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Detection of antibiotic-related genes from bacterial biocontrol agents with polymerase chain reaction

Y. Zhang, W.G.D. Fernando, T.R. de Kievit, C. Berry, F. Daayf, and T.C. Paulitz

Abstract: *Pseudomonas chlororaphis* PA23, *Pseudomonas* spp. strain DF41, and *Bacillus amyloliquefaciens* BS6 consistently inhibit infection of canola petals by *Sclerotinia sclerotiorum* in both greenhouse and field experiments. *Bacillus thuringiensis* BS8, *Bacillus cereus* L, and *Bacillus mycoides* S have shown significant inhibition against *S. sclerotiorum* on plate assays. The presence of antibiotic biosynthetic or self-resistance genes in these strains was investigated with polymerase chain reaction and, in one case, Southern blotting. Thirty primers were used to amplify (*i*) antibiotic biosythetic genes encoding phenazine-1-carboxylic acid, 2,4-diacetylphloroglucinol, pyoluteorin, and pyrrolnitrin, and (*ii*) the zwittermicin A self-resistance gene. Our findings revealed that the fungal antagonist *P. chlororaphis* PA23 contains biosynthetic genes for phenazine-1-carboxylic acid and pyrrolnitrin. Moreover, production of these compounds was confirmed by high performance liquid chromatography. *Pseudomonas* spp. DF41 and *B. amyloliquefaciens* BS6 do not appear to harbour genes for any of the antibiotics tested. *Bacillus thuringiensis* BS8, *B. cereus* L, and *B. mycoides* S contain the zwittermicin A self-resistance gene. This is the first report of *zmaR* in *B. mycoides*.

Key words: Pseudomonas, *Bacillus*, biocontrol, antibiotic genes.

Résumé : La souche PA23 de *Pseudomonas chlororaphis*, la souche DF41 de *Pseudomonas* spp. et la souche BS6 de *Bacillus amyloliquefaciens* ont régulièrement inhibé l'infection de pétales de canola par *Sclerotinia sclerotiorum* dans des expériences en serre et dans le champ. *Bacillus thuringiensis* BS8, *Bacillus cereus* L et *Bacillus mycoides* S ont démontré une inhibition significative de *S. sclerotiorum* dans des tests d'ensemencement. La présence de gènes de la biosynthèse d'antibiotiques ou d'auto-résistance chez ces souches fut évaluée à l'aide de la réaction de la polymérase en chaîne et dans un cas, de l'hybridation de type Southern. Trente amorces ont été utilisées pour amplifier (*i*) les gènes de la biosynthèse d'antibiotiques codant l'acide phenazine-1-carboxylique, le 2,4-diacetylphloroglucinol, la pyoluteorine, la pyrrolnitrine et (*ii*) le gène d'auto-résistance à la zwittermicine A. Nos découvertes révèlent que l'antagoniste fongique *P. chlororaphis* PA23 contient des gènes de la biosynthèse du l'acide phenazine-1-carboxylique et de la pyrrolnitrine. De plus, la production de ces composés fut confirmée par chromatographie liquide à haute performance. *Pseudomonas* spp. DF41 et *B. amyloliquefaciens* BS6 n'ont pas semblé renfermer de gènes d'aucun des antibiotiques analysés. *Bacillus thuringiensis* BS8, *B. cereus* L mycoides S contiennent le gène de l'auto-résistance à la zwittermicine A. Il s'agit de la première mention de *zmaR* chez *B. mycoides*.

Mots clés : Pseudomonas, *Bacillus*, biocontrôle, gènes d'antibiotiques.

