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Lipopeptide Surfactin Produced by *Bacillus amyloliquefaciens* KPS46 is Required for Biocontrol Efficacy Against *Xanthomonas axonopodis* pv. *glycines*

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ABSTRACT

The biological control of root and foliar diseases of soybeans caused by fungi and bacteria (e.g. *Xanthomonas axonopodis* pv. *glycines*, Xag) using the plant growth promoting rhizobacterium *Bacillus amyloliquefaciens* KPS46 has been previously reported. Disease suppression is thought to be due, in part, to the production of secondary metabolites. While a wide variety of these active compounds has been identified, their mode of action and mechanism of disease suppression on soybeans are not fully understood. The study used HPLC to identify secondary metabolites produced by *B. amyloliquefaciens* KPS46 and tested these compounds for biological control activity against soybean bacterial pustule disease caused by Xag. HPLC analyses indicated that a lipopeptide surfactin was present in KPS46 cell-free culture extracts, with maximum yields of ~550 ± 20.3 mg L^{-1}. Exogenous application of KPS46-produced surfactin to soybean plants directly that inhibited Xag, reduced disease severity and enhanced plant growth. UV mutagenesis of KPS46 and PCR assays were carried out to assess the role of surfactin production on the biocontrol activity. An independently-generated *srfAA* mutant of KPS46, strain M6, was unable to produce lipopeptide surfactin. The M6 mutant also was severely affected in its ability to produce extracellular enzymes, including endoglucanase, cellulase, and protease; and had reduced motility on the surface of agar compared to the wild-type strain KPS46. In contrast, the M6 mutant had enhanced production of α-amylase, and faster growth rate in nutrient broth, than did the parental strain. Soybean plant assays using the *srfAA* mutant and wild-type biocontrol agents against bacterial pustule disease indicated that the mutant strain M6 had significantly less effect on disease reduction compared to the wild-type parental strain. Results of this study suggest that the ability of *B. amyloliquefaciens* KPS46 to reduce bacterial pustule severity on soybeans is associated with the production of a lipopeptide surfactin encoded by *srfAA*, and that mutations in this locus also effect extracellular enzyme production.

Key words: biocontrol, UV mutagenesis, *srfAA* gene, extracellular enzymes, HPLC analysis, soybean bacterial pustule

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INTRODUCTION

Bacterial pustule disease caused by *Xanthomonas axonopodis* pv. *glycines* (Xag) is commonly observed in soybean fields in Thailand. Severe symptoms on susceptible cultivars include necrotic and raised spots on the leaf surface that are often surrounded by a chlorotic halo. Lesions coalesce during favorable environmental conditions resulting in leaf defoliation and yield loss (Prathuangwong *et al.,* 1990). Completely effective control measures for this disease are currently not available and bacterial pustule disease continues to be a persistent problem for soybean production. Biological control of this disease using *Bacillus amyloliquefaciens* KPS46, which was originally isolated from the soybean rhizosphere, has been demonstrated in several studies and represents an additional strategy for the effective and sustainable management of this disease (Prathuangwong and Kasem, 2003; Prathuangwong *et al.,* 2005b). The potential role by which KPS46 enhances disease suppression, and the ability to activate key elicitors, such as plant growth regulators and defense-related enzymes, have also been reported (Prathuangwong and Buensanteai, 2007; Buensanteai *et al.,* 2008). However, little is known about potential antimicrobial substances produced by this bacterium. Cell-free culture filtrates of strain KPS46 have proven to be effective in inhibiting Xag (Kasem, 2002). Subsequent studies have shown that three TLC-purified fractions were active against Xag *in vitro*. While these compounds were tentatively identified as lipopeptides, they have not yet been compared with metabolites from reference strains (Detmanee, 2004).

There have now been several reports showing that culture filtrates from KPS46 are effective against various plant pathogens (Kasem and Prathuangwong, 2001). The biocontrol ability of strain KPS46 is partly due to cyclic lipopeptides produced in a nonribosomal manner (Stein *et al.,* 1996). Some of these lipopeptides have been studied in detail, including surfactin, fengycins, and several iturins. Surfactin is a heptapeptide condensed, via a lactone bond, to a β-hydroxy fatty acid with 13 to 15 carbon atoms. Surfactin exerts its antimicrobial and antiviral effect by altering the membrane integrity (Peypoux *et al.,* 1999). Surfactin has exceptional antibiotic activity, and has surface active properties directed against microbial adhesion (Razafindralambo *et al.,* 1998). Lipopeptides have been postulated to influence the adhesiveness of *B. subtilis* by adsorbing to the bacterial surface (Ahimou *et al.,* 2000). It was reported that lipopeptides produced by other bacterial antagonistic strains also exhibit a wetting activity, involved in surface colonization by bacteria (Matsuyama and Nakagawa, 1996). Bacterial antagonists producing lipopeptide surfactins with antimicrobial and surfactant properties are a potentially attractive tool in plant disease management programs.

In the present study, the biologically active fraction from culture filtrates of the lipopeptide surfactin-producing bacterium *B. amyloliquefaciens* strain KPS46 was determined by HPLC analysis. When applied to soybeans as seed or foliar treatments, the active fraction was found to suppress infection of soybeans by Xag. In addition, the role of surfactin in biological control activity was evaluated using UV mutagenesis and PCR analyses.

