DISTRIBUTION, DIVERSITY, AND ACTIVITY OF ANTIBIOTIC-PRODUCING *PSEUDOMONAS* SPP.

JORGE T. DE SOUZA

Promotor:	Prof. Dr. ir. P.J.G.M. de Wit Hoogleraar in de Fytopathologie Wageningen Universiteit
Co-promotor:	Dr. J. M. Raaijmakers Universitair docent bij het Laboratorium voor Fytopathologie Wageningen Universiteit
Promotiecommissie:	Prof. Dr. ir. A. H. C. van Bruggen, Universiteit Wageningen Dr. P. Lemanceau, INRA, Dijon, France Prof. Dr. ir. L. C. van Loon, Universiteit Utrecht Prof. Dr. J. A. van Veen, Universiteit Leiden

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List of abbreviations

2,4-DAPG	2,4-diacetylphloroglucinol
aPCR	anchored polymerase chain reaction
CFU	colony forming unit
CMC	critical micelle concentration
CTAB	cetyltrimethylammoniumbromide
GC-FAME	gas chromatograph - fatty acid methyl ester
Ggt	*Gaeumannomyces graminis* var. *tritici*
HPLC	high performance liquid chromatography
IPTG	isopropylthio-β-D-galactosidase
KMB	King's medium B
LC-MS	liquid chromatography-mass spectrometry
MAPG	monoacetylphloroglucinol
MeCN	acetonitrile
N-AHLs	*N*-acylhomoserine lactones
PCA	phenazine-1-carboxilic acid
PCN	phenazine-1-carboxamide
PG	phloroglucinol
PHZ	phenazines
PKS	polyketide synthase
PLT	pyoluteorin
PRN	pyrrolnitrin
RAPD	random amplified polymorphic DNA
RFLP	random fragment length polymorphism
RP-HPLC	reverse phase-high performance liquid chromatography
TAD	take-all decline
TAPG	tri-acetylphloroglucinol
TEM	transmission electron microscopy
TLC	thin-layer chromatography
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactosidase

CONTENTS

CHAPTER 1

GENERAL INTRODUCTION AND OUTLINE

Part of this chapter has been accepted for publication as: Antibiotic production by bacterial biocontrol agents, by Raaijmakers, J.M., Vlami, M., and De Souza, J.T. in *Antonie van Leeuwenhoek* (*in press*).

Antibiotic-producing *Pseudomonas* spp.

Bacteria of the genus *Pseudomonas* are able to survive and prosper in a wide range of environmental conditions. This genus, not only contains plant, animal, and human pathogens, but also accomodates species of environmental interest, such as plant growth promoters, xenobiotic degraders, and biocontrol agents (Palleroni, 1992; Johnsen *et al.*, 1996). Among the biocontrol agents, the antibiotic-producing strains have received considerable attention.

Antibiotics encompass a chemically heterogeneous group of organic, low-molecular weight compounds. At low concentrations, antibiotics are deleterious to the growth or metabolic activities of other microorganisms (Fravel, 1988; Thomashow *et al.*, 1997). The fact that there is an abundant amount of studies on antibiotics produced by *Pseudomonas* spp. has several reasons: pseudomonads are common inhabitants of the rhizosphere and phyllosphere, are easily isolated from natural environments, utilize a wide range of substrates, are easy to culture and manipulate genetically, making them more amenable to experimentation (Leisinger and Margraff, 1979; Whipps, 2001).

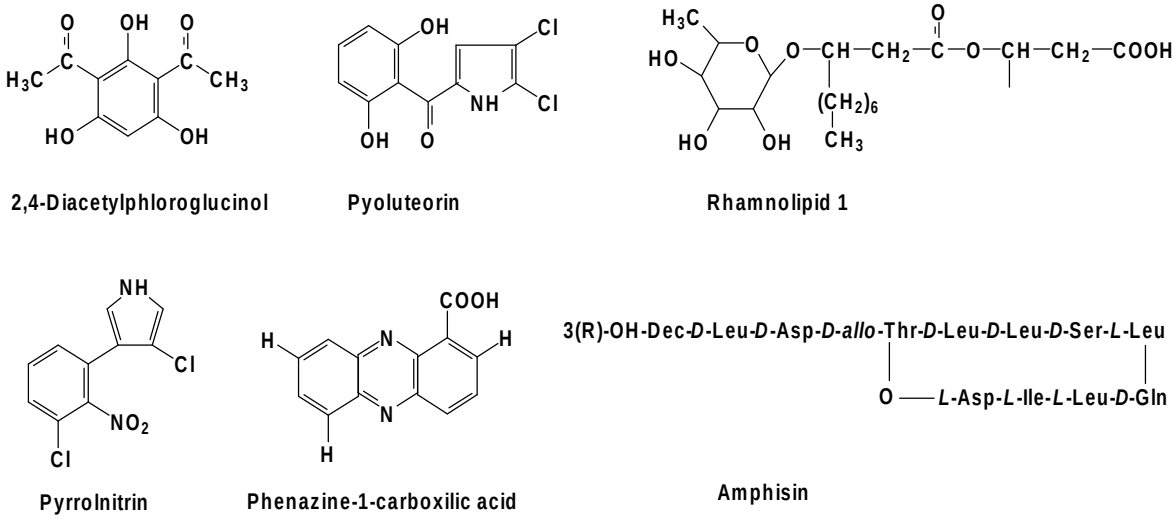

Figure 1. Chemical structure of some antibiotics produced by *Pseudomonas* species. In the primary structure of amphisin, Dec is hydroxydecanoic acid, Leu leucine, Asp aspartic acid, Thr threonine, Ser serine, Gln glutamine, and Ile isoleucine.

Pseudomonas biocontrol strains not only exhibit a wide range of diversity in the type but also in the number of antibiotics produced (Fig. 1). For example, *P. fluorescens* strains CHA0 and Pf5 (Keel *et al.*, 1996; Bender *et al.*, 1999) produce multiple antibiotics with overlapping or different degrees of activity against specific pathogens. This fact illustrates that for at least some biocontrol agents several antibiotics may account for the suppression of specific or multiple plant diseases. In this introductory chapter we will concentrate only on antibiotics produced by *Pseudomonas*, excluding siderophores and enzymes. Our attention will focus on the biosynthesis, regulation and activity of following antibiotics: 2,4-diacetylphloroglucinol (2,4-DAPG), phenazines (PHZ), pyrrolnitrin (PRN), pyoluteorin (PLT), and surface-active antibiotics. These five classes of antibiotics, in particular 2,4-DAPG, are subject of experiments described in this thesis.

2,4-diacetylphloroglucinol (2,4-DAPG)

Phloroglucinols are phenolic compounds produced by bacteria, algae, and plants (Reddi and Borovkov, 1969; Verrota *et al.*, 1999; Jimenez-Escrig *et al.*, 2001). More than 60 derivatives of phloroglucinol (PG) have been reported, but only three, monoacetylphloroglucinol (MAPG), 2,4-DAPG, and tri-acetylphloroglucinol (TAPG) are known to be produced by *Pseudomonas* species (Reddi and Borovkov, 1969; Shanahan *et al.*, 1992). In addition to MAPG and 2,4-DAPG, also PG has been isolated from cultures of a bacterium classified as *Aeromonas hydrophila* (Strunz *et al.*, 1978).

The antimicrobial activity of these compounds, and in particular of 2,4-DAPG has received considerable attention in the area of biological control of plant diseases. Numerous studies have demonstrated that 2,4-DAPG-producing *Pseudomonas* spp. can suppress a wide variety of plant pathogens, including fungi, bacteria and nematodes (Stutz *et al.*, 1986; Keel *et al.*, 1990; Vincent *et al.*, 1991; Fenton *et al.*, 1992; Levy *et al.*, 1992; Keel *et al.*, 1992; Cronin *et al.* 1997a, b; Duffy and Défago, 1997; Sharifi-Tehrani *et al.*, 1998). Among the fungi, *Gaeumannomyces graminis* on wheat, *Thielaviopsis basicola* on tobacco, *Fusarium oxysporum* f. sp. *radicis-lycopersici* on tomato, and the oomycete *Pythium ultimum* on sugar beet and cucumber have been shown to be controlled by 2,4-DAPG-producing *Pseudomonas* strains (Vincent *et al.*, 1991; Fenton *et al.*, 1992; Sharifi-Tehrani *et al.*, 1998; Stutz *et al.*, 1986; Duffy and Défago, 1997). The determinative role of 2,4-DAPG in disease suppression by *Pseudomonas* strains has been demonstrated by (1) the use of mutants deficient in 2,4-DAPG production (Vincent *et al.*, 1991; Shanahan *et al.*, 1992; Keel *et al.*, 1992; Cronin *et al.*,

1997a, b), (2) complementation of 2,4-DAPG- deficient mutants and subsequent restoration of biocontrol activity (Vincent *et al.*, 1991; Keel *et al.*, 1992; Cronin *et al.*, 1997a, b), and (3) expression of 2,4-DAPG biosynthetic genes in heterologous, nonproducing strains conferring biocontrol or enhanced activity (Vincent *et al.*, 1991; Shanahan *et al.*, 1993; Bangera and Thomashow, 1996). Reporter gene systems (Loper and Lindow, 1997) and analytical techniques (Thomashow *et al.*, 1997) have further demonstrated that 2,4-DAPG is produced *in situ* by both introduced strains and indigenous *Pseudomonas* populations (Keel *et al.*, 1992; Bonsall *et al.*, 1997; Duffy and Défago, 1997; Raaijmakers *et al.*, 1999; Notz *et al.*, 2001). Although 2,4-DAPG production is essential in the biocontrol activity of several *Pseudomonas* strains, it does not seem to play a determinative role in the survival and competitive ability of the producing strains (Carroll *et al.*, 1998; Chapter 4).

Despite the well-documented effects of 2,4-DAPG, its mode of action is still largely unclear. It was found that phloroglucinol derivatives are potent inhibitors of the photosystem II (Yoneyama *et al.*, 1990), however, little is known about its antibiotic mode of action against fungi and oomycetes. It has been suggested that 2,4-DAPG could also act as an inducer of the plant's defense mechanisms against pathogens. This assumption is based on the fact that herbicides, which are structurally similar to 2,4-DAPG, can induce resistance in plants (Altman and Campbell, 1977; Cohen *et al.*, 1986). We demonstrated that 2,4-DAPG does not affect the synthesis and composition of the cell wall of the oomycete *Pythium*, and that its deleterious action may reside in detrimental effects on the plasma membrane (Chapter 5).

Biosynthesis and regulation of 2,4-DAPG

The biosynthetic locus responsible for the synthesis of 2,4-DAPG by *Pseudomonas fluorescens* strain Q2-87 is composed of six genes (Bangera and Thomashow, 1996, 1999). The genes *phlACBD* constitute the biosynthetic operon and are flanked by *phlE* and *phlF*, which code for a putative transport and a repressor protein, respectively (Fig. 2). The gene *phlD* encodes a polyketide synthase responsible for the production of MAPG through condensation of an unknown precursor (Bangera and Thomashow, 1999). Genes *phlA*, *phlC*, and *phlB* encode proteins necessary for the acetylation of MAPG to 2,4-DAPG and may also function in the synthesis of MAPG (Table 1). Many strains of *Pseudomonas* produce TAPG in addition to 2,4-DAPG. Whether the known genes are also involved in the acetylation of 2,4-DAPG to TAPG remains to be determined.

The role of several environmental conditions influencing 2,4-DAPG production have been studied for a number of *Pseudomonas* strains (Shanahan *et al.*, 1992; Duffy and Défago,

Table 1. Open reading frames (ORFs) in the 2,4-DAPG biosynthetic gene cluster. (Bangera and Thomashow, 1996, 1999).

ORF	Size (bp)	Homology	Possible Function
phlA	1080	acyl carrier protein synthase	conversion of MAPG to 2,4-DAPG
phlC	1194	sterol carrier protein	conversion of MAPG to 2,4-DAPG
phlB	438	unknown	conversion of MAPG to 2,4-DAPG
phlD	1047	plant chalcone and stilbene synthases	synthesis of MAPG
phlE	1269	efflux protein	efflux of MAPG and 2,4-DAPG
phlF	606	repressor protein	repressor of 2,4-DAPG

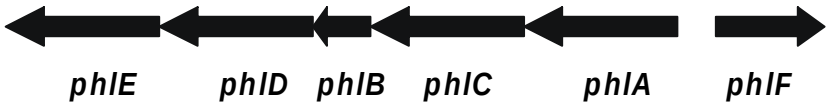

Figure 2. Organization of the 2,4-DAPG gene cluster in *P. fluorescens* Q2-87. Arrows indicate direction of transcription. (Bangera and Thomashow, 1996, 1999). Not drawn to scale.

1999; Notz *et al.*, 2001). These studies demonstrated that the regulation of this antibiotic through physiological factors may vary among strains. For example, 2,4-DAPG production was stimulated by glucose in many *P. fluorescens* strains, including strain CHA0, and repressed in strain F113 (Shanahan *et al.*, 1993; Duffy and Défago, 1997). In *P. fluorescens* S272, 2,4-DAPG production was stimulated by ethanol as a sole carbon source (Yuan *et al.*, 1998). Amendment of the growth medium with zinc sulfate and ammonium molybdate stimulates, whereas addition of inorganic phosphate inhibits 2,4-DAPG production. Fusaric acid, a toxin secreted by *Fusarium oxysporum* f. sp. *radicis-lycopersici* repressed production of 2,4-DAPG by *P. fluorescens* CHA0, resulting in inefficient performance of the biocontrol agent (Duffy and Défago, 1997). Studies employing the *lacZ* reporter gene fused to *phlA*, the first gene in the 2,4-DAPG biosynthetic operon (Fig. 2), showed that transcription of *phlA* in strain CHA0 is specifically stimulated by 2,4-DAPG and to a lesser extent by MAPG. Pyoluteorin and salycilate, other metabolites produced by the bacterium, repressed transcription of *phlA* (Schnider-Keel *et al.*, 2000). 2,4-DAPG production is, among others, regulated by *phlF*, a pathway-specific repressor gene located in the 2,4-DAPG biosynthetic locus (Fig. 2). This gene was found to encode a DNA-binding protein that represses production of the antibiotic at the transcriptional level. The protein was proposed to play an important role in preventing 2,4-DAPG production in early stages of growth, when the antibiotic could be detrimental to the cell (Delany *et al.*, 2000). The PhlF protein was also found to act as a mediator in the

autoinduction of 2,4-DAPG production and repression by pyoluteorin and salicylate. Mutants disrupted in *phlF* were insensitive to autoinduction by 2,4-DAPG and to repression of *phlA* by pyoluteorin and salicylate (Schnider-Keel *et al.*, 2000).

Phenazines (PHZ)

PHZs are nitrogen-containing compounds naturally produced exclusively by bacteria (Turner and Messenger, 1986). These antibiotics are very diverse, with more than 50 derivatives described. It is common to find several different strains producing the same PHZ compound and a single strain producing a variety of PHZs (Turner and Messenger, 1986). PHZs were found to be toxic to fungi, bacteria, and algae (Toohey *et al.*, 1965).

The proposed modes of action of PHZs include cell death or injury due to their capacity to undergo redox cycling in presence of several reducing agents and molecular oxygen, leading to the accumulation of toxic oxygen species (Hassan and Fridovich, 1980). Some PHZs were shown to intercalate into DNA strands, particularly with GC base pairs, to inhibit RNA synthesis (Turner and Messenger, 1986), and to disrupt electron transport and energy production (Baron *et al.*, 1989). Given the chemical diversity of PHZs, it is unlikely that a unifying mode of action among them exists (Turner and Messenger, 1986). PHZs produced by bacteria play important roles in virulence in humans and animals, biological control, and competitiveness. The PHZ compound pyocyanine produced by *P. aeruginosa* is a virulence factor in the infection of cystic fibrosis patients (Wilson *et al.*, 1987). *P. fluorescens* 2-79 produces phenazine-1-carboxilic acid (PCA) and *P. aureofaciens* 30-84 produces three PHZ compounds: PCA, 2-hydroxy-phenazine-1-carboxilic acid, and 2-hydroxy-phenazine. These PHZs were shown to play an important role in the competitive ability of the producing strains in the rhizosphere (Mazzola *et al.*, 1992).

PHZs also play a key role in biocontrol of several plant diseases. Mutants disrupted in PHZ biosynthesis could no longer control *G. graminis* var. *trtici* and *Septoria tritici* on wheat (Thomashow and Weller, 1988; Flaishman *et al.*, 1990; Pierson III and Thomashow, 1992), *Fusarium oxysporum* f.sp. *ciceris* on chickpea and *Pythium splendens* on beans and lettuce (Anjaiah *et al.*, 1998), and *F. oxysporum* f.sp. *radicis-lycopersici* on tomato (Chin-A-Woeng *et al.*, 1998). Mutants genetically restored in their ability to produce PHZs regained their ability to inhibit fungal growth on artificial medium and to control the disease *in planta* (Thomashow and Weller, 1988; Pierson III and Thomashow, 1992). Further evidence for the role of PHZs in biocontrol was provided by studies with reporter genes showing expression of

the PHZ biosynthetic operon in the rhizosphere (Chin-A-Woeng *et al.*, 1998) and its direct detection on roots of wheat treated with PHZ-producing strains (Thomashow *et al.*, 1990).

Biosynthesis and regulation of PHZs

The precise mechanisms of PHZ biosynthesis and the identity of all intermediates are still unknown, despite the amount of studies already performed. PHZs are derived from the shikimic acid pathway with chorismate as a probable branch point intermediate (Longley *et al.*, 1972). Phenazine-1,6-dicarboxylic acid was proposed as a common precursor to all other PHZs (Byng and Turner, 1977). Recent studies, however, indicate that phenazine-1-carboxylic acid is the common precursor (Mavrodi *et al.*, 2001). Genes involved in PHZ biosynthesis were studied in *P. aureofaciens* 30-84 (Pierson III *et al.*, 1995), *P. fluorescens* 2-79 (Mavrodi *et al.*, 1998), *P. chlororaphis* PCL1391 (Chin-A-Woeng *et al.*, 2001), and *P. aeruginosa* PAO1 (Mavrodi *et al.*, 2001). Seven genes were identified in these strains for biosynthesis of PCA. Initially, Pierson III *et al.* (1995) identified five genes, *phzCDEFG* (originally *phzFABCD*) from *P. aureofaciens* 30-84 for synthesis of PCA. The products of *phzD* and *phzE*, which have homology with isochorismatase and anthranilate synthase, respectively (Table 2), were hypothesized to catalyse the conversion of chorismate to phenazine-1,6-dicarboxylic acid (Pierson III and Thomashow, 1992). PhzF and PhzG were proposed to act in the conversion of phenazine-1,6-dicarboxylic acid to PCA. The proteins PhzA and PhzB were thought to act as stabilizers of a complex formed by PhzD and PhzE, which still work in the absence of PhzA and PhzB, but the specificity and efficiency decreases dramatically (Mavrodi *et al.*, 1998). In *P. aeruginosa* PAO1, two complete operons, *phzA1B1C1D1E1F1G1* and *phzA2B2C2D2E2F2G2* for PCA production were identified. These two PHZ operons are 98.3% identical at the DNA level and are homologous to the PHZ operons of *P.aureofaciens* 30-84, *P. fluorescens* 2-79, and *P. chlororaphis* PCL 1391. A screen of 30 other PHZ-producing strains by using the *phzA1B1C1D1E1F1G1* from *P. aeruginosa* PAO1 (Fig. 3) as a probe showed that the PHZ genes are highly conserved among *Pseudomonas* species and are different from the PHZ genes of *Burkholderia* species (Mavrodi *et al.*, 2001). According to the mechanism proposed for biosynthesis of PHZs, the operons *phzA1B1C1D1E1F1G1* and *phzA2B2C2D2E2F2G2* are responsible, by yet undefined intermediates, for the conversion of chorismic acid to PCA. The PHZ modifying genes *phzM*, *phzS*, and *phzH* further catalyze the conversion of PCA to other PHZs (Mavrodi *et al.*, 2001, Table 2).

PHZ antibiotics are only produced during late exponential growth phase and in the

Table 2. Open reading frames (ORFs) responsible for PHZ biosynthesis in *P. aeruginosa* PAO1. (Mavrodi *et al.*, 2001) and PAO1 genome (http://www.pseudomonas.com).

ORF [a]	Size (bp)	Homology [b]	Possible Function [c]
*phzA*1	489	*phzA* homologs, *phzX*	synthesis of PCA
*phzB*1	489	*phzB* homologs, *phzY*	synthesis of PCA
*phzC*1	1218	plant DAHP synthases	synthesis of PCA
*phzD*1	624	isochorismatases	synthesis of PCA
*phzE*1	1884	bacterial amidotransferases	synthesis of PCA
*phzF*1	837	*phzF*, and *phzC* homologs	synthesis of PCA
*phzG*1	645	bacterial pyridoxamine-5'-phosphate oxidases	synthesis of PCA
rhlI	606	autoinducer proteins	quorum sensing regulation
rhlR	726	transcriptional regulator	quorum sensing regulation
phzM	1005	O-methyltransferase	conversion of PCA to 5-CH-PCA
phzS	1209	bacterial mono-oxygenases	conversion of PCA to 1-OH-PHZ and 5-CH-PCA to pyocyanine
phzH	1833	bacterial asparagine synthetase	conversion of PCA to PCN

[a] ORFs *phzA1B1C1D1E1F1G1* comprise the core 1 biosynthetic operon of PAO1; *rhl*I and *rhl*R are quorum sensing regulators; *phz*M, *phz*S, and *phz*H are phenazine modifying genes.
[b] *phz*A, *phz*B, and *phz*F refer to homologs from *P. fluorescens* 2-79 and *P. chlororaphis* PCL1391; *phz*X, *phz*Y, and *phzC* refer to homologs from *P. aureofaciens* 30-84; DAHP is phospho-2-keto-3-deoxyheptamate aldolase.
[c] PCA is phenazine-1-carboxylic acid, 5-CH-PCA is 5-methyl phenazine-1-carboxylic acid betaine; 1-OH-PCA is 1-hydroxyphenazine, PCN is phenazine-1-carboximide.

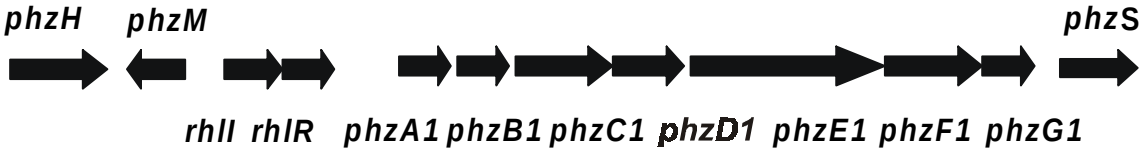

Figure 3. Organization of the PHZ gene cluster in *P. aeruginosa* PAO1. Arrows indicate direction of transcription. (Mavrodi *et al.*, 2001). Not drawn to scale.

stationary phase (Turner and Messenger, 1986). In natural environments, the expression of the PHZ operon can be influenced by seed and root exudates of several plant species (Georgakopoulos *et al.*, 1994). Long-term studies indicated that the expression levels of the PHZ operon are influenced by the developmental stage of wheat plants (Pierson III and Pierson, 1996).

Regulation of PHZ production and many other genes in bacteria is done, among others, through a process called quorum sensing (Fuqua *et al.*, 1996). Quorum sensing is a mechanism by which bacteria can communicate by using signal molecules, collectively known as *N*-acylhomoserine lactones (AHLs). By using these signal molecules produced by themselves or by other cells, bacteria can sense when a minimal unity or quorum of baterial cells has been

attained to exhibit multicellular behaviour in terms of gene expression (Fuqua *et al.*, 1996). Many genera of bacteria produce these diffusible molecules and regulate gene expression in a cell density-dependent manner. In *Pseudomonas*, traits such as production of toxins, enzymes, rhamnolipids, and PHZs are controlled by quorum sensing (Ochsner and Reiser, 1995). Quorum sensing systems generally comprise two proteins, such as RhlI/RhlR in *P. aeruginosa* PAO1 (Fig. 3). The autoinducer protein (RhlI) is responsible for the synthesis of AHLs, which diffuse freely out and into the cell. When the intercellular AHL concentration reaches a threshold, it interacts with the transcriptional regulator protein (RhlR), which in turn regulates specific gene expression (Passador *et al.*, 1993). Interestingly, only one of the two PHZ operons in *P. aeruginosa* PAO1 appears to be controlled by quorum sensing. The presence of two differentially regulated PHZ biosynthetic operons may give greater flexibility in modulating PHZ production depending on the growth phase or response to environmental signals (Mavrodi *et al.*, 2001). In *P. chlororaphis* strain PCL1391, PhzI/PhzR (RhlI/RhlR homologs) partially control PHZ production by quorum sensing. Expression levels of *phzI* are positively regulated by yet unidentified factor(s) secreted into the growth medium of overnight cultures. This positive regulation enables the strain to produce PCN at low cell densities and this factor(s) was shown not to be AHLs (C4-HSL or C8-HSL) or PCN itself (Chin-A-Woeng *et al.*, 2001).

The regulation of PHZ production by quorum sensing in *P. aeruginosa* PAO1 is very complex, involving also the LasI/LasR proteins, another set of autoinducer and transcriptional regulator proteins (Pesci *et al.*, 1997). These two quorum sensing systems do not act independently of one another, but a hierarchy exists with LasR required for the activation of *rhlR* and to some extent *rhlI*. Recently, a gene in *P. chlororaphis* PCL1391 which is homologous to the *lexA* gene of *P. aeruginosa* was shown to be involved in the regulation of PHZ (Chin-A-Woeng, 2000). Mutants disrupted in the *lexA* homolog exhibit a more than 10-fold increase in PHZ production levels as compared to the wild type strain. The introduction of a *lexA* mutation in *phzI* or *phzR* mutants restored production of PHZ to wild type levels and resulted in the reappearance of AHLs, which were not present in the background *phzI* mutant. The authors hypothesized that *lexA* represses a normally silent AHL synthase.

Pyrrolnitrin (PRN)

PRN (3-chloro-4-(2'-nitro-3'-chlorophenyl)-pyrrole) is a chlorinated phenylpyrrole antibiotic produced by many bacterial genera, including *Pseudomonas*, *Burkholderia*, *Enterobacter*, *Serratia*, and *Myxococcus* (Arima *et al.*, 1964; Elander *et al.*, 1968; Hammer *et al.*, 1999; Kalbe *et al.*, 1996).

The mode of action of PRN is primarily the inhibition of the respiratory electron transport system (Tripathi and Gottlieb, 1969). PRN has activity against several bacteria and fungi, in particular *Rhizoctonia solani* (Arima *et al.*, 1964; Cartwright *et al.*, 1995; Chernin *et al.*, 1996; El-Banna and Winkelmann, 1988; Hill *et al.*, 1994; Jayaswal *et al.*, 1993; Rosales *et al.*, 1995). PRN is also effective against post-harvest diseases caused by *Botrytis cinerea* on apple, pear and on cut flowers (Hammer and Evensen, 1993; Janisiewicz and Roitman, 1988), and has been used to treat infections in humans by opportunistic fungi (Tawara *et al.*, 1989). The role of PRN in biological control has been demonstrated in studies where mutants of *P. fluorescens* strains BL915 and Pf-5, defective in pyrrolnirin production, were unable to control *R. solani* on cotton and *Pyrenophora tritici-repentis* in axenically colonized wheat straw (Hill *et al.*, 1994; Pfender *et al.*, 1993). The antibiotic was also detected on wounded potatoes colonized by a *B. cepacia* strain (Burkhead *et al.*, 1994).

Biosynthesis and regulation of PRN

Four genes, *prnABCD*, which are organized in a single transcriptional unit are required for PRN synthesis by *P. fluorescens* BL915 (Fig. 4, Hammer *et al.*, 1997). The *prnA* gene contains a short region with high similarity to the NAD binding domain of the NADH dehydrogenases from *Haemophilus influenzae* and *Escherichia coli*, and the thioredoxin reductase from *Streptomyces clavuligerus*. The *prnB* gene had no homologies in the database. The gene *prnC* is highly homologous to the *chl* gene from *S. aureofaciens*, which encodes a chlorinating enzyme involved in the synthesis of tetracycline. Finally, *prnD* has similarity with a portion of the 3-chlorobenzoate-3,4-dioxygenase (*cbaA*) of *Alcaligenes* sp., phthalate 4,5-dioxygenase (*pht3*) from *P. putida*, and vanilate demethylase (*vanA*) from *Pseudomonas* sp. (Table 3, Hammer *et al.*, 1997). The two chlorinating enzymes encoded by *prnA* and *prnC* represent a new class of halogenating enzymes, since they were different from all previously described haloperoxidases (Hammer *et al.*, 1999). Further studies on the *prnABCD* gene cluster of four producing strains, *P. fluorescens* BL915, *B.cepacia* LT4-12-W, *B. pyrrocinia*, and

Table 3. PRN biosynthesis in *P. fluorescens* BL915. (Hammer *et al.*, 1997; Kirner *et al.* 1998).

ORF	Size (bp)	Homology [a]	Function [b]
prnA	1617	dehydrogenase from *H. influenzae*	conversion of L-T to 7-CT
prnB	1086	no homology	conversion of 7-CT to MDA
prnC	1704	*chl* gene from *S. aureofaciens*	conversion of MDA to APRN
prnD	1092	*cbaA* gene from *Alcaligenes* sp.	conversion of APRN to PRN

[a] Only highest level of homology shown.
[b] L-T is L-tryptophan, 7-CT is 7-chloro-L-tryptophan, MDA is monodechloroaminopyrrolnitrin, APRN is aminopyrrolnitrin.

Figure 4. Organization of the PRN biosynthetic operon of *P. fluorescens* BL915. Arrows indicate the direction of transcription. (Hammer *et al.*, 1997). Not drawn to scale.

Myxococcus fulvus showed that these genes are conserved among the studied strains (Hammer *et al.*, 1999).The biosynthetic pathway for PRN was clearly defined by using mutants defective in each of the genes in the PRN operon (Kirner *et al.*, 1998). Tryptophan was identified as the precursor of PRN, based on studies using isotopically labelled and substituted tryptophan to feed bacterial cultures (Chang *et al.*, 1981). The steps for the conversion of tryptophan to PRN, catalysed by the *prnABCD* gene products, are shown in Table 3.

The biosynthetic regulation of PRN is influenced by nutritional and environmental conditions. PRN production by *P. fluorescens* CHA0 was stimulated by fructose, mannitol, and amendment with a mixture of zinc and ammonium molybdate (Duffy and Défago, 1999). Studies with *B. cepacia* strains show that PRN is produced at higher concentrations when the bacteria were grown in media with low initial pH (Roitman *et al.*, 1990). The use of glycerol as a carbon source enhanced PRN production, whereas galactose, rhamnose, lactose and starch repressed production (El-Banna and Winkelmann, 1998). It was reported that PRN in *B. cepacia* was produced in the late growth phase and about 98% was inside the bacterial cells (Roitman *et al.*, 1990; El-Banna and Winkelmann, 1998).

Genetic engineerng of *P. fluorescens* BL915 for increased PRN production provides an example of the potential of such modifications in improving the performance of this strain as a biological control agent (Ligon *et al.*, 2000). The genetically modified strains provided better control of *R. solani* and *Pythium ultimum* than the wild type strain under controlled and field

conditions. The level of control provided by the modified strains was not different from fungicide checks and there was a correlation between increased PRN production and increased biocontrol activity.

Pyoluteorin (PLT)

PLT (4,5-dichloro-1 H-pyrrol-2-yl-2,6-dihydroxyphenyl ketone) is a phenolic polyketide antibiotic which consists of a resorcinol ring linked to a bichlorinated pyrrole moiety (Fig. 1). PLT was first isolated from *P. aeruginosa* (Takeda, 1958) and later from *P. aeruginosa* strain S10B2 (Ohmori *et al.*, 1978) and *P. fluorescens* strains Pf-5 and CHA0 (Bencini *et al.*, 1983; Bender *et al.*, 1999; Keel *et al.*, 1996). Most of the information on PLT biosynthesis, its role in biocontrol, and regulation stems from strains Pf-5 and CHA0.

The precise mode of action of pyolutorin is not clear, but it has bactericidal, herbicidal, fungicidal, and oomycidal activities, in particular against *Pythium* spp. (Maurhoffer *et al.*, 1992; Ohmori *et al.*, 1978; Takeda, 1958). Application of pure PLT to cotton seeds resulted in significant suppression of *Pythium ultimum*-induced damping-off (Howell and Stipanovic, 1980). Results from studies with derivatives of specific *Pseudomonas* strains, that were either defective in PLT production or produced higher levels of PLT, have indicated that the determinative role of PLT in biological control may vary among different plant hosts (Maurhoffer *et al.*, 1992, 1994, 1995; Kraus and Loper, 1992). PLT production in the rhizosphere by strain CHA0 and *plt* gene expression in the spermosphere by strain Pf-5 vary among different host plants, presumably affecting the biocontrol activity of PLT (Maurhoffer *et al.*, 1995; Kraus and Loper, 1995).

Biosynthesis and regulation of PLT

Ten genes for synthesis of PLT in *P. fluorescens* Pf-5 were identified. The genes *pltLABCDEFG* are organized in a single transcriptional unit, whereas *pltRM* comprise another transcriptional unit oriented in the opposite direction of *pltLABCDEFG* (Fig. 5). The *pltB* and *pltC* genes show similarity with the *eryA* gene of *Saccharopolyspora erythraea* that encode a bacterial Type I polyketide synthase necessary for erytrhromycin production (Nowak-Thompson *et al.*, 1997; Table 4). Genes *pltA*, *pltD*, and *pltM* are similar to genes encoding halogenating enzymes required for chlorotetracycline biosynthesis by *Streptomyces aureofaciens* (Cts4) and for pyrrolnitrin biosynthesis by strain BL915 (PrnC). The gene *pltE* is similar to

Table 4. Biosynthesis of PLT in *P. fluorescens* Pf-5. (Nowak-Thompson *et al.*, 1997, 1999).

ORF	Size (bp)	Homology [a]	Possible function
pltA	1347	halogenating enzymes	pyrrole chlorination
pltB	7374	Type I PKSs	resorcinol synthesis
pltC	5322	Type I PKSs	resorcinol synthesis
pltD	1632	halogenating enzymes	pyrrole chlorination
pltE	1140	acyl-CoA dehydrogenases	pyrrole synthesis
pltF	1494	peptide synthases	precursor activation
pltG	780	thioesterases involved in secondary metabolism	termination of resorcinol synthesis
pltL	264	no homology	unknown
pltM	1506	halogenating enzymes	pyrrole chlorination
pltR	1029	transcriptional regulator of *R. japonicum*	pathway specific regulation

[a] PKS is polyketide synthase; only highest level of homology is shown.

Figure 5. Organization of the PLT biosynthetic genes in *P. fluorescens* Pf-5. Arrows indicate transcription direction. (Nowak-Thompson *et al.*, 1997, 1999). Not drawn to scale.

many flavin-dependent acyl coenzyme A (acyl-CoA) dehydrogenases and specially to butyryl-CoA dehydrogenase from *Megasphaera elsdenii*. the *pltF* gene is similar to several peptide synthases including *grsB* from *Bacillus brevis*, *snbC* from *Streptomyces pristinaespiralis*, and *pvdD* from *P. aeruginosa*. The gene *pltG* is similar to several thioesterases involved in secondary metabolism, including thioesterase II from rat, *grsT* from *B. brevis*, and *CmaT* from *P. syringae*; *pltL* shows highest similarity to a hypothetical protein encoded by the *red* gene cluster of *S. coelicolor*. The *pltR* product resembles members of the LysR family of transcriptional regulators such as *gstR* of *Rhizobium japonicum*, and *pltL* did not have any match in the database (Nowak-Thompson *et al.*, 1999). The PLT biosynthetic gene cluster has not been completely delimited and little is known about the chemical intermediates involved in its biosynthesis. Proline and acetyl- coenzyme A are the precursors of PLT (Bender *et al.*, 1999). The available information on the synthesis of PLT is shown in Table 4.

PLT production is partially regulated by *pltR*, which encodes a transcriptional activator protein. Transcription of *pltB*, *pltE*, and *pltF* genes was reduced in mutants disrupted in *pltR* as compared to the wild type strain (Nowak-Thompson *et al.*, 1999). The physiological status of the bacterial cells and the environmental conditions greatly affect production of PLT.

Secretion of PLT was stimulated by zinc, cobalt, glycerol, and poor aeration, whereas glucose, phosphate, and tryptophan repressed PLT production (Bencini *et al.*, 1983; Duffy and Défago, 1999). The use of ethanol as a sole carbon source enhanced PLT production in *P. fluorescens* S272 (Yuan *et al.*, 1998).

Global Regulation of antibiotic production in *Pseudomonas* by *gacS*/*gacA*

In several strains that belong to the group of plant-associated *Pseudomonas* species, expression of genes involved in the biosynthesis of secondary metabolites and extracellular enzymes is positively controlled by the GacS/GacA two-component system (Heeb and Haas, 2001). This regulatory system consists of a membrane-bound sensor kinase protein (GacS) and a cytoplasmic response regulator protein (GacA). The current model proposes that GacS recognizes specific environmental stimuli and activates GacA which in turn triggers specific gene expression (Appleby *et al.*, 1996; Pernestig *et al.*, 2001; Heeb and Haas, 2001). Although a considerable amount of information has accumulated in the last decades on these two-component regulatory systems, the environmental signals responsible for the activation of GacS are still unknown.

The gene *gacS* was first described in *Pseudomonas syringae* pv. *syringae* as an essential factor for disease manifestation (Hrabak and Willis, 1992). The cognate response regulator gene *gacA* was first identified in the biocontrol strain *P. fluorescens* CHA0 as essential for the biocontrol activity of this strain (Laville *et al.*, 1992).

Many regulatory elements may be involved in the regulation of antibiotic-encoding genes, forming a complex regulatory cascade, with the *gacS*/*gacA* pair exerting control at a higher hierarchy (Sarniguet *et al.*, 1995; Chancey *et al.*, 1999; Blumer and Haas, 2000; Whistler *et al.*, 1998). Mutations in either *gacS* or *gacA* abolishes the production of 2,4-DAPG, PLT, PRN, and PHZ .

Production of 2,4-DAPG is influenced by the interactions of the *gacS*/*gacA* system with other regulatory elements, including the housekeeping sigma factor σ^{70} of RNA polymerase encoded by *rpoD*, and the stationary phase and stress sigma factor σ^{38} which is encoded by *rpoS* (Schnider *et al.*, 1995b; Whistler *et al.*, 1998; Blumer and Haas, 2000). Mutantional disruption of *rpoS* in *P. fluorescens* Pf-5 or introduction of multiple copies of *rpoD* in *P. fluorescens* CHA0 led to overproduction of 2,4-DAPG (Sarniguet *et al.*, 1995; Schnider *et al.*, 1995a).

PHZ production is regulated at multiple levels by *gacS*/*gacA*, including mediation of the transcription of *phzI*, which is necessary for AHL production in *P. aureofaciens* 30-84 (Chancey *et al.*, 1999). The *rpoS* gene was recently shown to regulate production of PHZ in *P. aeruginosa* PAO1 (Whiteley *et al.*, 2000).

Two global regulators are known to influence PRN production in *Pseudomonas*. The *rpoS* gene and GacS/GacA system were shown to be essential for PRN production by *P. fluorescens* Pf-5 (Sarniguet *et al.*, 1995; Corbell and Loper, 1995). Mutants disrupted in *rpoS* or *gacS*/*gacA* do not produce PRN (Gaffney *et al.*, 1994).

Genetic regulation of PLT production is complex, involving several regulators. The *gacS*/*gacA* regulatory system was shown to be essential for PLT production in strains Pf-5 and CHA0 (Laville *et al.*, 1992; Corbell and Loper, 1995). This two-component system is known to positively influence the accumulation of sigma factors in *Pseudomonas* (Whistler *et al.*, 1998). Inactivation of *rpoS* in *P. fluorescens* Pf-5 or the introduction of multiple copies of *rpoD* in *P. fluorescens* CHA0 also led to enhanced PLT production by these strains (Schnider *et al.*, 1995a; Sarniguet *et al.*, 1995). This mechanism of genetic regulation shows remarkable resemblance with that of 2,4-DAPG.

Most information available on *gacS*/*gacA* regulatory systems stems from studies on model strains and very little information is available on the distribution of these genes in rhizosphere environments. In this thesis, we investigated the usefullness of the *gacA* gene as a complementary genetic and phylogenetic marker for *Pseudomonas* (Chapter 3).

Biosurfactant antibiotics

Many surface-active molecules (surfactants) of biological origin have been described (Feichter, 1992). Their surfactant properties result from the presence of both hydrophilic and hydrophobic regions in the same molecule. There are several classes of biosurfactants with different chemical structures and surface properties. Biosurfactants can be classified as low-molecular-weight polymers that lower surface and interfacial tensions and high-molecular-weight polymers that bind to surfaces (Ron and Rosenberg, 2001). Several biosurfactants produced by *Pseudomonas* species have antibiotic activity in addition to their surfactant activity, including rhamnolipids and peptide biosurfactants. These two classes of biosurfactants are classified as low-molecular-weight polymers.