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Antibiosis has been widely studied as one of the most important biocontrol mechanisms inhibiting plant pathogens. Different antibiotics have been found to be responsible for this inhibition, including phenazines, 2,4-diacetylphloroglucinol (2,4-DAPG), pyoluteorin, pyrrolnitrin, zwittermicin A, and kanosamine. The biosynthetic genes for phenazine-1-carboxylic

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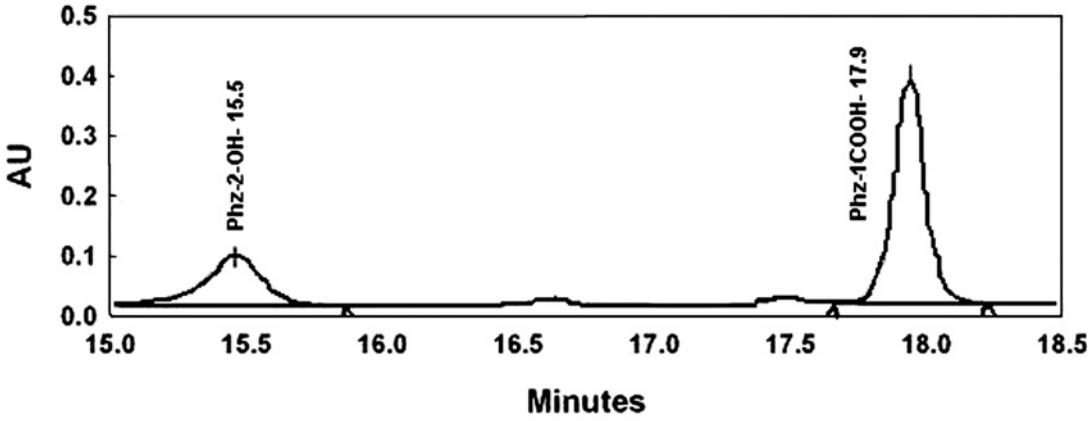
Fig. 1. High performance liquid chromatogram of culture filtrates from *Pseudomonas chlororaphis* PA23, showing peaks for 2-hydroxyphenazine and phenazine 1-carboxylic acid.



acid (PCA), 2,4-DAPG, pyrrolnitrin, and pyoluteorin, and the zwittermicin A self-resistance gene have been sequenced (Hammer et al. 1997; Nowak-Thompson et al. 1999; Stohl et al. 1999*b*; Mavrodi et al. 1998; Bangera and Thomashow 1999). Accordingly, availability of these sequences has enabled design of primers based on conserved regions for polymerase chain reaction (PCR) detection of antibiotic-producing bacteria (Bangera and Thomashow 1999; de Souza and Raaijmakers 2003; Raaijmakers et al. 1997; McSpadden Gardener et al. 2001; Raffel et al. 1996; Picard et al. 2000).

Previously, eight bacterial biocontrol agents screened against *Sclerotinia sclerotiorum* de Bary in vivo and (or) in vitro were shown to have significant control of fungal mycelial growth, sclerotial germination, ascospore germination, and to reduce stem rot disease incidence and severity on canola (*Brassica napus* L.) (Savchuck and Fernando 2004; Savchuck 2002; Zhang 2004). Three *Bacillus* strains were also effective against the wheat head blight pathogen *Fusarium graminearum* in planta (Fernando et al. 2002). *Pseudomonas chlororaphis* PA23 has been shown to inhibit several root pathogens in greenhouse and field experiments (Kavitha et al. 2005; Mathiyazhagan et al. 2004). The presence of a well-defined zone of inhibition of fungal growth on plates by all eight strains suggests that antibiosis is likely an important mechanism involved in antifungal activity and disease suppression. We report here our preliminary findings regarding the presence of antibiotic-specific genes in these eight biocontrol strains. High performance liquid chromatography (HPLC) analysis enabled us to detect three antibiotics produced by *P. chlororaphis* PA23.

