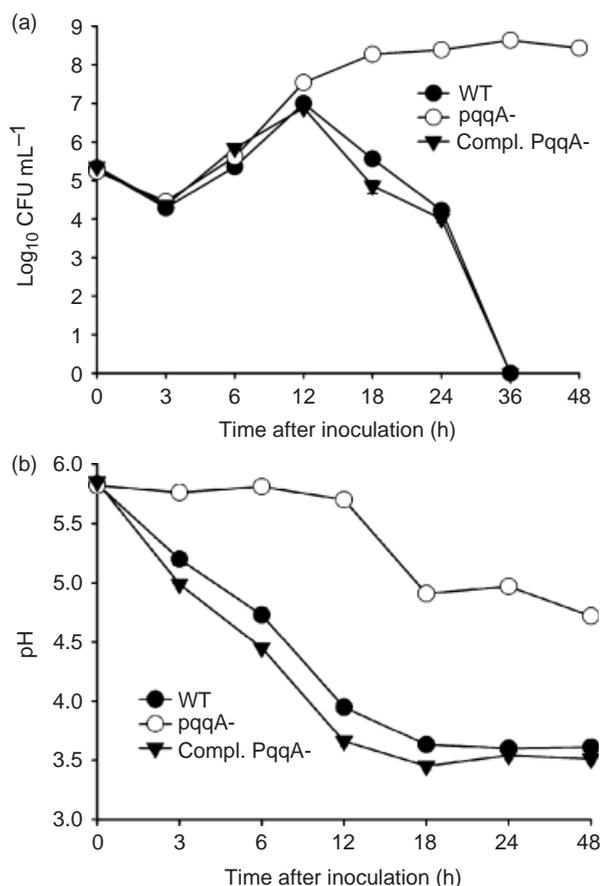


Fig 5. Growth (a) and pH change (b) of *Enterobacter intermedium* and *pqq* mutants in potato dextrose broth medium. Medium pH was measured using a pH meter equipped with a glass electrode. CFU of bacterial cultures were determined by plating serial dilutions of the culture on LB agar plates. The means of three experiments are presented, and the vertical bars represent the SEs. The *pqqB* mutant showed results similar to those of the *pqqA* mutant (data not shown).

which phosphorous is limiting (Seul *et al.*, 2007). We are currently attempting to determine the manner in which PQQ is involved in induced resistance.

In summary, PQQ, a cofactor for several dehydrogenases, including glucose dehydrogenase, was involved in a variety of beneficial functions of *E. intermedium* 60-2G including the solubilization of insoluble phosphorus, the production of antifungal activity, and the induction of systemic resistance. All of the beneficial functions negated by mutations of *pqq* genes in *E. intermedium* 60-2G, were restored by genetic complementation. Solubilization of insoluble phosphorus activity was restored by addition of gluconic acid the culture medium. However, gluconic acid itself did not have anti-fungal activity and did not induce systemic resistance. Our findings indicate that PQQ is involved either directly or indirectly in various metabolic pathways that control the beneficial traits of *E. intermedium* 60-2G.

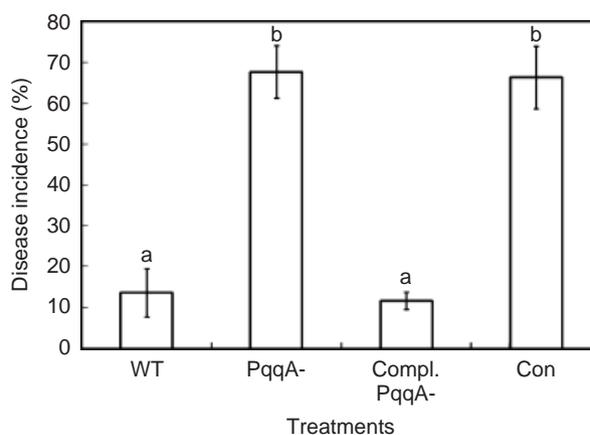


Fig. 6. Induced systemic resistance against soft-rot pathogen *Erwinia carotovora* SCC1 in tobacco seedlings. Seedlings grown in well plates and tobacco roots were inoculated with *Enterobacter intermedium* 60-2G (WT), *pqqA*- (*pqqA* mutant), compl. *pqqA*- (complemented *pqqA* mutant), or water as a control (Con). After 1 week, leaves were challenged with the pathogen and disease was evaluated 2 days later. Three independent experiments were conducted with 21 plants/treatment. Differences in letters indicate significant differences between the treated and control samples, based on Duncan's multiple range test at $P=0.05$. Treatment with the *pqqB* mutant and the complemented mutant gave results similar to those observed with the *pqqA* mutant (data not shown).

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