MATERIALS AND METHODS

Bacterial strains and culture conditions

The antagonistic bacterium *B. amyloliquefaciens* KPS46 and a virulent strain of the bacterial pustule pathogen *X. axonopodis* pv. *glycines* KU-K-46012 (No. 12-2) were obtained from the culture collection of Department of Plant Pathology, Kasetsart University, Thailand. Each bacterium was stored in 15% glycerol at -20°C and was grown by streaking onto nutrient agar

(NA) plates (Difco) and incubated at 28±2°C for 48 h. A loopful of each bacterium was inoculated into nutrient broth (NB) and incubated at room temperature for 48 h on a rotary shaker, at 150 rpm. Cells were harvested by centrifugation at 4,000xg for 20 min, washed in sterile water and resuspended in sterile water to a final concentration of ~1×10^6 to 1×10^8 cfu ml^{-1}, prior to application to plants. The remaining culture was kept at 10°C until used.

Culture filtrate assay of KPS46 strain against bacterial pathogen

Growth inhibition of the Xag strain KU-K-46012 (No. 12-2) by culture filtrates from *B. amyloliquefaciens* KPS46 was determined using a paper disc diffusion assay. Strain KPS46 was grown in 50 ml NB amended with 2% glucose (NGB) in a 300-ml Erlenmeyer flask for 48 h, with shaking, as described above. The culture was transferred to a 50-ml tube and cell-free supernatant was obtained by centrifugation at 13,000xg for 15 min at 4°C, followed by filtration through a 0.22 μm syringe microfilter. A 15 µl aliquot of the KPS46 supernatant was placed onto a 5 mm sterile filter paper disc, which was subsequently placed onto the surface of an NA plate, amended with 2% glucose (NGA), pre-spread with ~ 1×10^8 cfu ml^{-1} of the Xag KU-K-46012 pathogen. The plate was incubated for 48 h at 28°C and the zone of inhibition surrounding cell growth was measured with a ruler.

Generation of mutants deficient in lipopeptide synthesis

The *B. amyloliquefaciens* strain KPS46 was grown in NGB medium to stationary phase and ~ 10^4 cfu ml^{-1} was spread-plated onto NA plates. Petri plates were exposed to UV irradiation (65 watts) for 1, 2, 5, 10 or 15 min at a constant distance from the UV light source. The UV-irradiated plates were incubated at 28°C in the dark until surviving colonies were visible. Colonies from plates containing about 10-20% survivors were subsequently examined for deficiencies in lipopeptide synthesis using the paper disc assay described above. Only colonies not producing inhibition zone against the Xag strain KU-K-46012 (No. 12-2) were selected for mutant genotype confirmation, using PCR with strain-specific primers as discussed below.

To confirm that both mutant and wild-type strains studied were KPS46, a 500 bp PCR product from the 16S rDNA gene region was amplified using primers 16S-F 5' -TAATACGACT CACTA TAG GG- 3' and 16S-W 5' –CGATTTAG GTGACACTATAG-3' (Prathuangwong *et al.*, 2005a). Each 25 µl PCR reaction contained: distilled H_2O, 15.4 µl, 5X buffer, 5 µl, 25 mM $MgCl_2$ 1.5 µl, 10 µg/µl BSA, 0.4 µl, 0.5 µl of each primer, (0.2 µM), 10 µM dNTPsmix, 0.5 µl, 5 units/µl Taq, 0.2 µl and 1 µl of DNA from the wild-type and mutant strains. PCR reaction conditions were: initial denaturation at 98°C for 5 min, 35 cycles of denaturation at 95°C for 1 min, annealing at 58°C for 1 min and extension at 72° C for 1.5 min, a final extension at 72°C for 5 min and holding at 4°C. Aliquots (10 µl) of the PCR mixtures were analyzed on 1% TBE agarose gels following electrophoresis at 75 V for 2 h. Mutants producing the correct size 16S rDNA PCR product were *B. amyloliquefaciens* and were used for future studies.

Extraction and isolation of lipopeptide surfactin

Wild-type, lipopeptide surfactin-producing, strain KPS46 and UV mutants were inoculated into NGB medium, and incubated at 28°C, with shaking at 150 rpm, for 48 h. The culture suspension was centrifuged at 13,000xg for 15 min at 4°C, the resulting supernatant was acidified to pH 2.0 with concentrated HCl and the suspension was incubated in a refrigerator overnight to facilitate precipitation of lipoproteins. Lipopeptide was extracted from the precipitate using a modification of the method described by

Jacques *et al*. (1999) and Aruajo *et al*. (2002). The precipitates were collected by centrifugation at 13,000xg for 15 min at 4°C and washed three times with distilled water. The crude 1ipoipeptide was dissolved in butanol and 1oaded onto a silica gel column previously equilibrated with butanol. The column was washed with the same solvent and the 1ipopeptide was eluted with a 1iner gradient of 100% butanol. HPLC spectra were detected at 210 nm and compared with authentic lipopeptide surfactin purchased from Sigma Chemicals (St. Louis, MO, USA).

Analysis of *srfAA* gene disruption

Mutant KPS46 strains not producing inhibition zones against the Xag strain KU-K-46012 (No. 12-2) were examined in order to determine if the surfactin-deficient phenotype was due to disruption of the *srfAA* gene. The *srfAA* genes were PCR-amplified from genomic DNA of mutant and wild-type KPS46 strains using primers Srfkn-1 (5'-AGCCGTCCTGTCTGAC GACG-3') and Srfkn-2 (5'-TCTGCTGCCATA CCGCATAGTC-3'). The primers were previously shown to be complementary to conserved regions of *srfAA* from other strains of *B. amyloliquefaciens* (Koumouts *et al*., 2004). Each 25 µl reaction included: ddH_2O, 15.4 µl, 5X buffer, 5 µl, 25mM $MgCl_2$, 1.5 µl, 10 µg/µl BSA, 0.4 µl, 10 uM *srf1* and *srf2,* 0.5 µl each, 10uM dNTPsmix, 0.5 µl, 5 units/µl Taq, 0.2 µl and 1 µl of genomic DNA. PCR reaction conditions included an initial denaturation at 98°C for 5 min, 35 cycles, of denaturation at 95°C for 1 min, annealing at 58°C for 1 min and extension at 72°C for 1.5 min, a final extension at 72°C for 5 min, and holding at 4°C. The 1.75 kb PCR products were resolved by agarose gel electrophoresis as previously described (Koumouts *et al.,* 2004).