The eight bacterial strains examined in this study were *P. chlororaphis* PA23, *Pseudomonas* spp. DF41, *Bacillus amyloliquefaciens* BS6, *Bacillus thuringiensis* BS8, *Bacillus subtilis* H, *Bacillus cereus* L, *Bacillus mycoides* S, and *Bacillus* spp. B70 (Savchuck and Fernando 2004; Fernando et al. 2002; Zhang 2004). *Pseudomonas fluorescens* strains 2-79, Q2-87, and Pf-5 and *Bacillus cereus* UW85 were also included as positive controls for detecting antibiotic biosynthetic genes for PCA, 2,4-DAPG, pyoluteorin, pyrrolnitrin, and zwittermicin A resistance, respectively.

Thirty primers (Invitrogen™, Carlsbad, Carlifornia, USA) were synthesized and used to amplify specific antibiotic genes (Table 1). PCR cycling reactions were performed according to the original reference publications (Raaijmakers et al. 1997; McSpadden Gardener et al. 2001; de Souza and Raaijmakers 2003; Raffel et al. 1996; Giacomodonato et al. 2001; Delaney et al. 2001; Mavrodi et al. 2001). Amplifications were conducted with the positive control strains listed above to confirm that PCR conditions were optimal. PCR products were sequenced (University Core DNA & Protein Services, University of Calgary, Calgary, Alberta) and analyzed using the National Centre for Biotechnology Information nucleotide–nucleotide BLAST (blastn) and alignment (bl2seq) programs.

For growth conditions favouring phenazine and 2,4-DAPG production, *P. chlororaphis* A23 was grown in potato dextrose broth culture (25 mL in 50 mL Erlenmyer flasks) for 48 h on a rotary shaker. A 5 mL aliquot of broth culture was acidified to a pH of 2.0 with 56.5 µL of 10% trifluoroacetic acid (TFA). The acidified broth was then extracted twice with ethyl acetate, and the organic phases were removed and allowed to evaporate to dryness. The extract was resuspended in 2 mL of 30% acetonitrile (ACN) + 0.1% TFA and fractionated with HPLC at an injection volume of 5 µL. The HPLC system consisted of a Waters 717 auto sampler, a 600E solvent delivery system, a 600 controller, and a 996 photo diode array. Extracts were fractionated with a Waters NOVA-PAK C-18 Radial-Pak cartridge (4 µm, 8 mm × 100 mm) at a flow rate of 1 mL/min. Initial solvent conditions were 10% ACN + 0.1% TFA, followed by a 20 min linear gradient to 100% ACN + 0.1% TFA. HPLC profiles were monitored with the photo diode array at 270 nm (phloroglucinols) and 247 nm (phenazines). For pyrrolnitrin detection, cultures were grown on media 523 and nutrient agar + 2% glycerol media at 26 °C (Kado and Heskett 1970; Kraus and Loper 1995). Cells were scraped from plates after 48 h of growth, and pyrrolnitrin was extracted and analyzed by HPLC as described previously (Nowak-Thompson et al. 1999).

Analysis of *P. chlororaphis* PA23

Both *P. chlororaphis* PA23 and positive control strain *P. fluorescens* 2-79 showed a 1400 bp product when their DNA was used for amplification with primers PHZ1/PHZ2 (phenazine) (Table 1). A BLAST search of the PA23 sequence revealed that it had >90% identity (>500 bp) with phenazine biosynthetic genes from three strains of *P. chlororaphis*. It

Table 1. Polymerase chain reaction primers and expected amplification products from genes encoding enzymes involved in the biosynthesis of several antibiotics.