Rhamnolipids (RHLs) are glycolipid biosurfactants containing rhamnose and β-hydroxydecanoic acid and are produced by certain species of fluorescent pseudomonads, specifically *P. aeruginosa* (Itoh *et al.*, 1971; Van Dijke *et al.*, 1993). There are six known RHLs which differ in their rhamnose content and in the length and chemical nature of their lipid fraction (Jarvis and Johnson, 1949; Hirayama and Kato, 1982; Syldatk *et al.*, 1985). There is a considerable amount of information on the biosynthesis, genetics, and regulation of RHL production in *P. aeruginosa* (Hauser and Karnovsky, 1958; Ochsner and Reiser, 1995). RHLs produced by *P. aeruginosa* may serve as virulence factors during colonization of the human lung tissue (Iglewski, 1989). In addition, they show antibiotic effects, acting in solubilization of cell envelopes of competing microorganisms (Kurioka and Liu, 1967). RHLs also present antibacterial, mycoplasmacidal, cytotoxic, and antiviral activities *in vitro* (Jarvis and Johnson, 1949; Edwards and Hayashi, 1965; Hisatsuka *et al.*, 1971; Itoh *et al.*, 1971; Hirayama and Kato, 1982). The role of RHLs in the biocontrol of plant pathogens was recently demonstrated (Stanghellini and Miller, 1997; Kim *et al.*, 2000). RHLs were active against zoospores of oomycetes, causing cessation of motility and lysis of entire zoospore populations in less than 1 min. Introduction of a RHL-producing bacterium in a recirculating hydroponic system gave good control, although transient, of *Phytophthora capsici* on pepper (Stanghellini and Miller, 1997). *P. capsici* mycelial growth and *Colletotrichum orbicularie* spore germination were inhibited *in vitro* by RHL B produced by *P. aeruginosa* B5. The diseases caused by these pathogens were also suppressed in pepper and cucumber plants treated with purified RHL B (Kim *et al.*, 2000). RHLs are thought to act by intercalating into the plasma membrane of zoospores and cause its disruption (Stanghellini and Miller, 1997). However, their precise mode of action on structures protected by cell walls has not been demonstrated conclusively.

Lipopeptide surfactants are composed of a peptide moiety linked to a fatty acid. The peptide moiety, like many small peptides in microorganisms, is synthesized non-ribosomally by a multi-enzyme peptide synthase complex (Ron and Rosenberg, 2001). *Pseudomonas* species produce several lipopeptide surfactant, including viscosin (Hiramoto *et al.*, 1970), viscosinamide (Nielsen *et al.*, 1999), massetolides (Gerard *et al.*, 1997), tensin (Nielsen *et al.*, 2000), syringopeptins, syringomycins, syringostatins, syringotoxins, and pseudomycins (Hutchison and Gross, 1997; Serra *et al.*, 1999), tolaasin (Rainey *et al.*, 1991), the "white line inducing principle" (WLIP) (Mortshire-Smith *et al.*, 1991), amphisin (Sørensen *et al.*, 2001), pholipeptin (Ui *et al.*, 1997), arthrofactin (Morikawa *et al.*, 1993), and ecomycins (Miller *et al.*, 1998). All *Pseudomonas*-produced lipopeptides tested to date have antibiotic properties.

The lipopeptides syringomycins, syringopeptins, syringostatins, syringotoxins, and pseudomycins, produced by strains of *P. syringae* are virulence factors in the diseases caused by these phytopathogenic bacteria (Scholz-Schroeder *et al.*, 2001). Syringomycins, syringostatins, syringotoxins, and pseudomycins have marked antifungal activity and are potentially useful for the development of agents for the control of plant and human diseases, whereas syringopeptins have stronger phytotoxic and reduced antifungal activity as compared to the others (Lam *et al.*, 1987; Iacobellis *et al.*, 1992). Similarly, tolaasin, produced by *P. tolaasii*, is the primary bacterial compound responsible for eliciting disease symptoms on cultivated mushrooms (Godfrey *et al.*, 2001) and also activity against a range of basidiomycetes and Gram-positive bacteria (Rainey *et al.*, 1991). Interestingly,WLIP, produced by *P. reactans*, an opportunistic pathogen related to *P. tolaasii*, was proposed as an inhibitor of the symptoms caused by *P. tolaasi* on mushrooms (Soler-Rivas *et al.*, 1999). Viscosin, a lipopeptide with antifungal and surfactant properties, is an important virulence factor in the disease incited by pectolytic strains of *P. fluorescens* on brocoli (Hildebrand *et al.*, 1998). Tolaasin and viscosin are also proposed as biological agents for the control of pathogenic fungi (Nielsen *et al.*, 1999). Ecomycins, produced by *P. viridiflava*, were reported to be active against several human and plant pathogenic fungi (Miller *et al.*, 1998). The role of ecomycins in the weak disease inducing capacity of *P. viridiflava* is not yet clear.

Viscosinamide, and tensin, produced by soil-inhabiting *P. fluorescens* strains were reported to inhibit mycelial growth of several fungi and oomycetes, inducing hyphal swellings and stimulating hyphal branching (Thrane *et al.*, 1999; Nielsen *et al.*, 2000). *Pseudomonas* sp. from marine tube worms produce several cyclic depsipeptides called massetolides (Gerard *et al.*, 1997), which are related to other lipopeptide surfactants, such as viscosin and viscosinamide. Massetolides showed pronounced activity against *Mycobacterium* species.

Amphisin and pholipetin, related compounds produced by *P. fluorescens* (Ui *et al.*, 1997; Sørensen *et al.*, 2001), and arthrofactin produced by a *Pseudomonas* sp. (Morikawa *et al.*, 1993) did not yet have their biological activity investigated, but, because of their structural similarities to the other lipopeptides described above, similar activities might be expected.

The mode of action of several lipopeptide surfactants was shown to involve formation of ion channels in the cell wall and pertubations of the cell membrane structure (Nielsen *et al.*, 1999; Hutchison and Johnstone, 1993; Vanittanakom and Loeffler, 1986; Thrane *et al.*, 1999).

Biosynthesis and regulation of surfactant antibiotics

Little is known about the biosynthesis, regulation, and genetics of most lipopeptide surfactants. There is, however, some information available on the genetics of some *P. syringae* toxins (Scholz-Shroeder *et al.*, 2001), tolaasin (Rainey *et al.*, 1993), and viscosin (Braun *et al.*, 2001). From the few characterized examples, it is known that lipopeptides are encoded by relatively large DNA fragments (Scholz-Shroeder *et al.*, 2001; Braun *et al.*, 2001) and that peptide synthases are involved in the biosynthesis of these compounds (Marahiel *et al.*, 1997). It was reported that a massetolide-producing *Pseudomonas* sp. could incorporate amino acids artificially at various positions, thereby producing new types of massetolides (Gerard *et al.*, 1997). These results show that peptide synthases may be unspecific in their assembly of amino acids in depsipeptides. Some studies suggest that lipopeptide production is regulated in a cell-density dependent manner, because their production seems to be linked to cell proliferation (Ron and Rosenberg, 2001). Production of the toxin tolaasin commenced during exponential growth and continued into stationary phase (Rainey *et al.*, 1991). On the other hand, studies on the biosynthesis of viscosinamide showed that this compound was abundantly produced in the logarithmic phase of growth and abolished in the stationary phase (Nielsen *et al.*, 1999).This production pattern differs from that of the peptide antibiotic tolaasin, other antibiotics, and RHLs, which are considered secondary metabolites. The authors, based on their and other studies, proposed that viscosinamide should be considered a primary rather than a secondary metabolite (Nielsen *et al.*, 1999). Lipopeptide production patterns and regulation are expected to vary, especially when the different biological purposes for which *Pseudomonas* species produce lipopeptides (Ron and Rosenberg, 2001) are taken into consideration.

Distribution and detection of antibiotic-producing *Pseudomonas*

Numerous bacterial strains that produce antibiotics *in vitro* have been isolated from different soils and plant hosts. This apparent wide distribution suggests that antibiotic-producing bacteria are common constituents of the indigenous microflora in soil and plant-associated environments worldwide. To date, however, little is known about the frequency and ecology of indigenous antibiotic-producing bacteria. Knowledge about the ecology of naturally occurring strains that harbor specific biocontrol traits will significantly contribute to

improving the efficacy of existing biocontrol agents and may help to identify new strains that are better adapted to specific soils or host-pathogen systems.

Selection and identification of antibiotic-producing bacteria via random isolation from natural environments is very time-consuming and laborious. In fact, most of the model strains used to date were identified via screening huge numbers of isolates obtained by random procedures. The availability of cloned and sequenced antibiotic biosynthetic and regulatory genes, however, has facilitated the development of specific primers and probes that can be used for targeted detection and isolation of specific antibiotic-producing bacteria. A prerequisite for DNA-based detection and isolation is that the genes of interest must be conserved among a wide range of bacterial strains harboring this trait. By Southern hybridization with a 4.8-kb chromosomal DNA fragment of *P. fluorescens* strain Q2-87, Keel *et al.* (1996) showed that the biosynthetic locus for 2,4-DAPG production was highly conserved among 45 2,4-DAPG-producing *Pseudomonas* strains of worldwide origin. Subsequent development of primers and probes directed against *phlD*, a key gene in the biosynthesis of 2,4-DAPG, allowed targeted detection, isolation and enumeration of 2,4-DAPG-producing *Pseudomonas* spp. occurring naturally on roots of wheat (Raaijmakers *et al.*, 1997). Also the genes or gene clusters involved in the biosynthesis of PHZs (Mavrodi *et al.*, 2001) and PRN (Hammer *et al.*, 1999), respectively, appear to be conserved among different strains. In our studies, PCR analysis, Southern hybridization and restriction fragment length polymorphisms (RFLP) analyses showed that specific genes involved in PRN and PLT biosynthesis are highly conserved in a large collection of both *Pseudomonas* and *Burkholderia* species (Chapter 2).

The use of probes and primers directed against genes involved in antibiotic biosynthesis has proven to be a powerful technique to study the distribution and function of indigenous antibiotic-producing *Pseudomonas* spp. Colony hybridization followed by PCR analysis showed that root-associated fluorescent *Pseudomonas* spp. producing the antibiotic 2,4-DAPG were present on roots of wheat grown in several take-all suppressive soils, referred to as take-all decline soils (TAD), at densities ranging from approximately $5x10^5$ to $2x10^6$ CFU per gram of root. In the complementary conducive soils, 2,4-DAPG-producing pseudomonads were not detected or detected at densities at least 40-fold lower than in the TAD soils (Raaijmakers *et al.*, 1997). Moreover, 2,4-DAPG-producing *Pseudomonas* spp. were present on roots of wheat grown in at least three TAD soils at or above the threshold population density required for significant suppression of take-all of wheat. The specific suppression that operates in take-all suppressive soils was lost when indigenous 2,4-DAPG-producing fluorescent *Pseudomonas* spp. were eliminated by selective heat treatment, and

conducive soils gained suppressiveness to take-all when indigenous 2,4-DAPG-producing *Pseudomonas* strains were introduced via mixing in small amounts of raw take-all suppressive soil (Raaijmakers *et al.*, 1998).

Apart from genetic markers, also phenotypic markers may provide a source to rapidly isolate and identify bacteria with antagonistic potential. For example, Ellis *et al.* (2000) characterized a collection of 29 fluorescent pseudomonads with the intent of identifying conserved phenotypic or genotypic traits in strains with activity against *Pythium ultimum*. They found a significant correlation between biological control activity and the accumulation of a specific cyclopropane fatty acid (C17:O CFA) and hydrogen cyanide production. The authors concluded that screening isolates on the basis of elevated synthesis of C17:O CFA has the advantage that it does not depend on prior knowledge of secondary metabolite synthesis.

Diversity of antibiotic-producing *Pseudomonas*

The genotypic and phenotypic diversity that occurs in natural populations of biocontrol agents provides an enormous resource for improving biological control of plant diseases (Handelsman and Stabb, 1996; Thomashow and Weller, 1996). This approach has been widely used to select for better biocontrol agents of insects, and to improve the use of microorganisms in the production of fermented foods and biodegradation of xenobiotic compounds (reviewed in Stabb *et al.*, 1994). However, exploitation of genotypic diversity among bacterial biocontrol agents of plant pathogenic fungi, so far, has received much less attention. Knowledge of the diversity within a group of strains that share a common biocontrol trait may provide a new approach to identify biocontrol strains that are superior with respect to ecological competence and ability to suppress specific plant diseases.

Recent studies have shown that there is considerable genotypic diversity in antibiotic-producing *Pseudomonas* species (Keel *et al.*, 1996; McSpadden-Gardener *et al.*, 2000). Different genotypes of 2,4-DAPG-producing *Pseudomonas* spp. have been reported to differ in their ability to suppress Fusarium crown and root rot and Pythium root rot (Sharifi-Tehrani *et al.*, 1998), to produce other antibiotics in addition to 2,4-DAPG (Keel *et al.*, 1996), and to colonize roots of maize plants of different growth stages (Picard *et al.*, 2000). Among 101 isolates of indigenous 2,4-DAPG-producing *Pseudomonas* species, occurring on roots of wheat grown in a soil naturally suppressive to take-all disease of wheat, 16 different groups were identified by Random Amplified Polymorphic DNA (RAPD) analysis with two 10-mer

primers (Raaijmakers and Weller 2001). One RAPD-group made up 50% of the total population of 2,4-DAPG-producing *Pseudomonas* spp. Subsequent root-colonization and biocontrol studies indicated that this dominant genotype, exemplified by *P. fluorescens* Q8r1-96, was highly adapted to the wheat rhizosphere and was very effective in suppression of take-all disease of wheat (Raaijmakers and Weller, 2001). The observation that strain Q8r1-96 showed the same population dynamics during successive cycling of wheat in two other soils, both of which had different physical-chemical properties, suggested that its superior rhizosphere competence is not soil specific. This was supported by results obtained in a study by McSpadden-Gardener *et al.* (2000), who found that nearly one-third of the 2,4-DAPG-isolates obtained from soils of different wheat-growing areas in the United States were genotypically similar to strain Q8r1-96. Biochemical analyses indicated that the superior rhizosphere competence of Q8r1-96 was not related to elevated *in situ* 2,4-DAPG production levels but possibly to its ability to utilize specific substrates (Raaijmakers and Weller, 2001). These data and results obtained by Sharifi-Tehrani *et al.* (1998) illustrate that exploiting the diversity within a specific group of antagonistic microorganisms has potential for improving biological control. This approach capitalizes on existing knowledge concerning mechanisms, while exploiting differences among strains to face the biotic and abiotic complexity of natural environments.

OUTLINE OF THE THESIS

Bacteria of the genus *Pseudomonas* are able to survive and prosper in a wide range of environmental conditions. This genus not only contains plant, animal, and human pathogenic species, but also accomodates species that are of significant environmental importance, including plant growth promoters, xenobiotic degraders, and biocontrol agents. Their versatile metabolic activities and ability to produce a wide variety of secondary metabolites have stimulated numerous ecological, molecular and biochemical studies. In spite of significant progress in the understanding of the biosynthesis and regulation of several antibiotic metabolites (Chapter 1), little is known about the distribution and diversity of indigenous *Pseudomonas* populations harboring these traits. Furthermore, many studies focus on one or a limited number of model strains producing known antifungal compounds, whereas considerably less attention is given to the discovery of new metabolites that may have great potential for biological control of plant diseases. Our major objectives in this thesis are i) to study the distribution, diversity and activity of indigenous *Pseudomonas* populations producing the antbiotics 2,4-diacetylphloroglucinol (2,4-DAPG), phenazines (Phz), pyrrolnitrin (PRN) or pyoluteorin (PLT) (Chapters 2-5), and ii) to isolate, identify and characterize *Pseudomonas* strains and novel antifungal metabolites with biocontrol potential (Chapter 6).

In Chapter 2, the development and specificity of primers and probes for targeted isolation and enumeration of PRN- and PLT-producing *Pseudomonas* and *Burkholderia* species are described. The usefulness of these molecular markers for phylogenetic analysis of *Pseudomonas* and *Burkholderia* species was evaluated and compared to phylogeny inferred from 16S rDNA sequences. Finally, the frequencies of PRN- and PLT-producing *Pseudomonas* and *Burkholderia* species in the rhizosphere of wheat plants grown in soils suppressive to the take-all fungus *Gaeumannomyces graminis* var. *tritici* were assessed by colony hybridization followed by PCR.

Production of 2,4-DAPG, PHZ, PRN and PLT by several *Pseudomonas* strains is regulated by the two-component system GacA/GacS. Recent studies suggested that the response regulator gene *gacA* is widely distributed in *Pseudomonas* and other Gram-negative bacteria. In Chapter 3, the *gacA* gene was sequenced from ten different *Pseudomonas* strains and the evolutionary rates and patterns of nucleotide substitutions were studied. Phylogenies inferred from *gacA* gene and protein sequences were compared to the classic 16S rDNA-based phylogeny. The use of the *gacA* gene as a complementary genetic marker for detection of

Pseudomonas in environmental samples was evaluated on bacterial populations isolated from the rhizosphere of wheat grown in agricultural soils.

Antibiotic-producing *Pseudomonas* spp. have been implicated in the natural suppressiveness of soils to specific soil-borne fungi, including *G. graminis* var *tritici*. Chapter 4 aimed at disclosing the contribution of 2,4-DAPG-producing *Pseudomonas* spp. to the suppressiveness of two Dutch soils to *G. graminis* var. *tritici*. In these soils, 2,4-DAPG-producing *Pseudomonas* spp. were highly enriched on roots of wheat. Mutants defective in 2,4-DAPG production were constructed and their activities were compared to those of their parental strains to establish the determinative role of this metabolite in interactions with *G. graminis* var. *tritici* and in rhizosphere competence. In collaboration with USDA-ARS (Pullman, WA), comparative studies on major genotypic groups of 2,4-DAPG producers found in Dutch and US take-all decline soils were carried out.

Chapter 5 focuses on the activity of 2,4-DAPG against plant pathogenic fungi and in particular against *Pythium* species. This oomycete pathogen has a complex life cycle in which different structures play an important role. We focused on the asexual life cycle and determined the sensitivity of different structures to 2,4-DAPG. In collaboration with INRA (Dijon, France), transmission eclectron microscopy was performed to gain more insight into the mode of action of 2,4-DAPG.

The last experimental chapter of this thesis concentrated on the discovery and characterization of novel strains and metabolites with biocontrol potential. Strains producing surface-active compounds were isolated from the rhizosphere of wheat. The potential of one of these *Pseudomonas* strains to control Pythium root rot was studied in collaboration with the Applied Research Station (PPO) in Lisse, the Netherlands. Mutants defective in the production of the surface-active compound were constructed and partial sequences of genes involved in the biosynthesis were obtained by anchored PCR. The composition of the partially purified biosurfactants produced by this *Pseudomonas* strain was determined in collaboration with the Department of Organic Chemistry of Wageningen University (Chapter 6).

The results obtained in Chapters 2-6 are evaluated in a summarising discussion (Chapter 7).

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CHAPTER 2

POLYMORPHISMS WITHIN THE *prnD* AND *pltC* GENES FROM PYRROLNITRIN AND PYOLUTEORIN-PRODUCING *PSEUDOMONAS* AND *BURKHOLDERIA* SPP.

ABSTRACT

Pyrrolnitrin (PRN) and pyoluteorin (PLT) are broad-spectrum antibiotics produced by several strains of *Pseudomonas* and *Burkholderia* species, and play an important role in the suppression of multiple plant pathogenic fungi. Primers were developed from conserved nucleotide sequences and were able to amplify *prnD* and *pltC* fragments from eighteen *Pseudomonas* and four *Burkholderia* spp. of worldwide origin that either produce PRN or PLT or both. Subsequent RFLP (Restriction Fragment Length Polymorphisms) analysis of the 438-bp *pltC* fragment showed no polymorphisms among PLT-producing *Pseudomonas* strains. Polymorphisms within the 786-bp *prnD* fragment, however, allowed the assessment of the diversity among PRN-producing *Pseudomonas* and *Burkholderia* spp. to a level similar to that obtained by three 10-mer primers in RAPD (Random Amplified Polymorphic DNA) analysis. Phylogenetic analysis of 16S rDNA sequences of a number of strains representative of PRN-producing *Pseudomonas* and *Burkholderia* species correlated well with their taxonomic status. Phylogenetic relationships inferred from each of the four *prn* genes as well as from the complete sequence of the *prn* biosynthetic locus were similar to 16S rDNA based phylogeny for most strains except for *B. pyrrocinia* DSM10685. Both RFLP analysis and comparison of the *prn* gene sequences showed that *B. pyrrocinia* DSM10685 was more closely related to PRN-producing *Pseudomonas* strains, suggesting that lateral gene transfer may have occurred. Colony hybridization and PCR with PRN- and PLT-specific probes and primers showed that *Pseudomonas* and *Burkholderia* spp. harboring the *prnD* and *pltC* gene were not present at detectable levels on roots of wheat grown in five agricultural soils collected in the Netherlands, two of them being naturally suppressive to *Gaeumannomyces gramminis* var. *tritici*. These results suggest that PRN- and PLT-producing *Pseudomonas* and *Burkholderia* sp. do not contribute to the natural suppressiveness found in Dutch take-all decline soils.

Jorge T. de Souza and Jos M. Raaijmakers

FEMS Microbiology Ecology. accepted.

INTRODUCTION

Biological control of soil-borne diseases by plant growth-promoting rhizobacteria (PGPR) is a well-established phenomenon and antibiotics have been shown to play a major role in suppression of several plant pathogens (Handelsman and Stabb, 1996). Numerous bacterial strains that produce antibiotics *in vitro* have been isolated from different soils and host plants (O'Sullivan and O'Gara, 1992; Thomashow and Weller, 1996). This apparent wide distribution suggests that antibiotic-producing bacteria are common constituents of the indigenous microflora in soil and plant-associated environments worldwide. To date, however, little is known about the frequency and ecology of indigenous antibiotic-producing bacteria. Knowledge about the ecology of naturally occurring strains that harbor specific biocontrol traits will significantly contribute to improve the efficacy of existing biocontrol agents. Moreover, knowledge about the distribution of antibiotic genes in natural environments could lessen concerns about the environmental release of either non-indigenous strains containing these traits or transgenic biocontrol agents in which these traits have been introduced.

Selection and identification of antibiotic-producing bacteria via random isolation from natural environments is very time-consuming and laborious. Most of the model strains used to date were identified via screening huge numbers of isolates obtained by random procedures. The availability of cloned and sequenced antibiotic biosynthetic and regulatory genes, however, has facilitated the development of specific primers and probes that can be used for targeted detection and isolation of specific antibiotic-producing bacteria. For several antibiotics produced by strains of different bacterial genera, biosynthetic genes have been cloned and partially or fully sequenced. These include zwittermycin A produced by *B. cereus* (Stohl *et al.*, 1999), AFC-BC11 produced by *B. cepacia* (Kang *et al.*, 1998) and the antibiotics 2,4-diacetylphloroglucinol (2,4-DAPG), phenazines (PHZ), pyrrolnitrin (PRN), and pyoluteorin (PLT) produced by various *Pseudomonas* species (Nowak-Thompson *et al.*, 1999; Hammer *et al.*, 1997; Mavrodi *et al.*, 1998; Pierson *et al.*, 1995; Bangera and Thomashow, 1999; Chin-A-Woeng, 2000). A prerequisite for DNA-based detection and isolation is that the genes of interest must be conserved among a wide range of bacterial strains harboring this trait. By Southern hybridization (Keel *et al.*, 1996) and PCR analysis (Raaijmakers *et al.*, 1997; Ramette *et al.*, 2001; Mavrodi *et al.*, 2001), it was shown that the biosynthetic locus for 2,4-DAPG production, and in particular the *phlD* gene, was highly conserved among 2,4-DAPG-producing *Pseudomonas* strains of worldwide origin. Also some of the genes involved in the biosynthesis of PHZ appear to be conserved among different strains (Pierson *et al.*, 1995; Raaijmakers *et al.*, 1997; Mavrodi *et al.*, 2001).

Due to their broad-spectrum activity, PRN and PLT are among the antibiotics that have received considerable attention (Bender *et al.*, 1999; Hammer *et al.*, 1999; Ligon *et al.*, 2000; Thomashow and Weller, 1996; Whipps, 1997). PRN is a chlorinated phenylpyrrole antibiotic that was first isolated from *Burkholderia pyrrocinia* (Arima *et al.*, 1964) and later from other microorganisms, including *Pseudomonas fluorescens*, *P. chlororaphis*, *P. aureofaciens*, *B. cepacia*, *Enterobacter agglomerans*, *Myxococcus fulvus*, and *Serratia* species (Elander *et al.*, 1968; Hammer *et al.*, 1999; Kalbe *et al.*, 1996). PRN has activity against several bacteria and soil-borne fungi, in particular *Rhizoctonia solani* (Arima *et al.*, 1964; Cartwright *et al.*, 1995; Chernin *et al.*, 1996; El-Banna and Winkelmann, 1988; Hill *et al.*, 1994; Jayaswal *et al.*, 1993; Rosales *et al.*, 1995). PRN is also effective against post-harvest diseases caused by *Botrytis cinerea* on apple, pear and on cut flowers (Hammer and Evensen, 1993; Janisiewicz and Roitman, 1988), and has been used to treat humans infected by opportunistic fungi (Tawara *et al.*, 1989). PLT is a phenolic polyketide that was first isolated from *P. aeruginosa* (Takeda, 1958) and later from *P. aeruginosa* strain S10B2 (Ohmori *et al.*, 1978) and *P. fluorescens* strains Pf-5 and CHA0 (Bencini *et al.*, 1983; Bender *et al.*, 1999; Keel *et al.*, 1996). PLT has bactericidal, herbicidal, fungicidal, and oomycidal activities, in particular against *Pythium* spp. (Maurhoffer *et al.*, 1992; Ohmori *et al.*, 1978; Takeda, 1958). Application of pure PLT to cotton seeds resulted in significant suppression of *Pythium ultimum*-induced damping-off (Howell and Stipanovic, 1980).

Although the genes involved in the biosynthesis of PRN and PLT have been cloned and sequenced (Hammer *et al.*, 1999; Nowak-Thompson *et al.*, 1997, 1999), little information is available on the level of conservation of these genes in multiple PRN and PLT-producing *Pseudomonas* and *Burkholderia* species and strains. In the study by Hammer *et al.* (1999), sequencing of the PRN biosynthetic locus included only 4 strains, one *P. fluorescens*, two *Burkholderia* strains and one strain of *Myxococcus fulvus*. For PLT, only data for *P. fluorescens* strain Pf-5 are available in the database. In this study, we developed primers and probes from specific sequences within *prnD* and *pltC*, two genes encoding essential enzymes for the biosynthesis of PRN and PLT, respectively (Hammer *et al.*, 1999; Nowak-Thompson *et al.*, 1997, 1999). The specificity of these primers was tested with eighteen *Pseudomonas* and four *Burkholderia* strains of worldwide origin.

Table 1. Characteristics of *Pseudomonas* and *Burkholderia* strains used to test the specificity of PRN and PLT primers and diversity studies.

Strain [a]	Other designation (s) [a]	Origin/host plant	PRN production [b]	PLT production [b]	Source and/or reference
B. cepacia					
J2535	LMG 16671	rotting bark, Edinburgh, Scotland	+	–	Vandamme *et al.*, 1997
LMG1222	Palleroni 717; NCTC 10743	*Allium cepa*	+	–	Vandamme *et al.*, 1997
ATCC 51671	B37w; NRRL B-14858	*Sesbania exaltata* leaves	+	–	Burkhead *et al.*, 1994
J2540	LMG 16672	rhizosphere of a banana plant	–	–	Vandamme *et al.*, 1997
B. pyrrocinia					
DSM10685	ATCC 15958; LMG 14191	soil	+	–	Vandamme *et al.*, 1997; DSMZ
P. chlororaphis					
LMD 82.53	ATCC 43051; Strain ACN	mutant of ATCC 15926	+	–	Salcher and Lingens, 1980
P. fluorescens					
Pf-5		cotton, Texas, USA	+	+	Howell and Stipanovic, 1979
CHA0		tobacco, Morens, Switzerland	+	+	Keel *et al.*, 1992
Pf1		tobacco, Morens, Switzerland	+	+	Keel *et al.*, 1996
F113		sugar beet, Cork, Ireland	–	–	Shanahan *et al.*, 1992
Q8r1-96		wheat, Washington, USA	–	–	Raaijmakers and Weller, 2001
PF		wheat leaves, Oaklahoma, USA	+	+	Levy *et al.*, 1992
PFM2		derivative of PF	+	+	Levy *et al.*, 1992
PGNL1		tobacco, Ghana	+	+	Keel *et al.*, 1996
PGNR1		tobacco, Ghana	+	+	Keel *et al.*, 1996
PGNR2		tobacco, Ghana	+	+	Keel *et al.*, 1996
PGNR3		tobacco, Ghana	+	+	Keel *et al.*, 1996
PGNR4		tobacco, Ghana	+	+	Keel *et al.*, 1996
PILH1		tomato, Italy	–	–	Keel *et al.*, 1996
PINR2		tobacco, Italy	+	+	Keel *et al.*, 1996
PINR3		tobacco, Italy	+	+	Keel *et al.*, 1996

Table 1. Continued.

Pseudomonas sp.				
PHZ13	tomato, Dijon, France	+	–	This study
PHZ19	tomato, Dijon, France	+	–	This study
PHZ22	tomato, Dijon, France	+	–	This study
PHZ24	tomato, Dijon, France	+	–	This study
PHZ48	tomato, Dijon, France	+	–	This study

[a] ATCC, American Type Culture Collection, University Blvd, Manassas, Virginia, USA; DSMZ, Deutsche Sammlung von Mikroorganismen und Zelculturen GmbH, Braunschweig, Germany; LMD, Laboratorium voor Microbiologie, Technische Universiteit, Delft, The Netherlands; LMG, Laboratorium Microbiologie Gent Culture Collection, Universiteit Gent, Ghent, Belgium; NCPPB, National Collection of Plant Pathogenic Bacteria, Harpenden Laboratory, Hertfordshire, United Kingdom; NCTC, National Collection of Type Cultures, London, United Kingdom; NRRL, Northern Regional Research Laboratories, Peoria, Illinois, USA.

[b] Determined in this study by HPLC and/or TLC analysis.

Restriction fragment length polymorphisms (RFLP) among amplified PRN and PLT-fragments were evaluated to determine the diversity among different bacterial strains, species and genera. Random amplified polymorphic DNA (RAPD) analysis, 16S rDNA sequencing and *in silico* analysis of gene sequences involved in PRN biosynthesis were performed to study the diversity and phylogenetic relationships among PRN- producing bacteria. Finally, we determined the frequency of PRN- and PLT-producing *Pseudomonas* and *Burkholderia* spp. on roots of wheat grown in five agricultural soils collected in the Netherlands, including two soils that are naturally suppressive to *Gaeumannomyces graminis* var. *tritici* (Ggt). Determination of population densities of *Pseudomonas* spp. harboring genes for 2,4-DAPG or PHZ antibiotics was included for comparison purposes.

RESULTS

Antibiotic production. For many of the strains included in this study (Table 1), the ability to produce PRN was determined or confirmed by TLC or HPLC analysis. PRN was detected by TLC analysis in extracts of culture-filtrates of *B. cepacia* strains J2535, LMG 1222, and ATCC 51671; *B. pyrrocinia* DSM10685; *P. chlororaphis* LMD 82.53; *P. fluorescens* strains Pf-5, CHA0, Pf1, PF, PFM2, PGNL1, PGNR1, PGNR2, PGNR3, PGNR4, PINR2, PINR3, and *Pseudomonas* sp. strains PHZ13, PHZ19, PHZ22, PHZ24, PHZ48. In addition to PRN, strains PHZ13, PHZ19, PHZ22, PHZ24, and PHZ48 also produced phenazine antibiotics as was determined by HPLC analysis followed by spectral analysis of the peaks by photodiode array spectroscopy (data not shown).

PCR-amplification of *prnD* and *pltC* gene fragments. Primers PRND1 and PRND2 are directed against conserved sequences within the *prnD* genes of *B. pyrrocinia* DSM10685, *P. fluorescens* strain BL915, and *B. cepacia* strain LT4-12-W. PRND1 and PRND2 amplified the predicted 786-bp fragment from DNA of homologous strain *B. pyrrocinia* DSM10685 (Fig. 1A). The 786-bp fragment was digested with 7 different restriction enzymes and the sizes of the restriction fragments matched with the predicted sizes inferred from the *prnD* sequence of *B. pyrrocinia* DSM10685 available in the database. A fragment of the same size was amplified from DNA of all other known PRN-producing *Pseudomonas* and *Burkholderia* strains, whereas no 786-bp fragment was amplified from several non-PRN-producing strains, including J2540 and PILH1 (Fig. 1A). The PCR products amplified for PRN-producing *Pseudomonas* and

Table 2. Characteristics of the primers used for PCR analysis.

Primer	Sequence	Length (bp)	G+C (%)	T_m (°C)	Posit. [a]	Product Length (bp)	Reference
PRND1	GGGGCGGGCCGTGGTGATGGA	21	76.2	82.4	5269		
PRND2	YCCCGCSGCCTGYCTGGTCTG	21	66.6	74.2	6055	786	This study
PLTC1	AACAGATCGCCCCGGTACAGAACG	24	58.3	74.2	12282		
PLTC2	AGGCCCGGACACTCAAGAAACTCG	24	58.3	73.8	12720	438	This study
Phl2a	GAGGACGTCGAAGACCACCA	20	60	73	1915		
Phl2b	ACCGCAGCATCGTGTATGAG	20	55	72	2660	745	Raaijmakers *et al.*, 1997
PHZ1	GGCGACATGGTCAACGG	17	64.7	52.4	3428		
PHZ2	CGGCTGGCGGCGTATTC	17	70.6	57.3	4836	1408	Mavrodi *et al.*, unpubl.

[a] Position of the 5' end of the primer in the database sequence.

Burkholderia spp. all hybridized under high-stringent conditions with the PRN probe consisting of the DIG-labeled 786-bp fragment from the homologous strain *B. pyrrocinia* DSM10685 (Fig. 1B).

Primers PLTC1 and PLTC2 amplified the predicted 438-bp fragment from DNA of homologous *P. fluorescens* strain Pf-5 (Fig. 2A). The 438-bp PLT fragment was digested with 8 different restriction enzymes and the sizes of the restriction fragments matched the predicted sizes inferred from the *pltC* sequence from *P. fluorescens* Pf-5 available in the database. A fragment of the same size was amplified from all known PLT-producing strains, whereas no PCR product of that size was obtained from DNA of non-PLT-producing strains Q8r1-96, J2535, J2540, and F113 (Fig. 2A). The PCR products of all PLT producers hybridized under high-stringent conditions with the PLT probe consisting of the labeled 438-bp fragment amplified from strain Pf-5 (Fig. 2B).

Diversity of PRN-producing *Pseudomonas* and *Burkholderia* spp. Restriction patterns of the PRN-fragments (Fig. 3A), obtained by digestion with five endonucleases (*Hae*II, *Hinc*II, *Hinf*I, *Mnl*I, and *Pvu*II), yielded considerable polymorphisms (RFLPs) among the different strains. Digestion with *Acc*I and *BsmB*I yielded no additional polymorphisms. Cluster analysis of the restriction fragment patterns resulted in seven distinct groups. The largest group contained *Pseudomonas* strains that all produce both PRN and PLT (Fig. 3A, Table 1). There was considerable diversity among *B. cepacia* strains that only produce PRN (LMG 1222, J2535, ATCC 51671). The *Pseudomonas* strains that produce both PRN and phenazines (PHZ13, PHZ19, PHZ22, PHZ24, PHZ48, LMD 82.53) were clustered in three groups

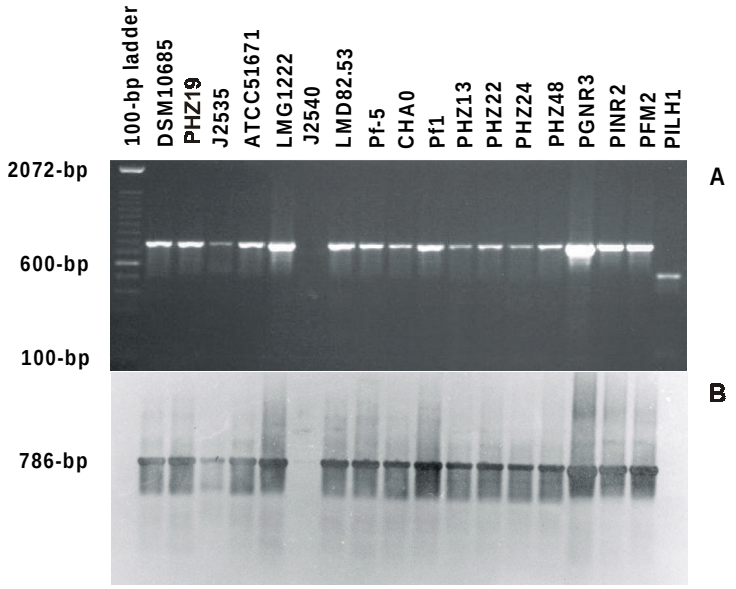

Figure 1. Agarose gel electrophoresis of the PCR products amplified from DNA of *Pseudomonas* and *Burkholderia* strains with primers PRND1 and PRND2 (A). Characteristics of the bacterial strains are shown in Table 1. Southern blot analysis of the PCR fragments shown in (A) with the PRN probe (786-bp fragment from *B. pyrrocinia* DSM10685) under high-stringent conditions (B).

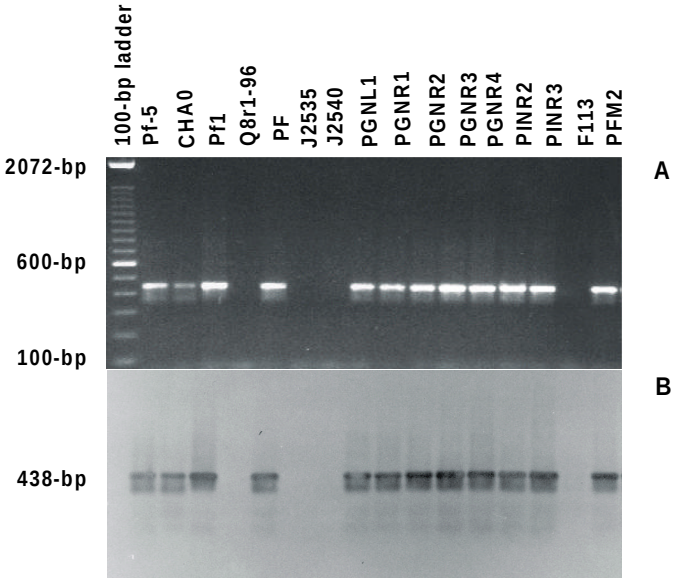

Figure 2. Agarose gel electrophoresis of the PCR products amplified from DNA of *Pseudomonas* and *Burkholderia* strains with primers PLTC1 and PLTC2 (A). Characteristics of the bacterial strains are shown in Table 1. Southern blot analysis of the PCR fragments shown in (A) with the PLT probe (438-bp fragment from *P. fluorescens* Pf-5) under high-stringent conditions (B).

with a relatively high level of similarity. Interestingly, *B. pyrrocinia* DSM10685 clustered more closely to *Pseudomonas* than to the other *Burkholderia* strains (Fig. 3A). RAPD analysis with primers M13, M12, and D7 gave rise to 72 polymorphic markers, which ranged in size from 190 to 2072-bp. *P. aeruginosa* strain PAO1 was included as an outgroup (Fig. 3B). The reproducibility of the amplification patterns was confirmed in three independent experiments. Although RAPD analysis allowed a higher level of discrimination among PRN-producing *Burkholderia* and *Pseudomonas* species than RFLP analysis of the PRN-fragment, the overall clustering of the isolates by RAPD analysis was very similar to that obtained by RFLP analysis (Fig. 3).

Relationship between phylogeny of *prn* genes and 16S rDNA classification. RFLP analysis showed that the *prnD* gene of *B. pyrrocinia* DSM10685 is more closely related to the *prnD* genes of *Pseudomonas* than to those of the other *Burkholderia* strains (Fig. 3A). To further investigate the phylogenetic relationships between PRN-producers of both genera, representative PRN-producing *Pseudomonas* (*P. fluorescens* Pf-5, *P. chlororaphis* LMD 8253, *Pseudomonas* sp. PHZ13 and PHZ22) and *Burkholderia* (*B. cepacia* LMG 1222, and J2535) strains were selected for 16S rDNA sequencing. The 16S rDNA sequences from *P. fluorescens* CHA0 and *P. aeruginosa* PAO1, were also included in the analysis. The phylogenetic trees inferred from these sequences with the neighbour-joining method yielded a similar topology as obtained by the UPGMA method; only the tree inferred with the latter method is presented (Fig. 4). 16S rDNA sequence analysis yielded two main clusters, one that comprised all six *Pseudomonas* strains and the second one containing all three *Burkholderia* strains, including *B. pyrrocinia* DSM10685. These results are in agreement with their taxonomic status. For most strains, phylogenetic *prn* biosynthetic locus were generally similar to 16S rDNA based phylogeny except for *B. pyrrocinia* DSM10685. *In silico* analysis of the *prnA-D* sequences of at least relationships inferred from each of the four *prn* genes as well as from the complete sequence of the four PRN-producing strains showed that each of the four *prn* genes as well as the complete *prn* biosynthetic locus of *B. pyrrocinia* DSM10685 is more closely related to *Pseudomonas* than to *B. cepacia* (Fig. 5). These results clearly point to a discrepancy between the phylogenies inferred from 16S rDNA sequences and *prn* biosynthetic genes.