Differential expression of *srfAA* target genes

To further investigate the surfactin-deficient phenotype of the UV mutants, bacterial growth rate, swarming motility, and the expression of extracellular enzymes were studied. For growth rate determination, wild-type and mutant strains were cultivated in 350 ml flasks containing NB for 24 h at 28°C, with shaking at 150 rpm. Bacterial growth was measured every 12 h over a three-day period by direct viable counts on the same medium.

The relationship between lipopeptide surfactin production and swarming motility was evaluated as previously described (Calvio *et al*. 2005). The KPS46 wild-type and UV mutant strains were grown in NB for 24 h at 28°C, culture suspension were adjusted to $OD_{600\ nm}$ = 0.2 and 10 µl aliquots of the cultures were spot-inoculated onto the center of an NA plate. Pertri plates were incubated at 28°C for 48 h and the diameters of cell growth due to bacterial migration, were measured.

Relative levels of extracellular enzyme production in wild-type and mutant *B. amyloliquefaciens* strains were assessed using radial diffusion assays (Wikström *et al.,* 1981). Cultures were grown overnight in NB at 28°C, cells were pelleted by centrifugation and the supernatant was sterilized by passage through 0.2 µm membrane filters. Aliquots (30 µl) of filter-sterilized supernatants were placed in a 0.5 cm well in agar medium on Petri plates. Media composition and incubation- and detection-conditions for each assay are specified below. Enzyme activity was estimated from the diameter of the zone surrounding the culture supernatant of each strain. All experiments were replicated. For endoglucanase activity, bacterial cells were grown in NYGA (5 g bacto-peptone, 3 g yeast extract, 20 g glycerol, 15 g agar, in 1 L water) supplemented with 0.125% carboxylmethyl cellulose (CMC) (Barber *et al*., 1991). The inoculated plate was stained with 0.1% congo red for 30 min, rinsed with water and washed twice with 1 M NaCl. Endoglucanase activity was observed by a pale yellow zone of clearing

contrasted with a red background. For cellulase activity, inoculated plates containing cellulose assay medium (0.1% CMC, 25 mM sodium phosphate pH 7.0, and 0.8% agarose) (Andro *et al.,* 1984) were incubated overnight at 28°C. Following growth, plates were stained with 0.1% Congo red for 20 min and washed twice with 1 M NaCl. Cellulase was visualized as halos surrounding the wells. For α-amylase production, inoculated plates containing alpha-amylase assay medium (0.5% yeast extract, 1.0% tryptone, 0.5% NaCl, 0.2% soluble starch, and 0.8% agarose) were incubated overnight at 28°C and stained with potassium iodine for 10 min as describe by Ray *et al*. (2002). The presence of α-amylase was detected as clear halos surrounding the wells. Proteases were investigated by inoculating plates containing NYGA medium supplemented with 0.5% skimmed milk as described by Barber *et al*. (1991). Plates were incubated at 28°C for 48 h and extracellular protease production was detected visually as clear halos surrounding the wells.

Biological control properties of surfactin-deficient mutants

The disease-suppression effects of vegetative cells, cell-fee supernatants and surfactin extracts from wild-type and mutant strains of KPS46 were evaluated using agar plate and soybean plant assays under laboratory and greenhouse conditions. The antibacterial activity of vegetative cells, supernatants, and lipopeptide surfactin against Xag was carried out using the paper disc diffusion method as described above. Cell-free culture filtrates and surfactin extracts were produced and assayed as indicated above. The size of the inhibition zone was determined after 48 h incubation at 28°C.

Soybean (cv. AGS292) seeds were treated with vegetative cells and cell-free supernatants of wild-type and mutant strains of KPS46. Soybeans were planted in 6-inch plastic pots containing standard mixed soil and grown in the greenhouse. There were 10 replications per treatment with three seeds per pot. At 7-14 days after seedling emergence, plants were harvested, and root and shoot length and fresh weights were measured. The experiment was conducted three times.

Vegetative cells, cell-free filtrates and lipopeptide surfactin extracts from filtrates from wild-type and mutant strains of *B. amyloliquefaciens* KPS46 were also sprayed onto cv. AGS292 plants at the R1 growth stage of plant growth (before the flowering stage). Controls consisted of nontreated plants grown under the same conditions. Plants were challenged with 10^8 cfu ml^{-1} of a suspension of the pathogenic Xag strain, 24 h after treatment with biocontrol strains or the substances described above. Treatments and bacteria were directly applied to the leaves of the entire plant using a manual hand sprayer. Plants treated with distilled water or a copper hydroxide bactericide (Funguran®) served as controls. Plants were evaluated for bacterial pustule severity 14 days after pathogen inoculation by estimating the percent area of each leaf exhibiting lesions and halos using the method described by Preecha (1988). The experiment was arranged in a complete randomized block design, with five replications. Measurements from all leaves in each pot were averaged prior to statistical analyses. Data from each planting were analyzed using the SAS statistical program (Cary, NC, USA).