Primer	Sequence	Gene (control strain)	Expected size of PCR product
Phenazine			
PHZ1[a]	GGCGACATGGTCAACGG	*phzFA* (*P. aureofaciens* 30-84); *phzCD* (*P. fluorescens* 2-79)	1400 bp (PA23)
PHZ2[a]	CGGCTGGCGGCGTATAT	*phzFA* (*P. aureofaciens* 30-84); *phzCD* (*P. fluorescens* 2-79)	1400 bp (PA23)
PHZX[a]	TTTTTTCATATGCCTGCTTCGCTTTC	*phzXY* (*P. aureofaciens* 30-84)	1100 bp (not detected)
PHZY[a]	TTTGGATCCTTAAGTTGGAATGCCTCC	*phzXY* (*P. aureofaciens* 30-84)	1100 bp (not detected)
PCA2a[b]	TTGCCAAGCCTCGCTCCAAC	*phzCD* (*P. fluorescens* 2-79)	1400 bp (not detected)
PCA3b[b]	CCGCGTTGTTCCTCGTTCAT	*phzCD* (*P. fluorescens* 2-79)	1400 bp (not detected)
2,4-Diacetylphloroglucinol			
Phl2a[c]	GAGGACGTCGAAGACCACCA	*phlD* (*P. fluorescens* CHA0; Pf-5; Q8r1l-96; 1M1-96; Q2-87)	745 bp (not detected)
Phl2b[c]	ACCGCAGCATCGTGTATGAG	*phlD*	745 bp (not detected)
BPF2[d]	ACATCGTGCACCGGTTTCATGATG	*phlD*	~470 bp (not detected)
B2BF[d]	ACCCACCGCAGCATCGTTTATGAGC	*phlD*	~470 bp (not detected)
BPF3[d]	ACTTGATCAATGACCTGGGCCTGC	*phlD*	~470 bp (PA23)
BPR2[d]	GAGCGCAATGTTGATTGAAGGTCTC	*phlD*	~470 bp (PA23)
BPR3[d]	GGTGCGACATCTTTAATGGAGTTC	*phlD*	~470 bp (PA23)
BPR4[d]	CCGCCGGTATGGAAGATGAAAAAGTC	*phlD*	~470 bp (PA23)
Pyrrolnitrin			
PrnAF[e]	GTGTTCTTCGACTTCCTCGG	*prnA* (*P. fluorescens* Pf-5)	1050 bp (PA23)
PrnAR[e]	TGCCGGTTCGCGAGCCAGA	*prnA* (*P. fluorescens* Pf-5)	1050 bp (PA23)
PRND1[f]	GGGGCGGGCCGTGGTGATGGA	*prnD* (*B. pyrrocinia* DSM10685; *P. fluorescens* BL915; *Burkholderia cepacia* LT4-12-W)	790 bp (PA23)
PRND2[f]	YCCCGCSGCCTGYCTGGTCTG	*prnD* (*B. pyrrocinia* DSM10685; *P. fluorescens* BL915; *Burkholderia cepacia* LT4-12-W)	720 bp (PA23)
PrnCf[g]	CCACAAGCCCGGCCAGGAGC	*prnC* (*P. fluorescens* BL915)	720 bp (PA23)
PrnCr[g]	GAGAAGAGCGGGTCGATGAAGCC	*prnC* (*P. fluorescens* BL915)	720 bp (PA23)
Pyoluteorin			
PltCreg1F[e]	AGGCAATCACTACCATCCGTGCGC	*pltC* (*P. fluorescens* Pf-5)	438 bp (not detected)
PltCreg2R[e]	ATGAGGAGCAGGAGGTGTCGAGCAC	*pltC* (*P. fluorescens* Pf-5)	438 bp (not detected)
PLTC1[f]	AACAGATCGCCCCGGTACAGAACG	*pltC* (*P. fluorescens* Pf-5)	438 bp (not detected)
PLTC2[f]	AGGCCCGGACACTCAAGAAACTCG	*pltC* (*P. fluorescens* Pf-5)	438 bp (not detected)
PltBf[g]	CGGAGCATGGACCCCCAGC	*pltB* (*P. fluorescens* Pf-5)	900 bp (PA23)
PltBr[g]	GTGCCCGATATTGGTCTTGACC	*pltB* (*P. fluorescens* Pf-5)	900 bp (PA23)
plt1[g]	ACTAAACACCCAGTCGAAGG	*pltB* (*P. fluorescens* Pf-5)	440 bp (not detected)
plt2[g]	AGGTAATCCATGCCCAGC	*pltB* (*P. fluorescens* Pf-5)	440 bp (not detected)

Table 1 (*concluded*).