Diversity among PLT-producing *Pseudomonas* and *Burkholderia cepacia*. Restriction of the PLT fragment with 8 different endonucleases (*Alw*I, *Ava*I, *Mnl*I, *Nae*I, *Pst*I, *Sac*II, *Taq*I, and *Xho*I) yielded no polymorphisms among twelve *Pseudomonas* strains (data not shown).

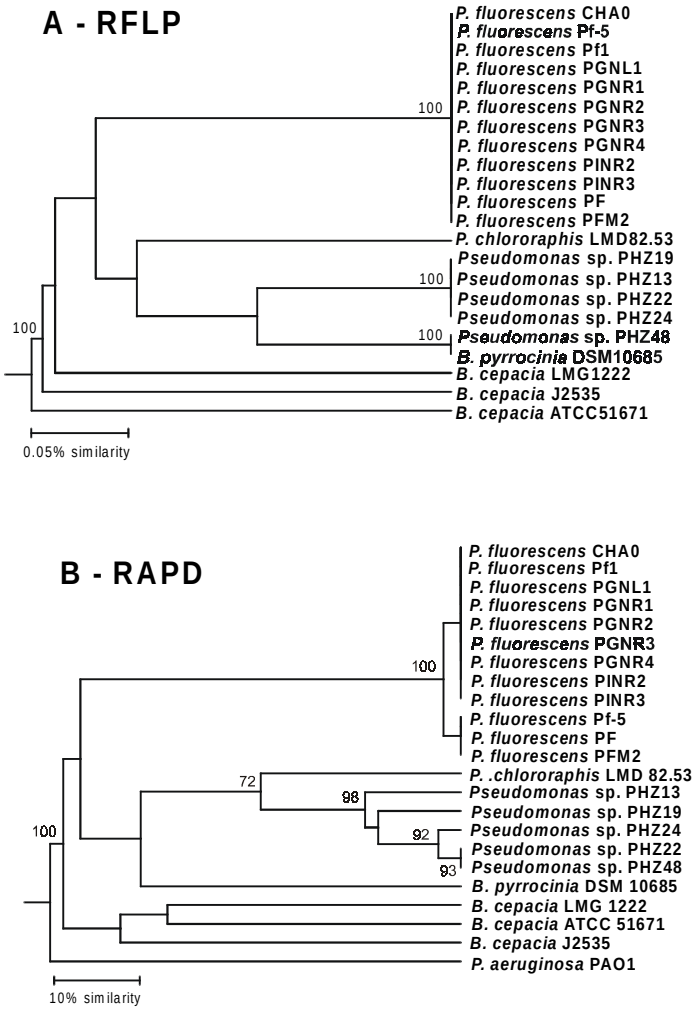

Figure 3. Dendrograms of different PRN-producing *Pseudomonas* and *Burkholderia* spp., based on restriction analysis of the 786-bp PRN fragment (A). Restriction analysis was performed with seven different restriction endonucleases. Dendrogram of the bacterial strains shown in (A), based on RAPD analysis with three different primers (B). Bootstrap values greater than 70% are shown at the branching points. Characteristics of the bacterial strains are shown in Table 1. Dendrograms constructed by using the UPGMA method are presented.

Frequency of *prnD* and *pltC*-harbouring *Pseudomonas* spp. in disease-suppressive soils. Several studies have implicated antibiotic-producing bacteria in the natural suppressiveness of certain soils to the take-all fungus *G. graminis* var. *tritici* (Charigkapakorn and Sivasithamparam, 1987; Raaijmakers *et al.*, 1997; Raaijmakers and Weller, 1998). To begin to address a possible role of PRN- and/or PLT-producing *Pseudomonas* spp. and *B. cepacia* in Dutch take-all suppressive soils (TAD soils), the initial step was to determine their densities in the rhizosphere of wheat grown in these soils. One of the five soils, designated CB, is

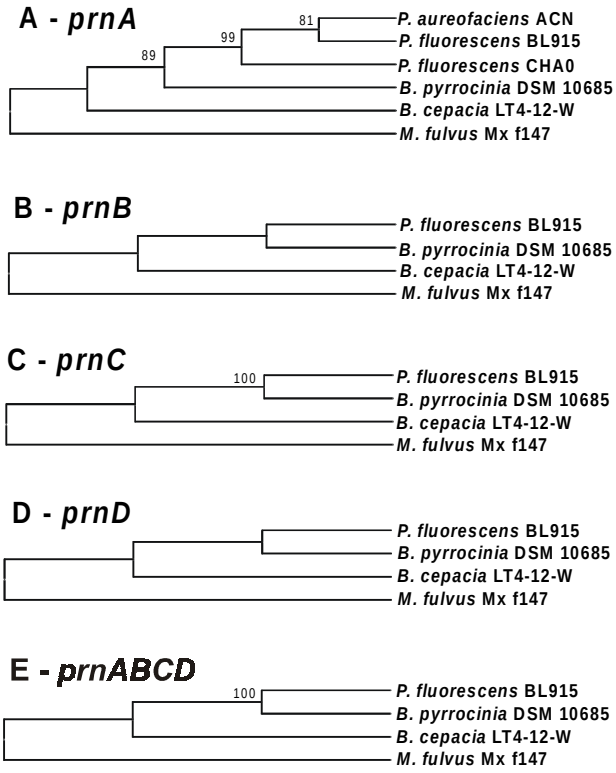

Figure 4. Most parsimonious phylogenetic trees generated with sequences of *prnA* (A), *prnB* (B), *prnC* (C), *prnD* (D), and the whole *prn* cluser (E). Position of the aligned nucleotides in relation to sequences of *P. fluorescens* BL915 were as follows: *prnA* (positions 615 to 2231), *prnB* (positions 2231 to 3316), *prnC* (3368 to 5068), *prnD* (5093 to 6205), *prn* (only coding regions were combined). Bootstrap values higher than 70% are shown in the appropriate branching points.

conducive to *G. graminis* var. *tritici*, and at least two of the other soils, designated SV and SSB, are suppressive to *G. graminis* var. *tritici* (Table 3). Colony hybridization with the *prnD* and *pltC* probes showed that *Pseudomonas* spp. harbouring these genes were not present at detectable levels on roots of wheat grown in any of the five soils. Some putative PRN-producing *Pseudomonas* spp. were initially isolated based on colony hybridization. However, these isolates gave a PCR product slightly bigger than the 786-bp PRN fragment, which did not hybridize at high- and low-stringent conditions with the PRN probe (data not shown). Furthermore, PRN production by these isolates was not detected in TLC and HPLC analysis (data not shown). In all soils, roots of wheat supported *B. cepacia* populations at densities ranging from 9.5×10^4 to 2.5×10^6 CFU per gram of root plus adhering soil. In PCR analysis of randomly selected *B. cepacia* isolates, no isolates that harbored *prnD* and *pltC* genes were detected in any of the five soils tested. For comparison purposes, we also determined the

frequencies of two other groups of antibiotic-producing *Pseudomonas* spp. *Pseudomonas* spp. harboring *phlD* (*phlD*+), a key gene involved in the biosynthesis of 2,4-DAPG, were found in all five soils tested (Table 4). The densities of *phlD*+ *Pseudomonas* spp. ranged from 5.0 X 10^4 to 3.6 X 10^6 CFU per gram of root plus adhering soil, representing on average 0.2 to 19% of the total population of culturable *Pseudomonas* spp. isolated from wheat roots. *Pseudomonas* spp. harboring *phzC-D*, two genes involved in the biosynthesis of phenazine antibiotics, were found only in SL soil at densities of 9.0 X 10^4 CFU g^{-1} root; these densities represent 0.5% of the total population of culturable *Pseudomonas* spp. Isolation of naturally occurring antibiotic-producing *Pseudomonas* spp. from roots in the seventh growth cycle of wheat growth cycle yielded similar results.

DISCUSSION

Pyrrolnitrin (PRN) and pyoluteorin (PLT) play a key role in the suppression of plant pathogens by several strains of *Pseudomonas* and *Burkholderia*. In this study, primers were developed from conserved sequences within the *prnD* and *pltC* genes, two genes that are essential in the biosynthesis of PRN and PLT, respectively. Both primer sets amplified the predicted fragments from DNA of a worldwide collection of PRN- and PLT-producing *Pseudomonas* and *Burkholderia* strains. The availability of these specific primers and probes allow to better understand the distribution and ecology of indigenous bacteria that produce these antibiotics as well as the behavior of introduced transgenic microorganisms in which these traits have been incorporated. For example, primers and probes directed against specific genes involved in the biosynthesis of 2,4-DAPG have shown that *Pseudomonas* spp. harboring this trait are present in different soils and plant-associated habitats (Keel *et al.*, 1996). The availability of primers and probes directed against antagonistic traits also enable a quick screening of large collections of bacterial strains that have given promising results in controlling plant diseases, thereby allowing site-directed mutagenesis to further unravel the relative contribution of those traits in disease suppression.

In addition to the ability of *Pseudomonas* and *Burkholderia* species to produce antibiotic compounds like PRN and PLT, their genotypic diversity has drawn considerable attention. Genotypic diversity not only is an interesting phenomenon with respect to host specificity and adaptation of microorganisms to changing abiotic conditions, but can also be

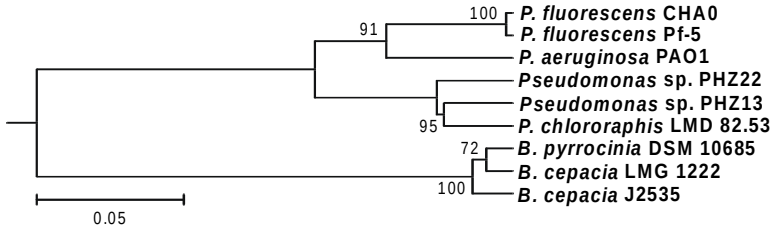

Figure 5. Dendrogram of selected strains based on 283-bp aligned sequences of the 16S rDNA (positions 28-310 of nucleotide sequence of *P. aeruginosa* strain PAO1). Scale indicates the number of nucleotide substitutions per site. The dendrogram was constructed by using the UPGMA method.

exploited to improve the efficacy of biological control (Sharifi-Tehrani *et al.*, 1998; Raaijmakers and Weller, 2001). RFLP analysis of the 438-bp *pltC* gene fragment showed no polymorphisms among PLT-producing *Pseudomonas* spp. Although this may be due to the fact that the size of the amplified fragment is relatively small to detect differences, similar-sized fragments have allowed the assessment of diversity among *Pseudomonas* strains to a level similar to that obtained with other markers (de Vos *et al.*, 1998; Dahllöf *et al.*, 2000). Another explanation for the lack of polymorphisms within the *pltC* fragment may be the fact that to date PLT production seems to be limited to a select group of *Pseudomonas* strains, most of which are genotypically very similar or even identical (Keel *et al.*, 1996; Sharifi-Tehrani *et al.*, 1998; McSpadden-Gardener *et al.*, 2000; Fig. 3).

In contrast to the *pltC* fragment, polymorphisms within the 786-bp *prnD* fragment allowed the assessment of diversity to a level similar to that obtained by RAPD analysis with three 10-mer primers. Although RAPD analysis provided a higher level of discrimination between strains than RFLP analysis of the *prnD* gene, a similar overall clustering of the antibiotic-producing strains was obtained (Fig. 3). Both RFLP and RAPD analyses showed that strains producing both PRN and PLT are grouped closely together (Fig. 3; Table 1). These results confirm and extend data obtained by Keel *et al.* (1996). Furthermore, the *Pseudomonas* strains that produce both PRN and phenazines were clustered in three groups with a relatively high level of similarity. Interestingly, *Pseudomonas* sp. PHZ48 and *B. pyrrocinia* DSM10685 gave identical RFLP patterns and the position of this *Burkholderia* strain was more close to *Pseudomonas* species than to the other *Burkholderia* strains included in this study (Fig. 3A). Subsequent phylogenetic analysis of 16S rDNA sequences of a number of strains representative of PRN-producing *Pseudomonas* and *Burkholderia* species correlated well with their taxonomic status. Also phylogenetic relationships inferred from each of the four *prn*

Table 3. Frequency of antibiotic-producing *Pseudomonas* spp. in the wheat rhizosphere.

Soil	Suppressive [a] to take-all	Densities (CFU g^{-1} root) [b]				
		Total pop.[c]	2,4-DAPG[d]	PHZ [e]	PRN [f]	PLT [g]
CB	-	2.3 X 10^7	5.0 X 10^4	n.d.	n.d.	n.d.
SL	n.t.	1.9 X 10^7	2.3 X 10^5	9.8 X 10^4	n.d.	n.d.
SU	n.t.	1.8 X 10^7	3.3 X 10^5	n.d.	n.d.	n.d.
SV	+	1.3 X 10^7	3.5 X 10^5	n.d.	n.d.	n.d.
SSB	+	1.9 X 10^7	3.6 X 10^6	n.d.	n.d.	n.d.

[a] - conducive, + suppressive.
[b] Mean values of four replicates are presented.
[c] Total population of *Pseudomonas* spp.
[d] Population of *Pseudomonas* spp. harboring the *phlD* gene.
[e] Population of *Pseudomonas* spp. harboring *phzCD* genes.
[f] Population of *Pseudomonas* spp. harboring the *prnD* gene.
[g] Population of *Pseudomonas* spp. harboring the *pltC* gene.
n.d.: not detected (detection limit was 10^4 CFU g^{-1} root).
n.t. : not tested.

genes as well as from the complete sequence of the *prn* biosynthetic locus were similar to 16S rDNA-based phylogeny for most strains, except for *B. pyrrocinia* DSM10685. *In silico* analysis of each of the four individual genes contained in the *prn* biosynthetic locus confirmed that in all cases *B. pyrrocinia* DSM10685 clustered closer to *Pseudomonas* species than to *B. cepacia* (Fig. 4). Comparable results have been obtained in *nodC* and *nifH* gene analysis of *Rhizobium* and *Sinorhizobium* (Laguerre *et al.*, 2001). Genetic rearrangements and lateral gene transfer were suggested as possible explanations for the discrepancy between phylogenies inferred from 16S rDNA sequences and *nodC* and *nifH* phylogenies (Laguerre *et al.*, 2001). The extent of occurrence of horizontal gene transfer among prokaryotes is now becoming more evident. Comparative studies of entirely sequenced genomes are showing that gene transfer plays a greater role in the diversification of microorganisms than thought before (Jain *et al.*, 1999). It is also known that operational genes, such as those involved in antibiotic production are more likely to be transferred than genes involved in transcription, translation, replication, and related processes (Rivera *et al.*, 1998). Although our results may suggest that horizontal transfer of the *prn* operon occurred between *Pseudomonas* and *B. pyrrocinia,* more *prn* biosynthetic loci of multiple *Burkholderia* and *Pseudomonas* species need to be sequenced to support this hypothesis. The genus *Pseudomonas* belongs to the γ-subclass of the Proteobacteria, whereas *Burkholderia* belongs to β-subclass, based on sequence analyses of the 16S rDNA gene, which is considered as a standard in phylogeny and taxonomy of living organisms (Maidak *et al.*, 1999; Anzai *et al.*, 2000). The discrepancies between *prnD* and 16S rDNA phylogenies in *B. pyrrocinia* DSM10685 ilustrate the limitations of the former in

phylogenetic studies. Conversely, Ramette *et al.* (2001) found a clear correspondence between polymorphisms within the *phlD* gene, one of the genes responsible for 2,4-DAPG biosynthesis in *Pseudomonas,* and phylogenetic analysis based on 16S rDNA sequences.

Molecular probes and primers have been used extensively in environmental microbiology to detect pathogenic bacteria and fungi, but have received very little attention in biocontrol research. Specific primers and probes not only allow targeted isolation of strains with specific biocontrol traits, but also can be very useful tools to study their role in natural disease-suppressive soils. Primers and probes developed for detection and isolation of *Pseudomonas* spp. that produce 2,4-DAPG or PHZ antibiotics (Raaijmakers *et al.*, 1997), were successfully used to study their diversity and population dynamics in wheat and maize rhizosphere (McSpadden-Gardener *et al.*, 2000; Picard *et al.*, 2000), and to elucidate their role in the natural suppression that operates in soils that are suppressive to take-all disease of wheat (Raaijmakers and Weller, 1998). Recently, a PRN-producing *P. fluorescens* strain was isolated from a *G. graminis* var. *tritici* suppressive soil in Japan and was shown to be effective in controlling take-all of wheat (Tazawa *et al.*, 2000). In this study, colony hybridization followed by PCR showed that *prnD*- and *pltC*-harboring *Pseudomonas* and *Burkholderia* spp. were not present at detectable levels in the rhizosphere of wheat grown in five soils obtained from two areas in the Netherlands. Two of these soils were shown to be suppressive to *G. graminis* var. *tritici* (Table 4). The fact that *prnD*- and *pltC*-harbouring *Pseudomonas* and *Burkholderia* spp. were not present at detectable levels in the wheat rhizosphere (Table 4) may suggest that they do not play a role in the natural suppresiveness of at least two Dutch take-all decline soils. However, this suggestion is based on the assumption that these bacteria need to reach threshold densities of at least 10^5 CFU g^{-1} root as reported earlier for 2,4-DAPG-mediated suppression of the take-all fungus (Raaijmakers and Weller, 1998) and suppression of Fusarium wilt of radish via siderophores or induced systemic resistance (Raaijmakers *et al.*, 1995). Whether similar threshold levels apply to PRN and PLT producers remains to be determined. *Pseudomonas* spp. harboring *phlD*, one of the genes involved in biosynthesis of the antibiotic 2,4-DAPG, were detected at densities similar to those reported by other authors for the wheat and maize rhizosphere (Raaijmakers *et al.*, 1997; Picard *et al.*, 2000). The occurrence of relatively higher populations of 2,4-DAPG-producing *Pseudomonas* spp. in the two take-all decline soils SV and SSB than in the take-all conducive soil CB (Table 4), point to a more prominent role of 2,4-DAPG-producing *Pseudomonas* spp. in Dutch take-all decline soils, a question that is currently being addressed.

Di Cello *et al.* (1997) demonstrated that *B. cepacia* occurs naturally on maize roots at densities of approximately 3.2 X 10^6 CFU g^{-1} root. In this study, similar densities were found for *B. cepacia* in the wheat rhizosphere. *Burkholderia* spp. have received considerable attention due to their nutritional versatility for bioremediation and their antibiotic properties for biocontrol (Homma and Suzui, 1989; Hebbar *et al.*, 1998; Vandamme *et al.*, 1997). However, because these bacteria are also implicated in infections of cystic fibrosis patients, their application in biological control has become very controversial and subject of many debates. In fact, with exception of *B. pyrrocinia*, all genomovars of *Burkholderia* (seven genomovars described) are able to infect or colonize cystic fibrosis patients (Parke and Gurian-Sherman, 2001). A reliable and conclusive technique which allows the differentiation between environmental *Burkholderia* spp. from those associated with cystic fibrosis is not yet available, although some differences have been reported (Bevivino *et al.*, 1994; Tabbachioni *et al.*, 1995). Therefore, their use in biological control should be avoided, at least until clear delimitations between human pathogenic and environmental *Burkholderia* strains are conclusively established.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Characteristics of the reference strains used in this study are given in Table 1. Naturally occurring *Pseudomonas* spp. were isolated from roots of wheat grown in raw soils on King's medium B [KMB] agar supplemented with chloramphenicol (13 µg ml^{-1}), ampicillin (40 µg ml^{-1}), and cycloheximide (100 µg ml^{-1}) [KMB^+] (Simon and Ridge, 1974). *Burkholderia cepacia* strains were isolated on trypan blue-tetracycline medium (TB-T) (Hagedorn *et al.*, 1987) amended with cycloheximide (100 µg ml^{-1}) to inhibit fungal growth. The combination of ingredients present in TB-T medium provides a high degree of selectivity to *B. cepacia* biotypes (Wise *et al.*, 1995; Tabacchioni *et al.*, 2000). All strains were stored at -80°C in Luria Bertani (LB) or KMB-broth supplemented with glycerol to a final concentration of 40% (v/v). For detection of PLT by high-performance liquid chromatography (HPLC) or by thin layer chromatography (TLC), bacterial strains were grown in 25 ml of KMB-broth in 50-ml screw cap vials for 14 days without shaking at 25°C. For detection of PRN by TLC, bacterial strains were grown in 25 ml of a minimal medium containing per liter: 30 g glycerol, 3 g K_2HPO_4, 0.5 g KH_2PO_4, 5 g NaCl, 0.5 g $MgSO_4.7H_2O$, 0.61 g D-tryptophan. Autoclaved medium was amended with filter-sterilized (0.2 µm) $ZnSO_4$ (0.35 mM) and $Mo_7(NH_4)_6O_{24}.4H_2O$ (0.5 mM) (Duffy and Défago, 1999; Salcher and Lingens, 1980). Bacterial isolates were shaken (180 rpm) for 24 h at 25°C and subsequently incubated at 25°C in the dark for four additional days without shaking. The oxygen-limiting conditions created by the absence of stirring are necessary to elicit production of phenolic compounds, including pyoluteorin (Bencini *et al.*, 1983). For DNA extractions to be used for 16S rDNA sequencing, bacteria were cultivated overnight at 24°C on nutrient broth yeast extract agar (Vidaver, 1967).

TLC and HPLC analysis. To detect PRN by TLC, 15 ml of bacterial culture was centrifuged (Sorvall RC-5, Du Pont, Wilmington, Delaware, USA) for 15 min at 8700 X g, supernatant was discarded and the cells were extracted twice with 5 ml of ethylacetate by sonicating (Bransonic 12, Branson Ultrasonics Corp., Geneva, Switzerland) the mixture for 3 min. For PLT, 15 ml of bacterial culture broth was extracted twice with 5 ml of ethylacetate. The organic phase was evaporated to dryness and the residue was resuspended in 150 µl ethylacetate. A volume of 50 µl was applied to precoated 0.25 mm silica gel plate (20 X 20 cm,

aluminium oxide 60 $F_{254,}$ Merck, Darmstadt, Germany). Separation was performed with chloroform-acetone (9:1 v/v) as the solvent system. The corresponding PRN spots were detected by spraying with 2% p-dimethylaminobenzaldehyde (Ehrlich's reagent, Sigma Aldrich Chemie B.V., Zwijndrecht, The Netherlands) dissolved in ethanol-sulfuric acid (1:1 v/v). PLT spots were detected by spraying with an aqueous 0.5% Fast Blue RR salt solution (Sigma Aldrich). The R*f* values were 0.80 for PRN and 0.50 for PLT, as determined by migration of pure PRN, and confirmed by identical color (crimson spot) and R*f* values reported for PLT (Bencini *et al.*, 1983; Burkhead, 1994). For detection of PRN and PLT by HPLC, 15 ml of the bacterial culture broth was extracted and analysed following protocols described previously (Bonsall *et al.*, 1997).

Primers. Characteristics of the primers used in PCR are listed in Table 2. Primers PRND1 and PRND2 were developed from conserved sequences within the *prnD* gene of *P. fluorescens* strain BL915 (GenBank U74493), *B. pyrrocinia* (GenBank AF161186), and *B. cepacia* strain LT4-12-W (GenBank AF161183) (Hammer *et al.*, 1999). Sequence alignment and primer location were performed with the DNAstar program (DNAstar Inc., Madison, WI, USA) and checked manually. The 1092-bp *prnD* gene of *P. fluorescens* strain BL915 encodes a 363-amino acid protein that shows homology to 3-chlorobenzoate-3,4-dioxygenase (CbaA) of *Alcaligenes* sp., phthalate 4,5-dioxygenase (Pht3) of *P. putida*, and vanilate demethylase (VanA) of *Pseudomonas* sp. (Hammer *et al.*, 1997). The *prnD* gene product catalyzes the oxidation of the amino group of aminopyrrolnitrin to a nitro group to form pyrrolnitrin (Kirner *et al.*, 1998). The PLT primers PLTC1 and PLTC2 were developed from sequences within the *plt*C gene from *P. fluorescens* strain Pf-5 (GenBank AF081920) (Nowak-Thompson *et al.*, 1999). The 5322-bp *plt*C gene has homology with *eryA* genes of *Saccharopolyspora erythraea*, which encode a polyketide synthase required for erythromycin production. The 1774-amino acid protein encoded by *pltC* is probably responsible for the formation of the resorcinol ring during pyoluteorin biosynthesis (Nowak-Thompson *et al.*, 1997). Primers were synthesized by Amersham Pharmacia Biotech (Roosendaal, The Netherlands).

PCR-analysis. Heat-lysed bacterial suspensions were prepared as described previously (Raaijmakers *et al.*, 1997) and diluted 20-fold in sterile distilled water. PCR amplifications were performed in a 25-µl reaction mixture containing 3 µl of the diluted heat-lysed cell suspension, 1X PCR buffer (Perkin-Elmer, Nieuwerkerk aan de IJssel, The Netherlands), 1.5 mM $MgCl_2$ (Perkin Elmer), 200 µM of each, dATP, dCTP, dGTP, and dTTP (Promega, Leiden, The Netherlands), 20 pmol of each primer, and 2.0 U of AmpliTaq polymerase (Perkin-Elmer). For RAPD analysis (see below), 40 pmol of a determined primer was used. Amplifications were carried out with an MJ Research PTC-200 thermocycler. The PCR program for PRN consisted of an initial denaturation at 95°C for 2 min followed by 30 cycles at 95°C for 1 min, annealing at 68°C for 1 min, and extension at 72°C for 1 min. For PLT, an initial denaturation at 95°C for 2 min was followed by 30 cycles of 95°C for 2 min, annealing at 67°C for 1 min, and extension at 72°C for 1 min. The PCR program for RAPD analysis was described by Keel *et al.* (1996). Samples (10-15 µl) of the PCR products were separated on a 1% agarose gel or on 2% agarose gels for RAPD analysis in 1X TBE buffer (90 mM Tris-borate, 2 mM EDTA [pH 8.0]) at 60-80 V for 1-3 h. The GIBCO-BRL 100-bp ladder (Life Technologies B.V., Breda, The Netherlands) was used as size marker. PCR products were visualized with a UV transilluminator.

RFLP and RAPD analysis. For all reference strains, PRN and PLT fragments were amplified with the PRND1/PRND2 and PLTC1/PLTC2 primers, respectively (Table 2). The 786-bp fragment for PRN and the 438-bp for PLT were the only DNA fragments amplified with these primers for all reference strains producing PRN and PLT (Fig. 1A and 2A). The PCR-fragments were precipitated with 4M LiCl and resuspended in sterile distilled water. Digestions were performed in a total volume of 15 µl, containing 1 µg of DNA, 1X reaction buffer, and 10 U of each enzyme separately (New England Biolabs Ltd., Hitchin, Hertfordshire, England; Promega Corporation, Leiden, The Netherlands; and Life Technologies B.V.). The enzymes used were *Acc*I, *BsmB*I, *Hae*II, *Hinc*II, *Hinf*I, *Mnl*I, *Pvu*II for digestion of the PRN-fragment and *Alw*I, *Ava*I, *Mnl*I, *Nae*I, *Pst*I, *Sac*II, *Taq*I, and *Xho*I for digestion of the PLT-fragment. The mixtures were incubated for 2 h at 37°C, with exception of *Taq*I, which was incubated at 65°C. Digestion products were separated on a 1.5% agarose gel.

RAPD analysis of the reference strains carrying genes for PRN (Table 1) was performed with primers M13, M12, and D7. These primers were selected among sixty-four other random primers based on the distinct and consistent banding pattern of the polymorphic markers produced (Keel *et al.*, 1996). The size of the bands was determined based on the 100-bp ladder as size marker. The presence or absence of fragments generated by RFLP and RAPD analysis (1 for the presence or 0 for the absence of a particular sized band in the gel) was used to calculate the pairwise coefficients of similarity (Nei-Li distances). Cluster analysis with the unweighted pair group method with arithmetic mean algorithm (UPGMA) and bootstrap analysis with a 1000 resampled data sets

were performed with the program FreeTree (Hampl *et al.*, 2001). The dendrograms were visualised and edited in TREEVIEW (Page, 1996) and TreeExplorer (Tamura, 1997). RAPD analysis was performed three times for each primer and RFLP was performed once.

Southern and colony hybridizations. DNA transfer from agarose gels and bacterial colony transfer to Hybond N^+ nylon membranes (Amersham Pharmacia Biotech) were performed according to standard methods (Sambrook and Russel, 2001). Membrane washes and hybridizations were performed following standard procedures (Sambrook and Russel, 2001). High-stringent conditions comprised prehybridization for 1.5 h at 65°C, hybridization for 12 h at 65°C, membrane washing twice each for 5 min with 2X SSC (1X SSC is 0.15 M NaCl plus 0.015 M sodium citrate)-0.1% SDS at room temperature, and membrane washing twice each for 30 min with 0.1X SSC-0.1% SDS at 65°C. Low stringent conditions consisted of prehybridization and hybridization at 55°C and two membrane washing steps with 0.5X SSC at 55°C. PRN- and PLT-probes were generated by random primed labeling of amplified PRN or PLT fragments with digoxigenin-dUTP after PCR or by direct labeling in PCR using the DIG labeling mix (Roche, Almere, The Netherlands). The 786-bp fragment amplified from *B. pyrrocinia* strain DSM10685 was used to generate the PRN-probe. The 438-bp fragment amplified from *P. fluorescens* Pf-5 was used to generate the PLT-probe. Hybridized probes were immunodetected according to the protocols provided by the supplier.

16S rDNA sequencing. Total DNA for use in PCR amplifications of the 16S rDNA genes was generated using a modification of the method used by Raaijmakers *et al.* (1997). Bacteria were placed in 0.6 ml micro-centrifuge tubes and suspended in 100 µl of lysis buffer and incubated in a water bath at 100°C for 15 min. Micro-centrifuge tubes were vortexed for 30 sec, and tubes were returned to the water bath for an additional 15 min. The cell suspension was centrifuged for 1 min at 14,000 rpm and the supernatant was transferred to a 1.5 ml micro-centrifuge tube and diluted with 1.4 ml sterile distilled water. Five microliters of the cell lysis suspension was used in each reaction which employed the primers 8f and 1406r (Amann *et al.*, 1995) using reaction conditions essentially as described by Liu *et al.* (1997). PCR-amplified products were analyzed by electrophoresis on 2% agarose gels. Gels were stained with ethidium bromide and visualized by UV illumination and photographed. Fragments of the appropriate size were cloned into the T/A vector pCR2.1 (Invitrogen, Carlsbad, CA), and transformed into *E. coli* strain INVαF'. Transformants were selected on LB agar amended with kanamycin (75 µg ml^{-1}) and X-Gal (20 µg ml^{-1}). Plasmid DNA was isolated from transformants using the S.N.A.P. mini-prep kit (Invitrogen). Cycle sequencing was conducted on a Perkin-Elmer model 480 thermal cycler (Norwalk, CT) and PRISM Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit (PE Biosystems, Warrington, UK). Reactions were conducted using cycling conditions recommended by the manufacturer with the T3 or T7 primer. Products of the sequencing reactions were purified using CENTRI-SEP spin columns (Princeton Separations, INC., Adelphia, NJ) and dried in a vacuum centrifuge. Samples were resuspended in 6 µl of a solution containing deionized formamide and blue dextran/EDTA in a ratio of 5:1. Samples were heated for 2 min at 90°C and 1.5 µl were loaded on an ABI 377 DNA sequencer (PE Biosystems, Foster City, CA). BLASTN searches were conducted on the nucleotide sequences obtained.

Nucleotide sequence accession numbers. Newly obtained sequences of the 16S rDNA gene were deposited with GenBank under the following accession numbers: *B. cepacia* J2535 (AF495866), *B. cepacia* LMG 1222 (AF495867), *P. chlororaphis* LMD 82.53 (AF495868), *Pseudomonas* sp. PHZ13 (AF495869), *Pseudomonas* sp. PHZ22 (AF495870), and *P. fluorescens* Pf-5 (AF495871). Additional sequences of the 16S rDNA were obtained from GenBank for the following strains: *P. aeruginosa* PAO1 (AE004091), *P. fluorescens* CHA0 (AJ278812), and *B. pyrrocinia* DSM10685 (U96930). Accession numbers for sequences of *prn* biosynthetic genes available in the database are: *P. fluorescens* BL915 (U74493), *B. pyrrocinia* DSM10685 (AF161186), *B. cepacia* LT4-12-W (AF161183), *M. fulvus* Mx fl47 (AF161185), *prnA* of *P. fluorescens* CHA0 (AF161184), and *prnA* from *P. aureofaciens* ACN (AF161182).

Sequence analyses. PRN and 16S rDNA sequences were aligned by CLUSTAL W 1.81 (Thompson *et al.*, 1994). Sites presenting alignment gaps were not considered in phylogenetic inferences. UPGMA dendrograms with the nucleotide substitution model of Jukes and Cantor (1969) and bootstrap analysis with 1000 resamplings were performed with the program MEGA (Kumar *et al.*, 2001). For the *in silico* analysis of the *prnABCD* genes, most parsimonious phylogenetic trees were obtained by using MEGA. Phylogenetic trees were constructed by the maximum likelihood method using DNAML contained in PHYLIP 3.5c Package (Felsenstein, 1993) after generation of 100 resampled datasets with PHYLIP's program SEQBOOT and finally obtaining the consensus tree with PHYLYP's program CONSENSE. Trees were also constructed by the maximum parsimony method using MEGA (Kumar *et al.*, 2001). Bootstrap analyses were performed with 1000

resamplings. Phylogenetic trees were visualized and edited as mentioned earlier.

Soils. Five soils were obtained from agricultural fields in the Netherlands. Soils CB, SU, SV, and SSB were collected in December 1997 and soil SL in June 1998 from the upper 50 cm of the soil profile, air dried for a week, and passed through a 0.5-cm mesh screen prior to use. Soils CB, SU, SV, and SSB were collected from a polder in the South-West of the Netherlands, located 10 km from the city of Bergen op Zom. These soils are physicochemically very similar, containing on average, 27% clay, 10% silt, and 51% sand. The organic matter content is on average 4.3% and the pH, determined after extraction with $CaCl_2$ is 7.5. Soil SL was obtained from a polder in the North-East of the Netherlands, at Lovinkhoeve, located 30 km from the city of Lelystad. SL soil contains 21% clay, 12% silt, 54% sand, 3.6 % organic matter, and pH, determined after extraction with $CaCl_2$ is 7.5. Under field conditions, all soils have been cropped continuously to wheat, with exception of CB soil, which was cropped to wheat and sugar beet in a rotation scheme (1:2).

Plant cultivation. Pots containing 200 g of sieved soil were sown with 15 seeds of wheat, cv. Bussard. Plants were grown in a climate chamber at 15°C with a 12-h photoperiod. Plants received 50 ml of 1/3 strength Hoagland's solution (macroelements only) twice a week. After 30 days, the roots were harvested and the soil remaining plus excised roots were mixed, returned to the same pot and sown again with 15 wheat seeds. The process of growing wheat and harvesting roots was repeated seven times. For each soil, four replicates were used and the whole experiment was performed twice.

Isolation of *Pseudomonas* spp. and *B. cepacia* from wheat roots. Population densities of total, and *prnD* and *pltC*-harbouring *Pseudomonas* spp. were determined on roots of wheat harvested from the first and seventh successive growth cycle. For *B. cepacia,* total populations and populations harboring *prnD* and *pltC* genes were determined in the second, third, fourth, fifth and sixth growth cycles. For each replicate, roots of five randomly selected plants were harvested and loosely adhering soil was gently removed. One gram of roots plus adhering rhizosphere soil was suspended in 5.0 ml of sterile distilled water, vortexed for 1 min and sonicated for 1 min in a ultrasonic cleaner. For isolation and enumeration of pseudomonads, samples were dilution plated onto KMB^+ and plates were incubated at 25°C for 48 h. For enumeration of *B. cepacia*, serial dilutions were plated onto TB-T medium and incubated for 4 days at 25°C. Population densities of *Pseudomonas* spp. harbouring *prnD* and/or *pltC* were determined by colony hybridization and PCR. Population densities of *B. cepacia* harbouring *prnD* and/or *pltC* were determined by PCR; for each soil, 216 randomly selected colonies were tested.

Suppressiveness of soils to *G. graminis* var. *tritici*. Soils were amended with 0.1% (wt/wt) of an oat grain inoculum of *G. graminis* var. *tritici* isolate R3-111a-1 (particle size 0.25 to 0.50 mm); sterilized oat grain inoculum was used as a control. Wheat seeds (cv. Bussard) were sown in PVC pots (8 cm high, 7 cm wide) containing 200 g of sieved soil, and covered with a 1-cm layer of soil without inoculum. Plants were grown as described above. Each treatment had five replicates with approximately 12 plants per replicate. Eight randomly selected plants were harvested, their root system washed and disease severity determined on a 0-to-8 scale, where "0" indicates no disease and "8" indicates dead plant (Thomashow and Weller, 1996). The experiment was performed twice. Data from disease index were ranked and analysed by ANOVA followed by Tukey's studentized range test, after certifying normal distribution and homogeneity of variances (SAS institute, Inc., Cary,N.C.).

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CHAPTER 3

CONSERVATION OF THE RESPONSE REGULATOR GENE *gacA* IN *PSEUDOMONAS* SPECIES

ABSTRACT

The response regulator gene *gacA* influences the production of several secondary metabolites in both pathogenic and beneficial *Pseudomonas* spp. In this study, we developed primers and a probe for the *gacA* gene of *Pseudomonas* species and sequenced *gacA* from ten *Pseudomonas* strains isolated from different plant-associated environments. PCR analysis and Southern hybridization showed that *gacA* is highly conserved within the genus *Pseudomonas*: multiple strains of different *Pseudomonas* species all responded positively to the probe, whereas no response was obtained from 18 other strains representing 14 species and 8 genera of Gram-negative bacteria other than *Pseudomonas*. Furthermore, from a total of approximately 550 indigenous bacterial isolates obtained from the rhizosphere of wheat, all isolates that hybridized with the GacA probe were classified as *Pseudomonas* spp. by group-specific primers. Isolates that did not respond to the GacA probe and primers were identified as bacterial genera other than *Pseudomonas*, including *Stenotrophomonas*, *Cryseomonas* and *Comamonas* spp. These results indicate that *gacA* can be used as a complementary genetic marker for detection of *Pseudomonas* spp. in environmental samples. Phylogenetic relationships inferred from the newly sequenced *gacA* genes and the sequences of *gacA* homologs present in the databases, showed six distinct clusters that correspond to the following bacterial groups: Pseudomonaceae, Enterobacteriaceae, Alteromonadaceae, Vibrionaceae, Burkholderia, and Xanthomonas. Within the Pseudomonadaceae and Enterobacteriaceae, polymorphisms within *gacA* and its homologs allowed identification of six and five subclusters, respectively. Comparison of the *gacA* gene and GacA protein-based trees with the tree inferred from 16S rDNA sequences yielded a similar overall clustering. These results suggest that *gacA* and its homologs may provide complementary genetic markers for phylogenetic studies of *Pseudomonas* spp. and Gram-negative bacteria other than *Pseudomonas*. Estimations of nonsynonymous to synonymous substitution rates (Ka/Ks ratios) showed that, for members of both, the Pseudomonaceae and Enterobacteriaceae, purifying selection is acting on *gacA*, indicating that there is selective pressure to avoid substitutions leading to functional changes in the GacA protein.

Jorge T. de Souza, Mark Mazzola, and Jos M. Raaijmakers

Submitted.

INTRODUCTION

Bacteria of the genus *Pseudomonas* are able to survive and prosper in a wide range of environmental conditions. This genus not only contains plant, animal, and human pathogenic species, but also accomodates species that are of significant environmental importance, including plant growth promoters, xenobiotic degraders, and biocontrol agents (Palleroni, 1992; Johnsen *et al.*, 1996; O'Sullivan and O'Gara, 1992). Their versatile metabolic activities and ability to produce a wide variety of secondary metabolites have stimulated numerous ecological, molecular and biochemical studies.

In several strains that belong to the group of plant-associated *Pseudomonas* species, expression of genes involved in the biosynthesis of secondary metabolites and extracellular enzymes is positively controlled by the GacS/GacA two-component system (reviewed by Heeb and Haas, 2001). This regulatory system consists of a membrane-bound sensor kinase protein (GacS) and a cytoplasmic response regulator protein (GacA). The current model proposes that GacS recognizes specific environmental stimuli and activates GacA which in turn triggers the expression of specific genes (Appleby *et al.*, 1996; Pernestig *et al.*, 2001; Heeb and Haas, 2001). Several other elements are also involved in the regulation of certain genes, forming a complex regulatory cascade, with the GacS/GacA pair controlling secondary metabolite production at a higher hierarchy (Sarniguet *et al.*, 1995; Chancey *et al.*, 1999; Whistler *et al.*, 1998; Blumer and Haas, 2000). For example, production of phenazine antibiotics, which are involved in the biocontrol activity and ecological competence of several *Pseudomonas* strains (Mazzola *et al.*, 1992), is partially regulated by the PhzI/PhzR quorum sensing system that uses *N*-acyl-homoserine lactone (Pierson III *et al.*, 1994). The GacS/GacA system interacts positively with the PhzI/PhzR quorum sensing system by regulating the synthesis of *N*-acyl-homoserine lactone thereby controlling phenazine production at the transcriptional level (Chancey *et al.*, 1999).