RESULTS

KPS46 produced antimicrobial compounds in liquid cultures

The relationship between growth suppression of Xag and the production of antimicrobial compounds by *B. amyloliquefaciens* KPS46 was analyzed using a paper disc diffusion assay. Both liquid cultures and the culture filtrates of strain KPS46 strongly suppressed growth of Xag on NA plates (Table 1). Undiluted filtrates

Table 1 Severity of bacterial pustule disease on soybean plants in a greenhouse experiment and inhibition of bacterial growth for *Xanthomonas axonopodis* pv. *glycines* (Xag) on NA following treatments with vegetative cells from *Bacillus amyloliquefaciens* KPS46 wild-type and *srfAA* mutant, and its cell-free filtrate and lipopeptide surfactin from wild-type.

Treatment	Disease symptom (%)[a]		Inhibition zone (mm)
	Severity	Reduction	
KPS46 cells	34.9[c]	30[a]	14.4
Cell-free filtrate	38.2[bc]	23[b]	7.1
KPS46 surfactin	35.5[bc]	29[a]	11.4
Surfactin mutant M6[b]	41.6[b]	16[c]	0
Copper hydroxide	36.6[bc]	26[ab]	13.2
Nontreated	49.7[a]	-	-

[a] Disease severity was assessed as the percentage of leaf area infection measured by the method of Preecha (1988). Percentage of disease reduction refers to values of leaf area infected by Xag on nontreated plants. Means in the column followed by the same letter are not significantly different, at P = 0.05, according to DMRT.

[b] Vegetative cells of surfactin mutant M6.

showed the strongest inhibition of pathogen growth, as revealed by a large inhibition zone (14.4 cm) surrounding the culture paper discs. Moreover, a six-fold dilution of culture filtrate still produced pathogen suppression, suggesting that the bacterium excreted large amounts of the inhibitory compounds (data not shown). This demonstrated that *B. amyloliquefaciens* KPS46 produced antimicrobial metabolites in the culture medium.

UV mutation in *srfAA* genes of the biocontrol strain KPS46

Six of 32 (18.7%) KPS46 colonies that survived UV mutagenesis lost the ability to suppress growth of the bacterial pustule pathogen Xag on NA plates. These six mutant strains, M6, M16, M17, M20, M24, and M33, were confirmed as being confide KPS46 by PCR analyses using strain-specific primers and gene sequencing. All strains produced a 500 bp fragment with primers 16S-F and 16S-W (Figure 1) and BLASTN analysis of the 16S rDNA gene sequences amplified from each mutant and the wild-type KPS46 strain indicated that the strains were *B. amyloliquefaciens*.

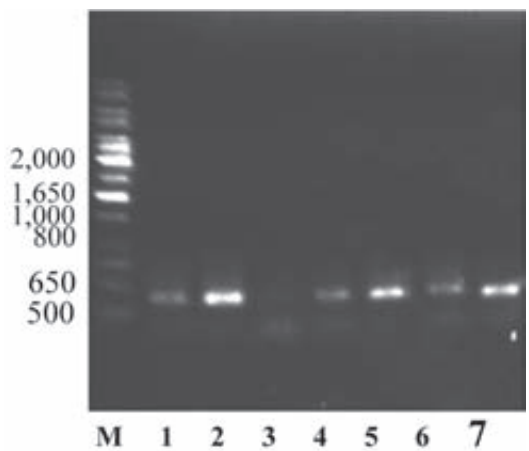

Figure 1 Gel electrophoresis of 16S rDNA region analysis. PCR amplification products of KPS46 mutant and wild-type strains obtained with the specific primers of *Bacillus amyloliquefaciens*, 16S-F and 16S-W. Strains M6, M16, M17, M20, M24, M33, and KPS46 are in lanes 1 to 7 respectively. Lane M contains a 100 bp molecular size marker ladder.

Among the mutants generated, strain M6 was selected for further study since culture filtrates from this mutant completely failed to suppress growth of Xag on agar plates. To test the hypothesis that production of lipopeptide surfactin produced by KPS46 could be responsible for the suppression of growth of the Xag pathogen, lipopeptide surfactin was recovered from culture filtrates of wild-type KPS46 and the M6 mutant strain and analyzed by analytical HPLC. Results in Figure 2 show that high concentrations of bioactive non-polar antibiotics were detected in culture filtrates. The antibiotic was identified as a surfactin-type lipopeptide based on the similarity retention times to authentic surfactin standards, such as the surfactin produced by *B. subtilis* ATCC21332 (Figure 2). Together, C18 homologues represented more than 50% of the