Primer	Sequence	Gene (control strain)	Expected size of PCR product
Zwittermicin A self resistance			
678[h]	ATGTGCACTTGTATGGGCAG	*zmaR* (*B. cereus*)	950 bp (*B. thuringiensis* BS8, *B. cereus* L, *B. mycoides* S)
667[h]	TAAAGCTCGTCCCTCTTCAG	*zmaR* (*B. cereus*)	950 bp (*B. thuringiensis* BS8, *B. cereus* L, *B. mycoides* S)

[a]Delaney et al. 2001. Positive control: *P. fluorescens* 2-79.
[b]Raaijmakers et al. 1997. Positive control: *P. fluorescens* 2-79.
[c]Raaijmakers et al. 1997. Positive control: *P. fluorescens* Q2-87.
[d]McSpadden Gardener et al. 2001. Positive control: *P. fluorescens* Q2-87.
[e]C. Press, personal communication; deSouza and Raaijmakers 2003. Positive control: *P. fluorescens* Pf-5.
[f]deSouza and Raaijmakers 2003. Positive control: *P. fluorescens* Pf-5.
[g]Mavrodi et al. 2001. Positive control: *P. fluorescens* Pf-5.
[h]Milner et al. 1996. Positive control: *B. cereus* UW85.

also showed 93% identity (>700 bp) with the *phzCD* sequence from the positive control strain *P. fluorescens* 2-79.

DNA fragments of approximately 1050, 790, and 720 bp were amplified from *P. chlororaphis* PA23 and positive control strain *P. fluorescens* Pf-5, using pyrrolnitrin-specific primers PrnAF/PrnAR, PRND1/PRND2, and PrnCf/PrnCr, respectively (Table 1). The 1050 bp PCR product amplified from *P. chlororaphis* PA23 by primers PrnAF/PrnAR showed high similarity (>90% identity, >700 bp) to pyrrolnitrin biosynthetic genes (*prnABCD*) of *P. fluorescens*, *Burkholderia pyrrocinia*, and *P. chlororaphis*.

Primers PltBf/PltBr, specific for pyoluteorin, amplified the expected 850 bp PCR product from positive control strain *P. fluorescens* Pf-5 and a slightly larger (900 bp) product from *P. chlororaphis* PA23 (Table 1). The sequence of the PA23 product showed no similarity with that from the positive control strain *P. fluorescens* Pf-5 or any bacterial pyoluteorin biosynthetic genes in GenBank. Since the other pyoluteorin-specific primers tested (PltCreg1F/PltCreg2R, PLTC1/PLTC2, plt1/plt2; Table 1) did not yield a PCR product, we concluded that strain PA23 does not contain pyoluteorin biosynthetic genes.

BPF2, BPF3, and B2BF were designed as forward primers and BPR2, BPR3, and BPR4 were designed as reverse primers within a 745 bp region (McSpadden Gardener et al. 2001). All nine combinations of the three forward primers and the three reverse primers were used for PCR amplification. BPF3 was the only forward primer that generated PCR products in combination with the three reverse primers. PCR with DNA from *P. chlororaphis* PA23 and positive control *P. fluorescens* Q2-87 produced a 470 bp band, with additional weak bands, when amplified with primers BPF3/BPR2 specific for 2,4-DAPG biosynthetic genes (Table 1). However, sequence analysis of the *P. chlororaphis* PA23 470 bp PCR product did not reveal any significant homology with the published sequences of *phlD* gene. As an alternative approach to determining whether PA23 harbors genes for this antibiotic, Southern analysis was performed following previously described methods (de Kievit et al. 1995). A *phlD*-specific probe was generated by PCR amplification of *P. fluorescens* Q2-87 genomic DNA. As expected, the probe hybridized to genomic DNA from *P. fluorescens* Q2-87 and Pf-5; however, no hybridization to PA23 DNA was observed (data not shown).