The sensor kinase gene *gacS* was first described in *Pseudomonas syringae* pv. *syringae* as an essential factor for disease manifestation (Hrabak and Willis, 1992). Since then, *gacS* homologs have been identified in other *Pseudomonas* strains, *Azotobacter vinelandii*, *Vibrio cholerae*, and several enteric bacteria, including *E. coli*, *Salmonella enterica* serovar *typhimurium*, and *Erwinia carotovora* subsp. *carotovora* (Heeb and Haas, 2001). Alignment of GacS homologs resulted in three groups: the pseudomonads, the enteric bacteria, and *Vibrio/Shewanella* (Heeb and Haas, 2001). Interestingly, within a total of nine *Pseudomonas* strains included in their analyses, *P. fluorescens, P. chlororaphis, P. tolaasii, P. viridiflava* and *P.*

syringae belonged to a subgroup, whereas GacS homologs from *P. aeruginosa* and *P. putida* clustered at some distance (Heeb and Haas, 2001).

The response regulator gene *gacA* (global activator of antibiotic and cyanide production) was first identified in the biocontrol strain *P. fluorescens* CHA0 and was shown to be essential for the biocontrol activity and ecological fitness of this strain (Laville *et al.*, 1992; Natsch *et al.* 1994). Homologs of GacA have been identified in several other Gram-negative bacterial genera. Within the group of *Pseudomonas* spp., Heeb and Haas (2001) described GacA homologs in a total of eight *Pseudomonas* strains, including *P. aeruginosa, P. viridiflava, P. syringae* pv. *syringae, P. fluorescens,* and *P. aureofaciens*. Subsequent alignment of the GacA proteins placed these eight *Pseudomonas* strains at some distance from GacA homologs of enteric bacteria, and *Xylella* and *Shewanella*. The results from these *in silico* analyses suggested that the *gacA* gene may be conserved within Pseudomonadaceae. However, most information on *gacS*/*gacA* regulatory systems stems from studies on model strains and little information is available on the distribution of these genes in populations of rhizosphere-associated bacteria.

In this study, we developed primers and a probe for the *gacA* gene of *Pseudomonas* species and sequenced the *gacA* gene from 10 *Pseudomonas* strains isolated from different plant-associated environments. The newly sequenced *gacA* genes as well as *gacA* homologs currently present in databases were used in phylogenetic analyses. Delineation of *Pseudomonas* species based on *gacA* gene and protein sequences was compared to classification based on 16S rDNA sequences. Sequence data were also used to compare the rate of evolution of the *gacA* gene as well as the ratio of synonymous and nonsynonymous substitutions in *gacA* sequences within and between Pseudomonaceae and Enterobacteriaceae. The potential usefulness of the *gacA* gene as a complementary genetic marker for *Pseudomonas* was evaluated on bacterial populations isolated from the rhizosphere of wheat. Approximately 550 isolates were genotypically and phenotypically identified, and characterized for the production of specific extracellular enzymes and secondary metabolites known to be regulated by GacA.

RESULTS

Design and evaluation of the GACA primers and probe. Primers GACA1 (5'-GBATCGGMGGYCTBGARGC) and GACA2 (5'-MGYCARYTCVACRTCRCTGSTGAT) were developed from conserved sequences within the *gacA* genes of *P. fluorescens* strains BL915 (GenBank L29642), CHA0 (GenBank

M80913), Pf-5 (GenBank U30858), *P. aeruginosa* PAO1 (GenBank U27988), *P. syringae* pv. *syringae* B728a (GenBank U09767), *P. chlororaphis* 30-84 (GenBank AF115381), and the *repB* gene of *P. viridiflava* PJ-08-6A (GenBank L30102). Primers GACA1 and GACA2 are complementary to positions 489 and 914, respectively, in the *gacA* sequence of *P. fluorescens* strain CHA0. Primers GACA1 and GACA2 amplified the predicted 425-bp fragment of homologous strain *P. fluorescens* CHA0. Restriction analysis of the amplified fragment with enzymes *Ava*II, *Alu*I, *Fsp*I, *Hinf*I, *Nae*I, and *Sty*I resulted in restriction fragments with sizes identical to those deduced from the restriction map of the *gacA* sequence of CHA0. The GACA primers amplified a 425-bp fragment from all other *Pseudomonas* strains, representing different species, except from *P. agarici* DSM11810, and *P. fluorescens* strains 2-79RN10 and SS101 (Table 1). The PCR products obtained from all positive strains hybridized under high stringent conditions with the GacA probe consisting of the labeled 425-bp PCR fragment obtained from strain CHA0. In Southern hybridization with *EcoR*I-digested genomic DNA, all *Pseudomonas* strains tested, including DSM11810, 2-79RN10 and SS101, hybridized under high-stringent conditions with the GacA probe. A single hybridized band was detected for all strains. No PCR products or hybridization signals were obtained from 18 other strains, representing 14 species and 8 genera of Gram-negative bacteria other than *Pseudomonas* (Table 1).

Sequence analysis of *gacA* and 16S-rDNA. Ten *Pseudomonas* strains were selected for sequencing of the *gacA* gene. The ten strains were *P. fluorescens* strains Q2-87, SSB17, F113, and SSB33; *P. chlororaphis* LMD 82.53 and PHZ26, *Pseudomonas* sp. PHZ13; *P. putida* WCS358R; *P. aeruginosa* DSM 1128, and *P. syringae* pv. *pisi* 24.7. The overall base composition of the newly obtained *gacA* sequences was *T* = 20.4% (± standard deviation 0.5), *C* = 31.3% (± 0.6), *A* = 21.0% (± 0.3), and *G* = 27.4% (± 0.4). The identity of the new *gacA* sequences to the *gacA* sequence from strain CHA0 ranged from 79.6 to 89.2% and the similarity of the predicted GacA protein ranged from 85.9 to 98.5%. The 16S rDNA sequences were determined over a total of approximately 300 - 600 bp to verify or identify the following strains: *P. fluorescens* Pf-5, SSB33, and SSB17, *P. aeruginosa* DSM 1128, *P. chlororaphis* LMD 82.53, and PHZ26, *Pseudomonas* sp. PHZ13, *P. putida* WCS358R, and *P. syringae* pv. *pisi* 24.7. The sequence identity of the 16S rDNA in relation to that of strain CHA0 was 64.2% (399 nucleotides overlap) for strain SSB17, 73.5% (328 nucleotides overlap) for PHZ13, 81.5% (336 nucleotides overlap) for PHZ26, 82.3% (322 nucleotides overlap) for WCS358R, 83.3% (270 nucleotides overlap) for LMD 82.53, 87.3% (378

Table 1. Specificity of the *gacA* primers and probes.

Species	Strain	Secondary metabolites [a]	Specificity [b]	
			PCR	Hybrid.
Pseudomonas aeruginosa	PAO1	RHL, pro	+	+
	DSM1128	RHL, pro	+	+
	DSM939	RHL	+	+
P. agarici	DSM11810		-	+
P. aureofaciens	LMD82.53	PRN, PHZ, pro, phc	+	+
P. fluorescens	CHA0	2,4-DAPG, PRN, PLT, pro, phc	+	+
	Pf-5	2,4-DAPG, PRN, PLT, pro, phc	+	+
	PGNR1	2,4-DAPG, PRN, PLT, pro, phc	+	+
	PINR2	2,4-DAPG, PRN, PLT, pro, phc	+	+
	F113	2,4-DAPG, pro	+	+
	Q8r1-96	2,4-DAPG, pro	+	+
	PILH1	2,4-DAPG, pro	+	+
	2-79RN10	PHZ, pro, phc	-	+
	SSB33		+	+
	SS101		-	+
P. chlororaphis	PHZ50	PHZ, pro	+	+
	PHZ26	PHZ, pro, phc	+	+
Pseudomonas sp.	PHZ13	PHZ	+	+
P. syringae pv. *pisi*	24.7	pro	+	+
P. putida	WCS358R		+	+
Burkholderia cepacia	LMG1222	not determined	-	-
	ATCC51671	PRN, pro, phc	-	-
	J2535	PRN, pro, phc	-	-
	J2540	PRN, pro	-	-
	J2553	pro, phc	-	-
B. pyrrocinia	DSM10685	PRN, pro	-	-
B. multivorans	LMG13010	phc	-	-
B. vietnamiensis	LMG10929	phc	-	-
Escherichia coli	JM107	not determined	-	-
Stenotrophomonas maltophilia	DSM8573	pro	-	-
S. nitritireducens	DSM12575	not determined	-	-
Xanthomonas fragariae	DSM3587	not determined	-	-
X. campestris pv. *campestris*	DSM 3586	pro	-	-
Flavobacterium aquatile	DSM1132	pro	-	-
Pantoea agglomerans	DSM8570	not determined	-	-
Serratia marcescens	DSM30121	pro, phc	-	-
S. plymuthica	DSM8571	pro, phc	-	-
Rhizobium radiobacter	DSM30205	not determined	-	-

[a] Secondary metabolites – 2,4-DAPG (2,4-diacetylphloroglucinol), PHZ (phenazines), PRN (pyrrolnitrin), PLT (pyoluteorin), RHL (rhamnolipids), pro (protease), and phc (phospholipase C) were detected by using HPLC, specific primers, skim milk medium (pro), and egg yolk plates (phc). PRN and PLT detection (Keel *et al.*, 1996, De Souza and Raaijmakers, submitted).

[b] Amplification in PCR with primers GACA1 and GACA2 and/or hybridization under high and low stringent conditions with a 425-bp *gacA* probe derived from *P. fluorescens* CHA0.

nucleotides overlap) for SSB33, 87.4% (496 nucleotides overlap) for Pf-5, and 88.1% (503 nucleotides overlap) for *P.syringae* pv. *pisi* 24.7.

Phylogenetic analyses. The neighbour-joining tree inferred from the newly sequenced *gacA* genes plus the sequences of *gacA* homologs already present in the databases (Table 2), showed

Table 2. Accession numbers of *gacA* and 16S rDNA sequences.

Species	Strain	Accession number [a]	
		GacA	16S rDNA
Pseudomonaceae			
Azotobacter vinelandii	ATCC 9046	AF382827	-
Pseudomonas sp.	PHZ13	AF502416	AF495869
Pseudomonas aeruginosa	PAO1	U27988	AE004091
	DSM1128	AF502413	AF502423
P. aureofaciens	30-84	AF115381	-
P. chlororaphis	LMD82.53	AF502415	AF495868
	PHZ26	AF502417	AF502424
P. fluorescens	CHA0	M80913	AJ278812
	Pf-5	AF065156	AF495871
	F113	AF502414	AJ278814
	Q2-87	AF502419	AJ278813
	SSB33	AF502420	AF502427
	BL915	L29642	-
	SSB17	AF502421	AF502426
	PfO-1	unfinished	unfinished
	FPT9601	AB054363	-
P. putida	KT2420	unfinished	unfinished
	WCS358R	AF502422	AF502428
P. syringae pv. *pisi*	24.7	AF502418	AF502425
P. syringae pv. *syringae*	B728a	U09767	-
P.syringae pv. *tomato*	DC3000	unfinished	unfinished
P. viridiflava	PJ-08-6A	L30102	-
Enterobacteriaceae			
Escherichia coli	K-12 MG1655	M24615	AE000129
	RIMD0509952	AP002559	AP002566
	O157 EDL933	AE005414	AE005195
	CFT073	unfinished	unfinished
Klebsiella pneumoniae	M6H 78578	unfinished	unfinished
Pectobacterium caratovorum	SSC3193	X95564	-
Salmonella typhi	CT18	AL627272	AL627282
	TY2	unfinished	unfinished
S. typhimurium	LT2	AE008786	AE008706
	ATCC 14028	U67869	-
Shigella flexneri	2a	unfinished	unfinished
Yersinia pestis	CO-92	AJ414150	AJ414156
	KIM5 P12	unfinished	unfinished
Y. enterocolitica	0:8	unfinished	unfinished
Alteromonadaceae			
Shewanella putrefasciens	MR-1	unfinished	unfinished
Vibrionaceae			
Vibrio cholerae	O395	AF071105	-
	N16961	AE004201	AE004157
Xanthomonas group			
Xylella fastidiosa	9a5c	AE004068	AE003870
	Ann-1	unfinished	unfinished
	Dixon	unfinished	unfinished
	Temecula 1	unfinished	unfinished
Burkholderia group			
Burkholderia cepacia	J2315	unfinished	unfinished

[a] Unfinished refers to non-annotated data obtained from genome sequencing projects. (-) refers to absence of data on these strains in the databases.

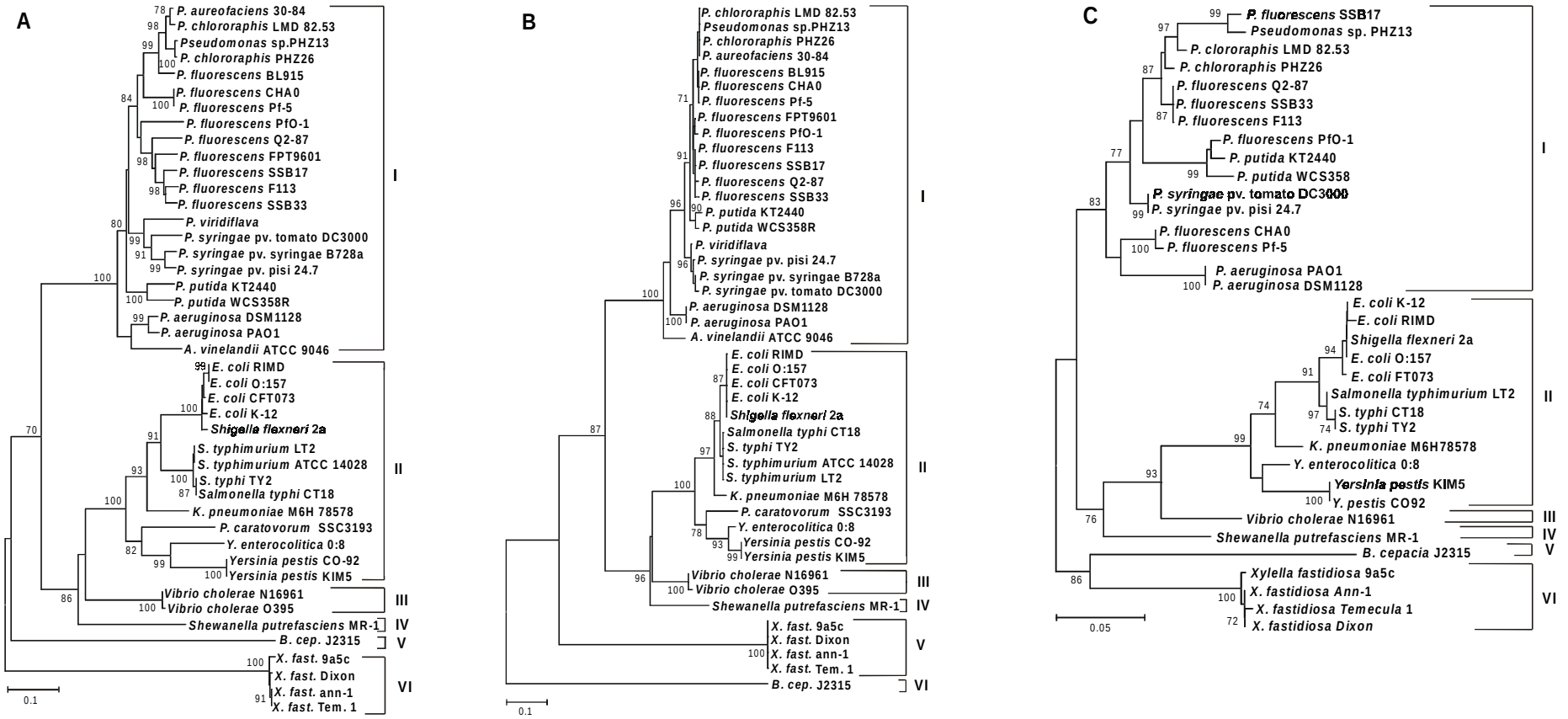

Figure 1. Phylogenetic trees inferred by the neighbour-joining method with sequences of *gacA* (A), gacA (B), and 16S rDNA (C). A: Based on 406-bp aligned *gacA* nucleotides (positions 501-906 of the *gacA* sequence of *P. fluorescens* CHA0). B: Based on 135 amino acids of GacA (positions 63-197 of gacA sequence of *P. fluorescens* CHA0). C: Based on 270-bp aligned 16S rDNA sequences (positions 1-270 of the nucleotide sequence of *P. fluorescens* CHA0). Bootstrap values greater than 70% are shown.

six distinct clusters that correspond to the following bacterial groups: Pseudomonaceae (I), Enterobacteriaceae (II), Alteromonadaceae (III), Vibrionaceae (IV), Burkholderia (V), and Xanthomonas (VI) groups (Fig. 1A). Within the Pseudomonadaceae, six subclusters can be distinguished based on significant bootstrap values (>80%): the first contained *P. chlororaphis*, *P. aureofaciens*, and *P. fluorescens* CHA0, Pf-5, and BL915; the second subcluster contained only *P. fluorescens* strains; the third contained *P. viridiflava* and *P. syringae* strains; the fourth was composed of two *P. putida* strains, the fifth of *P. aeruginosa* strains, and the sixth of *Azotobacter vinelandii*. Within the enteric bacteria, five subclusters were distinguished: the first one comprised *E. coli* isolates and *Shigella flexneri*, which were tightly clustered; the second contained only *Salmonella* species; the third subcluster contained *Klebsiella pneumoniae*; the fourth, the plant pathogenic *Pectobacterium caratovorum*, and the fifth contained *Yersinia* species (Fig 1A). Other major clusters contained the Vibrionaceae, represented by *Vibrio* (cluster III), Alteromonadaceae, represented by *Shewanella putrefaciens* (cluster IV), the Burkholderia group, represented by *B. cepacia* (V), and the Xanthomonas group, represented by *Xylella fastidiosa* isolates (cluster VI). Cluster VI was the most distant of all clusters obtained (Fig. 1A). In all cases, with exception of clusters V and VI, the bootstrap values were highly significant. Similar results were obtained with the neighbour-joining tree inferred from the predicted GacA protein sequences (Fig. 1B), although the length of the branches defining clusters were shorter than those in the tree inferred from the *gacA* gene sequences. Another difference is that *B. cepacia* was clustered as the most distant group, whereas *Xylella* is the most distant group in the tree inferred from DNA sequences. Comparison of the *gacA* gene and GacA protein-based trees with the tree inferred from 16S rDNA sequences yields a similar overall clustering (Fig. 1C), although some incongruences were noted. *P. fluorescens* SSB17 clustered closer to *P. chlororaphis* and *P. aureofaciens* than to other *P. fluorescens* strains. Furthermore, *P. fluorescens* strains CHA0 and Pf-5 were clustered closer to *P. aeruginosa*. The 16S rDNA clustering of *B. cepacia* was in agreement with the clustering based on *gacA* gene sequences (Fig. 1A). Phylogenetic analyses of the *gacA* gene, predicted GacA protein and 16S rDNA sequences with the maximum likelihood method yielded similar results as obtained by neighbour-joining.

Rate of evolution of *gacA*. The measure of nonsynonymous relative to synonymous substitution rates (Ka/Ks) provides insight into the level of selective constraint acting on proteins (Li *et al.*, 1985). Protein-encoding genes evolving at a neutral rate have Ka/Ks ratios equal to 1, indicating that the protein evolves without constraint on amino acid replacements.

When Ka/Ks < 1, the protein is under selective constraints and protein-encoding genes are subjected to purifying selection. Finally, when Ka/Ks > 1, positive selection has acted to promote amino acid replacements (Hughes and Nei, 1988; Li *et al.*, 1985). The Ka/Ks ratio for *gacA* genes was determined for different categories and was shown always to be smaller than 1 (Table 3). Fisher's exact test (P=0.05) and Z-test confirmed that purifying selection is operating on the *gacA* genes of both Pseudomonadaceae and Enterobacteriaceae.

Tajima's relative rate test was performed to test the molecular clock hypothesis. According to this hypothesis, sequences evolve at the same rate, irrespective of the substitution model and whether or not the substitution rate varies with site (Tajima, 1993). The test was done in 45 pairwise combinations with a third *gacA* sequence as outgroup. Prior to this analysis, majority sequences of *gacA* of species belonging to the Pseudomonaceae and Enterobacteriaceae were computed to reduce the number of two-by-two combinations. Tajima's test showed that there were no statistically significant differences in every possible pairwise comparison between members of the Pseudomonaceae, with *K. pneumoniae* as outgroup (data not shown). Comparisons of members of the Pseudomonaceae with members of the Enterobacteriaceae, with *B. cepacia* as outgroup, showed that 13 out of 24 possible combinations gave statistically different evolutionary rates (Table 4). In all these 13 statistically different combinations, members of the Enterobacteriaceae presented significantly higher substitution rates than members of the Pseudomonaceae (i.e. $m_2 > m_1$). Within the Enterobacteriaceae, with *A. vinelandii* as outgroup, 5 out of 10 possible pairwise comparisons showed significantly different substitution rates (Table 4). Collectively, these results indicate that all tested *gacA* sequences from Pseudomonadaceae evolve at the same rate. In contrast,

Table 3. Nonsynonymous (Ka) and synonymous (Ks) substitution rates in *gacA* sequences.

Categories [a]	N [b]	Ka	Ks	Ka/Ks
All sequences	44	0.304 ± 0.017	0.437 ± 0.029	0.696
Pseudomonaceae and Enterobacteriaceae	36	0.165 ± 0.017	0.643 ± 0.018	0.257
Pseudomonaceae	22	0.034 ± 0.001	0.519 ± 0.009	0.063
Enterobacteriaceae	14	0.055 ± 0.004	0.582 ± 0.029	0.074

[a] Comparisons of the *gacA* sequences in different categories. Categories of the *gacA* gene and its homologs were: 1) all *gacA* sequences together; 2) *gacA* sequences from the Pseudomonadaceae and Enterobacteriaceae; 3) only *gacA* sequences from Pseudomonadaceae; and 4) only *gacA* sequences from Enterobacteriaceae.
[b] Number of sequences per category.

Table 4. Tajima's 1D relative rate test between pairs of bacterial species.

Species 1 [a]	Species 2 [a]	Outgoup	m_1	m_2	X^2 [b]
Pseudomonadaceae X Enterobacteriaceae					
P. syringae	*P. caratovora*	*B. cepacia*	41	62	4.28 *
P. syringae	*Yersinia* spp.	*B. cepacia*	43	66	4.85 *
P. fluorescens	*P. caratovora*	*B. cepacia*	43	66	4.85 *
P. fluorescens	*Yersinia* spp.	*B. cepacia*	41	68	6.69 **
P. putida	*P. caratovora*	*B. cepacia*	52	75	4.17 *
P. putida	*Yersinia* spp.	*B. cepacia*	48	74	5.54 *
P. aeruginosa	*P. caratovora*	*B. cepacia*	41	66	5.84 *
P. aeruginosa	*Yersinia* spp.	*B. cepacia*	42	69	6.57 **
P. aeruginosa	*E. coli*	*B. cepacia*	41	62	4.28 *
P. aeruginosa	*Salmonella* spp.	*B. cepacia*	37	58	4.64 *
A. vinelandii	*P. caratovora*	*B. cepacia*	44	69	5.53 *
A. vinelandii	*Yersinia* spp.	*B. cepacia*	43	70	6.45 *
A. vinelandii	*E. coli*	*B. cepacia*	38	59	4.55 *
Enterobacteriaceae X Enterobacteriaceae					
P. caratovora	*Salmonella* spp.	*A. vinelandii*	41	21	6.45 **
P. caratovora	*K. pneumoniae*	*A. vinelandii*	44	26	4.63 *
Yersinia spp.	*E. coli*	*A. vinelandii*	40	19	7.47 **
Yersinia spp.	*Salmonella* spp.	*A. vinelandii*	46	19	11.22 **
Yersinia spp.	*K. pneumoniae*	*A. vinelandii*	42	17	10.59 ***

[a] Majority sequences for the *gacA* gene were obtained for the compared bacterial species followed by spp.
[b] Significant at 5% (*), 1% (**), and 0.01% (***).

approximately 50% of the comparisons within the Enterobacteriaceae and between Enterobacteriaceae and Pseudomonaceae gave significantly different substitution rates, suggesting higher evolutionary rates for the *gacA* gene in the Enterobacteriaceae.

Evaluation of *gacA* as a complementary genetic marker for *Pseudomonas* spp. Populations of indigenous bacteria harbouring homologs of *gacA* found in *Pseudomonas* spp. were isolated from roots of wheat by colony hybridization with the GacA probe and subsequent PCR with the GACA primers. On KMB$^+$, a medium semi-selective for *Pseudomonas* (Simon and Ridge, 1974), their densities ranged from approximately 5.0 X 10^6 to 1.8 X 10^7 CFU g^{-1} root and represented on average 35.8 to 73.5% of the total bacterial population recovered on this medium (Table 5). Based on colony hybridization and subsequent PCR, 494 *gacA* positive (referred to as '*gacA*+') and 58 *gacA* negative (referred to as '*gacA*-') isolates were selected for further genotypic and phenotypic analyses (Table 6). RAPD analysis with three 10-mer primers showed 116 and 34 unique groups among the '*gacA*+' and '*gacA*-' isolates, respectively. Characterization of representatives from all RAPD groups, employing Southern hybridization

of *EcoR*I-digested genomic DNA, confirmed that all '*gacA*+' isolates responded positively with the GacA probe, whereas all 58 '*gacA*-' isolates responded negatively, even at low-stringent conditions (data not shown). Based on PCR with group-specific primers (Widmer *et al.*, 1998), all '*gacA*+' isolates were identified as *Pseudomonas*, whereas none of the 58 '*gacA*-' isolates gave a positive response. Based on GC-FAME-analysis of the '*gacA*-' isolates, 62% were classified as *Stenotrophomonas maltophilia*, 14% as *Comamonas acidovorans*, 5% as *Cryseomonas indologenes*, and 19% could not identified conclusively due to very low matching coefficients. Subsequent characterization of the isolates for the production of specific secondary metabolites and extracellular enzymes, known to be regulated by *gacA,* showed that none of the '*gacA*-' isolates displayed fluorescence, produced surfactants, or harboured biosynthetic genes for 2,4-diacetylphloroglucinol and phenazine antibiotics (Table 6).

Fluorescence and surfactant production was found in 88 and 0.6% of the '*gacA*+' isolates, respectively, and genes for 2,4-diacetylphloroglucinol and phenazine antibiotics were found in 34 and 3% of the '*gacA*+' isolates, respectively. Both '*gacA*+' and '*gacA*-' isolates produced protease and phospholipase C, enzymes that are under the control of *gacA* in the biocontrol strain *P. fluorescens* CHA0 (Sacherer *et al.*, 1994). Protease production was found in similar proportions of '*gacA*+' and '*gacA*-' isolates (~70%), whereas phospholipase C was detected in a higher proportion of the '*gacA*-' isolates. Chitinase was detected only in '*gacA*-' isolates and in particular in the isolates that were identified by GC-FAME analysis as *Stenotrophomonas maltophilia*.

Table 5. Frequency of *gacA*+ bacterial isolates in the rhizosphere of wheat grown in different soils.

Soil	Total population [a b] (CFU g^{-1} root)	GacA+ [b] (CFU g^{-1} root)	GacA/ tot. (%)
CB	1.9 X 10^7	9.8 X 10^6	52.4
SL	1.5 X 10^7	5.4 X 10^6	35.8
SU	2.9 X 10^7	1.5 X 10^7	51.0
SV	2.5 X 10^7	1.8 X10^7	73.5
SSB	2.4 X 10^7	1.7 X 10^6	69.4

[a] Total population of bacteria isolated on *Pseudomonas* semi-selective medium (Simon and Ridge, 1974).
[b] Colony hybridization followed by PCR was used to determine the frequencies. Mean values of four replicates are shown.

DISCUSSION

The response regulator gene *gacA* influences the production of several secondary metabolites in both pathogenic and beneficial *Pseudomonas* spp. (Heeb and Haas, 2001). In this study, we demonstrated that *gacA* is highly conserved within the genus *Pseudomonas*. In PCR analysis and Southern hybridization with the *gacA* primers and probe, multiple strains of different *Pseudomonas* species all responded positively, whereas no response was obtained from 18 other strains representing 14 species and 8 genera of Gram-negative bacteria other than *Pseudomonas* (Table 1). Furthermore, from a total of approximately 550 indigenous bacterial isolates obtained from the rhizosphere of wheat, all isolates that responded positively to the GacA probe and primers (referred to as *gacA*+) were classified as *Pseudomonas* spp., whereas isolates that did not hybridize with the GacA probe (referred to as *gacA*-) were identified as bacterial genera other than *Pseudomonas*, including *Stenotrophomonas*, *Cryseomonas* and *Comamonas* spp. (Table 6). Based on RAPD analyses, both *gacA*+ and *gacA*- isolates represented a large number of genotypically different groups. These results indicate that *gacA* can be used as a complementary genetic marker for detection of *Pseudomonas* spp. in rhizosphere samples. They also show that the selectivity of King's medium B for *Pseudomonas* spp. is limited, although all fluorescent bacteria recovered on this medium responded

Table 6. Phenotypic and genotypic characteristics of *gacA*+ and *gacA*- strains from the wheat rhizosphere.

Analysis [a]	***gacA*+**	***gacA*-**
Number of isolates	494	58
Unique RAPD groups [b]	116	34
2,4-diacetylphloroglucinol [c]	33.8	0.0
Phenazines [c]	3.0	0.0
Fluorescence [c]	88.0	0.0
Protease [c]	71.0	69.0
Phospholipase C [c]	19.6	70.7
Surfactants [c]	0.6	0.0
Chitinase [c]	0.0	55.2
Identity [d]	*Pseudomonas* (100%)	*Stenotrophomonas* (62%), *Comamonas* (14%), *Cryseomonas* (5%), unidentified (19%)

[a] Percentage in relation to the number of isolates.
[b] RAPD analysis made with three primers and 100% similarity was used to define a unique group.
[c] Secondary metabolites were detected as mentioned in Table 1.
[d] Identity determined with *Pseudomonas* group-specific primers targeting 16S rDNA (Widmer *et al.*, 1998), API test, and GC-FAME analysis.

positively to hybridization and PCR with the GacA probe and primers and were identified as *Pseudomonas* spp. by group-specific primers.

Although many studies have focused on the genus *Pseudomonas*, their taxonomy and phylogenetic relationships are not completely resolved (Bossis *et al.*, 2000). Among the various methods used to identify and classify pseudomonads, DNA-DNA hybridization and 16S rDNA sequencing are the most advocated ones (Wayne, *et al.*, 1987; Anzai *et al.*, 2000). The use of both methods, however, may present certain limitations. DNA-DNA homology has not been powerfull enough to reveal phylogenetic relationships (Anzai *et al.*, 1997). Also, sequences of 16S rDNA present limited power when analyzing closely related organisms that diverged at almost the same time (Fox *et al.*, 1992; Woese, 1987). Furthermore, 16S rDNA sequences occur in multiple copies in a single cell and, interestingly, these copies were shown to evolve at different rates (Ueda *et al.*, 1999).

In this study, phylogenetic relationships inferred from *gacA* sequences revealed six distinct bacterial groups, including the Pseudomonaceae, Enterobacteriaceae, Alteromonadaceae, Vibrionaceae, Burkholderia, and Xanthomonas group (Fig. 1). Within the Pseudomonaceae, polymorphisms within the *gacA* gene allowed further distinction of at least six subclusters. Although similar overall phylogenetic relationships were found for 16S rDNA sequences, some differences were noted. For example, *P. fluorescens* strains CHA0 and Pf-5 were clustered closer to *P. aeruginosa* and *P. syringae* based on 16S rDNA sequences, whereas clustering based on *gacA* gene and GacA protein sequences placed both strains closer to the other *P. fluorescens* strains. Discrepancies between phylogenies of *Pseudomonas* inferred from 16S rDNA and other genes were also observed for *gyrB* and *rpoD* (Yamamoto and Harayama, 1998; Yamamoto *et al.*, 2000). Given the relatively small size of the *gacA* gene and its apparent occurrence as a single copy in *Pseudomonas* makes the *gacA* gene not only a useful complementary genetic marker for detection of *Pseudomonas* but also eligible for phylogenetic studies. It is remarkable that a relatively small sized gene like *gacA* has such strong resolving capacity for phylogenetic purposes. Comparable results were obtained with the 249-bp *oprI* gene, which allowed molecular taxonomic studies in pseudomonads belonging to the rRNA group I, a group that contains the genuine *Pseudomonas* species (De Vos *et al.*, 1998). Interestingly, polymorphisms within *gacA* homologs also allowed distinction of five subclusters in the group of enteric bacteria, including *E. coli*, *Salmonella, K. pneumoniae, P. caratovorum*, and *Yersinia* species (Fig. 1). These results suggest that *gacA* homologs also may provide a complementary genetic marker for phylogenetic studies of Gram-negative bacteria other than

Pseudomonas. More sequences of *gacA* homologs of representative strains of different enteric and other bacterial genera will be required to further support this hypothesis.

Estimations of nonsynonymous to synonymous substitution rates (Ka/Ks) indicated that, for members of both the Pseudomonaceae and Enterobacteriaceae, purifying selection is acting on *gacA* (Table 3). These results indicate that there is selective pressure to avoid substitutions leading to functional changes in the GacA protein. In this context, it is interesting to note that for several *P. fluorescens* strains spontaneous mutations in *gacA* and also *gacS* have been described to occur frequently under laboratory conditions (Duffy and Défago, 2000; Whistler *et al.*, 1998). In *P. fluorescens* strain CHA0, grown in nutrient-rich conditions, spontaneous mutants accumulated over a period of several days to levels of 1.25% of the population, and half of the mutants tested were restored by *gacA* (Bull *et al.*, 2001). Subsequent competition experiments showed that the *gacA* mutant had only a temporary selective advantage over the wildtype that was restricted to an early phase of the stationary phase (Bull *et al.*, 2001). The role of the *gacA/gacS* pair in surviving exposure to oxidative stress (Whistler *et al.*, 1998), and in production of secondary metabolites that play a significant role in the ecological fitness of certain *Pseudomonas* strains (Mazzola *et al.*, 1992; Natsh *et al.*, 1994) provide, at least in part, an explanation for the relatively low rate of synonomous substitutions in *gacA.* Tajima's relative rate test showed that in 50% of the comparisons between members of the Enterobacteriaceae and Pseudomonadaceae, evolutionary rates in *gacA* were higher (Table 4). So, in several members of the Enterobacteriaceae the *gacA* gene appears to evolve faster than in members of the Pseudomonaceae, even though purifying selection is acting on *gacA* of both bacterial groups. The biological relevance of this difference is not clear. One may speculate that many enteric bacterial strains have a parasitic life-style and a higher exposure to therapeutic antibiotics for which a higher adaptive capacity may be required. However, the parasitic life-style also applies to several plant and human pathogenic bacterial species of the Pseudomonaceae, including *P. syringae* and *P. aeruginosa*. More studies will be necessary to elucidate the biological relevance, if any, of the relatively higher evolutionary rates of *gacA* in several members of the Enterobacteriaceae as compared to Pseudomonaceae.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Naturally occurring *Pseudomonas* spp. were isolated from roots of wheat grown in raw soils on the semi-selective King's medium B (KMB) agar supplemented with chloramphenicol (13 µg ml^{-1}), ampicillin (40 µg ml^{-1}), and cycloheximide (100 µg ml^{-1}) [KMB^+] (Simon and Ridge, 1974). Bacterial isolates were routinely grown on KMB medium. For DNA extractions, bacteria were cultivated overnight at 24°C on nutrient broth yeast extract agar (Vidaver, 1967). *Escherichia coli* strains were grown in LB (Luria Bertani). All bacterial strains were stored at -80°C in LB or KMB broth supplemented with 40% glycerol (v/v).

DNA extractions. For all PCR analyses, including RAPD analysis, heat-lysed bacterial suspensions were used and prepared as described previously (Raaijmakers *et al.*, 1997). Total DNA for use in PCR amplifications of the 16S rDNA genes was obtained as described by Raaijmakers *et al.* (1997). Total genomic DNA, used for Southern blot analysis and *gacA* amplifications, was extracted from bacterial strains by a modified version of a cetyltrimethylammoniumbromide (CTAB)-based protocol (Ausubel *et al.*, 1995). A 1.5-ml sample of bacterial cultures grown overnight was centrifuged for 3 min at 14,000 rpm; supernatant was discarded and the pellet was resuspended in 550 µl of TE buffer (Tris 10mM, EDTA 10mM, pH 8.0) amended with lysozyme (1.82 mg ml^{-1}); the mixture was incubated at 37°C for 30 min. Seventy-five µl of 10% SDS amended with proteinase K (0.86 mg ml^{-1}) was added to the bacterial suspension and thoroughly mixed. After 15 min incubation at 65°C, 100 µl of 5 M NaCl and 80 µl CTAB/NaCl (0.3 M CTAB, 0.7 M NaCl) were added. After 10 min of incubation at 65°C, DNA was obtained by extraction with chloroform/isoamyl alcohol (24:1, vol/vol), isopropanol precipitation and subsequent washes with 70% ethanol. The extracted DNA was dissolved in 100 µl of 10 mM Tris pH 8.0 containing RNAase (20 µg ml^{-1}) and stored at –20°C. Restrictions of genomic DNA were performed with 5U of *EcoR*I (Promega), an enzyme without restriction sites in the *gacA* genes of *P. fluorescens* strains BL915, CHA0, and Pf-5, *P. aeruginosa* PAO1, *P. syringae* pv. *syringae* B728a, *P. chlororaphis* 30-84, and the *repB* gene of *P. viridiflava* PJ-08-6A. Digestions were performed in a total volume of 100 µl containing 2.0 µg of DNA; digested DNA was precipitated with 4M LiCl, washed with 70% ethanol, dissolved in 15 µl of sterile distilled water and separated on 1% agarose gels in TBE.

PCR amplification of the *gacA* gene. Primers GACA1 and GACA2 were used to amplify the 425-bp fragment from different *Pseudomonas* spp. strains. Amplifications were performed in 25-µl reaction mixtures containing 3 µl of diluted, heat-lysed cell suspension, 1X PCR buffer (Perkin-Elmer, Nieuwerkerk aan de IJssel, The Netherlands), 1.5 mM $MgCl_2$ (Perkin Elmer), 200 µM of each, dATP, dCTP, dGTP, and dTTP (Promega, Leiden, The Netherlands), 20 pmol of each primer, and 2U of AmpliTaq polymerase (Perkin-Elmer). The PCR program for *gacA* consisted of an initial denaturation at 95°C for 2 min followed by 30 cycles at 95°C for 1 min, annealing at 61°C for 1 min, and extension at 72°C for 1 min. Amplifications were carried out in a MJ Research PTC-200 thermocycler.

Sequencing of the *gacA* gene. Amplifications of the *gacA* gene with primers GACA1 and GACA2 were performed with the Expand High Fidelity PCR system, following the supplier's recommendations (Roche Diagnostics GmbH, Mannheim, Germany). PCR products were run in a 1% agarose gel, bands of the appropriate size were excised from the gel and purified by using the PCR purification kit (Qiagen, Hilden, Germany). These fragments were sequenced directly by loading on an ABI sequencer.

Sequencing of 16S rDNA. Five microliters of the cell lysis suspension were used in each reaction with the primers 8f and 1406r (Amann *et al.*, 1995) using reaction conditions as described by Liu *et al.* (1997). PCR-amplified products were analyzed by electrophoresis on 2% agarose gels. Fragments of the appropriate size were cloned into the T/A vector pCR2.1 (Invitrogen, Carlsbad, CA), and transformed into *E. coli* strain INVαF'. Transformants were selected on LB agar amended with kanamycin (75 µg ml^{-1}) and X-Gal (20 µg ml^{-1}). Plasmid DNA was isolated from transformants using the S.N.A.P. mini-prep kit (Invitrogen). Cycle sequencing was conducted on a Perkin-Elmer model 480 thermal cycler (Norwalk, CT) and PRISM Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit (PE Biosystems, Warrington, UK). Reactions were conducted using cycling conditions recommended by the manufacturer with the T3 or T7 primer. Products of the sequencing reactions were purified using CENTRI-SEP spin columns (Princeton Separations, INC., Adelphia, NJ) and dried in a vacuum centrifuge. Samples were resuspended in 6 µl of a solution containing de-ionized formamide and blue dextran/EDTA in a ratio of 5:1. Samples were heated for 2 min at 90°C and 1.5 µl was loaded on an ABI 377 DNA sequencer (PE Biosystems, Foster City, CA). BLASTN searches were conducted on the nucleotide sequences obtained.

Nucleotide sequence accession number of *gacA* and 16S rDNA. Newly obtained sequences were deposited with GenBank under accession numbers shown in Table 2.