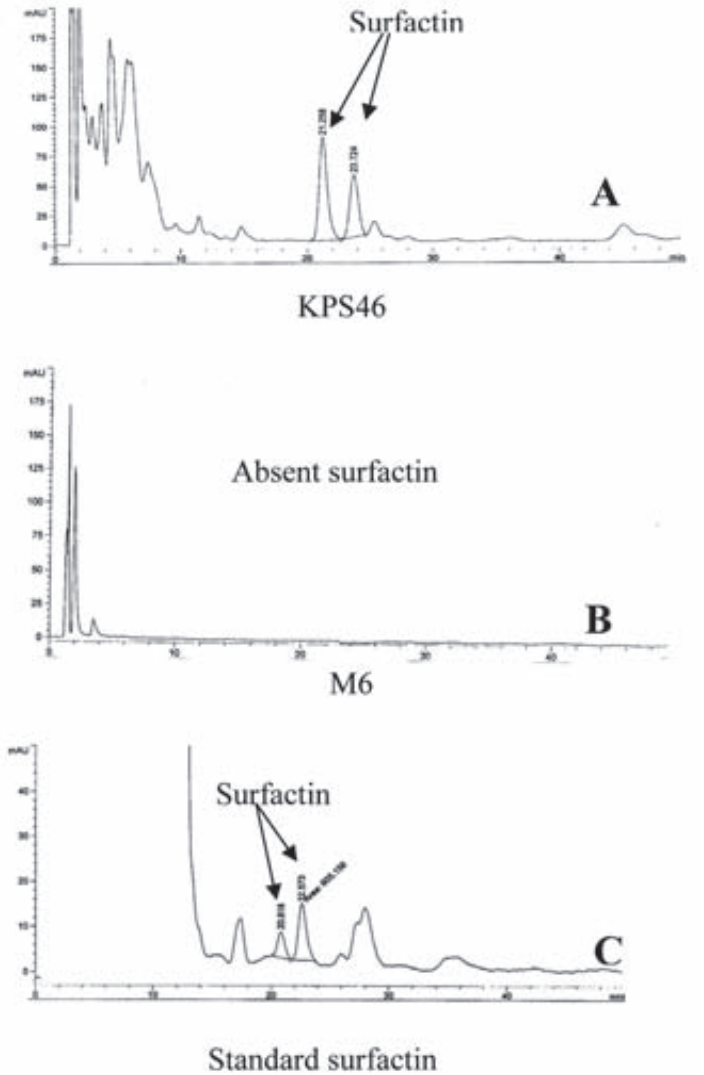

Figure 2 Identification of lipopeptodes present in extracts from liquid cultures of the biocontrol agent *Bacillus amyloliquefaciens* KPS46. HPLC profile of lipopeptide surfactin produced by strain KPS46 in NGB medium (A) and absence of lipopeptide surfactin produced by mutant M6 (B) compared with surfactin standard produced by *Bacillus subtilis* ATCC21332 (C).

total amount of surfactin lipopeptides present in the extract. Based on HPLC peak areas of the surfactin lipopeptide extract, relative to values obtained from standard curves, the total amount of surfactins produced by strain KPS46 was ~550±20.3 mg L^{-1} (mean and standard deviation calculated from three independent cultures).While surfactin lipopeptide was detected by HPLC analysis in extracts from strain KPS46, the mutant M6 did not produce detectable surfactin by HPLC analysis (Figure 2). Moreover, the inability of extracts of mutant M6 to suppress growth of pathogenic Xag in disk assays was always correlated with a lack of detection of surfactin by HPLC analysis.

Distinct phenotypes influenced by *srfAA* in KPS46

To determine if mutations in *srfAA* were related to bacterial phenotypes impacting biocontrol efficacy, wild-type KPS46 and mutant M6 were evaluated for growth and survival, biocontrol ability, motility, and ability to produce extracellular enzymes. Results in Figure 3 show the survival of bacterial cells grown in NB medium. The M6 mutant grew to significantly greater numbers (P=0.05) than did the KPS46 wild-type strain in every growth phase measured. At stationary phase, after 36 h of incubation, growth of the M6 mutant strain was approximately 2.0 times greater than that of the wild-type strain.

The effect of *srfAA* on cell motility was examined to test the hypothesis that the lipopeptide surfactin may down regulated flagella genes. Results in Table 2 and Figure 4 show that the KPS46 wild-type strain was motile, as shown by an increased colony diameter after 48 h. In contrast, the M6 mutant strain was not capable of swarming. The mutation in the *srfAA* strain M6 resulted in a reduction in the ability to swarm on both LB and NB supplemented with 0.5% agar. Interestingly, however, the M6 mutant strain produced larger-sized colonies on 1.5% agar media than did the wild-type KPS46 strain (Table 2). This result suggests that the presence of SrfAA may alter the expression of motility genes, but a functioning gene is not required for swarming.

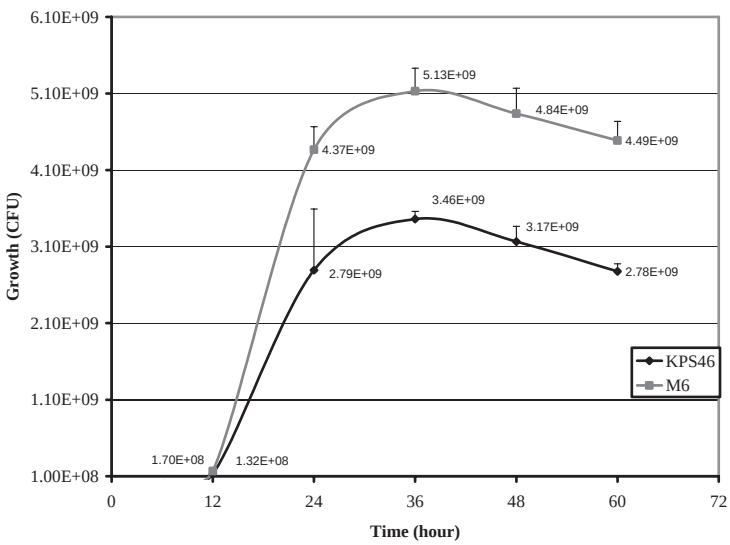

Figure 3 Survival of *Bacillus amyloliquefaciens* KPS46 wild-type and M6 mutant during growth in NB medium. Strain M6 is carrying a UV disruption mutation in *srfAA*. Each growth curve is based on measurements every 12 h for three days and represents the average of six replicates.

Table 2 Motility expression associated with SrfAA of *Bacillus amyloliquefaciens* KPS46 wild-type and the M6 mutant strain.

Strain/incubation	Colony size (cm)[a]					
	LBA (% agar)			NA (% agar)		
	0.5	1.0	1.5	0.5	1.0	1.5
24 h						
M 6 mutant	32.5	23.5	22.0	29.0	26.0	24.5
KPS46 wild-type	48.0	32.0	17.0	82.5	21.5	15.0
48 h						
M 6 mutant	46.6	35.0	31.5	51.8	46.0	28.3
KPS46 wild-type	51.6	38.3	21.6	90.0	59.0	53.0

[a] Strains were assayed on Luria-Bertani agar (LBA) and nutrient agar (NA) using three different agar concentrations. The relative expression was measured as the diameter in colony size after 48 h of incubation.