Our PCR findings are consistent with HPLC analysis, revealing PCA and 2-hydroxyphenazine in PA23 culture supernatants, with higher levels of the former (Fig. 1). PA23 was also found by HPLC analysis to produce pyrrolnitrin when grown on media 523 and nutrient agar + 2% glycerol media (data not shown). Finally, we were unable to detect 2,4-DAPG biosynthetic genes by PCR or Southern blotting, and this antibiotic was not detected in PA23 culture supernatants (data not shown).

Extracts from *P. chlororaphis* PA23 have been shown to inhibit sclerotial and (or) spore germination by several plant pathogens, including *S. sclerotiorum*, *Macrophomina phaseolina*, *Rhizoctonia solani*, *Sclerotium rolfsii*, *Fusarium oxysporum*, *Alternaria solani*, and *Botryodiplodia theobromae* (Kavitha et al. 2005). Our findings indicate that PA23 is capable of producing PCA, 2-OH phenazine, and pyrrolnitrin. These antibiotics are major determinants of the

biological control of plant pathogens, which might explain the wide-spectrum disease suppression observed with PA23. Whether PCA or pyrrolnitrin antibiotics are involved in sclerotinia disease suppression has yet to be determined. Additional mutagenesis studies and subsequent testing of mutants and the wild-type strains on plants, together with gene fusion studies, will shed light on the role these antibiotics play in biological control.

Detection of *Pseudomonas* spp. DF41 antibiotic genes

Pseudomonas spp. DF41 did not show amplification with primers specific for antibiotic biosynthetic genes encoding PCA, pyrrolnitrin, pyoluteorin, and 2,4-DAPG, or for the zwittermicin A self-resistance gene. In light of these findings, we believe that the aforementioned antibiotics do not contribute to the antifungal activity exhibited by DF41. Tn*5*-mutagenesis analysis of this strain has revealed a gene with 91% identity to the *syrB* gene of the *P. syringae* syringomycin biosynthetic cluster (C. Berry, W.G.D. Fernando, and T.R. de Kievit, unpublished data). A Tn insertion in this gene completely abolishes antifungal activity, suggesting a molecule similar to syringomycin may be responsible for antibiosis in strain DF41. Using 16S rDNA analysis and Biolog microbial typing, we have been unable to assign a species eponym to DF41. However, since DF41 is oxidase positive and *P. syringae* is oxidase negative, this strain is a bacterium other than *P. syringae*.

Detection of a zwittermicin A self-resistance gene in *Bacillus* spp.

A 950 bp PCR product was amplified from *B. thuringiensis* BS8, *B. cereus* L, and *B. mycoides* S, along with positive control *B. cereus* UW85, using zwittermicin A self-resistance gene-specific primers 678/677 (Table 1). PCR products amplified with primers 678/677 from *B. thuringiensis* BS8, *B. cereus* L, and *B. mycoides* S showed >98% identity with the *B. cereus* zwittermicin A resistance gene (*zmaR*), which indicates that these strains may produce zwittermicin A. No PCR product was amplified from *B. amyloliquefaciens* BS6, *B. subtilis* H, or *Bacillus* spp. B70 using any of the 30 primers tested. The zwittermicin A self-resistance gene, *zmaR*, and the production of zwittermicin A have only been found in *B. thuringenensis* and *B. cereus* strains from the natural environment (Milner et al. 1996; Raffel et al. 1996; Stohl et al. 1999*a*, 1999*b*; Emmert et al. 2004). In this study, *B. mycoids* S was found to have a *zmaR* gene. This is the first report that a *Bacillus* species other than *B. thuringiensis* and *B. cereus* contains *zmaR*.