Phylogenetic analysis. Prior to analysis, sequences of the *gacA* gene and its homologs were separated into the following categories: 1) all *gacA* sequences together (44 sequences); 2) *gacA* sequences from the Pseudomonadaceae and Enterobacteriaceae (36 sequences); 3) only *gacA* sequences from Pseudomonadaceae (22 sequences); and 4) only *gacA* sequences from Enterobacteriaceae (14 sequences). Amino acid and nucleotide sequences were aligned by using the CLUSTAL W 1.81 program (Thompson *et al.*, 1994). Pairwise nonsynonymous (Ka) and synonymous (Ks) nucleotide substitutions in the *gacA* sequences were calculated according to the Nei-Gojobori's method (Nei and Gojobori, 1986) as implemented in MEGA (Kumar *et al.*, 2001). Fisher's exact test and Z-test for selection based on codon usage were performed by the Nei-Gojobori's method with the number of synonymous differences per synonymous site and the number of nonsynonymous differences per nonsynonymous site. Significance values for the Z-test were determined by using 1,000 bootstrap replicates.

Majority sequences of *gacA* of species belonging to the Pseudomonaceae and Enterobacteriaceae were computed using Megalign (DNAstar Inc., WI, USA) to reduce the number of two-by-two comparisons by the Tajima's test. Tajima's 1D relative rate test (Tajima, 1993) was used to estimate pairwise rate variation between majority sequences of *gacA* with the closest relative as outgroup. Phylogenetic trees of *gacA* and 16S rDNA were constructed by the PHYLIP 3.5c Package (Felsenstein, 1993), MEGA, and Treecon (Van de Peer and De Wachter, 1994) using the Neighbour-joining method (Saitou and Nei, 1987) with the nucleotide substitution model of Jukes and Cantor (1969). For comparison purposes, similar analyses were performed with the maximum likelihood method. Phylogenetic trees with the GacA protein sequences were constructed using the Neighbour-joining method with the PAM distance matrix of Dayhoff (Dayhoff *et al.*, 1978) and were computed with the program PROTDIST of PHYLIP. Confidence of the tree topology was performed by bootstrap analysis on 1,000 resamplings (Felsenstein, 1985).

Southern and colony hybridizations. DNA transfer from agarose gels and bacterial colony transfer to Hybond N^+ nylon membranes (Amersham Pharmacia Biotech) were performed according to standard methods (Sambrook and Russel, 2001). Membrane washes and hybridizations were performed following standard procedures (Sambrook and Russel, 2001). High-stringent conditions comprised pre-hybridization for 1.5 h at 65°C, hybridization for 12 h at 65°C, membrane washings twice each for 5 min with 2X SSC (1X SSC is 0.15 M NaCl plus 0.015 M sodium citrate)-0.1% SDS at room temperature followed by membrane washings twice each for 30 min with 0.1X SSC-0.1% SDS at 65°C. Low stringent conditions consisted of pre-hybridization and hybridization at 55°C and two membrane washings with 0.5X SSC at 55°C. The GacA probe was generated by direct labeling in PCR using the DIG labeling mix (Roche, Almere, The Netherlands). Hybridized probes were immunodetected according to the protocols provided by the supplier.

Soils. Five soils were obtained from agricultural fields in the Netherlands. All soils were collected from the upper 50 cm of the soil profile, air dried for a week, and passed through a 0.5-cm mesh screen prior to use. Soils CB, SU, SV, and SSB were collected in December 1997 from a polder in the South-West of the Netherlands, 10 km from the city of Bergen op Zoom. These soils are physicochemically very similar, containing on average, 27% clay, 10% silt, and 51% sand. The organic matter content is on average 4.3% and the pH, determined after extraction with $CaCl_2$ is 7.5. Soil SL was collected in June 1998 from a polder in the North-East of the Netherlands, at Lovinkhoeve, located 30 km from the city of Lelystad. SL soil contains 21% clay, 12% silt, 54% sand, 3.6 % organic matter, and pH, determined after extraction with $CaCl_2$ is 7.5.

Plant cultivation. Pots containing 200 g of sieved soil were sown with 15 seeds of wheat, cv. Bussard. Plants were grown in a climate chamber at 15°C with a 12-h photoperiod. Plants received 50 ml of 1/3 strength Hoagland's solution (macroelements only) twice a week. After 30 days of plant growth, the roots were harvested and used for isolation of root-associated bacteria.

Isolation of bacteria from the wheat rhizosphere. Three to five plants of each replicate were randomly harvested and loosely adhering soil was removed. Root samples of 0.2 to 1.0 gram were vortexed for 1 min, sonicated for 1 min in a ultrasonic cleaner (Bransonic 12, Branson Ultrasonics Corp., Geneva, Switzerland), and dilution plated onto KMB^+. Plates were incubated at 25°C for 48 h. Populations of bacteria harbouring *gacA* homologs were determined by colony hybridization with the GacA probe followed by confirmation by PCR with the GacA primers. Colonies that reacted positively in hybridization with the GacA probe and primers (*gacA*+ isolates) were isolated and purified for further analysis; several colonies that reacted negatively in hybridization and subsequent PCR with the GacA probe and primers (*gacA*- isolates) were also included.

Characterization of rhizosphere bacteria harbouring *gacA* homologs. Isolates obtained from the rhizosphere of wheat were genotypically identified and characterized for the production of specific extracellular enzymes and secondary metabolites known to be regulated by *gacA*. The first genotypic identification of the isolates was by PCR analysis with primers specific for the 16S rDNA of the genus *Pseudomonas* (sensu stricto) (Widmer *et al.*, 1998). Secondly, isolates were clustered in genotypic groups by Random Amplified Polymorphic DNA (RAPD) analysis with primers M13, M12, and D7 (Keel *et al.*, 1996). The presence or absence of fragments generated by RAPD analysis (1 for the presence or 0 for the absence of a particular sized band in the gel) was used to calculate the pairwise coefficients of similarity (Nei/Li distances). RAPD analysis was performed three times for each primer. Gas chromatograph-fatty acid methyl ester (GC-FAME) analysis was performed to identify *gacA*- bacterial isolates. For GC-FAME analysis, isolates were cultivated on tryptic soy broth agar (Becton Dickinson, Cockeysville, MD) and incubated for 24 h at 28°C. Cells were collected with a 4 mm diameter transfer loop and processed for extraction of fatty acids using the procedures as outlined by the manufacturer (Microbial ID, Inc., Newark, DE). Fatty acid methyl esters were analyzed using a Microbial Identification System equipped with an HP5890 series II gas chromatograph, HP3365 Chem Station and version 3.9 of the aerobe library (Microbial ID, Inc.).

For all isolates, presence of genes involved in the biosynthesis of specific antibiotics was performed with primers Phl2a and Phl2b for 2,4-DAPG (Raaijmakers *et al.*, 1997) and primers PHZ1 (5'-GGCGACATGGTCAACGG) and PHZ2 (5'-CGGCTGGCGGCGTATTC) for phenazine antibiotics (Mavrodi *et al.*, unpublished data). Protease, phospholipase C, and chitinase activities were detected by growing the bacterial isolates on skim milk agar, egg yolk agar (Sacherer *et al.*, 1994), and chitin-agar (De Boer *et al.*, 1998), respectively. Production of biosurfactants and fluorescence of the isolated bacteria was determined by growth on medium containing CTAB-methylene blue (Siegmund and Wagner, 1991) and *Pseudomonas* medium F (Cho and Tiedje, 2000), respectively.

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CHAPTER 4

FREQUENCY, DIVERSITY, AND ACTIVITY OF 2,4-DIACETYLPHLOROGLUCINOL-PRODUCING FLUORESCENT *PSEUDOMONAS* SPP. IN DUTCH TAKE-ALL DECLINE SOILS

ABSTRACT

Natural suppressiveness of soils to take-all disease of wheat, referred to as take-all decline (TAD), occurs worldwide. It has been postulated that different microbial genera and mechanisms are responsible for TAD in soils from different geographical regions. We demonstrated that fluorescent *Pseudomonas* spp. that produce the antibiotic 2,4-diacetylphloroglucinol (2,4-DAPG) play a key role in the natural suppressiveness of two Dutch TAD soils. First, 2,4-DAPG-producing fluorescent *Pseudomonas* spp. were present on roots of wheat grown in both of the TAD soils at densities at or above the threshold density required to control take-all of wheat; in a complementary take-all conducive soil, population densities of 2,4-DAPG-producing *Pseudomonas* spp. were below this threshold level. Second, introduction of 2,4-DAPG-producing strain SSB17, a representative of the dominant genotypic group found in the Dutch TAD soils, into the take-all conducive soil at population densities similar to the densities of indigenous 2,4-DAPG-producers found in TAD soils provided control of take-all to the same level as found in the TAD soil. Third, a mutant of strain SSB17 deficient in 2,4-DAPG-production was not able to control take-all of wheat, indicating that 2,4-DAPG is a key determinant in take-all suppression. These results show that in addition to the physicochemically different TAD soils from Washington State (USA), 2,4-DAPG-producing fluorescent *Pseudomonas* spp. are also a key component of the natural suppressiveness found in Dutch TAD soils. Furthermore, it is the first time since the initial studies of Gerlagh (Neth. J. Plant Pathol. (1968) 74:1-97) that at least part of the mechanisms and microorganisms that operate in Dutch TAD soils are identified. In spite of quantitative similarities, 2,4-DAPG-producing *Pseudomonas* spp. found in the Dutch TAD soils are genotypically different from those found in TAD soils from Washington State.

Jorge T. de Souza, David M. Weller, and Jos M. Raaijmakers

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INTRODUCTION

Take-all, caused by the fungus *Gaeumannomyces graminis* var. *tritici*, is an important root disease of wheat worldwide (Asher and Shipton, 1981). Although wheat is particularly susceptible to the take-all fungus, many other Gramineae can be infected (Shipton, 1975; Gutteridge *et al.*, 1993). Take-all decline (TAD) is defined as the spontaneous decrease in the incidence and severity of take-all that occurs with monoculture of wheat or other susceptible host crops after one or more severe outbreaks of the disease (Cook and Baker, 1983; Hornby, 1983; Shipton *et al.*, 1973; Shipton, 1975). Soils in the state of TAD are naturally suppressive to the take-all fungus. The biological basis of the specific suppression associated with TAD has been demonstrated in a series of experiments: suppressiveness is eliminated by treating the soil with moist heat (pasteurization, 60°C for 30 min.), methyl bromide or chloropicrin, and is transferable by adding small amounts of TAD soil to raw conducive, fumigated or pasteurized soil (Cook and Rovira, 1976; Cook and Baker, 1983; Raaijmakers and Weller, 1998). The mechanisms responsible for TAD involve microbiological changes in the bulk soil or rhizosphere environment resulting in suppression of the pathogen. Repeated introduction of *G. graminis* var. *tritici* into soil also induced suppression of take-all (Gerlagh, 1968; Zogg and Jaggi, 1974; Wildermuth, 1980), but the relation of this form of suppressiveness to that of TAD is not clear.

The occurrence of TAD throughout the world is remarkable in view of the broad range of soil types, climates and agronomic conditions under which wheat is cultivated (Shipton, 1975). Field studies have clearly indicated that the development of TAD follows a consistent pattern everywhere requiring the presence of three components: monoculture of a take-all susceptible host, the presence of *G. graminis* var. *tritici*, and at least one occurrence of severe disease. Factors such as soil type and previous cropping history seem only to modulate the extent and speed of development of TAD (Shipton, 1975). Previous work on TAD has suggested that different microbial genera and mechanisms are responsible for suppressiveness in soils from different geographical regions. First, TAD develops in multiple agro-ecosystems (Shipton, 1975; Brown, 1981). Second, the length of time for TAD to develop varies among fields and the crop grown in monoculture (Shipton, 1975). Finally, antagonists from many different taxonomic groups have been isolated from TAD soils and, when introduced into take-all conducive soils, provide a certain level of control of take-all (Deacon, 1976; Shipton, 1975; Hornby, 1983; Wong and Baker, 1984; Aberra *et al.*, 1998; Weller *et al.*, 1988). Despite the wide range of microorganisms implicated in TAD, several of them do not fit one or more of the biological properties of TAD (Cook, 1985). For example, the sensitivity of the TAD factor(s) to pasteurization with moist heat (60°C, 30 min) rules out the involvement of heat-resistant,

spore-forming bacteria like *Bacillus* spp. and probably also actinomycetes (Cook and Baker, 1983).

Among the antagonistic bacteria, the fluorescent *Pseudomonas* spp. have been implicated in TAD soils throughout the world (Cook and Rovira, 1976; Smiley, 1978; Weller *et al.*, 1988; Sarniguet *et al.*, 1992; Raaijmakers and Weller, 1998). Characteristics that suggest involvement of pseudomonads in TAD include: they are well adapted to the rhizosphere environment and produce a variety of secondary metabolites, including antibiotics and siderophores, that inhibit the growth of *G. graminis* var. *tritici* (Weller *et al.*, 1988; Thomashow and Weller, 1990), their population densities increase considerably on roots with take-all lesions (Weller, 1983; Smiley, 1978; 1979; Brown, 1981; Sarniguet *et al.*, 1992; Barnett *et al.*, 1999; Charigkapakorn and Sivasithamparam, 1987), and they are eliminated by soil pasteurization, which also eliminates specific suppression (Cook and Baker, 1983; Raaijmakers and Weller, 1998). Recent studies demonstrated that fluorescent *Pseudomonas* spp. producing the antibiotic 2,4-diacetylphloroglucinol (2,4-DAPG) play a key role in the suppressiveness of TAD soils in Washington State (Raaijmakers *et al.*, 1997; Raaijmakers and Weller, 1998, 1999). Using specific primers and probes directed against sequences within the biosynthetic locus of 2,4-DAPG have clearly indicated that fluorescent *Pseudomonas* spp. producing this antifungal metabolite are highly enriched in TAD soils from Washington State (Raaijmakers *et al.*, 1997). Elimination of 2,4-DAPG producers resulted in loss of suppressiveness (Raaijmakers and Weller, 1998). When a 2,4-DAPG-producing strain was introduced via seed treatment into a take-all conducive soil, it provided control of take-all of wheat to levels similar to that obtained in the complementary TAD soil (Raaijmakers and Weller, 1998).

One key question has been whether 2,4-DAPG-producing *Pseudomonas* spp. also play a key role in the specific suppression found in TAD soils from other geographical regions. *Pseudomonas* spp. harboring *phlD* (*phlD*+), one of the key genes in the biosynthesis of 2,4-DAPG, were found on roots of wheat grown in other soils with a history of wheat monoculture (McSpadden-Gardener *et al.*, 2000), however, the level of take-all suppressiveness of these soils and the role of 2,4-DAPG in take-all suppression were not addressed. In this study, we determined the frequency, diversity and activity of 2,4-DAPG-producing *Pseudomonas* spp. found in Dutch polder soils with a history of wheat monoculture. Dutch polder soils were among the first TAD soils reported (Gerlagh, 1968), however, the exact mechanisms operating in these soils have not been elucidated. Phenotypic and genotypic analyses were performed to characterize 2,4-DAPG-producing *Pseudomonas* isolates obtained from Dutch soils. The biocontrol efficacy and rhizosphere competence of *P. fluorescens* SSB17, a representative of the

major genotypic group of 2,4-DAPG-producers found in a Dutch soil, were compared to that of Q8r1-96, a representative of the major genotypic group of 2,4-DAPG-producers found in TAD soils from Washington State (Raaijmakers and Weller, 2001). The role of 2,4-DAPG in control of take-all and in rhizosphere competence was determined for both strains by generating mutants deficient in 2,4-DAPG production. Finally, we determined whether populations of 2,4-DAPG-producing *Pseudomonas* spp. are specifically enriched upon infection of wheat roots by the take-all fungus.

RESULTS

Natural suppressiveness of soils to *G. graminis* var. *tritici*. Characteristics of soils CB, SV, and SSB are shown in Table 1. Physical and chemical properties of these soils differ from TAD soils from Washington State (Raaijmakers *et al.*, 1997). Major differences are that the Dutch soils have lower levels of nitrate, phosphorus, potassium, and silt content, and higher levels of organic matter and clay content compared to the four soils from Washington State. The three Dutch soils were tested in bioassays for suppressiveness to *G. graminis* var. *tritici*. When these soils were inoculated with *G. graminis* var. *tritici*, the disease index was significantly lower on roots of plants grown in SV and SSB soils as compared to plants grown in CB soil (Fig. 1). No take-all symptoms were found on roots of wheat grown in soils not inoculated with *G. graminis* var. *tritici* (data not shown). Based on these results, and the occurrence of long term wheat monoculture in the SV and SSB soils, they were classified as TAD soils, whereas CB soil was regarded as a *G. graminis* var. *tritici* -conducive soil.

Frequency and diversity of 2,4-DAPG-producing *Pseudomonas* spp. in Dutch TAD soils. In CB soil, wheat rhizosphere population densities of *phlD*+ *Pseudomonas* spp. were relatively low and ranged from less than 10^4 to 2.7 x 10^4 CFU g^{-1} root (Table 2). In the SV and SSB soils, populations of *phlD*+ fluorescent *Pseudomonas* spp. ranged from 5.1 x 10^4 to 1.4 x 10^6 CFU g^{-1} root (Table 2). These populations represented approximately 5 to 14% of the total population of fluorescent pseudomonads isolated from wheat roots and were, on average, 5 to 52-fold higher than populations of *phlD*+ fluorescent *Pseudomonas* spp. found on roots of wheat grown in the CB soil. These results demonstrate a correlation between relatively high populations of *phlD*+ fluorescent *Pseudomonas* spp. and suppressiveness to take-all of wheat.

Analysis of the genotypic diversity of the isolated *phlD*+ fluorescent *Pseudomonas* spp. by RAPD analysis with primers M13, M12 and D7 generated a total of 58, 56 and 48

consistent RAPD markers, respectively, ranging in size from 190 to 2070 bp. Reproducibility of the RAPD amplifications was confirmed in three independent experiments and the reliability of the dendrogram topology (Fig. 2) was evaluated by bootstrap analysis using 1000 resampled data sets. Among a total of 161 isolates of *phlD*+ fluorescent *Pseudomonas* spp. obtained from roots of wheat grown in SV and SSB soil, 33 unique groups were identified and only one RAPD group, represented by isolates SSB14 and SV7, was found in both soils (Fig. 2; Table 3). The major genotypic groups found on roots of wheat grown in SV and SSB soils represented on average 40 and 26%, respectively, of the total number of *phlD*+ fluorescent *Pseudomonas* spp. isolates from SV and SSB soils (Table 3). The major genotypic group (SV8) found in SV soil clustered very distantly from the major genotypic group (SSB17) found in SSB soil (Fig. 2). Isolates from SV and SSB soils clustered distantly from 2,4-DAPG-producing reference strains CHA0, Pf5 and Q8r1-96, and from 2,4-DAPG-nonproducing isolates (groups SV12, SV13, and SSB24) obtained from SV and SSB soils (Fig. 2). The Shanon-Weaver's diversity index was higher for populations of *phlD*+ fluorescent *Pseudomonas* spp. isolated from SSB soil than for those from SV soil (Table 3), indicating a higher level of genotypic diversity in SSB soil.

In addition to genotypic clustering by RAPD analysis, also data from plate inhibition assays were used to phenotypically group the isolates on the basis of their capacity to inhibit *G. graminis* var. *tritici* and *R. solani*. Based on *G. graminis* var. *tritici* mycelial inhibition, a total of seven clusters was obtained. Five of these clusters (I to V) contained isolated 2,4-DAPG producers and reference strains Pf-5, CHA0, and Q8r1-96, and two clusters (VI and VII) contained isolates that did not produce 2,4-DAPG. Thirteen clusters were obtained based on inhibition of mycelial growth of *R. solani*. Clusters I to XI contained isolated 2,4-DAPG producers and reference strains and clusters XII and XIII contained non-2,4-DAPG producers (Fig. 3). Comparison of the cluster analyses indicates that 2,4-DAPG-producing isolates are more inhibitory *in vitro* to *G. graminis* var. *tritici* than to *R. solani* (Fig. 3). The mean inhibition of mycelial growth by *phlD*+ fluorescent *Pseudomonas* spp. isolates and 2,4-DAPG-producing reference strains ranged from approximately 35 to 58% for *G. graminis* var. *tritici* and from approximately 11 to 43% for *R. solani*. For 2,4-DAPG non-producing isolates, inhibition of *G. graminis* var. *tritici* ranged from 0 to 30% and for *R. solani* inhibition ranged from 0 to 20%. Most phenotypic groups contained more than one RAPD group and isolates belonging to the same RAPD group (based on 100% similarity) were always grouped in the same phenotypic group (Fig. 2; Fig. 3). Strain SSB17, which is a representative of the major RAPD group found on roots of wheat grown in SSB soil (Fig. 2; Table 3), clustered in the

phenotypic group that was most inhibitory to *G. graminis* var. *tritici* (Fig. 3) and was selected for further studies. Strain Q8r1-96, which represents the major RAPD group among 2,4-DAPG-producing *Pseudomonas* spp. isolated from wheat grown in the Quincy TAD soil from Washington State (USA) (Raaijmakers and Weller, 2001) was included for comparison purposes. Q8r1-96 clustered in the same *G. graminis* var. *tritici* -phenotypic group as SSB17 (Fig. 3), but is genotypically different (Fig. 2).

Role of 2,4-DAPG-producing *Pseudomonas* spp. in Dutch TAD soils. If 2,4-DAPG-producing fluorescent *Pseudomonas* spp. play a determinative role in the natural suppressiveness of Dutch TAD soils, then introduction of a 2,4-DAPG-producing strain into the conducive soil should give suppressiveness to *G. graminis* var. *tritici* to a level similar to that obtained in the complementary TAD soil. Furthermore, if 2,4-DAPG is a key metabolite in suppression of *G. graminis* var. *tritici* then 2,4-DAPG-deficient mutants should have a reduced ability to suppress take-all of wheat. Tn*5* mutagenesis of *P. fluorescens* strains SSB17 and Q8r1-96 generated 1078 and 686 transformants, respectively. For strains SSB17 and Q8r1-96, 4 and 2 transformants, respectively, did not produce the red pigment characteristic of 2,4-DAPG producers. RAPD analysis confirmed strain integrity of all 6 mutants and HPLC analysis showed that all 6 mutants were defective in 2,4-DAPG production. In plate inhibition assays wildtype strains SSB17 and Q8r1-96 caused clear inhibition of mycelial growth of *G. graminis* var. *tritici*, *R. solani*, and *P. ultimum* var. *sporangiiferum,* whereas the mutants caused no inhibition of these pathogens (data not shown). Southern blot analysis revealed that all mutants had a single transposon insertion. Mutants 9H4, derived from SSB17, and 4C5, derived from Q8r1-96, were selected for the bioassays.

Strain SSB17 and its 2,4-DAPG-deficient mutant 9H4 were introduced via seed treatment into the take-all conducive CB soil, amended with *G. graminis* var. *tritici* inoculum, at initial densities of approximately 10^3 CFU seed^{-1}. SSB17 and 9H4 established rhizosphere population densities of 5.0 x 10^6 and 9.0 x 10^6 CFU g^{-1} root, respectively, after 4 weeks of plant growth. Population densities of the wildtype and mutant were not significantly different and were similar to the density of indigenous 2,4-DAPG-producers (5.0 x 10^6 CFU g^{-1} root) found on roots of wheat grown in the SSB soil amended with *G. graminis* var. *tritici* inoculum. Treatment of seeds with strain SSB17 significantly reduced take-all severity and provided control to the same level as found in the SSB TAD soil (Fig. 4A). Mutant 9H4 did not reduce disease severity caused by *G. graminis* var. *tritici* (Fig. 4A). These results provide further evidence that *phlD*+ fluorescent *Pseudomonas* spp. are a key component of the suppressiveness

Table 1. Cropping history, physicochemical properties of the soils used in this study.

Soil	Cropping history	Wheat cultivar	pH [a]	Content (µg g^{-1} of soil)					Content (%)			
				NO_3-N	NH_4-N	Na	P	K	OM [b]	Sand	Silt	Clay
CB	Rotation wheat/sugar beet	Bussard	7.6	3	2	14	1	74	4.4	49	12	27
SV	14 years continuous wheat	Monopol	7.4	4	2	10	2	74	4.4	52	9	27
SSB	27 years continuous wheat	Bussard	7.5	3	2	9	2	89	4.4	50	10	28

[a] pH was determined after extraction with $CaCl_2$.
[b] Organic matter.

Table 2. Frequency of *phlD*+ fluorescent *Pseudomonas* spp. in the wheat rhizosphere.

Soil	First experiment			Second experiment		
	Total [a] fluorescent *Pseudomonas* spp.	*phlD*+ [b] fluorescent *Pseudomonas* spp.	% *phlD*+ [c] fluorescent *Pseudomonas* spp.	Total [a] fluorescent *Pseudomonas* spp.	*phlD*+ [b] fluorescent *Pseudomonas* spp.	% *phlD*+ [c] fluorescent *Pseudomonas* spp.
CB	2.7×10^5	$< 10^4$	< 0.1	1.2×10^6	2.7×10^4	2.3
SV	5.7×10^5	5.1×10^4	9.0	4.5×10^6	2.1×10^5	4.8
SSB	1.1×10^6	1.1×10^5	14.0	1.1×10^7	1.0×10^6	12.7

[a] Total population of fluorescent *Pseudomonas* spp. (CFU g^{-1} root).
[b] Population of fluorescent *Pseudomonas* spp. harboring the *phlD* gene (CFU g^{-1} root).
[c] Percentage of fluorescent *Pseudomonas* spp. harboring the *phlD* gene in relation to the total fluorescent *Pseudomonas* population.

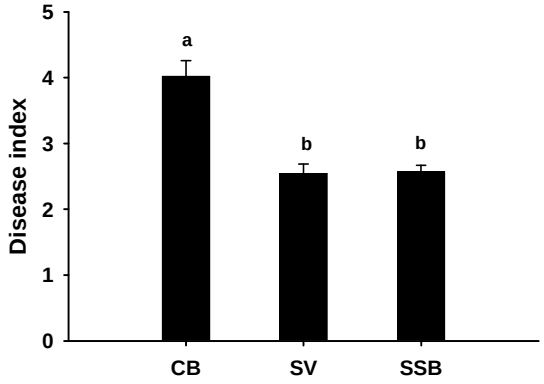

Figure 1. Natural suppressiveness of soils CB, SV, and SSB to *G. graminis* var. *tritici*. Soils were amended with 0.1% of an oat grain inoculum of *G. graminis* var. *tritici*. Plants were grown for one month under controlled conditions and disease caused by *G. graminis* var. *tritici* was scored on a 0 to 8 scale, where 0 indicates no disease and 8 indicates dead plant. Means of five replicates are shown. Means with the same letter are not statistically different according to Tukey's studentized range test (P = 0.05). Error bars represent the standard error of the mean. The experiment was repeated 4 times and representative results are shown.

that operates in the SSB TAD soil and that 2,4-DAPG is the major determinant of take-all suppression by strain SSB17. In independent experiments, similar results were found for strain Q8r1-96 and its 2,4-DAPG deficient mutant 4C5 (Fig. 4B). Although there were no differences between strains SSB17 and Q8r1-96 in their ability to suppress take-all, there was a significant difference in their rhizosphere competence (Fig. 5). In spite of the fact that strain Q8r1-96 was isolated from a different wheat cultivar grown in a physicochemically different soil from another geographical region, it was more rhizosphere competent than strain SSB17. Dose-response studies showed that strain Q8r1-96 required lower initial densities on seeds than strain SSB17 to reach the same final population on roots (Fig. 5). When comparing dose-response kinetics of the wildtype strains SSB17 and Q8r1-96 with those obtained for their respective 2,4-DAPG-deficient mutants, 9H4 and 4C5, no differences were found (Fig. 5). These results suggest that 2,4-DAPG production does not contribute to the rhizosphere competence of strains SSB17 and Q8r1-96.

Effect of take-all and *Rhizoctonia* root rot on rhizosphere population densities of 2,4-DAPG-producing fluorescent *Pseudomonas* spp. The influence of *G. graminis* var. *tritici* and *R. solani* infections on wheat root colonization by indigenous and introduced *phlD*+ fluorescent *Pseudomonas* spp. was studied. Population densities of indigenous pseudomonads and *phlD*+ fluorescent *Pseudomonas* spp. were assessed on healthy and *G. graminis* var. *tritici*- or *R. solani*-infected roots of wheat grown in SV and SSB soils, and in more detail on non-

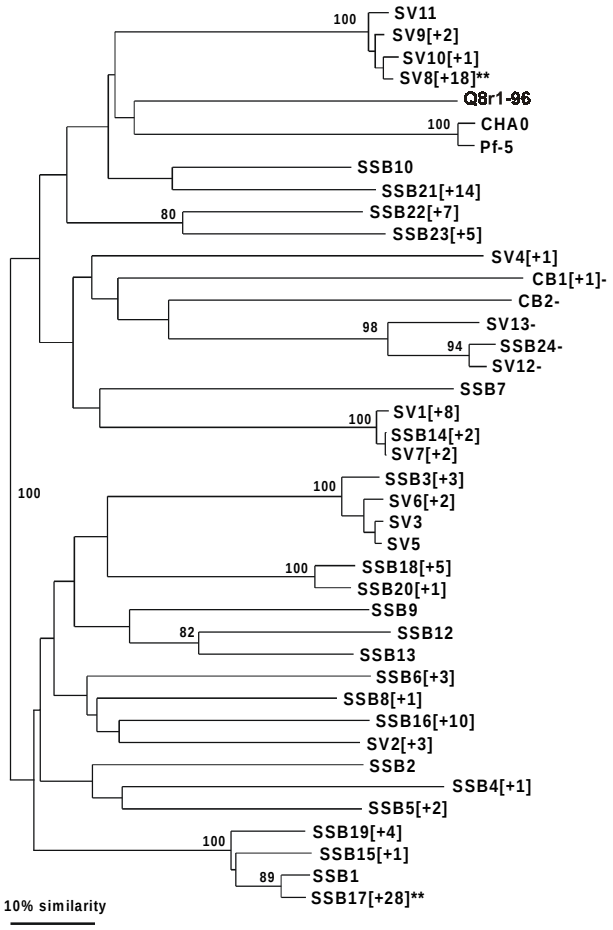

Figure 2. Dendrogram of *phlD*+ fluorescent *Pseudomonas* spp. strains based on RAPD markers. The pairwise coefficients of similarity (Nei/Li distances) were clustered using the neighbour-joining method. On roots of wheat grown in soil, SV, 11 (SV1 to SV11) different genotypic groups of *phlD*+ isolates were found on the basis of 100% similarity. In soil SSB, 23 (SSB1 to SSB23) different groups were found. Each branch represents a unique genotypic group. Isolates followed by a "-" are *phlD*- fluorescent *Pseudomonas* spp. Numbers between brackets represent the additional number of isolates in the RAPD group. Asteriscs indicate the major genotypic groups in SV and SSB soils. Bootstrap values were calculated for 1000 resampled data sets and only values equal or higher than 80% are shown.

lesioned and lesioned root sections of *G. graminis* var. *tritici*- or *R. solani*-infected roots (Tables 4, and 5). When roots of wheat grown in SV and SSB soils were infected with *G. graminis* var. *tritici*, or *R. solani*, densities of *phlD*+ fluorescent *Pseudomonas* spp. on the whole root systems increased significantly by 5 to 8-fold. Identical increases were observed for the total fluorescent *Pseudomonas* population on *G. graminis* var. *tritici* -infected roots and on roots infected with *R. solani.* When population densities of both total and *phlD*+ fluorescent *Pseudomonas* spp.

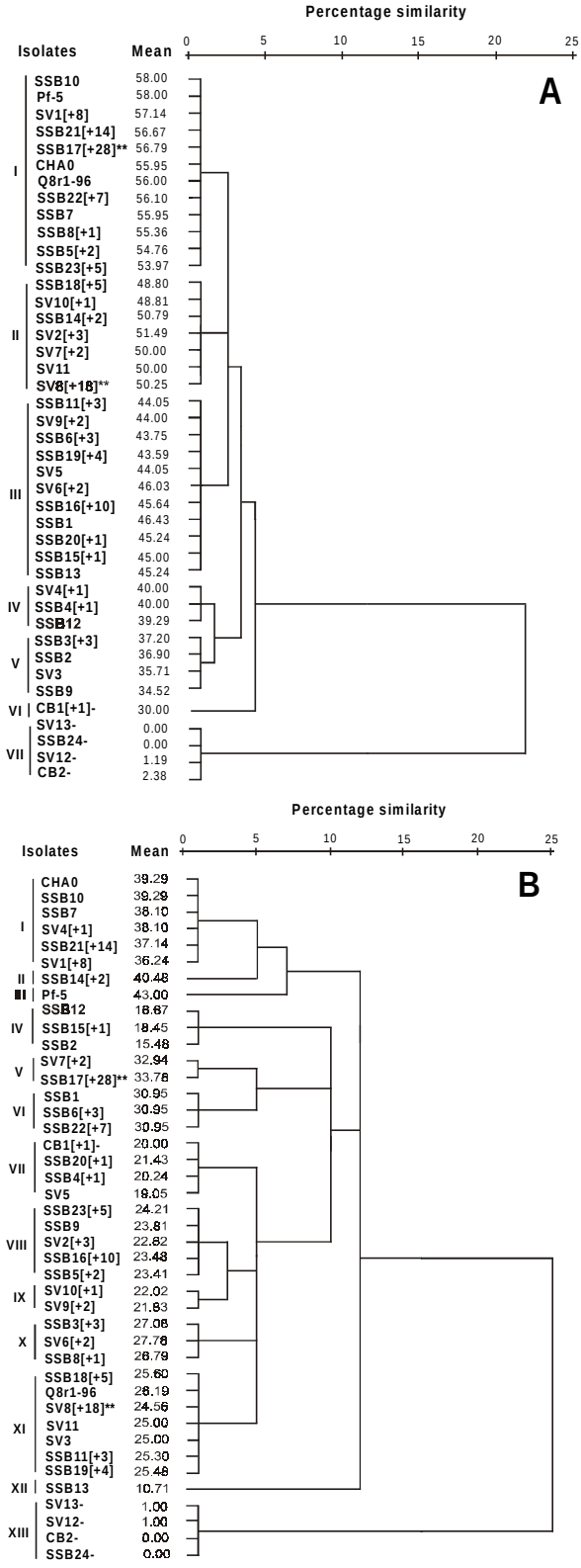

Figure 3. Cluster analysis of *in vitro* inhibition of *G. graminis* var. *tritici* (A) and *R. solani* (B) by *phlD*+ fluorescent *Pseudomonas* spp. Hierarchical, single linkage cluster analysis was computed using the mean inhibition of mycelial growth on plates given by a certain *Pseudomonas* isolate when compared to the control (the fungus only). Numbers between brackets represent the number of additional isolates in the cluster. Asteriscs indicate the major genotypic groups in SV and SSB soils. Isolates followed by a "-" are *phlD*- fluorescent *Pseudomonas* spp. Mean refers to the mean percentage inhibition relative to the control.

Table 3. Clustering of *phlD*+ fluorescent *Pseudomonas* spp. by RAPD analysis[a].

Origin/type of isolate	Number of isolates	RAPD groups [b]	Major groups [c] (%)	Shanon-Weaver index [d]
SV soil	48	11	39.6 (SV8)	32.2
SSB soil	113	23	25.7 (SSB17)	84.2
SV and SSB soils [e]	6	1	-	-
Non-2,4-DAPG producers [f]	6	5	-	-
Reference strains [g]	3	3	-	-

[a] *phlD*+ isolates were obtained from roots of wheat grown in SV and SSB soils.
[b] RAPD groups were defined with primers D7, M12, and M13 on the basis of 100% similarity.
[c] Major group refers to the percentage of the total number of *phlD*+ fluorescent *Pseudomonas* represented by a single dominant genotype.
[d] Calculated by using the formula $H=\sum[pi\text{-}ln(pi)]$, where pi is the proportion of each RAPD group (based on 100% similarity) in relation to the total number of RAPD groups per soil.
[e] Present in both, SV and SSB soils.
[f] *phlD*- isolates include CB1, CB2, CB3, SV12, SV13, and SSB24 and were obtained from roots of wheat grown in CB, SV and SSB soils.
[g] Reference strains include *P. fluorescens* strains CHA0, Pf-5, and Q8r1-96.

were compared in more detail on lesioned and non-lesioned root sections of infected roots, *G. graminis* var. *tritici* infection led to increases ranging from 4 to 15-fold. Again, no significant differences were found between increases in population densities of total and *phlD*+ fluorescent *Pseudomonas* spp. for the SV soil (P = 0.60) and SSB soil (P = 0.60). Population densities of *phlD*+ fluorescent *Pseudomonas* spp. were up to 6 fold greater on *R. solani*-lesioned root sections than on non-lesioned root sections. These increases were not statistically significant different from the increases observed on *G. graminis* var. *tritici* -lesioned root sections for both SV and SSB soils (P = 0.26 and P = 0.47, respectively).

The effect of *G. graminis* var. *tritici* on root colonization was also determined for *P. fluorescens* strains SSB17, Q8r1-96, and their respective 2,4-DAPG-defective mutants, 9H4 and 4C5, all of which were introduced at two different initial densities (Table 6). After 3 weeks of plant growth, rhizosphere population densities of the wildtype strains and their respective mutants were not significantly different in presence or in absence of *G. graminis* var. *tritici*. Population densities of SSB17 and 9H4 were significantly lower than population densities of Q8r1-96 and 4C5, irrespective of the presence of *G. graminis* var. *tritici*. For both initial densities of the introduced strains, infections of wheat roots by *G. graminis* var. *tritici* did not lead to significant increases in their population densities.

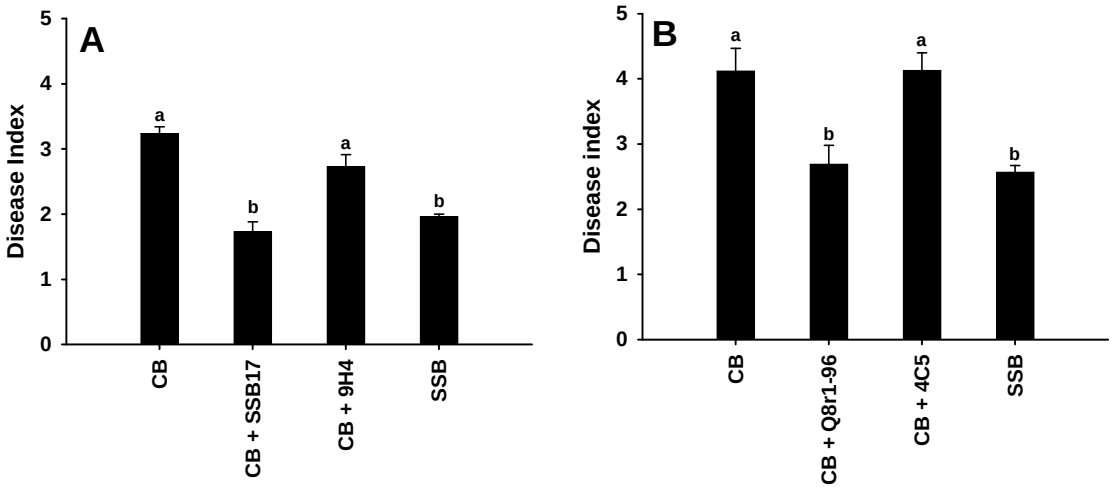

Figure 4. Effect of *P. fluorescens* strains SSB17, and its 2,4-DAPG-deficient mutant, 9H4, (A) and Q8r1-96, and its 2,4-DAPG-deficient mutant, 4C5, (B) on infection of wheat by *G. graminis* var. *tritici*. Seeds treated with bacterial strains at densities of approximately 10^3 CFU $seed^{-1}$ were sown in CB soil amended with *G. graminis* var. *tritici* inoculum. Untreated seeds sown in CB and SSB soils served as controls. Plants were grown for one month and the severity of take-all was assessed on a 0 to 8 scale. Means of five replicates are shown. Means with the same letter are not statistically different according to Tukey's studentized range test (P = 0.05). Error bars represent standard errors of the means. The experiment was repeated 2 times and representative results are shown.

DISCUSSION

The results of this study show that 2,4-DAPG-producing *Pseudomonas* spp. play a key role in the natural suppressiveness of two Dutch TAD soils. First, 2,4-DAPG-producing *Pseudomonas* spp. were present on roots of wheat grown in both TAD soils at densities up to approximately 10^6 CFU g^{-1} root (Table 2), densities that are equal or higher than the threshold density (10^5 CFU g^{-1} root) of 2,4-DAPG-producing fluorescent *Pseudomonas* strains required to control take-all (Raaijmakers and Weller, 1998). In the complementary take-all conducive soil, population densities of 2,4-DAPG-producing fluorescent *Pseudomonas* spp. were below this threshold density. Second, introduction of 2,4-DAPG-producing strain SSB17, representative of the dominant genotypic group found in one of the Dutch TAD soils, into the take-all conducive soil at rhizosphere population densities similar to the densities of indigenous 2,4-DAPG-producing fluorescent *Pseudomonas* spp. found in TAD soils, provided control of take-all to the same level as found in the complementary TAD soil (Fig. 4). Third, a mutant of SSB17 deficient in 2,4-DAPG-production was not able to control take-all of wheat, indicating that 2,4-DAPG is a key determinant in take-all suppression (Fig. 4). Previously the

role of 2,4-DAPG-producing fluorescent *Pseudomonas* spp. in TAD soils had been demonstrated only in Washington State (USA). Gerlagh (1968) postulated from his elegant and classic studies of TAD in Dutch polders that specific suppression involves antibiotic production by soil microorganisms. This report identifies for the first time, at least in part, the mechanism and microorganisms that contribute to the natural suppressiveness of Dutch TAD soils.