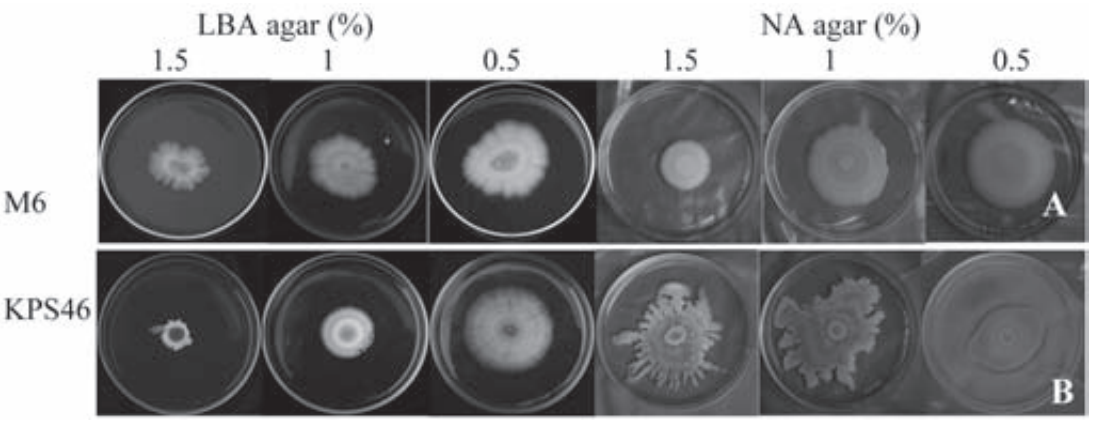

Figure 4 Swarming motility tests on a three-plate set containing Luria-Bertani agar (LBA: left); and nutrient agar (NA: right) media with 1.5, 1, and 0.5% agar (from left to right). Plates were spotted with 10 μl of a stationary culture of *Bacillus amyloliquefaciens* strain M6 mutant (A) and KPS46 wild-type (B) respectively. For the swarming test, colony diameter in millimeters was measured after 48 h of incubation and the mean was calculated from 24 measurements.

Since extracellular molecules produced by bacterial antagonists are thought to be involved in biocontrol efficacy, wild-type KPS46 and the *srfAA* mutant strain M6 were examined for the production of extracellular enzymes. The *srfAA* mutant strain M6 exhibited reduced production of endoglucanase, cellulases, and proteases compared to the parental wild-type KPS46 strain (data not shown). In contrast, the *srfAA* mutant produced a larger zone (18.3 mm diameter) of α-amylase activity than did the wild-type strain (12.5 mm diameter) on the test medium (Figure 5).

Biological control analysis of surfactin-deficient mutants

The ability of vegetative cells, cell-free supernatants and lipopeptide surfactin from wild-type KPS46 and the M6 mutant to inhibit bacterial growth of Xag was assessed *in vitro*. Results in Table 1 show that inhibition zones were never observed in the presence of Xag for the mutant strain M6 by 48 h after co-inoculation. The reduction in radial growth of the pathogenic strain Xag by the KPS46 wild-type strain and its metabolites ranged from 7.1 to 14.4 mm inhibition

diameter. Vegetative cells of the KPS46 strain exhibited the greatest inhibition, followed by the lipopeptide surfactin and cell-free supernatant, respectively (Table 1).

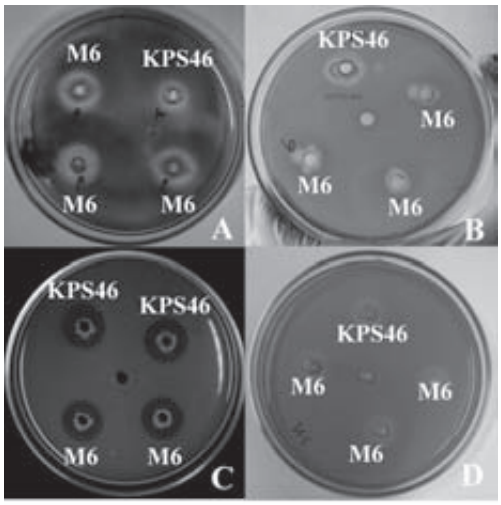

Figure 5 Exoenzyme activity of *Bacillus amyloliquefaciens* KPS46 and *srfAA* mutant strain M6. Filtrates from bacterial cultures were added to wells in assay media to assess: A, alpha-amylase; B, CMCase; C, protease; and D, endoglucanase activity. Assays were completed twice, once with four replicates, and a representative plate for each assay is shown.

KPS46 has been reported to secrete phytohormone, like indole-3-acetic acid (IAA), resulting in the direct enhancement of soybean growth (Buensanteai *et al.,* 2009). To determine if other compounds secreted by KPS46, including cell-free filtrates and the lipopeptide surfactin, have a direct role in regulating plant growth, crude extracellular lipopeptide surfactin and cell-free filtrates from wild-type KPS46 and the mutant strain M6 were evaluated for their ability to promote growth of soybean seedlings under greenhouse conditions. Results in Table 3 show that vegetative cells and culture extracts, when applied to soybean seeds, significantly (P=0.05) increased root and shoot lengths, but did not significantly increase fresh weight. Interestingly, seed treatment with vegetative cells from the *srfAA* mutant M6 significantly increased shoot and root length, and the number of lateral roots, whereas the lipopeptide surfactin significantly increased seed germination relative to controls. Furthermore, seed treatment with KPS46 vegetative cells and its cell-free culture extracts were significantly better than, or equivalent to, treatment with the copper hydroxide bactericide in enhancing plant growth (Table 3). Taken together, these data confirm that the *B. amyloliquefaciens* strain KPS46 is both a plant growth promoting rhizosphere (PGPR) and a biocontrol agent that is compatible with soybean production, and that

Table 3 Vigor of soybean seedlings treated with *Bacillus amyloliquefaciens* KPS46 and its culture filtrates, mutant M6, or copper hydroxide bactericide.