In summary, we used 30 different PCR primers to identify antibiotic-related genes in previously isolated bacteria exhibiting good biocontrol activity. Isolation and identification of antibiotic-producing strains from natural environments is a slow, laborious process. Using PCR to detect antibiotic genes enables isolates to be quickly classified into different groups based on the genes present, expediting further characterization. From our studies, it is clear that utilizing multiple primer sets for PCR is essential to ensure accurate results. For example, although three primer pairs were used to detect PCA from strain PA23, which has been shown through HPLC analysis to produce this antibiotic, only PHZ1/PHZ2 amplified a product. This was a surprising finding since primers PCA2a/PCA2b were reported to detect *phzCD* from a number of soil isolates (Raaijmakers et al. 1997), and these genes are believed to be highly conserved among the phenazine-producing strains *P. fluorescens*, *P. aureofaciens*, and *P. chlororaphis.* Regardless, these analyses have advanced our understanding of the antifungal activities of the eight strains examined. As we learn more about the susceptibility of plant pathogens to various antibiotics, the ability to screen for biocontrol qualities becomes even more important in the pursuit of new strains adaptive to particular soils.

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References

Bangera, M.G., and Thomashow, L.S. 1999. Identification and characterization of a gene cluster for synthesis of the polyketide antibiotic 2,4-diacetylphloroglucinol from *Pseudomonas fluorescens* Q2-87. J. Bacteriol. **181**: 3155–3163.

de Kievit, T.R., Dasgupta, T., Schweizer, H., and Lam, J.S. 1995. Molecular cloning and characterization of the *rfc* gene of (*Pseudomonas aeruginosa* serotype O5). Mol. Microbiol. **16**: 565–574.

Delaney, S.M., Mavrodi, D., Bonsall, V.R.F., and Thomashow, L.S. 2001. *phzO*, a gene for biosynthesis of 2-hydroxylated phenazine compounds in *Pseudomonas aureofaciens* 30-84. J. Bacteriol. **183**: 318–327.

de Souza, J.T., and Raaijmakers, J.M. 2003. Polymorphisms within the *prnD* and *pltC* genes from pyrrolnitrin and pyoluteorin-producing *Pseudomonas* and *Burkholderia* spp. FEMS Microbiol. Ecol. **43**: 21–34.

Emmert, E.A.B., Klimowicz, A.K., and Thomas, M.G. 2004. Genetics of zwittermicin A production by *Bacillus cereus*. Appl. Environ. Microbiol. **70**: 104–113.

Fernando, W.G.D., Chen, Y., and Parks, P. 2002. Effect of three *Bacillus* sp. from wheat on FHB production. National Fusarium Head Blight Forum Proceedings – Chemical and Biological Control. 7–9 December 2002, Erlanger, Kentucky, USA. pp. 73–75.

Giacomodonato, M.N., Pettinari, M.J., Souto, G.I., Mendez, B.S., and Lopez, N.I. 2001. A PCR-based method for the screening of bacterial strains with antifungal activity in suppressive soybean rhizosphere. World J. Microbiol. Biotechnol. **17**: 51–55.

Hammer, P.E., Hill, D.S., Lam, S.T., Pee, K.H., and Ligon, J.M. 1997. Four genes from *Pseudomonas fluorescens* that present the biosynthesis of pyrrolnitrin. Appl. Environ. Microbiol. **63**: 2147–2154.

Kado, C.I., and Heskett, M.G. 1970. Selective media for isolation of *Agrobacterium*, *Corynebacterium*, *Erwinia*, *Pseudomonas*, and *Xanthomonas*. Phytopathology, **60**: 969–976.

Kavitha, K., Mathiyazhagan, S., Sendhilvel, V., Nakkeeran, S., Chandrasekar, G., and Fernando, W.G.D. 2005. Broad spectrum action of phenazine against active and dormant structures of fungal pathogens and root knot nematode. Arch. Phytopathol. Plant Protection, **38**: 69–76.

Kraus, J., and Loper, J.E. 1995. Characterization of a genomic locus required for production of the antibiotic pyoluteorin by the biological control agent *Pesudomonas flurorescens* Pf-5. Appl. Environ. Microbiol. **61**: 849–854.