The densities of indigenous 2,4-DAPG-producing *Pseudomonas* spp. found on roots of wheat grown in the Dutch TAD soils were quantitatively similar to the densities found in four TAD soils from Washington State (USA) (Raaijmakers *et al.*, 1997; Raaijmakers and Weller, 1998). However, the genotypes of 2,4-DAPG-producers responsible for TAD in the Dutch soils differed from those in Washington State TAD soils (McSpadden-Gardener *et al.*, 2000; Raaijmakers and Weller, 2001). For example, Raaijmakers and Weller (2001) identified 16 RAPD groups among 101 *phlD*+ isolates obtained from roots of wheat grown in Quincy, Washington TAD soil, but one group comprised 50% of the isolates. This genotype also was dominant on roots of wheat grown in Moses Lake and Lind, Washington TAD soils and belonged to BOX-PCR group D as defined by McSpadden *et al.* (2000). In the current study, 33 distinct RAPD groups were identified among 161 *phlD*+ isolates from the two Dutch soils, but the SV and SSB soils had different dominant groups which represented 40% and 26%, respectively, of the isolates. All of the Dutch isolates clustered distantly from Q8r1-96, a representative of the dominant *phlD*+ isolates from the three Washington TAD soils. Furthermore, the dominant Dutch isolates corresponded to BOX-PCR groups M and F, which do not occur in Washington TAD soils.

The Shanon-Weaver's index indicated that the diversity among 2,4-DAPG producers in the SSB soil was higher than in the SV soil (Table 3). This was unexpected since we had hypothesized that the longer history of wheat monoculture in the SSB soil as compared to SV soil, would have enriched for a narrower range of genotypes. One explanation for the difference in diversity index between the two soils, may be the fact that different wheat cultivars were grown in these soils under field conditions. The wheat cultivar Bussard, used in the experiments described in this study, was also the cultivar grown in SSB soil in the field, whereas cv. Monopol was grown successively in the SV soil under field conditions. The influence of the host plant and cultivar on the composition of the microflora is well established for several systems. For example, certain wheat cultivars select for specific populations of *P. putida* that are antagonistic to the causal agents of apple replant disease (Mazzola and Gu, 2000), and different tomato genotypes provide better support for the

Table 4. Influence of *G. graminis* var. *tritici* on wheat root colonization by indigenous fluorescent *Pseudomonas* spp.

	Healthy X diseased roots [a]						Non-lesioned X lesioned root sections of diseased plants [b]					
	Total pseudomonads [c]			*phlD*+ [d]			Total pseudomonads [c]			*phlD*+ [d]		
Soil	-Ggt	+Ggt	Increase [e]	- Ggt	+ Ggt	Increase [e]	-Ggt	+Ggt	Increase [e]	-Ggt	+Ggt	Increase [e]
SV	3.9×10^6	3.4×10^7 *	8.7	7.6×10^5	3.7×10^6 *	5.0	5.6×10^6	5.6×10^7	10.1	4.9×10^5	4.8×10^6	9.7
SSB	8.1×10^6	4.9×10^7 *	6.1	7.3×10^5	4.3×10^6 *	6.0	8.8×10^6	3.8×10^7 *	4.3	2.7×10^5	4.0×10^6 *	14.8

[a] Roots of wheat growing in CB soil non-amended (- Ggt) or amended (+ Ggt) with *G. graminis* var. *tritici*.
[b] Non-lesioned and lesioned root sections of plants infected by *G. graminis* var. *tritici*.
[c] Populations of total fluorescent *Pseudomonas* spp. (CFU g^{-1} root).
[d] Population of *phlD*+ (CFU g^{-1} root). Mean values of five replicates are presented. Means marked with an asterisc (two means in a row) are significantly different according to Wilcoxon's two sample test ($P = 0.05$).
[e] Increases in populations of total fluorescent *Pseudomonas* spp. or *phlD*+ fluorescent *Pseudomonas* spp.

Table 5. Influence of *R. solani* on wheat root colonization by indigenous fluorescent *Pseudomonas* spp.

	Healthy X diseased roots [a]						Non-lesioned X lesioned root sections of diseased plants [b]					
	Total pseudomonads [c]			*phlD*+ [d]			Total pseudomonads [c]			*phlD*+ [d]		
Soil	-Rhiz.	+Rhiz.	Increase [e]	- Rhiz.	+ Rhiz.	Increase [e]	-Rhiz.	+Rhiz.	Increase [e]	-Rhiz.	+Rhiz.	Increase [e]
SV	1.5×10^7	1.1×10^8 *	7.7	8.5×10^5	3.9×10^6 *	4.6	8.7×10^7	1.4×10^8	1.6	5.5×10^6	2.3×10^6	0.4
SSB	1.7×10^7	1.0×10^8 *	6.3	1.7×10^6	1.1×10^7 *	6.6	5.3×10^7	1.6×10^8 *	3.1	3.0×10^6	1.9×10^7 *	6.2

[a] Roots of wheat growing in soil non-amended (- Rhiz.) or amended (+ Rhiz.) with *R. solani*.
[b] Non-lesioned and lesioned root sections of plants infected by *R. solani*.
[c] Populations of total fluorescent *Pseudomonas* spp. (CFU g^{-1} root).
[d] Population of *phlD*+ (CFU g^{-1} root). Mean values of five replicates are presented. Means marked with an asterisc (two means in a row) are sgnificantly different according to Wilcoxon's two sample test ($P = 0.05$).
[e] Increases in populations of total fluorescent *Pseudomonas* spp. or *phlD*+ fluorescent *Pseudomonas* spp.

growth and biocontrol activity of *Bacillus cereus* (Smith *et al.*, 1999). Furthermore, the significance of the plant cultivar has been recently demonstrated at the physiological level. Signficant changes in the expression of the *phl* biosynthetic operon of *P. fluorescens* strain CHA0 were detected on different maize cultivars (Nots *et al.*, 2001). Studies using different profiling techniques (Mavrodi *et al.*, 2001) suggested a significant level of endemism for wheat-associated *phlD*+ fluorescent *Pseudomonas* spp. (Wang *et al.*, 2001). Given the influence a cultivar may have on the composition and activity of *phlD*+ isolates, the differences between genotypes found in SV and SSB soils as well as between Dutch and US TAD soils may be due to the different wheat cultivars used in different geographical regions and in different fields within a specific region. The effect of wheat cultivars on the genotypic diversity of 2,4-DAPG-producing *Pseudomonas* spp. will be addressed in future studies.

Comparison of the genotypic clustering of the isolates with the clustering based on *in vitro* inhibition of mycelial growth of *G. graminis* var. *tritici* and *R. solani* revealed that this aproach was less discriminatory than genotypic clustering (Figs. 2 and 3). In several host-pathogen systems, antibiotic production by antagonistic microorganisms *in vitro* does not correlate well with the level of suppression of diseases obtained *in situ* (Wong and Baker, 1984; Sharifi-Tehrani *et al.*, 1998; Ellis *et al.*, 2000). However, good correlation was found between *in vitro* inhibition and suppression of *G. graminis* var. *tritici* *in situ* by pseudomonads isolated from wheat roots grown in a TAD soil from Washington State (Weller

Table 6. Influence of *G. graminis* var. *tritici* on rhizosphere colonization by introduced *P. fluorescens* strains.

Isolate	Initial density [a] (CFU seed^{-1})	Final density (CFU g^{-1} root) [b]				Increase
		-*G. graminis* var. *tritici*		+*G. graminis* var. *tritici*		
SSB17	2.1 X 10^4	1.0 X 10^7	a	1.9 X 10^7	a	1.9
SSB17	1.3 X 10^6	1.9 X 10^7	a	3.1 X 10^7	a	1.6
9H4	3.2 X 10^4	2.3 X 10^7	a	2.4 X 10^7	a	1.0
9H4	1.5 X 10^6	1.9 X 10^7	a	2.6 X 10^7	a	1.4
Q8r1-96	2.5 X 10^4	4.7 X 10^7	b	3.7 X 10^7	b	0.8
Q8r1-96	3.9 X 10^6	7.4 X 10^7	b	9.1 X 10^7	b	1.2
4C5	3.9 X 10^4	4.2 X 10^7	b	5.7 X 10^7	b	1.4
4C5	9.3 X 10^6	7.4 X 10^7	b	7.1 X 10^7	b	1.0

[a] *P. fluorescens* strains SSB17, Q8r1-96, and their respective 2,4-DAPG-deficient mutants 9H4 and 4C5 were applied to seeds at two different initial densities.

[b] Plants were grown in CB soil for one month and roots were harvested to determine the final densities in the rhizosphere. Means of five replicates are shown. Means followed by the same letter (columns) are not significantly different according to Wilcoxon's two sample test (P = 0.05).

et al., 1988). In this context, it is interesting to note that the dominant genotypic group found in the Dutch TAD soil, represented by strain SSB17, clustered closely together with Q8r1-96 in the phenotypic groups that were most inhibitory to the take-all fungus. In contrast, SSB17 and Q8r1-96 did not belong to the phenotypic group that was most inhibitory to *R. solani* (Fig. 3). More isolates representative of the genotypic and phenotypic groups need to be tested in biocontrol assays to support the earlier results obtained by Weller *et al.* (1988).

Strain SSB17 and strain Q8r1-96, when introduced into the conducive soil (CB) via seed treatment at low densities of approximately 10^3 CFU per seed were able to suppress *G. graminis* var. *tritici* to the same extent as occurred naturally in the suppressive soil (SSB). However, their 2,4-DAPG-deficient mutant 9H4 and 4C5, respectively, did not protect wheat plants against take-all (Fig. 4). Although 2,4-DAPG plays a key role in take-all suppression, it does not contribute to the rhizosphere competence of 2,4-DAPG producers, since SSB17 and Q8r1-96 colonized wheat roots to the same extent as their respective 2,4-DAPG deficient mutants. Similar results were obtained in experiments employing *P. fluorescens* strain F113 and its 2,4-DAPG-defective mutant (Carroll *et al.*, 1995). In contrast to 2,4-DAPG, biosynthesis of phenazine antibiotics was shown to significantly contribute to the ecological competence of *P. fluorescens* 2-79 and *P. aureofaciens* 30-84 (Mazzola *et al.*, 1992). Recently, Raaijmakers and Weller (2001) demonstrated that Q8r1-96 is much more aggressive as a colonist of wheat roots than other genotypes (Raaijmakers and Weller, 2001) and it is now known that this unique colonizing ability is shared by strains from other soils that are genotypically similar to strain Q8r1-96. Strain Q8r1-96 was more rhizosphere competent than SSB17 (Fig. 5; Table 6) even though the studies were conducted in raw Dutch soil, a finding which supports further suggestion that the rhizosphere competence of Q8r1-96 is minimally affected by the physicochemical characteristics of the soil (Raaijmakers and Weller, 2001) in which it is growing.

One of the key questions about the phenomenon of TAD is the basis of the enrichment of the antagonist responsible for specific suppression and the effect of the soil environment on the development and expression of suppressiveness. We found no quantitative differences in the population densities of 2,4-DAPG-producing fluorescent pseudomonads on roots of wheat grown in Dutch TAD soils as compared to densities reported on roots of wheat grown in Washington State TAD soils (Raaijmakers *et al.*, 1997; Raaijmakers and Weller, 1998). These findings suggest that the enrichment is independent of the physical and chemical characteristics of the soil. Classical studies of TAD demonstrated that TAD is a field phenomenon requiring the take-all pathogen, a susceptible host and at least one outbreak of

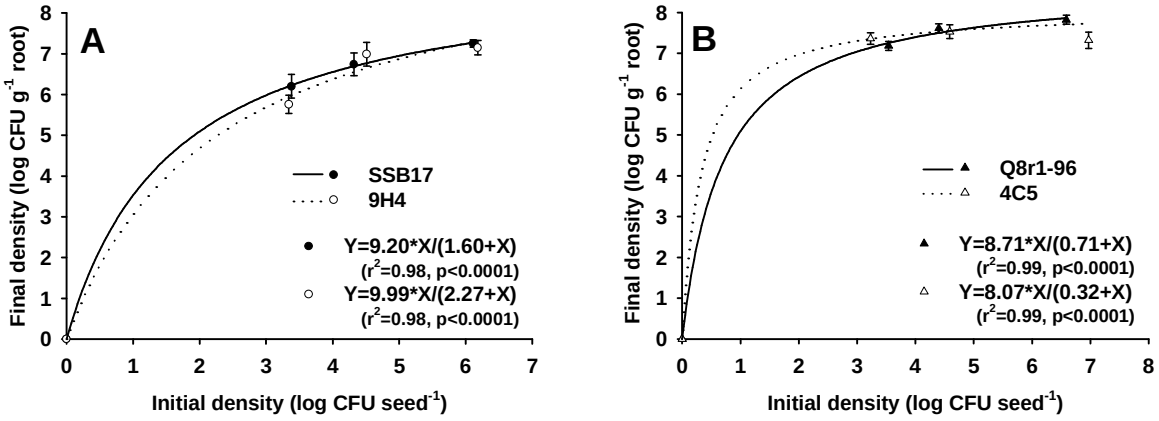

Figure 5. Relationships between the initial density on seeds (log CFU seed^{-1}) and the final densities on roots (log CFU g^{-1} root) for *P. fluorescens* strains SSB17, Q8r1-96, and their respective 2,4-DAPG-defective mutants, 9H4, and 4C5. Comparison of the rhizosphere competence of *P. fluorescens* strain SSB17 (solid line) and its 2,4-DAPG-deficient mutant 9H4 (dashed line), (A) and Q8r1-96 (solid line) and its 2,4-DAPG deficient mutant 4C5 (dashed line), (B). Wheat seeds were treated with each of the strains at densities of approximately 0, 10^3, 10^4, and 10^6 CFU seed^{-1}. Plants were grown for one month in CB soil and rhizosphere populations of the introduced strains were determined by dilution plating. Means of five replicates are shown. Error bars represent the standard error of the mean. Non-linear regression was performed to determine the relationship between the initial and final densities of the introduced strains. The equation used was Y=a*X/(X+b), where Y is the final density, X is the initial density, *a* is the maximum final density and *b* is the initial density necessary to reach half of the maximum final density. The experiment was repeated twice and representative results are shown.

severe disease. Some early studies suggested that the take-all pathogen, rather than the host plant is primarily responsible for the selection of specific antagonists (Gerlagh, 1968; Zogg and Jaggi, 1974; Wildermuth, 1980; Smiley, 1978; Brown, 1981; Barnett *et al.*, 1999; Sarniguet *et al.*, 1992). For example, Gerlagh (1968) and Zogg and Jaggi (1974) induced suppressiveness to take-all by repeatedly adding mycelium of *G. graminis* var. *tritici* to soil, but the relationship of this type of suppression to TAD is still not known. In an attempt to begin to address the question about the basis of enrichment of 2,4-DAPG producers in Dutch TAD soils, we compared populations of fluorescent pseudomonads on healthy and diseased roots, and on segments of roots from diseased plants with and without lesions. As has been demonstrated many times in the literature (Weller, 1983; Vojinovic, 1972; Brown, 1981), we found that roots with take-all lesions supported significantly larger populations of fluorescent pseudomonads than healthy roots. However, 2,4-DAPG producers were not enriched on diseased roots to a greater extent than the total population of fluorescent *Pseudomonas* spp. Furthermore, no significant increases in population densities of introduced strains SSB17 and

Q8r1-96 were observed upon infection of wheat roots by *G. graminis* var. *tritici*. Based upon these limited studies, at this time we conclude that increased nutrient availability upon fungal infection is primarily responsible for the enrichment of 2,4-DAPG producers.

In light of the results of this and earlier studies (Raaijmakers and Weller, 1998; Raaijmakers *et al.*, 1997), the specificity of the suppression that operates in TAD soils becomes interesting especially when considering the broad-spectrum activity of 2,4-DAPG against many fungal pathogens (Keel *et al.*, 1992). Australian TAD soils were shown to have also certain levels of suppressiveness to *Rhizoctonia solani*, *Giberella zeae*, *Pythium irregulare*, *Cochliobolus sativus* and *Fusarium culmorum* (Wildermuth, 1982). The results of our study, however, indicated that the two Dutch TAD soils were not suppressive to *R. solani*. Given that *R. solani* did not adversely affect population densities of 2,4-DAPG-producers (Tables 4, 5), a possible explanation for a lack of suppressiveness to *R. solani* may be the relative insensitivity of this fungus to 2,4-DAPG (Keel *et al.*, 1992). Furthermore, fungi can change patterns of bacterial gene expression (Fedi *et al.*, 1997) and more specifically interfere with the production of 2,4-DAPG (Duffy and Défago, 1997; Notz *et al.*, 2001). The suppressiveness of Dutch TAD soils to pathogens other than *R. solani* and their interaction with 2,4-DAPG-producing *Pseudomonas* spp. are currently under investigation.

MATERIALS AND METHODS

Microorganisms. Naturally occurring populations of fluorescent *Pseudomonas* spp. were isolated from the wheat rhizosphere on King's medium B (KMB) agar supplemented with chloramphenicol (13 µg ml^{-1}), ampicillin (40 µg ml^{-1}), and cycloheximide (100 µg ml^{-1}) [KMB^{+}] (Simon and Ridge, 1974). All *Pseudomonas* strains described in this study, including SSB17 (this study), Q8r1-96 (Raaijmakers and Weller, 2001), Pf-5 and CHA0 (Keel *et al.*, 1996) were grown on KMB. Spontaneous rifampicin resistant derivatives of SSB17 and Q8r1-96 were selected on KMB supplemented with rifampicin (100 µg ml^{-1}). Strains 9H4 and 4C5 are 2,4-DAPG deficient mutants derived from strain SSB17 and Q8r1-96, respectively. 2,4-DAPG deficient mutants are resistant to rifampicin (100 µg ml^{-1}) and kanamycin (100 µg ml^{-1}). *Escherichia coli* strain S17 λ *pir* was obtained from Dr. L. S. Thomashow (USDA-ARS, WA) and contained the mini-Tn*5lacZ* element in plasmid pUT (De Lorenzo *et al.*, 1990). S17 λ *pir* was grown in liquid Luria Bertani (LB) amended with kanamycin (25 µg ml^{-1}). All bacterial strains were stored at -80°C in LB or KMB-broth supplemented with 40% (v/v) glycerol.

Isolate R3-111a-1 of *G. graminis* var. *tritici* (the causal agent of take-all) was originally isolated from wheat grown in a soil near Moses Lake, Washington State, USA. Isolate AG-8 of *Rhizoctonia solani* (the causal agent of Rhizoctonia root rot) was obtained from the collection maintained by the USDA-ARS, Pullman, Washington State. Isolate CBS 219.65 of *Pythium ultimum* var. *sporangiiferum* (the causal agent of Pythium root rot) was obtained from the Dutch collection of microorganisms (CBS, Baarn, The Netherlands). *G. graminis* var *tritici*, *R. solani*, and *P. ultimum* var. *sporangiiferum* were routinely grown on potato dextrose agar (Oxoid Ltd., Basingstoke, Hampshire, England) [PDA]. Mycelial plugs were immersed in sterile mineral oil and stored at 15°C.

Soils. Soils CB, SV, and SSB were obtained in December 1997, 1999 and 2001 from agricultural polder fields at Woensdrecht, The Netherlands. Woensdrecht is located in the South-West of the Netherlands, 10 km from the city Bergen op Zoom.

In 1997, the SV and SSB soil were continuously cropped to wheat for 14 and 27 years, respectively; the CB soil was grown to wheat and sugarbeet in a 1:2-year rotation scheme. Soils were collected from the top 50-cm of the soil profile, air-dried for a week, and passed through a 0.5-cm mesh screen prior to use. Chemical and physical properties of the soils used in this study are listed in Table 1.

Natural suppressiveness of soils to *G. graminis* var. *tritici* and *R. solani*. Soils were amended with 0.1 to 0.4% (wt/wt) of an oat grain inoculum of *G. graminis* var. *tritici* isolate R3-111a-1 (particle size 0.25 to 0.50 mm) (Raaijmakers and Weller, 1998); sterilized oat grain inoculum was used as a control. Wheat seeds (cv. Bussard) were sown in PVC pots (8 cm high, 7 cm wide) containing 200 g of soil and covered with a 1-cm layer of soil without inoculum. Plants were grown for four weeks in climate chambers at 15°C with a 12-h photoperiod. Twice a week, plants received 50 ml of 1/3 strength Hoagland's solution (macroelements only). Each treatment had five replicates with approximately 12 plants per replicate. Eight randomly selected plants were harvested, their root systems washed and the severity of take-all determined on a 0-to-8 scale, where "0" indicates no disease and "8" a dead plant (Pierson and Weller, 1994). Similar to assays performed with *G. graminis* var. *tritici*, suppressiveness of the soils to *R. solani* was assessed by introducing 0.5% (wt/wt) of a millet grain inoculum of *R. solani* AG-8; sterilized millet grain was used as a control. To assess the level of suppressiveness to *R. solani* infections, roots of eight randomly selected plants were harvested, washed and root dry weight determined. From each replicate, three to four randomly selected plants were collected for isolation of naturally occurring pseudomonads from the rhizosphere.

Isolation and enumeration of *phlD*+ fluorescent *Pseudomonas* spp. After 4 weeks of plant growth, three to five plants of each replicate were harvested and loosely adhering soil was removed from the root system. Naturally occurring fluorescent *Pseudomonas* spp. were isolated from uninfected wheat roots and roots infected by *G. graminis* var. *tritici* or *R. solani*. From the infected roots both, non-lesioned and lesioned root sections were seleted. Root samples of 0.2 to 1 g fresh weight were vortexed for 1 min and then sonicated for 1 min in a ultrasonic cleaner (Bransonic 12, Branson Ultrasonics Corp., Geneva, Switzerland). Root suspensions were dilution plated onto KMB^+ and incubated at 25°C for 48 h. Population densities of *phlD*+ *Pseudomonas* spp., a key gene in the biosynthesis of 2,4-DAPG, were determined by colony hybridization followed by PCR (Raaijmakers *et al.*, 1997). *phlD*+ colonies were purified and stored at -80°C for further analyses.

RAPD analysis. RAPD analysis of *phlD*+ isolates was performed with primers M13, M12, and D7. These primers were selected by Keel *et al.* (1996) among sixty-four other random primers based on the distinct and consistent banding patterns of the polymorphic markers produced. PCR was carried out in a 25-µl reaction mixture as described previously (Raaijmakers *et al.*, 1997). PCR amplifications were carried out in a MJ Research PTC-200 thermocycler. Samples (10-15 µl) of the PCR products were separated on 2% agarose gels in 1X TBE buffer (90 mM Tris-borate, 2 mM EDTA [pH 8.0]) at 80 V for 3 h. Gels were stained with ethidium bromide and bands were visualized on a UV transilluminator. RAPD analysis was repeated at least three times for each primer. The presence or absence of bands that were consistently generated by RAPD analysis (1 for the presence and 0 for the absence of a particular sized band in the gel) was used to calculate the pairwise coefficients of similarity (Nei-Li distances). Cluster analysis with the neighbour joining method and bootstrap analysis were performed with the program FreeTree (Hampl, *et al.*, 2001). The dendrogram was edited and visualised in TREEVIEW (Page, 1996). Data from the RAPD analyses were also used to calculate the Shanon-Weaver's diversity index, using the formula $H=\Sigma[pi\text{-}ln(pi)]$, where pi is the proportion of each RAPD group (based on 100% similarity) in relation to the total number of RAPD groups per soil.

Plate inhibition assays. *phlD*+ *Pseudomonas* isolates obtained from roots of wheat grown in Dutch agricultural soils were inoculated at the edges of 9-cm plates containing $1/5^{th}$ strength PDA (initial pH = 6.5) and incubated for two days at 25°C. Mycelial plugs (5 mm diameter) of *G. graminis* var. *tritici*, *R. solani*, and *Pythium ultimum* var. *sporangiiferum* were tranferred to the centre of the plate. Plates were incubated at 25°C and the radial growth of the fungus toward the bacterial isolate was measured after 3 to 5 days and compared with the control (fungi or oomycete only). For each isolate, growth inhibition of both *G. graminis* var. *tritici* and *R. solani* was determined in duplicate. Data from plate inhibition assays were used to construct similarity matrices, where a hierarchial cluster analysis was performed by the single linkage method (Davies *et al.*, 2001).

Tn*5*-mutagenesis. 2,4-DAPG-deficient mu-tants of spontaneous rifampicin-resistant derivatives of *P. fluorescens* strains SSB17 and Q8r1-96 were obtained by biparental mating with *E. coli* strain S17 λ *pir* harbouring the mini-Tn*5lacZ* element in plasmid pUT, according to protocols described by Sambrook and Russel (2001). Transformants were selected on KMB supplemented with rifampicin and kanamycin and subsequently transferred to 98-well microtiter plates containing KMB broth. Transformants unable to produce a red pigment, which is not 2,4-DAPG, but is characteristic for

2,4-DAPG producing *Pseudomonas* strains (Bangera and Thomashow, 1996), were selected after one week of growth at 25ºC. High-performance liquid chromatography (HPLC) was used to confirm the inability of these transformants to produce 2,4-DAPG (Bonsall *et al.*, 1997). Plate inhibition assays with *G. graminis* var. *tritici*, *R. solani* and *P. ultimum sporangiiferum* were used to further confirm the inability of mutant strains to inhibit these pathogens *in vitro*. RAPD analysis with primers M13, M12, and D7, was performed to confirm strain integrity. Southern blot analysis was used to determine the number of copies of the Tn*5lacZ* element in the 2,4-DAPG-deficient mutants.

Southern analysis and colony hybridizations. For Southern blot analysis, genomic DNA of wildtype and mutant strains was extracted with the DNA Wizard Kit (Promega, Leiden, The Netherlands). Samples containing 2.0 µg of DNA were digested with 5 U of *KpnI* and *EcoRI* (Promega), two enzymes for which restriction sites are not present in the kanamycin gene of the mini-Tn*5lacZ* element (De Lorenzo *et al.*, 1990). Restrictions were performed in a total volume of 100 µl at 37°C for 12 h. Digested DNA was precipitated with 4M LiCl, washed with 70% ethanol, dissolved in 15 µl of sterile distilled water and separated on 1% agarose gels in 1X TBE. DNA and colony transfer to Hybond N^+ nylon membranes (Amersham Pharmacia Biotech) were performed according to standard methods (Sambrook and Russel, 2001). Membrane washes and hybridizations were performed following standard procedures (Sambrook and Russel 2001). High-stringent conditions comprised prehybridization for 1.5 h at 65°C, hybridization for 12 h at 65°C, membrane washing twice for 5 min each with 2X SSC (1X SSC is 0.15 M NaCl plus 0.015 M sodium citrate)-0.1% SDS at room temperature, and membrane washing twice each for 30 min with 0.1X SSC-0.1% SDS at 65°C. A 575-bp probe, specific for the kanamycin gene contained within the Tn*5LacZ* element, was obtained by direct PCR DIG-labelling (Roche Corp., Basel, Switzerland) of the fragment using primers KM1 (5'-CCCGATGCGCCAGAGTTGTT) and KM2 (5'-TCACCGAGGCAGTTCCATAGG).

Treatment of seeds with *Pseudomonas*. For bioassays and rhizosphere competence studies, wheat seeds (cv. Bussard) were coated with SSB17, Q8r1-96, or their 2,4-DAPG-deficient mutants in 1% methylcellulose to final densities of approximately 10^3, 10^4, and 10^6 CFU per seed; seeds treated with 1% methylcellulose served as a control. Population densities of introduced *Pseudomonas* spp. strains SSB17, Q8r1-96 and their 2,4-DAPG deficient mutants were assessed on uninfected wheat roots and on roots infected by *G. graminis* var. *tritici*.

Statistical analysis. Prior to ANOVA, data from population counts were $\log_{10}$-transformed and disease index data were ranked. Root dry weight data were analysed directly by ANOVA followed by Tukey's studentized range test, after certifying normal distribution and homogeneity of variances (SAS institute, Inc., Cary, N.C.). Data from bacterial population counts on *G. graminis* var. *tritici*- and *R. solani*-lesioned roots, which did not present normal distribution after transformations $\log_{10}$ (CFU), were analysed by the one-way non-parametric Kruskal-Wallis test and comparison of means of two treatments was performed by Wilcoxon's two sample test. Data from population increases were also analysed by the one-way non-parametric Kruskal-Wallis test followed by Wilcoxon's two sample test. Data from rhizosphere competence assays were analysed by non-linear regression analysis to determine the relationship between the initial density of strains SSB17 and Q8r1-96 on seeds and their final density on roots of 1-month old wheat plants. The equation used was $Y=a*X/(b + X)$, where Y represents the final density (log CFU g^{-1} root), X the initial density (log CFU $seed^{-1}$), a the maximum final density, and b the initial density necessary to reach half of the maximal final density (Raaijmakers and Weller, 2001). All experiments were performed at least twice and representative results are shown.

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CHAPTER 5

EFFECT OF 2,4-DIACETYLPHLOROGLUCINOL ON *PYTHIUM* SPECIES: CELLULAR RESPONSES AND VARIATION IN SENSITIVITY AMONG PROPAGULES AND SPECIES

ABSTRACT

The antibiotic 2,4-diacetylphloroglucinol (2,4-DAPG) plays an important role in the suppression of plant pathogens by several strains of *Pseudomonas* spp. The results of this study show that within and among *Pythium* species there is variation in sensitivity to 2,4-DAPG. Also various propagules of *P. ultimum* var. *sporangiiferum* (*Pus*), that are part of the asexual stage of the life cycle, differ considerably in their sensitivity to 2,4-DAPG. Mycelium was the most resistant structure, followed by zoosporangia, zoospore cysts and zoospores. Additionally, we report, for the first time, that pH has a significant effect on the activity of 2,4-DAPG, with a higher activity at low pH. Furthermore, the level of acetylation of phloroglucinols is also a major determinant of their activity. Transmission electron microscopy (TEM) studies revealed that 2,4-DAPG causes different stages of disorganisation in hyphal tips of *Pus*, including alteration (proliferation, retraction and disruption) of the plasma membrane, vacuolisation and cell content disintegration. The implications of these results for the efficacy and consistency of biological control of plant pathogenic *Pythium* species by 2,4-DAPG-producing *Pseudomonas* spp. are discussed.

Jorge T. de Souza, Christine Arnould, Chrystel Deulvot, Philippe Lemanceau, Vivienne Gianinazzi-Pearson, and Jos M. Raaijmakers

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INTRODUCTION

Phloroglucinols (PG) are phenolic, secondary metabolites produced by plants, algae and bacteria (Vincent *et al.*, 1991; Bangera and Tomashow, 1996; 1999; Ishiguro *et al.*, 1988; Bokesch *et al.*, 1999; Verrota *et al.*, 1999; Jimenez-Escrig *et al.*, 2001). More than sixty PG derivatives have been described and were reported to have antiviral, antimicrobial, ichthyotoxic, insect and mammal antifeedant, antihelminthic, phytotoxic, antioxidant, cytotoxic, anti-tumor, and plant growth-regulating activities (James, 1979; Yajima and Munakata, 1979; Arisawa *et al.*, 1990; Tada *et al.*, 1990; 1992; Keel *et al.*, 1992; Cronin *et al.*, 1997a; Te-Chato and Lim, 1999; Lawler *et al.*, 1999; Ito *et al.*, 2000; Debabrata and Naik, 2000; Jimenez-Escrig *et al.*, 2001). The antimicrobial activity of these compounds, and in particular of 2,4-diacetylphloroglucinol (2,4-DAPG), has received considerable attention in the area of biological control of plant diseases.

Numerous studies have demonstrated that 2,4-DAPG-producing *Pseudomonas* spp. can suppress a wide variety of plant pathogens, including fungi, bacteria and nematodes (Stutz *et al.*, 1986; Keel *et al.*, 1990; Vincent *et al.*, 1991; Fenton *et al.*, 1992; Levy *et al.*, 1992; Keel *et al.*, 1992; Cronin *et al.*, 1997a, b; Duffy and Défago, 1997; Sharifi-Tehrani *et al.*, 1998). Among the plant pathogenic fungi, *Gaeumannomyces graminis* var. *tritici*, *Thielaviopsis basicola*, *Fusarium oxysporum* f. sp. *radicis-lycopersici*, and the oomycete *Pythium ultimum* can be effectively controlled by 2,4-DAPG-producing *Pseudomonas* strains (Vincent *et al.*, 1991; Fenton *et al.*, 1992; Sharifi-Tehrani *et al.*, 1998; Stutz *et al.*, 1986; Duffy and Défago, 1997). The determinative role of 2,4-DAPG in disease suppression by *Pseudomonas* strains has been demonstrated by (1) the use of mutants deficient in 2,4-DAPG production (Vincent *et al.*, 1991; Shanahan *et al.*, 1992; Keel *et al.*, 1992; Cronin *et al.*, 1997a, b), (2) complementation of 2,4-DAPG deficient mutants and concomittant restoration of biocontrol activity (Vincent *et al.*, 1991; Keel *et al.*, 1992; Cronin *et al.*, 1997a, b), and (3) expression of 2,4-DAPG biosynthetic genes in heterologous, nonproducing strains thereby conferring biocontrol activity or enhanced activity (Vincent *et al.*, 1991; Shanahan *et al.*, 1993; Bangera and Thomashow, 1996).

Reporter gene systems (Loper and Lindow, 1997) and analytical techniques (Thomashow *et al.*, 1997) have further demonstrated that 2,4-DAPG is produced *in situ* by both introduced *Pseudomonas* strains as well as indigenous *Pseudomonas* populations (Keel *et al.*, 1992; Bonsall *et al.*, 1997; Duffy and Défago, 1997; Raaijmakers *et al.*, 1999; Notz *et al.*, 2001). *Pseudomonas* strains that produce 2,4-DAPG also produce monoacetylphloroglucinol (MAPG) and depending on the nutritional environment the ratio of MAPG to 2,4-DAPG

may change (Duffy and Défago, 1997). In addition to MAPG and 2,4-DAPG, also PG has been isolated from cultures of a bacterium found on decayed roots of red pine seedlings and classified as *Aeromonas hydrophila* (Strunz *et al.*, 1978).

In spite of significant progress in our understanding of the biosynthesis and regulation of 2,4-DAPG-production in *Pseudomonas* strains (Bangera and Thomashow, 1996, 1999; Sarniguet *et al.*, 1995; Whistler *et al.*, 1998; Blumer and Haas, 2000), little attention has been given to fungal responses to 2,4-DAPG and to its mode of action. Most studies on the sensitivity of plant pathogenic fungi to 2,4-DAPG take into account only one species or isolate of the target pathogen (Keel *et al.*, 1992; Levy *et al.*, 1992). Furthermore, most studies on sensitivity of pathogens to 2,4-DAPG consider only one particular stage in the life cycle of a pathogen, usually mycelial growth (Keel *et al.*, 1992; Levy *et al.*, 1992; Mazzola *et al.*, 1995). Most life cycles of pathogens, however, are complex and comprise numerous pathogen structures and infectious propagules. For example, oomycete pathogens can infect host tissues by means of multiple propagules, including mycelium, zoosporangia, zoospores and oospores. Understanding the variation in sensitivity of different phases of the life cycle of a pathogen to a specific biocontrol trait will give more insight into the potential efficacy of biocontrol agents harbouring that particular trait.

In this study, we investigated the response of *Pythium* species to 2,4-DAPG. *Pythium* species are ubiquitous pathogens of many important crops around the world and possess a high level of diversity with more than 200 species described (Van der Plaats-Niterink, 1981; Dick, 1990). Many of these species can occur simultaneously at the same site and often more than one species infect a certain host plant (Chamswarng and Cook, 1985). We determined the variation in sensitivity of 14 *Pythium* isolates to 2,4-DAPG; the isolates were obtained from multiple hosts and represent eight species. The sensitivity of various infectious propagules of *Pythium ultimum* var. *sporangiiferum* to 2,4-DAPG was studied in detail with special emphasis on those propagules that are part of the asexual life cycle. The effects of pH and level of acetylation on activity of phloroglucinols against mycelial growth of *P. ultimum* var. *sporangiiferum* were also assessed. To understand the mode of action of 2,4-DAPG, ultrastructural changes induced by 2,4-DAPG in hyphal tips of *Pythium ultimum* var. *sporangiiferum* were studied by transmission electron microscopy.

RESULTS

Effect of 2,4-DAPG on mycelial growth of different *Pythium* species. Mycelial growth of fourteen *Pythium* isolates, obtained from multiple host plants and representing eight different species, was completely inhibited at 2,4-DAPG concentrations ranging from 8 to more than 300 µg ml^{-1} (Table 1). *P. volutum* CBS 699.83 was the most sensitive isolate, whereas *P. deliense* V1-PPO was relatively insensitive to 2,4-DAPG. Mycelial growth of the other *Pythium* isolates was completely inhibited at 2,4-DAPG concentrations ranging from 32 to 72 µg ml^{-1}. *P. ultimum* var. *sporangiiferum* isolate CBS 219.65, further referred to as *Pus*, was used for a detailed analysis of the effect of 2,4-DAPG on different propagules, including zoosporangia, zoopores and zoospore cysts.

Effect of 2,4-DAPG on zoosporangia. 2,4-DAPG had a significant effect on both the formation and the diameter of zoosporangia of *Pus* (Fig. 2). The number of newly formed zoosporangia decreased by approximately 50% when exposed to 2.0 µg ml^{-1} of 2,4-DAPG. The diameter of the newly formed zoosporangia, an indicative measure of their ripening, decreased from 26 to 20 µm with increasing concentrations of 2,4-DAPG.

Effect of 2,4-DAPG on zoospores and zoospore cysts. 2,4-DAPG adversely affected the behaviour of *Pus* zoospores at concentrations of 0.8 ng ml^{-1} and higher (Fig. 3). At a concentration of 0.8 ng ml^{-1}, all zoospores stopped swimming and approximately 50% of the zoospores disintegrated (Fig. 4). The time for disintegration to occur depended on the concentration of 2,4-DAPG. At concentrations of 0.8 ng ml^{-1}, disintegration of the zoospores occurred in approximately 20 min and at concentrations of 3.2 ng ml^{-1} it only took 3 min (data not shown).

The effect of 2,4-DAPG on germination of encysted zoospores was studied in water and in wheat root exudates. Wheat root exudates were included since responses to antibiotics may differ depending on the nutritional conditions. In experiments conducted in water, 15.8% of the total number of encysted zoospores had germinated prior to addition of 2,4-DAPG. There was no significant effect of 2,4-DAPG on the percentage of germinated zoopore cysts at concentrations up to 2.8 µg ml^{-1} (Fig. 5A). At a 2,4-DAPG concentration of 3.2 µg ml^{-1}, there was a significant increase in germination of encysted zoospores, which was observed in all replicate experiments. At 2,4-DAPG concentrations higher than 3.2 µg ml^{-1}, germination of encysted zoospores of *Pus* significantly decreased.

Table 1. Effect of 2,4-DAPG on mycelial growth of *Pythium* spp.

Species	Isolate [a]	Origin	EC_{50} (µgml^{-1})[b]	MIC (µg ml^{-1}) [c]
P. aphanidermatum	311 – PPO	cucumber	32-40	48
P. aristosporum	CBS 263.38	wheat	16-24	32
P. deliense	V1 – PPO	cucumber	300	>300
P. intermedium	294 – LBO	lily	32-40	56
P. intermedium	LBO	hyacinth	24-32	48
P. irregulare	ATCC 1120	wheat	24-32	48
P. irregulare	LBO	crocus	16-24	56
P. macrosporum	111 – LBO	lily	16-24	40
P. ultimum	P17 – LBO	tulip	24-32	72
P. ultimum	266	lily	24-32	72
P. ultimum	IRS	sugar beet	24-48	56
P. u. var. *sporangiiferum*	CBS 219.65	wheat	16-24	32
*P. u.*var. *ultimum*	CBS 725.94	wheat	16-24	32
P. volutum	CBS 699.83	wheat	< 8	8

[a] CBS, Collection of Fungal Cultures, Utrecht, The Netherlands; LBO, Applied Plant Research, section Flower Bulbs, Lisse, The Netherlands; PPO, Applied Plant Research, Naaldwijk, The Netherlands; ATCC, American Type Culture Collection, University Blvd, Manassas, Virginia, USA; IRS, Institute for Rational Sugar Production, Bergen op Zoom, The Netherlands.
[b] Concentration of 2,4-DAPG necessary to inhibit radial mycelial growth by 50%.
[c] Minimal concentration of 2,4-DAPG causing 100% inhibition of mycelial growth.
Medium used was PDA (pH 6.5) and inhibition was assessed every 24 hours during 7 days of incubation at 25°C. 2,4-DAPG concentrations varied from 0 to 300 µg ml^{-1} with intervals of 8 µg ml^{-1}. Each value is based on three experiments with 6 replicates per treatment.