Treatment	Seedling growth promotion[a]				
	Seed germination (%)	Plant height (cm)	Root length (cm)	No. of lateral root	Fresh weight (g)
KPS46 cell	83.0b	12.9ab	14.8ab	33.3ab	24.1a
Cell-free filtrate	89.0a	12.2b	14.3bc	32.0b	25.1a
KPS46 surfactin	87.0ab	12.7ab	13.7c	29.3c	23.8a
Surfactin mutant M6[b]	82.0bc	13.4a	15.4a	34.5a	23.2a
Copper hydroxide	77.0bc	12.8ab	13.7c	29.8c	22.4ab
Nontreated	73.0c	11.9b	13.4c	26.9d	20.1b

[a] Means in the column followed by the same letter are not significantly different at P = 0.05 according to DMRT.

[b] Vegetative cells from surfactin mutant M6.

srfAA does not directly affect the genes involving plant growth regulation.

Results in Table 1 show that the frequency of disease severity due to bacterial pustule disease varied significantly among the treatments examined. Significant (P=0.05) treatment effects on disease severity were only detected with non-treated controls and not with the other treatments examined. However, all treatments significantly reduced the percentage of diseased leaf areas caused by Xag compared with the non-treated control. The vegetable cells of wild-type KPS46 consistently suppressed soybean infection by Xag and this result was similar to that observed when bioassays of growth inhibition were carried out. The least significant disease reduction occurred with the mutant strain M6, and this was significantly less than plants receiving culture filtrates and extracellular surfactin from the wild-type strain KPS46. The amount of disease suppression resulting from plants sprayed with wild-type strain KPS46, and its extracts, was equivalent to the suppression observed from using copper hydroxide bactericide (Funguran®). In contrast, vegetative cells from the mutant strain M6 showed lower levels of disease suppression compared to all the treatments examined.

DISCUSSION

Antimicrobial metabolites produced by bacteria may act as potential biocontrol agents. In the study presented here, cell-free culture filtrates from *Bacillus amyloliquefaciens* KPS46 were shown to be involved in the inhibition of several fungal pathogens, including *Sclerotium rolfsii*, *Colletotrichum truncatum*, and *Fusarium* sp. (data not shown), and the bacterial pustule pathogen, *X. axonopodis* pv. *glycines*, causal agents of soybean diseases (Prathuangwong and Kasem, 2003). Culture filtrates of KPS46 also inhibited mycelial growth of various taxonomically diverse phytopathogenic fungi (Prathuangwong *et al.,* 2004), indicating that compounds in the filtrates exhibit a wide spectrum of antimicrobial activity. *Bacillus* spp., particularly *B. subtilis,* have been reported to produce many types of antimicrobial peptide substances, such as iturin A and surfactin (Leclere *et al.,* 2005). Of these compounds, lipopeptide surfactin has been postulated to have antibiotic properties and also to modify the attachment of microbes to different surfaces, which, together contribute to the survival of bacterial cells in their native habitats (Razafindralambo *et al.,* 1998). These properties prompted further study to determine if the strain KPS46 produced lipopeptide surfactin as an effective activity against Xag.

HPLC analyses of culture supernatants from strain KPS46 clearly showed that this bacterium produced a lipopeptide antibiotic and/or surfactant that had antimicrobial effects against fungi and bacteria. Surfactin has previously been shown to exhibit effective control against several plant pathogens (Prathuangwong and Buensanteai, 2007), probably by destroying the cell wall of these deleterious microorganisms (Carrillo *et al.,* 2003). Moreover, surfactin has also been shown to disintegrate the envelope and capsid of viruses, through the formation of ion channels (Heerklotz and Seelig, 2001). Lipopeptides may also influence the adhesiveness of bacteria by adsorbing to their surfaces (Ahimou *et al.,* 2000). The lipopeptides produced by bacterial antagonist strains also exhibit a wetting activity, which is involved in surface colonization by microorganisms (Matsuyama and Nakagawa, 1996).

The genome sequences of pathogenic and saprophytic bacteria indicate that multiple adhesive functions are encoded in their genomes. This may be because it helps them to have functional redundancy in terms of attachment to any particular host surface and also because they require multiple adhesives at different stages of their life cycles (Marco *et al.,* 2005). In *Bacillus* spp. strains, SrfAA has been reported to be

involved in colonization of plant surface and protect plants from pathogen infection (Koumouts *et al*., 2004). To clarify the involvement of antimicrobials in the biocontrol performance, a reverse genetic approach was adopted to understand the role of *srfAA* in strain KPS46. A UV-mutagenesis strategy was used here to construct a collection of KPS46 mutants with alterations in lipopeptide surfactin production. The biocontrol results obtained in assays using vegetative cells and extracted surfactin showed the expected role for lipopeptide surfactin in the disease suppression ability by strain KPS46. Moreover, growth inhibition assays done using extracts from supernatants from the mutant strain M6 confirmed those data, and supported the conclusion that lipopeptide surfactin is essential for the biocontrol ability of this bacterium against Xag.