Mathiyazhagan, S., Kavitha, K., Nakkeeran, S., Chandrasekar, G., Manian, K., Renukadevi, P., Krishnamoorthy, A.S., and Fernando, W.G.D. 2004. PGPR mediated management of stem blight of *Phyllanthus amarus* (Schum and Thonn) caused by *Corynespora cassiicola* (Berk and Curt) Wei. Arch. Phytopath. Plant Protection, **37**: 183–199.

Mavrodi, D.V., Ksenzenko, V.N., Bonsall, R.F., Cook, R.J., Boronin, A.M., and Thomashow, L.S. 1998. A seven-gene locus for synthesis of phenazine-1-carboxylic acid by *Pseudomonas fluorescens* 2-79. J. Bacteriol. **180**: 2541–2548.

Mavrodi, O.V., McSpadden Gardener, B.B., Mavrodi, D.V., Bonsall, R.F., Weller, D.M., and Thomashow, L.S. 2001. Genetic diversity of *phlD* from 2,4-diacetylphloroglucinol-producing fluorescent *Pseudomonas* spp. Phytopathology, **91**: 35–43.

McSpadden Gardener, B.B., Mavrodi, D.V., Thomashow, L.S., and Weller, D.M. 2001. A rapid polymerase chain reaction-based assay characterizing rhizosphere populations of 2,4-diacetylphloroglucinol-producing bacteria. Phytopathology, **91**: 44–54.

Milner, J.L., Stohl, E.A., and Handelsman, J. 1996. Zwittermicin A resistance gene from *Bacillus cereus*. J. Bacteriol. **178**: 4266–4272.

Nowak-Thompson, B., Chaney, N., Wing, J.S., Gould, S.J., and Loper, J.E. 1999. Characterization of the pyoluteorin biosynthetic gene cluster of *Pseudomonas fluorescens* Pf-5. J. Bacteriol. **181**: 2166–2174.

Picard, C., Di Cello, F., Ventura, M., Fani, R., and Guckert, A. 2000. Frequency and biodiversity of 2,4-diacetylphloroglucinol-producing bacteria isolated from the maize rhizosphere at different stages of plant growth. Appl. Environ. Microbiol. **66**: 948–955.

Raaijmakers, J.M., Weller, D.M., and Thomashow, L.S. 1997. Frequency of antibiotic-producing *Pseudomonas* spp. in natural environments. Appl. Environ. Microbiol. **63**: 881–887.

Raffel, S.J., Stabb, E.V., Milner, J.L., and Handelsman, J. 1996. Genotypic and phenotypic analysis of zwittermicin A-producing strains of *Bacillus cereus*. Microbiology, **142**: 3425–3436.

Savchuck, S.C. 2002. Evaluation of biological control of *Sclerotinia sclerotiorum* on canola (*Brassica napus*) in the laboratory, in the greenhouse and in the field. M.Sc. thesis, University of Manitoba, Winnipeg, Man., Canada.

Savchuck, S., and Fernando, W.G.D. 2004. Effect of timing of application and population dynamics on the degree of biological control of *Sclerotinia sclerotiorum* by bacterial antagonists. FEMS Microbiol. Ecol. **49**: 376–388.

Stohl, E.A., Brady, S.F., Clardy, J., and Handelsman, J. 1999*a*. ZmaR, a novel and widespread antibiotic resistance determinant that acetylates zwittermicin A. J. Bacteriol. **181**: 5455–5460.

Stohl, E.A., Milner, J.L., and Handelsman, J. 1999*b*. Zwittermicin A biosynthetic cluster. Gene, **237**: 403–411.

Zhang, Y. 2004. Biocontrol of sclerotinia stem rot of canola by bacterial antagonists and study of biocontrol mechanisms involved. M.Sc. thesis, University of Manitoba, Winnipeg, Man., Canada.