In wheat root exudates, germination of encysted zoospores of *Pus* was approximately 100% (Fig. 5B). At concentrations up to 2.4 µg ml^{-1} there was no adverse effect of 2,4-DAPG on germination, whereas concentrations ranging from 2.8 to 6.4 µg ml^{-1} led to a significant decrease in cyst germination. 2,4-DAPG concentrations of 6.4 µg ml^{-1} reduced the percentage of germinated cysts even below the initial percentage of 11% germinated zoospore cysts that were present prior to addition of 2,4-DAPG, which may suggest that relatively high concentrations of 2,4-DAPG cause lysis of germ tubes.

Effect of pH and level of acetylation on activity. In absence of 2,4-DAPG, pH had no significant effect on the growth rate of *Pus*: growth rates were on average 23 mm day^{-1} at a pH ranging from 4.5 to 8.0 (Fig. 6). In presence of 2,4-DAPG, growth rates of *Pus* decreased significantly and this decrease was highly dependent on pH. At pH 4.5, mycelial growth of *Pus* was completely inhibited at a 2,4-DAPG concentration of 16 µg ml^{-1}, whereas a 2,4-DAPG concentration of 40 µg ml^{-1} was not sufficient to completely inhibit mycelial growth at pH 7.5 and 8.0.

2,4-DAPG was significantly more inhibitory than MAPG and PG (Fig. 7). Mycelial

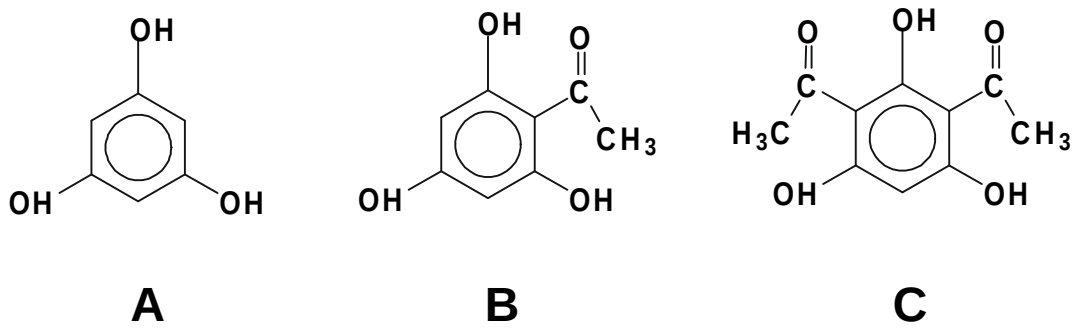

Figure 1. Chemical structure of the phloroglucinol derivatives used in this study. (A) Phloroglucinol – PG, (B) Monoacetylphloroglucinol – MAPG, (C) 2,4-Diacetylphloroglucinol – 2,4-DAPG.

growth of *Pus* was completely inhibited at a 2,4-DAPG concentration of 32 µg ml^{-1}, whereas for MAPG almost 10-fold higher concentrations were necessary (Fig. 7). In presence of PG, *Pus* could still grow at concentrations of 300 µg ml^{-1}. Collectively, these results indicate that the higher the number of acetyl groups the greater the activity of phloroglucinol derivatives.

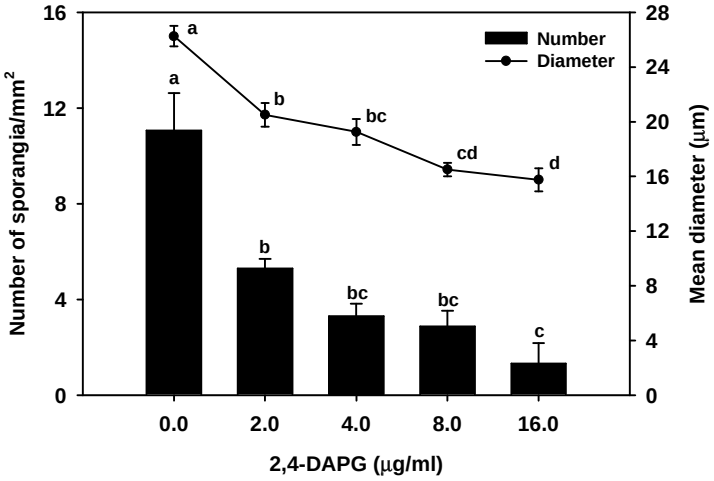

Figure 2. Effect of 2,4-DAPG on formation and diameter of zoosporangia of *P. ultimum* var. *sporangiiferum* (*Pus*). Mycelial plugs of *Pus* grown on medium without the antibiotic were flooded with solutions of different 2,4-DAPG concentrations. After four days of incubation at 25°C the number and diameter of 10 sporangia formed per treatment was determined microscopically. Mean values of five replicates are shown. Error bars represent the standard error of the mean. For each of the two parameters, means followed by the same letter are not significantly different according to Tukey's studentized range test (P=0.05).

Static effect of 2,4-DAPG on mycelial growth. *P. ultimum* var. *sporangiiferum* (*Pus*) was grown for 7 days on PDA plates amended with different concentrations of 2,4-DAPG. *Pus* could grow, although at reduced level, in presence of 2,4-DAPG up to a concentration of 32 µg ml^{-1}. Mycelial plugs from *Pus* cultures that failed to grow at concentrations higher than 32 µg ml^{-1} were transferred to PDA plates without 2,4-DAPG. The plugs exposed to 48, 64, and 96 µg ml^{-1} re-grew on PDA without 2,4-DAPG, whereas the ones exposed to 128 µg ml^{-1} 2,4-DAPG or higher were not able to grow on PDA plates free of 2,4-DAPG.

TEM studies. For studying ultra-structural changes in hyphal tips of *Pus* exposed to 2,4-DAPG, samples were taken at the periphery of *Pus* colonies growing in absence and presence of 2,4-DAPG. The concentration of 2,4-DAPG used was 400 µg per paper disc, which was placed on the surface of the agar medium. In absence of 2,4-DAPG, most hyphal tips (72%) showed normal cell morphology, characterised by dense cytoplasm containing numerous organelles (mitochondria, ribosomes, endoplasmic reticulum and some vacuoles (Table 2; Fig. 8A, B). In the presence of 2,4-DAPG, different stages of disorganisation in hyphal tips were observed. A relatively low percentage (10%) of the hyphal tips had dense cytoplasm (Table 2) which contained numerous mitochondria, ribosomes and small vacuoles (Fig. 8C, D). Recurrent features observed in the cell contents of hyphal tips exposed to 2,4-DAPG were 1) a well-defined rough endoplasmic reticulum, 2) the localised alteration (proliferation or

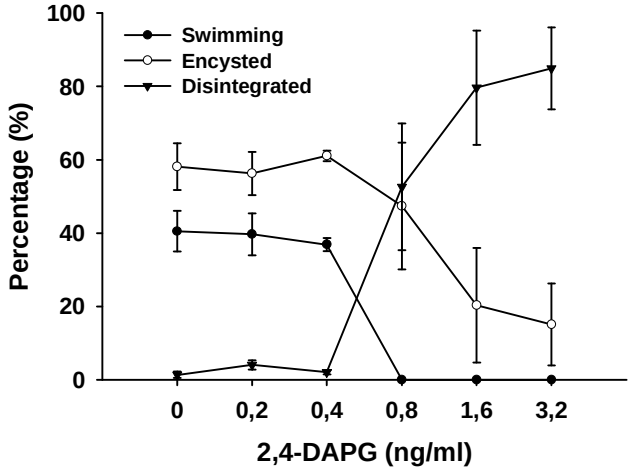

Figure 3. Effect of 2,4-DAPG on zoospores of *P. ultimum* var. *sporangiiferum* (*Pus*). Zoospores were exposed to different concentrations of 2,4-DAPG and the percentage of swimming, encysted, and disintegrated zoospores were determined microscopically. Mean values of 5 replicates are given. Error bars represent the standard error of the mean.

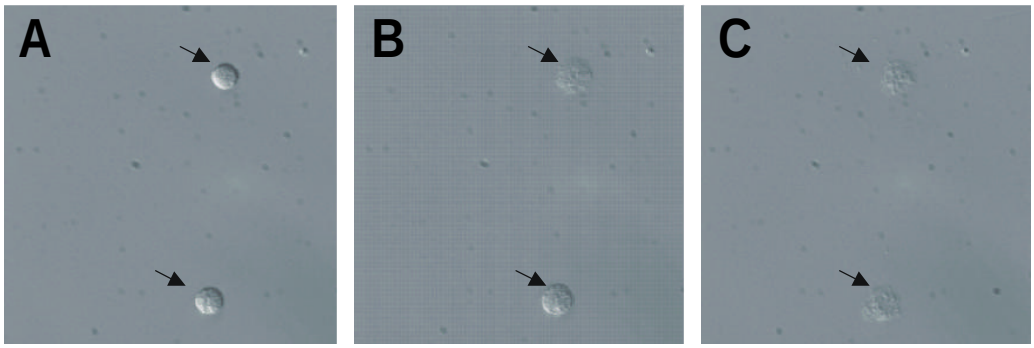

Figure 4. Effect of 2,4-DAPG on zoospores of *P. ultimum* var. *sporangiiferum* (*Pus*). (A): round-shaped zoospores (B): disintegration. (C): disappearance. Arrows indicate zoospores of *Pus* (magnification 100X). The concentration of 2,4-DAPG was 3.2 ng ml^{-1} and the zoospore suspension contained 10^4 zoospores ml^{-1}.

disruption) in plasma membrane organisation, and 3) the development of an extensive network of smooth membranous vesicles which appeared to be closely associated with the endoplasmic reticulum (Fig. 8C). A large proportion of the hyphae (66%) showed cell content disorganisation (Table 2), a degenerated cytoplasm which was bordered by a retracted plasma membrane (Fig. 8E). Hyphal senescence was accompanied by vacuolisation and degeneration of its contents (Fig. 8F). *Pus* hyphal tips growing in absence of 2,4-DAPG were delimited by a thin electron translucent wall where the cellulosic components were strongly stained by the PATAg reaction (Fig. 8B). The PATAg reaction occurred similarly in cell walls of *Pus* growing in presence of 2,4-DAPG (Fig. 8D). These results suggest that 2,4-DAPG does not affect the β(1-4), and β(1-6) glucan content of the cell wall of *Pus*.

Observations of sections immunolabelled to locate β(1-3) glucans revealed the presence of these compounds in both the membrane and the cytoplasm of *Pus* growing in absence (Fig. 9B) or in presence of 2,4-DAPG (Fig. 9C). No immunolabelling was present in control sections without primary antibody (Fig. 9A). These results indicate that 2,4-DAPG does not adversely affect synthesis of β (1-3) glucans.

DISCUSSION

The results of this study show that within and among *Pythium* species there is variation in sensitivity to 2,4-DAPG, a broad-spectrum antibiotic produced by *Pseudomonas* biocontrol strains (Table 1). Also various propagules of *P. ultimum* var. *sporangiiferum* (*Pus*), that are part of the asexual stage of the life cycle, can differ considerably in their sensitivity to 2,4-DAPG (Table 1, Figs. 2, 3, 5). Mycelium was the most resistant structure, followed by zoosporangia, zoospore cysts and zoospores. Additionally, we report for the first time that pH has a significant effect on the activity of 2,4-DAPG, with a higher activity at low pH. Furthermore, the level of acetylation of phloroglucinols also is a major determinant of their activity. Finally, TEM studies revealed that 2,4-DAPG causes different stages of disorganisation in hyphal tips of *Pus*, including alteration (proliferation, retraction and disruption) of the plasma membrane, vacuolisation and cell content degeneration. Collectively, these results give a better understanding of the mode of action and activity of 2,4-DAPG, which contributes to further improve the efficacy of biological control of plant pathogenic *Pythium* species by 2,4-DAPG-producing *Pseudomonas* spp.

Pythium species have a complex life cycle with multiple infectious propagules. Depending on the environmental conditions, zoosporangia may be very important propagules in the interaction between *Pythium* species and their hosts (Nelson and Hsu, 1994). The formation and diameter of *Pus* zoosporangia were significantly reduced at a 2,4-DAPG concentration of 2.0 μg ml^{-1} (Fig. 2). The diameter of zoosporangia is an indicator of their developmental stage since they need to reach a certain diameter to ripen and liberate zoospores (Van der Plaats-Niterink, 1981). Several *Pythium* species reproduce asexually, forming mobile biflagellate zoospores, which are responsible for the dispersal under moist conditions. In hydroponic systems or humid soils, zoospores locate new hosts by responding to chemo-attractants produced by the plant (Zhou and Paulitz, 1993). Zoospores swim, accumulate on root surfaces and encyst in response to root polysaccharides that interact with receptors on the zoospore surface (Deacon and Donaldson, 1993). We found that zoospores of *Pus* stopped swimming at a 2,4-DAPG concentration of 0.8 ng ml^{-1} and almost all disintegrated at 3.2 ng ml^{-1} (Fig. 3). The high sensitivity of zoospores to 2,4-DAPG may be explained by the lack of a protective cell wall (Deacon and Donaldson, 1993). This is supported by the observation that encysted zoospores of *Pus*, which have a cell wall, were significantly more resistant to 2,4-DAPG than non-encysted zoospores (Figs. 3, 5). Encysted zoospores germinate in response to host-produced amino acids and polysaccharides, causing root, stem, or seed infections

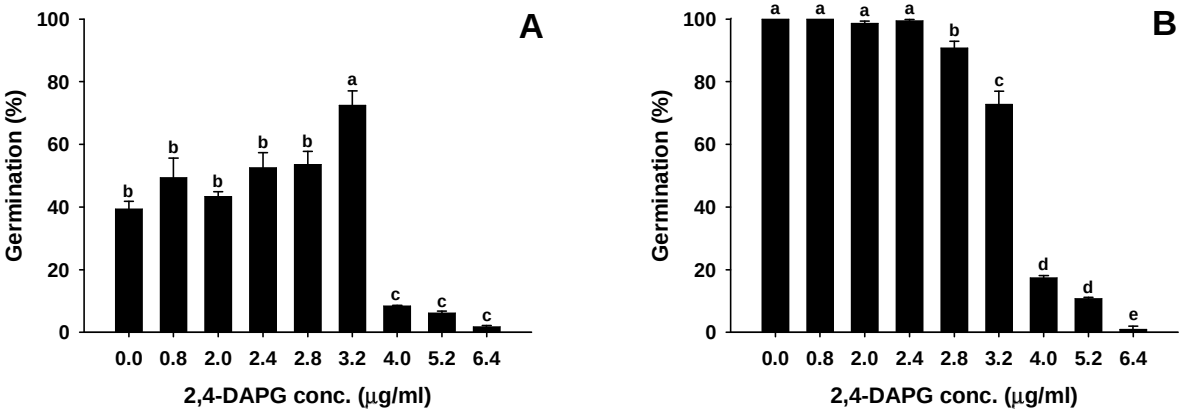

Figure 5. Effect of 2,4-DAPG on germination of encysted zoospores of *P. ultimum* var. *sporangiiferum* (Pus). (A) Germination of encysted zoospores in water after two hours of exposure to different 2,4-DAPG concentrations. (B) Germination of encysted zoospores in wheat root exudates after two hours exposure to different 2,4-DAPG concentrations. The germination percentage was determined microscopically. Means of five replicates are presented. Error bars represent the standard error of the means. Means followed by the same letter are not significantly different according to Tukey's studentized range test (P=0.05).

(Estrada-Garcia *et al.*, 1990; Zhou and Paulitz, 1993; Deacon and Donaldson, 1993). The results of this study clearly illustrate the stimulatory effect of root exudates on cyst germination (Fig. 5). The significant increase in zoospore cyst germination in water at a 2,4-DAPG concentration of 3.2 µg ml^{-1} could be explained by stimulation at sub-lethal concentrations commonly observed with fungicides (Kenyon *et al.*, 1997). For example, pyrazophos increased the sporulation of *Erysiphe* sp. on *Rhododendron* at relatively low fungicide concentrations (Kenyon *et al.*, 1997). Similar results were reported for benomyl, carbendazim, and sulphur, enhancing both the growth and sporulation of *Drechslera teres* (Toubia-Rahme *et al.*, 1995). The causes of this phenomenon are not known. However, it may be interpreted as a last survival effort of the pathogen. Collectively, the results of our study suggest that 2,4-DAPG-producing *Pseudomonas* spp. may be much more effective in biological control of *Pythium* species that infect host plants primarily by means of zoospores, or under specific environmental conditions where zoospores play a much more prominent role in the disease cycle.

Within the relatively limited number of different *Pythium* species tested in this study, considerable variation in sensitivity of mycelial growth to 2,4-DAPG was observed. Work by Mazzola *et al.* (1995) showed that various isolates of the take-all fungus *G. graminis* var. *tritici* can differ substantially in their sensitivity toward 2,4-DAPG; isolates that were relatively

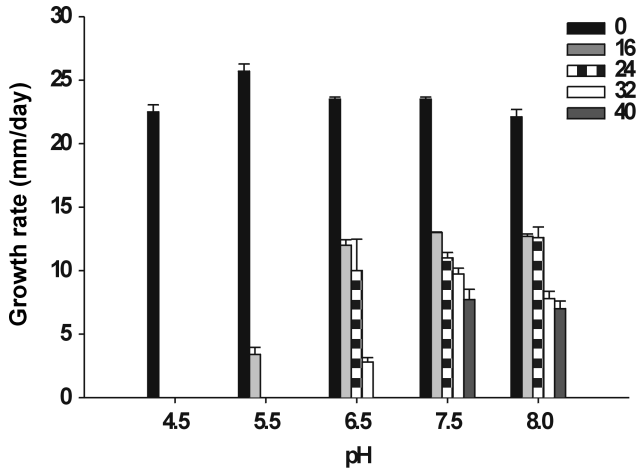

Figure 6. Effect of pH on activity of 2,4-DAPG against *P. ultimum* var. *sporangiiferum* (*Pus*). Growth rate of *Pus* on PDA with buffered pH amended with different concentrations of 2,4-DAPG was determined by measuring the radial mycelial growth every 24 hours during seven days of incubation at 25°C. Means of ten replicates are shown. Bars of different shading correspond to different concentrations of 2,4-DAPG (µg ml^{-1}). Error bars represent the standard error of the mean.

insensitive to 2,4-DAPG *in vitro* could no longer be suppressed *in situ* by a 2,4-DAPG-producing *Pseudomonas* strain. Their work clearly illustrated that variation in sensitivity within target pathogen populations to specific metabolites produced by antagonistic microorganisms can be an important factor in the inconsistency of biological control encountered under field conditions. The variation in sensitivity among and within *Pythium* species to 2,4-DAPG combined with the fact that many pathogenic species can occur simultaneously at the same site (Chamswarng and Cook, 1985), may have a significant impact on the success, or at least the consistency, of biological control provided by 2,4-DAPG producing strains.

The role of environmental conditions in 2,4-DAPG production has been studied both *in vitro* and *in situ* for a number of *Pseudomonas* strains (Shanahan *et al.*, 1992; Duffy and Défago, 1999; Notz *et al.*, 2001). These studies demonstrated that the regulation of production of this antibiotic through physiological factors may vary among strains. For example, 2,4-DAPG production was stimulated by glucose in *P. fluorescens* strain CHA0 and repressed in strain F113. In this study, we showed that pH has a significant effect on the activity of 2,4-DAPG against mycelial growth of *Pus*: the lower the pH the higher the activity of 2,4-DAPG (Fig. 6). These results suggest that the temporal and spatial changes that may occur in the nutritional and physical conditions of microsites where 2,4-DAPG- producing strains are operating will influence not only the production but also the activity of 2,4-DAPG. Changes in pH in the rhizosphere of plants, growing in agricultural soils, by as much as 2 pH units may occur (Nye, 1981). In general, the pH changes induced by roots lead to

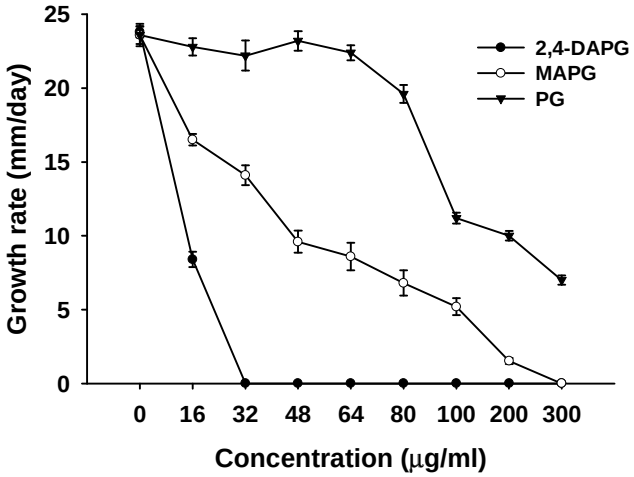

Figure 7. Activity of different phloroglucinol derivatives against mycelial growth of *P. ultimum* var. *sporangiiferum (Pus)*. 2,4-DAPG, MAPG, and PG were added to PDA plates (pH 6.5) at different concentrations. *Pus* mycelial growth was measured every 24 hours during 7 days of incubation at 25°C. Mean values of five replicates are shown. Error bars represent the standard error of the mean.

acidification, with more pronounced effects observed for dicot plants (Marschner and Römheld, 1983). Also microorganisms, including pathogenic fungi, can alter the pH to make nutrients or trace elements more readily available (Cunningham and Kuiack, 1992). As a result of these changes in pH, the activity of antimicrobial compounds produced by competing microorganisms may change. In this study, we observed that *P. ultimum* var. *sporangiiferum* acidifies unbuffered, liquid medium (potato dextrose broth) in a 7-day period from pH 6.5 to 4.5 (data not shown). This reduction in pH and coordinate increase in toxicity of 2,4-DAPG may explain some of the discrepancies in inhibitory concentrations of 2,4-DAPG reported in the different experiments presented in this study (Table 1, Fig. 6). To our knowledge, this is the first time that the effect of pH on the toxicity of 2,4-DAPG has been reported. The lower activity of 2,4-DAPG at high pH is possibly related to a dissociation of the hydroxyl-substituents of 2,4-DAPG, which may hamper accumulation into the target cell (M.A. de Waard, pers. comm.). Similar results have been described for another antibiotic, phenazine-1-carboxilic acid (PCA), produced by *P. fluorescens* 2-79, a strain that was more active against *G. graminis* var. *tritici* at lower pH (Brisbane *et al.*, 1987). A comparison between PCA from strain 2-79 and phenazine-1-carboxamide (PCN) from *P. chlororaphis* PCL 1391 showed that both phenazines have the same activity at pH levels lower than 5.0. However, PCN remained active at higher pH, whereas the activity of PCA decreased significantly (Chin-A-Woeng *et al.*, 1998). Nevertheless, no determinative effect of pH was found when strain 2-79 was introduced in soil (Ownley *et al.*, 1992). Probably, pH exerts greater influence in microsites in the rhizosphere, which are considered to be the courts of interaction between biocontrol agents

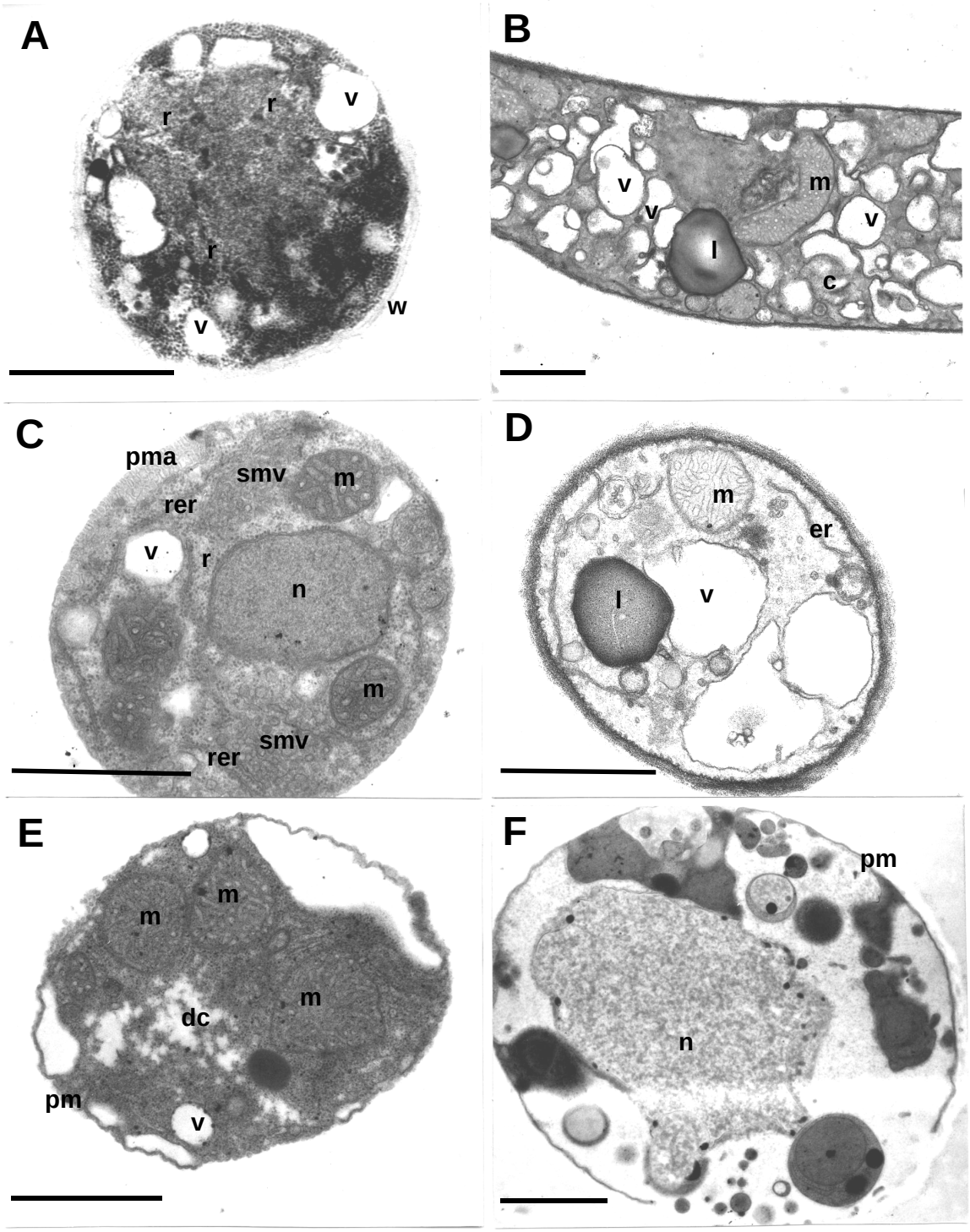

Figure 8. Electron micrographs of hyphae of *P. ultimum* var. *sporangiiferum* growing on King's B medium in absence (A, B) or in presence of 2,4-DAPG (C - F). Sections were stained either by double contrast (A, C, E, F) or with the PATAg reaction (B, D). c = cytoplasm, dc = degenerated cytoplasm, er = endoplasmic reticulum, rer = rough endoplasmic reticulum, l = lipid granule, m = mitochondria, n = nucleus, pm = plasma membrane, pma= plasma membrane alteration, r = ribosomes, smv = smooth membranous vesicles, v = vacuole, w = electron translucent wall. Bar = 1 µm.

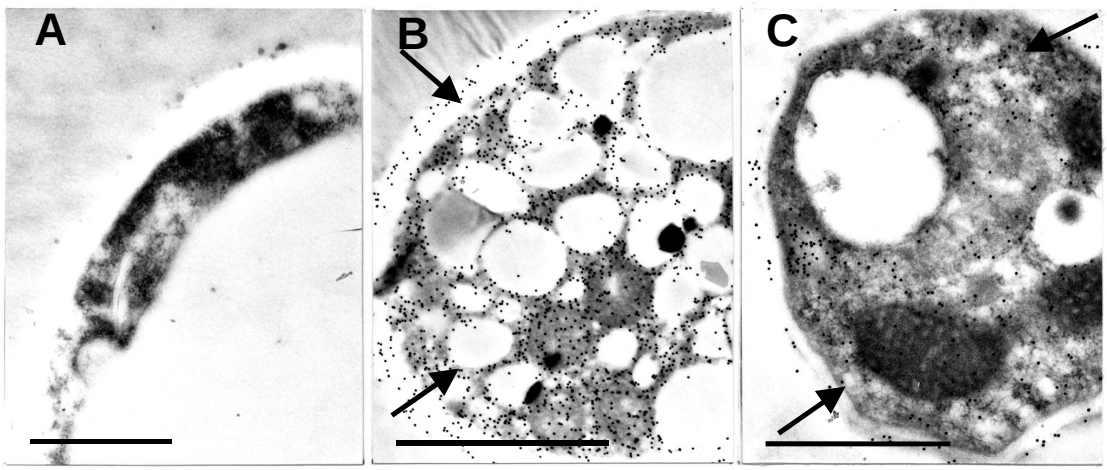

Figure 9. Electron micrographs of hyphae of *P. ultimum* var. *sporangiiferum* (*Pus*) immunolabelled with a β(1-3) glucopyranose monoclonal antibody. Control sections without primary antibody (A), hyphae of *Pus* growing in absence (B) or in presence of 2,4-DAPG (400 µg $disc^{-1}$) (C). Arrows indicate immunolabelling. Bar=1 µm.

and root pathogens. A better understanding of the interactions in these microsites is being explored by molecular techniques including the use of reporter genes (Bloemberg *et al.*, 2000; Sankarasubramanian and Kaushik, 2001; Notz *et al.*, 2001).

The differences in activity of the phloroglucinol derivatives PG, MAPG, and 2,4-DAPG indicated that the higher the level of acetylation the higher the activity against mycelial growth of *Pus* (Fig. 7). Given that in several *Pseudomonas* strains, the amounts produced and the ratio of MAPG to 2,4-DAPG may vary depending on biotic and abiotic factors (Duffy and Défago, 1999), one may envisage that also the biocontrol activity will change accordingly.

Table 2. Effect of 2,4-DAPG on cell structure of hyphal tips of *P. ultimum* var. *sporangiiferum* (*Pus*). Cell structures were scored by transmission electron microscopy and divided into 4 categories.

Cell structure	Control (N=50)	2,4-DAPG (N=29)
Dense cytoplasm with few vacuoles	16	3
Many vacuoles in the cell lumen	56	7
Beginning of cell disorganisation	24	66
Deteriorated or empty cell content	4	24

Although many activities of 2,4-DAPG are well documented (James, 1979; Yajima and Munakata, 1979; Arisawa *et al.*, 1990; Tada *et al.*, 1990; 1992; Keel *et al.*, 1992; Cronin *et al.*, 1997a; Te-Chato and Lim, 1999; Lawler *et al.*, 1999; Ito *et al.*, 2000; Debabrata and Naik, 2000; Jimenez-Escrig *et al.*, 2000), its mode of action was unclear. Phloroglucinol derivatives were reported as potent inhibitors of the photosystem II in plants (Yoneyama *et al.*, 1990; Trebst *et al.*, 1984). Typical ultrasructural changes observed in fungi exposed to other phenolic fungicides such as quintozene, chloroneb, and phenylphenol, include lysis of the inner mitochondrial membranes, vacuolization of the nuclear envelope and increases in cell wall thickness (Lyr, 1995). In our studies, however, no such symptoms were observed in *Pus* hyphal tips exposed to 2,4-DAPG. Instead, TEM observations (Figs. 8, 9) showed that 2,4-DAPG does not affect the cell wall structure and composition of hyphal tips of *Pus*, since β(1-3), β(1,4), and β(1,6) glucans were equally present in hyphal tips exposed or not to 2,4-DAPG (Figs. 8B, D, 9B, C). The extensive network of the membranous vesicles closely associated with the rough endoplasmic reticulum are likely to be transport or secretion vesicles which may accumulate as a result of stimulation of the development and activity of the Golgi apparatus by 2,4-DAPG. Alternatively, the accumulation of secretion vesicles derived from Golgi bodies may result from inhibition of hyphal elongation through a yet unknown mechanism of action of 2,4-DAPG. Localised alteration in plasma membrane organisation may indicate disruption or proliferation, due to increased Golgi vesicle trafficking.

MATERIALS AND METHODS

Organisms and culture conditions. Characteristics of the *Pythium* spp. used in this study are shown in Table 1. *Pythium* spp. were routinely grown on potato dextrose agar (PDA; Oxoid Ltd, Basingstoke, Hampshire, England) or on V8-juice agar (V8; N.V.Campbell Foods, Puurs, Belgium) at 25°C. For production of zoospores, *P. ultimum* var. *sporangiiferum* (*Pus*) was grown on V8 amended with 10 g l^{-1} $CaCO_3$ (V8+). Mycelial plugs of all *Pythium* isolates were stored in sterile mineral oil at 15°C. *Pseudomonas fluorescens* strain P60 was isolated from the wheat rhizosphere and identified with the API 20 NE test (BioMérieux sa., Lyon, France). Strain P60 was grown on Pseudomonas agar F (PSA; Difco laboratories, Detroit, MI, USA) or on King's medium B (KMB) agar (King *et al.*, 1954). The bacterial strain was stored at – 80°C in KMB broth supplemented with glycerol to a final concentration of 40% (vol/vol). For transmission electron microscopy studies, *Pus* was grown on KMB agar.

Antibiotics. The antibiotics used were phloroglucinol (PG), mono-acetylphloroglucinol (MAPG), and 2,4-diacetylphloroglucinol (2,4-DAPG). PG (1,3,5-trihydroxybenzene) and MAPG (2,4,6-trihydroxyacetophenone monohydrate) were obtained from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). 2,4-DAPG was purified from cultures of *P. fluorescens* strain P60 by high pressure liquid chromatography (HPLC) followed by photodiode array spectroscopy (PDA) to determine peak purity and identity. Purification protocols and the HPLC-PDA system were described previously (Raaijmakers *et al.*, 1999). All antibiotics were dissolved in 96% ethanol (Merck KGaA, Darmstadt, Germany). In experiments where activity of the antibiotics was determined, all treatments including controls had the same concentration of ethanol.

Wheat root exudates. Seeds of wheat cultivar Pagode were surface sterilized by soaking in 96%

ethanol for 30 sec, washing once with sterile demineralised water followed by agitation in 1.5% NaOCl supplemented with 1% Tween 20. After 1.5 h, seeds were washed three times in sterile demineralised water, incubated again for 15 min in 1.5% NaOCl and washed three times in sterile demineralised water. Seeds were germinated on 1/5th strength PDA plates at 18°C in a climate chamber with a 12-h photoperiod. Plates showing any sign of contamination were discarded. After 11 days, germinated seedlings without seed remnants were transferred to culture tubes (De Wit culture tubes, LAB Associated BV, Oudenbosch, The Netherlands) containing 5 ml of sterile diluted (2:3, vol/vol) Hoaglund's solution. After 7 days incubation at 18°C in a climate chamber with a 12-h photoperiod, plants were removed and the nutrient solutions containing the root exudates were checked for contamination by plating samples onto KMB and PDA. Contaminated samples were discarded. Sterile root exudates were stored at -20°C.

Effect of 2,4-DAPG on mycelial growth of different *Pythium* species. Effect of 2,4-DAPG on mycelial growth of fourteen different *Pythium* isolates was tested on PDA plates amended with 2,4-DAPG. The initial pH of the agar was adjusted to 6.5. 2,4-DAPG concentrations ranged from 0 to 300 µg ml^{-1} with intervals of 8 µg ml^{-1}. Mycelial plugs (0.5-cm diameter) were obtained from 3-day old cultures grown on PDA and transferred to PDA plates amended with different concentrations of 2,4-DAPG. Mycelial growth was recorded every 24h for seven days. The EC_{50} value was assessed and is defined as the concentration necessary to inhibit 50% of the mycelial growth. The minimal inhibitory concentration (MIC) was determined and is defined as the minimum concentration necessary to completely (100%) inhibit mycelial growth. Each treatment had six replicates and the experiment was performed three times.

Effect of 2,4-DAPG on zoosporangia. Mycelial plugs of 1.5 cm-diameter were cut from *Pus* agar cultures grown for 3 days on V8+ at 25°C. Mycelial plugs were transferred to petri-plates and flooded with 15 ml of solutions containing 2,4-DAPG at final concentrations of 0.0, 2.0, 4.0, 8.0, and 16.0 µg ml^{-1}. After four days at 25°C, the number of zoosporangia formed in one microscopic field at magnification of 100X (Leitz Wetzlar Dialux 20 EB, Germany), corresponding to an area of 3.14 mm^2, was measured. The diameter of 10 randomly selected zoosporangia formed per treatment after four days was measured with an ocular micrometer (Periplan 10X M, Leitz). Each treatment had five replicates and the experiment was performed twice.

Effect of 2,4-DAPG on zoospores and zoospore cysts. Zoospores of *Pus* were produced using a modification of a method described by Zhou and Paulitz (1993). *Pus* was grown on V8+ for three days at 25°C. The full-grown agar plates were cut into 2-cm wide strips and one half of the strips was transferred to another petri plate; both plates were flooded with 20 ml of sterile water and kept at 25°C. After 1 h, the water was discarded and replaced with the same volume of water. Plates were incubated at 18°C for four days, water was removed and replaced with the same volume and incubated at 18°C for 2 h; zoospores were collected and concentrated using a 0.45 µm nylon filter (ZapCap; Schleicher & Schuell GmbH, Dassel, Germany). Supensions containing 20,000 to 40,000 zoospores ml^{-1} were typically obtained. Aliquots of 50 µl of a suspension containing 25,000 zoospores ml^{-1} of *Pus* were transferred to 96-well microtiter plates (Greiner BV, Alphen aan den Rijn, The Netherlands) and mixed in a 1:1 ratio with solutions of 2,4-DAPG to obtain final 2,4-DAPG concentrations of 0.0, 0.2, 0.4, 0.8, 1.6, and 3.2 ng ml^{-1}. The number of swimming, encysted, germinated, and disintegrated zoospores were counted under an inverted microscope (Axiovert 10, Carl Zeiss, Jena, Germany). Treatments had five replicates and the experiment was performed five times.

The effect of 2,4-DAPG on germination of encysted zoospores was studied in more detail in separate experiments. These experiments were performed both in water and in wheat root exudates. To study germination in water, encystment of zoospores of *Pus* was induced by vortexing a suspension containing 25,000 zoospores ml^{-1} for 25 sec. Encysted zoospores were transferred to 96-well microtiter plates and mixed with 2,4-DAPG solutions to obtain final 2,4-DAPG concentrations of 0, 0.8, 1.6, 2.0, 2.4, 2.8, 3.2, 4.0, 5.2, and 6.4 µg ml^{-1}. To study the effect of wheat root exudates, a zoospore suspension containing 50,000 zoospores ml^{-1} was vortexed for 25 sec; encysted zoospores were transferred to microtiter plates and diluted with root exudates to obtain the same final 2,4-DAPG concentrations described above. The final concentration of root exudates was half of the initial root exudates obtained as described previously. Microtiter plates were incubated at 25°C and after 2 h, a drop of 96% ethanol was added to each well to stop germination. The number of germinated zoospore cysts was determined microscopically. Prior to addition of 2,4-DAPG and wheat root exudates to zoospore suspensions, the initial number of germinated encysted zoospores was determined microscopically. Treatments had five replicates each and the experiment was performed twice.

Effect of acetylation on activity of phloroglucinol derivatives. The effect of the level of acetylation on the activity of phloroglucinol derivatives was tested on mycelial growth of *Pus*. PG, MAPG, and 2,4-DAPG have no, one, and two acetyl groups, respectively (Fig. 1). *Pus* was grown on PDA

amended with PG, MAPG, or 2,4-DAPG to final concentrations of 0.0, 16.0, 32.0, 48.0, 64.0, 80.0, 100.0, 200.0, and 300.0 µg ml^{-1}. Mycelial growth was measured every 24 h for seven days and growth rates (mm day^{-1}) were calculated. Treatments had five replicates and the experiment was performed three times.

Effect of pH on activity of 2,4-DAPG. *Pus* was grown on PDA buffered with 0.0015 M citric acid (0.001 M $C_6H_8O_7.1H_2O$; 0.002 M $Na_2HPO_4.2H_2O$) to a pH of 4.5, 5.5, 6.5, 7.5, or 8.0. PDA plates with different pH were amended with 2,4-DAPG to final concentrations of 0.0, 16.0, 24.0, 32.0, and 40.0 µg ml^{-1}. Plates were incubated at 25°C and Mycelial growth was measured every 24 h for seven days. Each treatment had 10 replicates and the experiment was performed three times.

Static effect of 2,4-DAPG on mycelial growth. *Pus* was grown on PDA for three days and 0.5-cm-diameter mycelial plugs were transferred to PDA plates amended with 2,4-DAPG to final concentrations of 0, 8, 16, 32, 48, 64, 96, 128, 200, and 300 µg ml^{-1}. After seven days, mycelial plugs that failed to grow on PDA amended with 2,4-DAPG were again transferred to PDA (pH 6.5) without 2,4-DAPG. Growth was recorded up to seven days after transfer. Treatments had five replicates and the experiment was perfrmed twice.