In this study, crude extracellular lipopeptide surfactin was found to be also significantly effective in promoting the growth of soybean seedlings under greenhouse conditions, compared with the non-treated control. If purified surfactin also has a direct effect in causing the promotion of plant growth, this would be a new function for lipopeptide surfactin, which is better known for its effects on surface hydrophobicity, bacterial adhesion and movement on surfaces, including microbial activity (Bonmatin *et al*., 2003). However, there is evidence that certain synthetic surfactants can stimulate plant growth by synergizing auxin action, activating certain plant enzyme systems or affecting plant cell membrane permeability, thereby increasing water or nutrient uptake or excretion of plant factors such as riboflavin (Ernst *et al*., 1971).

The genomic organization of *srfAA* in KPS46 responsible for surfactin production was confirmed by PCR analysis, and the mutation in the *srfAA* gene was studied to determine if it could influence other genes that might be co-transcribed or located downstream of *srfAA*. The results also indicated that a mutation in the *srfAA* gene of strain KPS46 also affected production of extracellular enzymes, such as endoglucanase, cellulase, and protease. It is believed that *srfAA* has not been previously known to influence the pectolytic activity of *Bacillus* spp. strains. However, in *X. campestris* pv. *campestris* (He *et al*., 2006), *X. axonopodis* pv. *citri* (Andrade *et al*., 2006), *Xylella fastidiosa* (Chatterjee *et al*., 2008), and Xag (Thowthampitak *et al*., 2008), it has been reported that DSF production or *rpf*F influences transcript abundance of gene encoding DSF-regulated products. In *X. campestris* pv. *campestris*, the DSF regulon influences transcription of 165 genes, including those involved in extracellular enzyme production (He *et al*., 2006). As in *X. campestris* pv. *campestris,* genes encoding for extracellular, protease and pectate lyase were positively influenced by *rpf*F genes. According to this model, SrfAA may serve as a primary signal in a signal transduction pathway that links the sensing of the genes in the same transcriptional orientation (Dow *et al*., 2003). Linkage was also found of endoglucanase, cellulase, and protease activity to *srfAA* in KPS46, whereas α-amylase production was found to be upregulated in the M6 mutant. The *srfAA* mutation that led to increased α-amylase production might be unavoidable as a product of the mechanisms involved in improved growth or survival under stress conditions and the M6 mutant may have lost lipopeptide surfactin secretion for ecological fitness (Marco *et al*., 2005). Several reports demonstrated that production of amylase was directly corrected with growth or survival of most *Bacillus* spp., including *B. amyloliquefaciens* (Kelly *et al*., 1977).

In some bacteria, such as *Sinorhizobium meliloti*, the strict regulation of motility results in migration towards chemo-attachments under nutrient-sufficient or -excess conditions, and the development of the vegetative stage and biofilms occur upon encountering stress or starvation conditions (Wei and Bauer, 1998). Similarly, the

B. amyloliquefaciens strain KPS46 was found to be motile throughout the active growth period when nutrients were readily available, whereas different types of motility might be expressed by KPS46 when it is faced with stresses, particularly in association with deficiencies in lipopeptide surfactin production. Studies done with mutant M6 demonstrated that SrfAA influences expression of motility genes in strain KPS46 and this was required for swarming. In this study, NA seemed to be suitable for testing migration of strain KPS46, compared with LBA lacking glucose. The current results also suggest that lipopeptide surfactin produced by *B. amyloliquefaciens* KPS46 played an important role in adhesion of this biocontrol agent to plant surfaces. In association with the antimicrobial properties of lipopeptides, this aspect appeared essential for the biological control of plant diseases. This result was in agreement with previous reports that had pointed out the multiple diseases caused by various pathogens can be efficiently targeted by the antibiotic-producing strain KPS46 (Prathuangwong *et al.,* 2004)

Additional assays done in this study revealed that the purified lipopeptide surfactin had lower antimicrobial activity against Xag than vegetative cells of strain KPS46. In this aspect, *Bacillus* spp. has been demonstrated to produce various antibiotics in addition to surfactin, including iturin and fengicin (Kloepper *et al.,* 2004). Detmanee (2004) also reported that the strain KPS46 produced at least three antibiotics effective against Xag. This suggests that surfactin and other antibiotics likely act synergistically to inhibit plant pathogens (Romeo *et al.,* 1988). This is also likely reflected in the current results that showed that surfactin-deficient M6 mutant failed to inhibit Xag on agar plates, but still reduced disease infection by Xag on soybean plants under greenhouse conditions.

CONCLUSION

The *B. amyloliquefaciens* strain KPS46 was shown to be an excellent biocontrol agent for enhancing plant health in soybean production systems. Since the experiments carried out here showed an antimicrobial effect of cell-free culture filtrates and the presence of the antimicrobial compound surfactin in liquid cultures, our results support the contention of previous reports that antibiosis is likely the main mechanism by which the strain KPS46 controls plant diseases. Furthermore, mutational analyses supported the proposition that lipopeptide surfactin plays a major role in the biocontrol activity of the antagonistic strain KPS46. Lipopeptide surfactin altered the KPS46 surface hydrophobicity and consequently, microbial adhesion to solid surfaces. When incorporated into growth medium, lipopeptide surfactin inhibited the rate of Xag growth, while the surfactin-deficient M6 mutant still expressed disease suppression on soybean plants. This suggests that induced systemic resistance also played a role in the biocontrol of Xag. Taken together, these results suggest that the lipopeptide surfactin plays a role in adhesion of this antagonist to plant surfaces, and together with the antimicrobial properties of this lipopeptide, strain KPS46 is an effective biological control agent for soybean bacterial pustule disease caused by Xag.

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