TEM studies. Mycelial plugs of 6 mm-diameter were cut from *Pus* agar cultures grown for 3 days on KMB at 25°C. Mycelial plugs were transferred to the center of Petri plates containing KMB. Sterile antibiotic assay discs of 9 mm-diameter (Whatman, Springfield Mill, England) were imbibed with solutions containing 2,4-DAPG at final concentrations of 0.0, 25, 50, 100, 200, and 400 µl. Discs were placed at a distance of 3 cm from the mycelial plugs. Petri dishes were sealed with parafilm and incubated 5 to 6 days in the dark at 25°C. Five samples of 2 mm^2 of agar were collected at the margin of *Pus* cultures exposed or not to 400 µg of 2,4-DAPG per disc. Samples were fixed overnight at 4°C in 2% glutaraldehyde buffered in 0.1M cacodylate (pH 7.2), washed in the same buffer, then dehydrated in a graded ethanol series and embedded in Epon (Prolabo, France) or LR White medium grade resin (Oxford instrument, France).

Epon embedded samples were postfixed with 1% osmium tetroxide before ethanol dehydration (Gianinazzi and Gianinazzi- Pearson, 1992). Ultrathin sections (0.1µm) were cut with a diamond knife (Reicher Ultracut microtone). For ultrastructural observations, Epon sections were cut onto copper grids and double contrasted by post staining with 3% uranyl acetate in 50% ethanol (Valentine, 1961) followed by lead citrate (Reynolds, 1963). To locate β(1-4) and β(1-6) glycosylated compounds (glycoproteins and polysaccharides), Epon embedded samples were subjected to the periodic acid thiocarbohydrazide silver proteinate reaction (PATAg) according to Thiéry (1967).

LR White embedded sections were collected on golden grids coated with collodion for analytical immunocytochemistry using an indirect immunogold labelling technique for the detection of β(1-3) glucans (Lemoine *et al.*, 1995). Samples on grids were incubated overnight at 4°C in a mouse monoclonal antibody raised against β(1-3) glucopyranose polymers (Biosupplies Australia, Australia) which has previously been shown to be specific for β(1-3) glucans of the cell wall (Lemoine *et al.*, 1995). The antibody was diluted 1000 or 500 times in Tris buffer saline with 1% bovine serum albumin. After rinsing, sections were incubated for 1h in a gold-labelled (15 nm) goat antimouse polyclonal antibody (Tebu Biocell, France) diluted 20 times to detect the primary antibody. Imunological control sections were prepared by omitting the monoclonal antibody during the first incubation step. All sections were counter stained for 10 min with 3% aqueous uranyl acetate before observation in a transmission electron microscope (Hitachi H7500) at 80 kV.

The effect of 2,4-DAPG on *Pus* mycelium was evaluated by quantifying hyphal responses on Epon-embeded double contrasted sections in the following categories: 1) dense cytoplasm with few vacuoles (young); 2) many vacuoles in the cell lumen; 3) beginning of cell content disorganisation; 4) deteriorated or empty cell contents (dead). The effect of 2,4-DAPG on *Pus* cell wall synthesis was determined by observing LR White imunogold labelled sections. All experiments were repeated twice.

Statistical analysis. Data were analysed by ANOVA followed by Tukey's studentized range test after certifying normal distribution and homogeneity of variances (SAS institute, Inc., Cary, N.C.). All experiments were repeated at least twice and representative results are shown.

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CHAPTER 6

ACTIVITY OF BIOSURFACTANTS PRODUCED BY *PSEUDOMONAS FLUORESCENS* AGAINST OOMYCETE PATHOGENS

ABSTRACT

Biosurfactants are amphipathic compounds produced by microorganisms that reduce surface and interfacial tensions. In this study, we demonstrated that biosurfactant-producing fluorescent *Pseudomonas* species occur in the wheat rhizosphere at densities of approximately 10^5 CFU g^{-1}. We report the discovery of six *Pseudomonas* isolates that produce biosurfactants with lytic activity against zoospores of multiple oomycete pathogens. Zoospores exposed to cell suspensions or cell-free culture supernatant of these isolates lost their motility within 30 sec and exploded within 60 sec. One of these isolates, *P. fluorescens* SS101, reduced the surface tension of water from 73 mN m^{-1} to approximately 30 mN m^{-1}. Application of cell suspensions of strain SS101 to soil or hyacinth bulbs provided protection against root rot caused by *Pythium intermedium*. Five mutants of SS101, obtained by random Tn*5* mutagenesis, lacked the ability to reduce the surface tension of water and to cause lysis of zoospores. Subsequent genetic characterization of the Tn*5*-flanking regions of two surfactant-deficient mutants, 10.24 and 17.18, showed that the transposons had integrated in condensation domains of peptide synthetases. When applied to soil, mutant 10.24 was not able to protect hyacinth against Pythium root rot, whereas wildtype strain SS101 gave significant control. The partially purified surfactants from *P. fluorescens* SS101 lowered the surface tension of water from 73 to approximately 30 mN m^{-1} and reached the critical micelle concentration at 25-50 µg ml^{-1}. Reverse-phase high performance liquid chromatography (RP-HPLC) and liquid chromatography–mass spectrometry (LC-MS) yielded eight different fractions of which five had surface activity and caused cessation of motility and lysis of zoospores. Collectively, the results indicate that biosurfactants produced by *P. fluorescens* SS101 play an important role in interactions with zoosporic pathogens and in control of Pythium root rot of hyacinth.

Jorge T. de Souza, Marjan de Boer, Pieter de Waard, Teris A. van Beek, and Jos M. Raaijmakers

To be submitted.

INTRODUCTION

Microbial compounds that alter the conditions prevailing at a surface or interface are often referred to as adjuvants, bioemulsifiers or biosurfactants (Cooper and Zajic, 1980; Zajic and Seffens, 1984; Fiechter, 1992). A variety of microorganisms, including bacteria, fungi and yeasts, have been described to produce biosurfactants. Several of these biosurfactants are well-described chemically and catagorized into high- and low-molecular mass compounds. The low-molecular biosurfactants include glycolipids and lipopeptides, such as rhamnolipids and surfactin. The high-molecular compounds include proteins, lipoproteins or complex mixtures of these polymers (Ron and Rosenberg, 2001). Although biosurfactants are structurally diverse, they all have an amphipathic nature, i.e. they contain both hydrophobic and hydrophylic groups. The hydrophobic moieties are usually saturated, unsaturated or hydroxylated fatty acids, and the hydrophylic moieties consist of mono-, di-, or polysaccharides, carboxylic acids, amino acids or peptides (Ron and Rosenberg, 2001).

Among the bacterial genera, *Pseudomonas* spp. have been reported to produce biosurfactants (Fiechter, 1992; Ron and Rosenberg, 2001). *Pseudomonas* spp. are common inhabitants of soil and rhizosphere environments and have received considerable attention in the areas of bioremediation of xenobiotics and biological control of plant pathogenic fungi. In the area of bioremediation, surfactant-producing *Pseudomonas* spp. have been implicated in facilitating degradation of ubiquitous pollutants such as polycyclic aromatic hydrocarbons (PAHs) and *n*-alkanes (Arino *et al*., 1996; Déziel *et al*., 1996). In the area of biological control of plant pathogenic fungi, the potential of biosurfactants produced by *Pseudomonas* spp. was only recently described. Rhamnolipids produced by strains of *P. aeruginosa* were shown to be highly effective against plant pathogens, including *Pythium aphanidermatum*, *Plasmopara lactucae-radicis* and *Phytophthora capsici* (Stanghellini and Miller, 1997). Purified rhamnolipids caused cessation of motility and lysis of entire zoospore populations within less than 1 min. Introduction of a rhamnolipid-producing strain in a recirculating hydroponic system gave good control, although transient, of *P. capsici* on pepper (Stanghellini and Miller, 1997). Kim *et al*. (2000) confirmed and extended these observations by showing that rhamnolipid B produced by *P. aeruginosa* B5 not only has lytic effects on zoospores, but also inhibitory activity against spore germination and hyphal growth of several other pathogens. Mycelial growth of *P. capsici* and spore germination of *Colletotrichum orbiculare* were inhibited *in vitro* and the diseases caused by these pathogens were suppressed in pepper and cucumber plants, respectively, by application of purified rhamnolipid B to leaves (Kim *et al.*, 2000).

Several lipopeptide surfactants with antibiotic properties were recently proposed as biological compounds for the control of plant pathogenic fungi (Nielsen *et al.*, 1999). Viscosinamide and tensin, produced by soil-inhabiting *P. fluorescens* strains, were shown to affect mycelium of *Rhizoctonia solani* and *Pythium ultimum*, causing reduced growth and intracellular activity, hyphal swellings, increased branching and rosette formation (Thrane *et al.*, 1999; Nielsen *et al.*, 2000). These studies clearly indicated the potential of biosurfactant-producing *Pseudomonas* spp. for crop protection.

To date, however, little is known about the frequencies of biosurfactant-producing *Pseudomonas* spp. in natural environments and more specifically in the plant rhizosphere. In this study, we determined the frequency of biosurfactant- producing *Pseudomonas* spp. in the rhizosphere of wheat. We report the discovery of *P. fluorescens* strains that produce surfactants with lytic activity against zoospores of oomycetes, including *Pythium* species and *P. infestans*. The role of biosurfactants in suppression of Pythium root rot of hyacinth by representative *P. fluorescens* strain SS101 was determined by generating mutants defective in the production of biosurfactants. Mutants were genetically and phenotypically characterized and their activity against *Pythium* spp. was compared to that of their parental strain. The composition and activity of the surface-active extract was determined by reverse phase high-performance liquid chromatography (RP-HPLC) and bioassays, respectively.

RESULTS

Frequencies of surfactant-producing *Pseudomonas* spp. Population studies were performed for seven successive cycles of wheat grown in three soils. Only results from three growth cycles are shown (Table 1). The population densities of surfactant-producing *Pseudomonas* spp. was determined by randomly transferring approximately 10% of the fluorescent *Pseudomonas* colonies, isolated from the wheat rhizosphere on KMB^+ medium, to SW medium. Bacterial colonies showing a halo on SW medium were considered as putative surfactant producers. Population densities of putative surfactant-producing *Peudomonas* spp. ranged from 1.0×10^5 to 7.0×10^5 CFU g^{-1} root and represented approximately 1 to 5% of the total population of fluorescent *Pseudomonas* isolated from roots of wheat. Cycles 4 to 7 and cycles 1 to 7 of the repeated experiment showed similar results.

Table 1. Population densities of surfactant-producing *Pseudomonas* spp. recovered from roots of wheat grown in natural soils for three successive cycles of four weeks each.

Soil	First cycle [a]		Second cycle [a]		Third cycle [a]	
	Fluoresc. [b] Pseudom.	Surfact. [c] Produc.	Fluoresc. [b] Pseudom.	Surfact. [c] Produc.	Fluoresc. [b] Pseudom.	Surfact. [c] Produc.
CB	1.7×10^7	1.7×10^5	8.5×10^6	2.6×10^5	5.3×10^6	1.1×10^5
SV	1.9×10^7	3.8×10^5	5.3×10^6	2.1×10^5	1.5×10^7	1.5×10^5
SSB	1.4×10^7	7.0×10^5	5.6×10^6	1.1×10^5	5.2×10^6	1.0×10^5

[a] Wheat seedlings were grown in CB, SV and SSB soils for three successive cycles of 4 weeks each.
[b] Total population densities of fluorescent *Pseudomonas* spp. (CFU g^{-1} root) were determined by plating rhizosphere suspensions on KMB^+ medium. Mean values of four replicates are given.
[c] Populations of surfactant-producing *Pseudomonas* spp. (CFU g^{-1} root) were determined by randomly transferring approximately 10% of the colonies recovered on KMB^+ to SW medium. Colonies forming a halo after 3 to 5 days of growth at 25 °C were considered surfactant producers. Mean values of four replicates are given.

Selection and characterization of strain SS101. From a total of 375 *Pseudomonas* isolates that produced a halo on SW medium, 6 isolates were able to lyse zoospores of *Pythium ultimum* var. *sporangiiferum* (Fig. 1). All 6 isolates were obtained from roots of wheat grown in SSB soil. A cell suspension (10^9 CFU ml^{-1}) of these isolates caused cessation of zoospore motility within 30 sec and the shape of the zoospores was changed from bean-shaped to round (Fig. 1A). The bacterial cell suspension caused lysis of the entire zoospore populations within 60 sec (Fig. 1B). Identical responses were observed for zoospores from *Phytophthora infestans*, *Pythium intermedium*, and *Albugo candida*. From the six isolates that caused lysis of zoospores, isolate SS101 was selected for further studies. Biochemical characterization by API 20 NE test and GC-FAME analysis classified isolate SS101 as *P. fluorescens* Biovar II. Strain SS101 exhibited multiple characteristics indicative of surfactant production, including halo formation on SW medium, foam formation, drop-collapse, and the ability to lower the surface tension (Table 2). The other *Pseudomonas* strains, including reference strains WCS358, PAO1, and DSM1128, exhibited similar characteristics, however, none of these isolates caused lysis of zoospores (Table 2). Only isolate SV7 caused cessation of zoospore motility, but no subsequent lysis.

Activity of *P. fluorescens* SS101 against *P. intermedium*. The activity of *P. fluorescens* SS101 against *P. intermedium*, causal agent of Pythium root rot of hyacinth, was tested in a bioassay at 9°C for 8 weeks. *P. intermedium* P52 significantly decreased the root fresh weight of hyacinth by at least 4-fold (Fig. 2). Treatment of hyacinth bulbs or soil with *P. fluorescens* SS101 provided substantial protection of roots of hyacinth grown in soil treated with *P. intermedium* P52 (Fig. 2A). In a second experiment, treatment of both bulbs and soil resulted

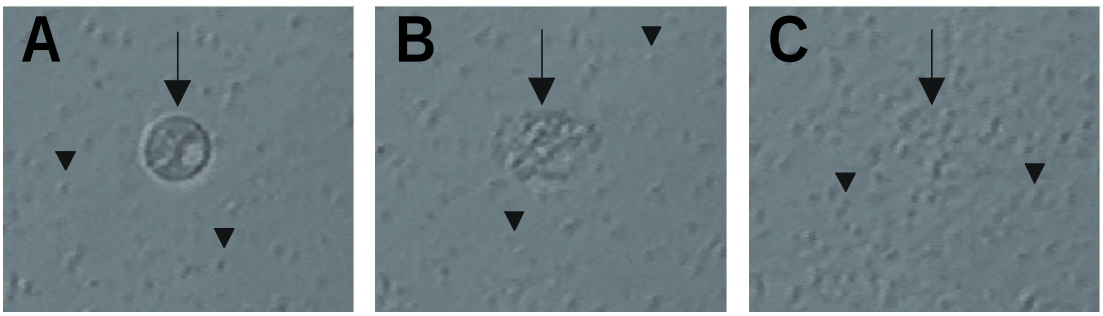

Figure 1. Effect of *P. fluorescens* SS101 on zoospores of *P. ultimum* var. *sporangiiferum*. A: within 30 sec after exposure to strain SS101, zoospores become immotile and round-shaped. B: within 60 sec, lysis of zoospores occurs; C: within an additional 10-30 sec, the remnants of the zoospores completely disappear. Arrows indicate a zoospore of *P. ultimum* var. *sporangiiferum* and arrowheads indicate cells of strain SS101 (magnification 400X). The density of the bacterial cell suspension was 10^9 CFU ml^{-1} and zoospore suspensions contained 10^4 zoospores ml^{-1}.

in complete protection of hyacinth roots against *P. intermedium* P52 (Fig. 2B).

Tn*5* mutagenesis and characterization of surfactant-deficient mutants. A total of 520 transformants of *P. fluorescens* SS101 were obtained following mutagenesis with Tn*5lacZKm*. Five of these transformants were not able to form a halo on SW medium (Table 3). Characterization of these mutants with RAPD analysis using three 10-mer primers confirmed strain integrity. Southern blot analysis with a probe targeting the kanamycin resistance gene showed the presence of a single Tn*5*-integration for each of the five mutants. These mutants gave a negative reaction in the drop-collapse test, were unable to form foam after vigorously shaking, did not lower the surface tension of water to the same extent as wild-type strain SS101, and could no longer affect the motility and cause lysis of zoospores of multiple oomycetes (Table 3). In contrast to the wild-type, all mutants presented β-galactosidade activity, indicating integration of the promotorless Tn*5lacZKm* element in a coding region. All mutants still produced wild-type levels of protease, phospholipase C, and siderophores, suggesting that a global regulatory gene was not inactivated.

Role of surfactants in activity against *P. intermedium*. To determine the possible role of surfactant production in the activity of strain SS101 against *P. intermedium*, bioassays with mutant 10.24 were performed. Application of wild-type strain SS101 restored the root weight of hyacinth to levels not significantly different from the untreated control (Fig. 3A). In

Table 2. Phenotypic characteristics of *P. fluorescens* strain SS101 and other *Pseudomonas* strains.

Strain	Halo [a] formation	Drop [b] collapse	Foam [c]	Surface [d] tension (mN m^{-1})	Zoospore [e] motility	Zoospore [e] lysis
Water	- *	-	-	73.5	-	-
P. fluorescens SSB17	-	-	-	70.5	-	-
P. fluorescens SS101	+ *	+	+	29.7	+	+
P. fluorescens SV7	+	+	+	54.0	+	-
P. putida WCS358	+	+	+	30.5	-	-
P. aeruginosa PAO1	+	+	+	56.5	-	-
P. aeruginosa DSM1128	+	+	+	49.5	-	-

* + positive reaction; - negative reaction.

[a] Evaluated on SW medium after 3 to 5 days of growth at 25°C.

[b] Cell suspensions (OD_{600}=1)were tested on an oily surface for their ability to collapse a drop of water.

[c] Foam formation after vigorous shaking cell suspensions (OD_{600}=1).

[d] Surface tension of cell suspensions (OD_{600}=1)was measured with a tensiometer.

[e] Zoospore motility and lysis were observed microscopically after mixing bacterial cell suspensions (OD_{600}=1) with zoospore suspensions of *P. ultimum* var. *sporangiiferum* (10^4 zoospores ml^{-1}).

contrast, the surfactant-deficient mutant 10.24 was not effective against Pythium root rot of hyacinth (Fig. 3A). The disease level was significantly reduced in the treatment with strain SS101, whereas mutant 10.24 failed to control Pythium root rot (Fig. 3B). These results indicate that surfactant production by strain SS101 plays an important role in control of Pythium root rot of hyacinth.

Identification of genes involved in surfactant biosynthesis. The flanking regions of Tn*5* transposon were cloned and sequenced for surfactant-deficient mutants 10.24 and 17.18 by anchored PCR (aPCR). Sequences of mutant 10.24 yielded a 852-bp fragment with a deduced protein sequence that has similarity with the condensation domain of peptide synthase genes from several bacteria and eukaryotes (Fig. 4). The deduced protein sequence showed 54% (161/293) similarity with a condensation domain in the syringopeptin synthetase gene *sypA* from *P. syringae* pv. *syringae* B310D and varying degrees of similarity to five condensation domains of the syringomycin synthetase *syrE* gene of *P. syringae* pv. *syringae* B310D.

The *syrE* gene encodes a 9376-aa protein and the similarities of its different condensation domains to the condensation domain of *P. fluorecens* SS101 were 48% (109/226) for positions 65 to 283, 38% (112/288) for positions 4270 to 4557, 36% (107/293) for positions 5332 to 5623, 35% (106/295) for positions 6388 to 6681, and 39% (117/295) for positions 8548 to 8841. Similarities were 44% (121/271) for positions 11 to 279 and 32% (71/210) for positions 1053 to 1258 of a 1278-aa protein involved in the synthesis of the siderophore 2,3-dihydroxybenzoate (DHB) by *B. subtilis*, and 41% (121/281) for positions

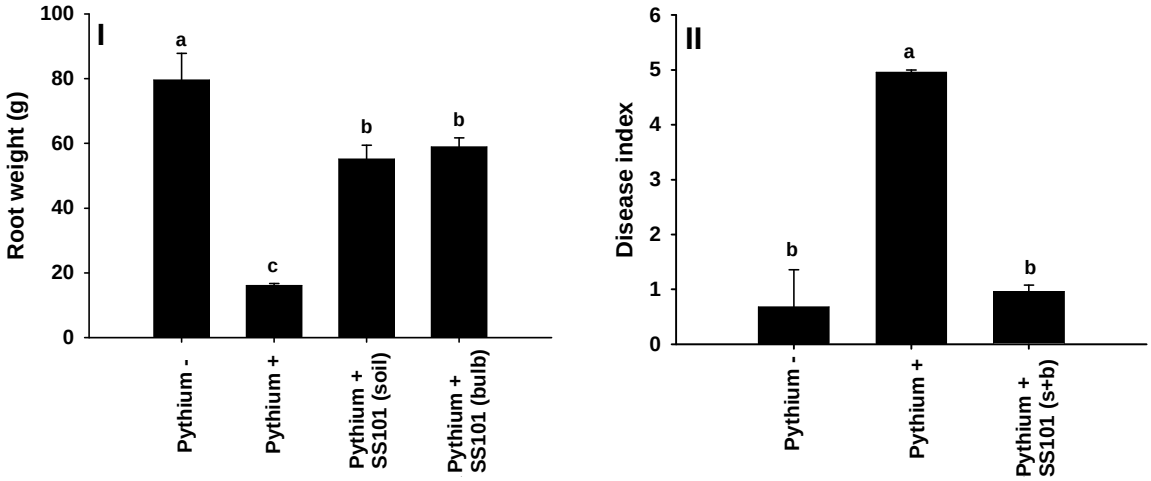

Figure 2. Effect of *P. fluorescens* SS101 on Pythium root rot of hyacinth. Inoculum of *P. intermedium* P52 was mixed through soil (Pythium +). Soil not treated with *P. intermedium* (Pythium -) served as a healthy control. *P. fluorescens* SS101 was applied to soil [SS101(soil)], bulbs [SS101(bulb)], or both [SS101(s + b)]. Cell suspensions for bulb treatment were 10^7 CFU ml^{-1} and the initial density of SS101 in soil treatments was 10^7 CFU g^{-1} soil fresh weight. Plants were grown at 9°C in the dark. After 8 weeks, root weight was determined in experiment I and disease index was scored in experiment II. Disease index was scored on a 0 to 5 scale, where 0 indicates no disease and 5 indicates 80 to 100% loss of root biomass relative to the control. Means of five replicates are shown. Means with the same letter are not statistically different according to Tukey's studentized range test (P = 0.05). Error bars represent the standard error of the mean. The experiment was repeated 4 times and representative results of two experiments are shown.

1990 to 2270 and 45% (83/189) for positions 3145 to 3322 of a 3487-aa protein encoded by the gene *mcyE*, which is responsible for the synthesis of the cyclic heptapeptide microcystin in the bacterium *Microcystis aeruginosa* K-139. A majority sequence obtained from the alignement of 43 different condensation domains, including proteins involved in the synthesis of gramicidin S by *Brevibacillus brevis*, surfactin by *Bacillus subtilis* and peptide synthetases from several fungi, including *Cochliobolus carbonum*, *Penicillium chrysogenum*, *Emericella nidulans*, and *Acremonium chrysogenum*, showed that the active-site motif (HHXXXDG) is conserved in all aligned condensation domains, including the partial sequence obtained from strain SS101 (Fig. 6).

Sequence analysis of the Tn*5* flanking region of mutant 17.18 yielded a 453-bp fragment with a deduced protein sequence that presents 61% (90/145) similarity with a 3310-aa protein from *Bradyrhizobium japonicum* 110spc4, 66% (96/144) similarity for positions 415 to 558 and 61% (90/146) similarity for positions 2550 to 2695 of a 5953-aa peptide synthase protein of the bacterial pathogen *Ralstonia solanacearum* GMI1000, 63% (92/144) similarity with the 3316-aa protein XpsB from *Xenorhabdus bovienii* T228, and similarity with

Table 3. Characteristics of surfactant-deficient mutants of *P. fluorescens* strain SS101.

Test	SS101	Mutants				
		9.26	10.24	11.17	13.3	17.18
Halo formation [a]	+ *	- *	-	-	-	-
Drop-collapse [b]	+	-	-	-	-	-
Foam formation [c]	+	-	-	-	-	-
Surface tension (mN m^{-1}) [d]	29.7	64.3	56.0	60.0	56.5	62.0
β-galactosidase activity [e]	-	+	+	+	+	+
Protease [f]	+	+	+	+	+	+
Phospholipase C [g]	+	+	+	+	+	+
Fluorescence [h]	+	+	+	+	+	+
Zoospore motility [i]	+	-	-	-	-	-
Zoospore lysis [i]	+	-	-	-	-	-

* + positive reaction; - negative reaction.

[a] Evaluated on SW medium after 3 to 5 days of growth at 25°C.

[b] Cell suspensions (OD_{600}=1) were tested on an oily surface for their ability to cause the collapse of a drop of water.

[c] Cell suspensions (OD_{600}=1) were observed for foam formation after vigorous shaking.

[d] Surface tension of cell suspensions (OD_{600}=1) was measured with a tensiometer.

[e] Evaluated on KMB plates containing X-gal and IPTG after 48h of growth at 25 °C.

[f] Tested on skim milk plates.

[g] Tested on egg yolk plates.

[h] Tested on Pseudomonas agar F plates under near UV light.

[i] Zoospore motility and lysis were observed microscopically after mixing bacterial cell suspensions with zoospore suspensions of *P. ultimum* var. *sporangiiferum*, *A. candida*, or *P. infestans*.

four domains of the 9376-aa syringomycin synthetase protein from *P. syringae* pv. *syringae* B310D (Fig. 7). Similarity levels for these domains were 58% (86/146) for positions 1337 to 1478, 57% (84/144) for positions 2425 to 2563, 57% (84/144) for positions 3512 to 3650, and 56% (85/148) for positions 7794 to 7941 of syringomycin synthetase. A database search and comparison of these sequences showed that the transposon is integrated in a condensation domain of peptide synthetases. However, the flanking sequence of mutant 17.18 was not long enough to identify conserved active-site motifs of condensation domains. Collectively, these results show that in both mutants 10.24 and 17.18 the transposon is integrated in condensation domains of putative peptide synthetases.

Isolation and characterization of surfactants produced by *P. fluorescens* SS101. Extraction of the surfactant(s) produced by *P. fluorescens* SS101 yielded a white precipitate, whereas no such precipitate was obtained from the surfactant-deficient mutant 10.24. After extraction, the composition was investigated by RP-HPLC with both UV and evaporative light scattering (ELS) detection. With an acidified water – acetonitrile gradient, eight fractions were observed and collected (Table 4). Fraction 8 was the main component and accounted for at least 95% of the extract. There was a good correspondence between UV detection at 210 nm and ELS

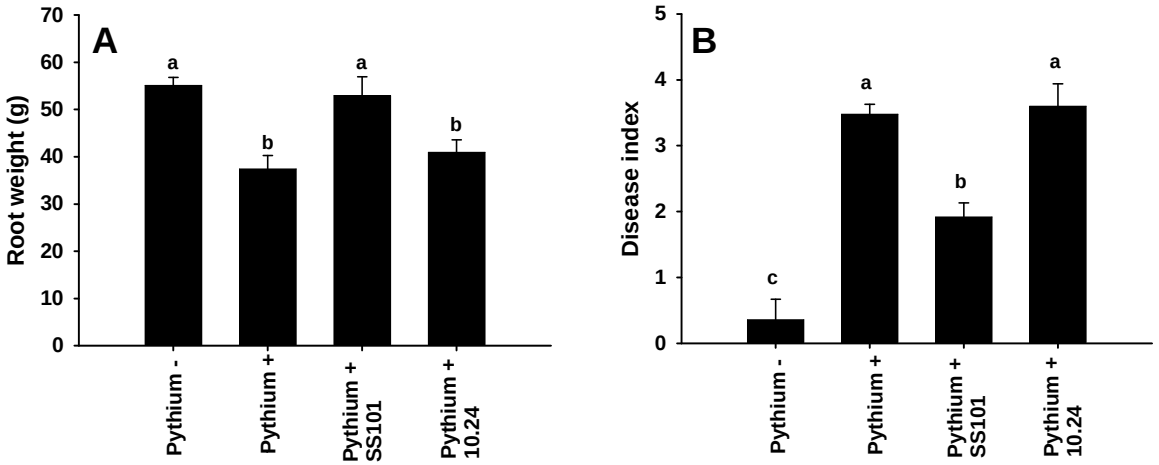

Figure 3. Effect of *P. fluorescens* SS101 and its surfactant deficient mutant 10.24 on Pythium root rot of hyacinth. Inoculum of *P. intermedium* P52 was mixed through soil (Pythium +). Soil not treated with *P. intermedium* (Pythium -) served as a healthy control. *P. fluorescens* SS101 and surfactant-deficient mutant 10.24 were applied to the soil at densities of 10^7 CFU g^{-1} soil fresh weight. Plants were grown at 9°C in the dark. After 8 weeks, root weight (A) and disease index (B) were determined. Disease caused by *P. intermedium* P52 was scored on a 0 to 5 scale, where 0 indicates no disease and 5 indicates 80 to 100% loss of root biomass relative to the control. Means of five replicates are shown. Means with the same letter are not statistically different according to Tukey's studentized range test (P = 0.05). Error bars represent the standard error of the mean. The experiment was repeated 2 times and representative results are shown.

detection. All fractions, with exception of fraction 1, were positive in the drop-collapse test, indicating a decrease in the surface tension. Only fractions 5 and 8 were positive in the foam formation test. Fractions 4 to 8 caused cessation of zoospore motility and subsequent lysis. Fraction 8, which was the most abundant fraction, caused lysis of zoospores in a much shorter time frame than the other fractions. Zoospores were rendered immotile within 10 sec and lysed within 30 sec after contact with fraction 8, whereas for the other fractions it took approximately 30 sec for cessation of motility and approximately 60 sec for lysis. The lysis caused by fraction 7 was not a characteristic explosion observed with the other fractions, but resembled leaking at one point in the zoospore membrane.

The critical micelle concentration (CMC value) is defined as the solubility of a surfactant in an aqueous phase. At concentrations above the CMC, surfactant molecules aggregate to form polymers called mycelles which solubilize oil or water into the other phase, creating a microemulsion. No further drop of the surface tension occurs as the amount of surfactant exceeds the CMC value because the emulsion is already saturated (Georgiou *et al.*, 1992). The CMC value is one of the measures of the strength of the surface activity of a

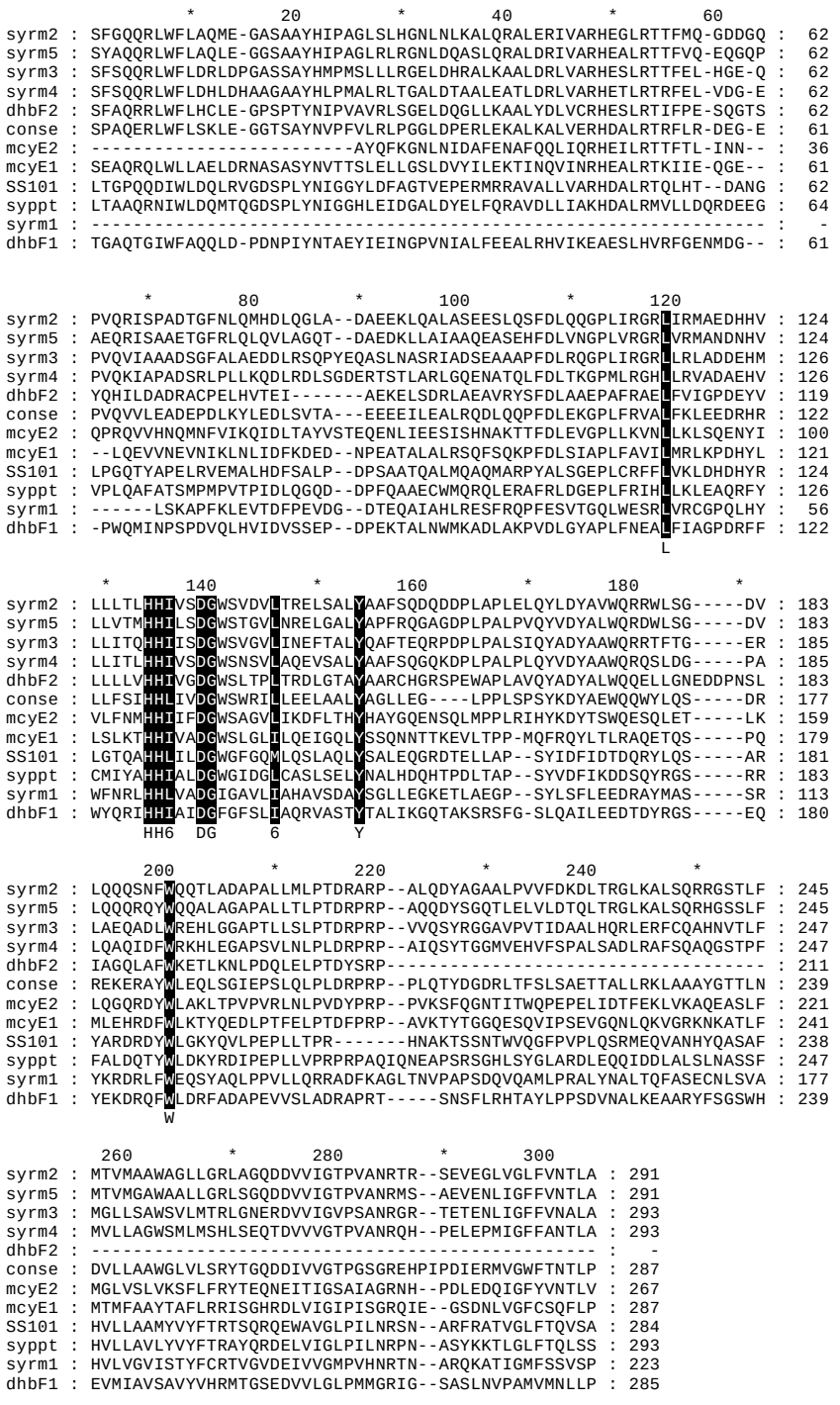

Figure 4. Alignment of the deduced protein sequences of *P. fluorescens* SS101 and other condensation domains of peptide synthetase sequences. Sequence of *P. fluorescens* SS101 was obtained from the Tn*5* flanking region of surfactant-deficient mutant 10.24. Syrm1 to syrm5 are condensation domains from the syringomycin synthetase protein of *P. syringae* pv. *syringae* B310D (Accession number T14593). Syppt is a condensation domain of the syringopeptin synthetase protein from *P. syringae* pv. *syringae* B310D (AAF99707). Conse represents a majority sequence obtained from the alignement of 43 condensation domains of different peptide synthetases (PF00574). DhbF1 and dhbF2 are condensation domains of a peptide synthetase involved in the synthesis of the siderophore 2,3-dihydroxybenzoate (DHB) by *B. subtilis* (NP_391076), and myc1 and myc2 are condensation domains of a protein responsible for the synthesis of microcystin by *Microcystis aeruginosa* K139 (BAB12211). Black-shaded residues represent high levels of similarity.

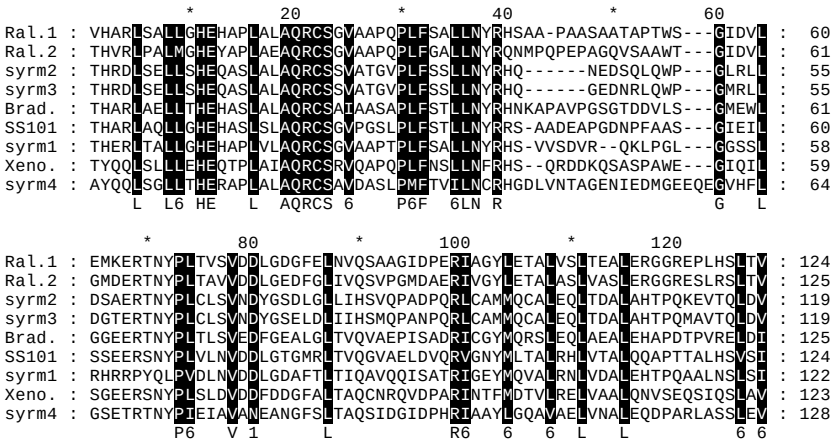

Figure 5. Alignment of deduced protein sequences of *P. fluorescens* SS101 and other proteins found in the database. For *P. fluorescens* SS101, the sequence was obtained from the Tn*5* flanking region of surfactant-deficient mutant 17.18. Ral.1 and Ral.2 are different domains of a peptide synthetase protein from *Ralstonia solanacearum* GMI1000 (NP_522203). Syrm1 to syrm4 are different domains of of the syringomycin synthetase protein from *P. syringae* pv. *sringae* B310D (T14593). Brad. is a protein from *Bradyrhizobium japonicum* 110spc4 (AAG61082), and Xeno. is a domain from the XpsB protein from *Xenorhabdus bovienii* T228 (AAL57600). Black-shaded residues are similar in all aligned sequences.

surfactant. The CMC of the partially purified surfactant extract from strain SS101 was shown to be between 25 and 50 μg ml^{-1} (Fig. 6). The effect on zoospores (motility and lysis) was observed at concentrations of 25 μg ml^{-1} and higher (Fig. 6).

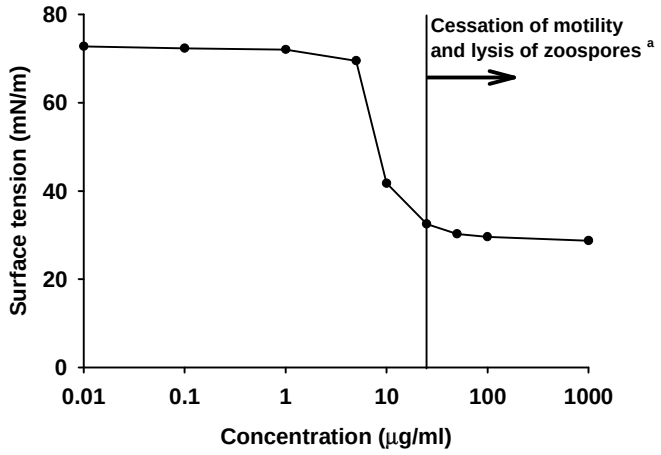

Figure 6. Relationships between the concentration of the partially purified extract obtained from *P. fluorescens* SS101, surface tension, and zoospore motility and lysis. The partially purified extract was dissolved in sterile water (pH 8.0) at different concentrations and surface tension was measured with a tensiometer. [a] At a concentration of 25 μg ml^{-1} and higher, the surface-active extract caused cessation of motility and subsequent lysis of zoospores of *P. ultimum* var. *sporangiferum*.

DISCUSSION

Biosurfactants are surface-active molecules produced by microorganisms. In this study, we demonstrated that biosurfactant-producing fluorescent *Pseudomonas* species occur in the wheat rhizosphere at densities of approximately 10^5 CFU g^{-1} root, representing on average 3% of the total population of culturable rhizospheric *Pseudomonas* spp. (Table 1). To our knowledge, studies on the frequency of biosurfactant-producing *Pseudomonas* species in the rhizosphere of wheat have not been reported before. To date, most biosurfactant producers were isolated from oil-contamined soils (Arino *et al.*, 1996; Déziel *et al.*, 1996) and in these environments up to 72% of the indigenous bacterial population appeared to produce biosurfactants (Khalid and Malik, 1997).

Further characterization of the surfactant-producing *Pseudomonas* isolates obtained from the wheat rhizosphere showed that six had lytic activity against zoospores of oomycetes (Fig. 1). Comparison of representative isolate SS101 with other surfactant producers showed that reduction of the surface tension alone is not indicative for causing lysis of zoospores (Table 2). To date, rhamnolipids are the only biosurfactants reported with lytic activity against zoospores (Stangellini and Miller, 1997). These compounds are produced by *P. aeruginosa* strains (Itoh *et al.*, 1971) and may serve as pathogenicity factors during the colonization of the human lung tissue (Iglewski, 1989). There is a considerable amount of information available on the biosynthesis and regulation of rhamnolipid production by *P. aeruginosa* strains (Ochsner *et al.*, 1994; Ochsner and Reiser, 1995). These studies led to the identification of a cluster of two biosynthetic genes, *rhlA* and *rhlB*. In addition, *rhlR* and *rhlI*, encoding a transcriptional activator and an autoinducer, respectively, positively regulate rhamnolipid production. The autoinducer encoded by *rhlI* binds and activates RhlR (Ochsner and Raiser, 1995). Biochemical characterization of strain SS101 showed that this isolate was not classified as *P. aeruginosa* but as *P. fluorescens* biovar II. Subsequent characterization by Southern hybridization and PCR with primers specific for all rhamnolipid biosynthetic and regulatory genes revealed the absence of these genes in *P. fluorescens* strain SS101 and other biosurfactant-producing isolates from the wheat rhizosphere (data not shown).

Our results show that *P. fluorescens* strain SS101 significantly and consistently controlled Pythium root rot of hyacinth (Figs. 1, 2). The role of biosurfactants produced by *Pseudomonas* spp. in suppression of plant pathogenic fungi have been inferred from studies with purified compounds or microcosm studies in which *in situ* production was correlated with fungal inhibition (Stanghellini and Miller, 1997; Kim *et al.* 2000; Thrane *et al.* 2000). A crucial and more conclusive step toward determining the role of biosurfactants in interactions

Table 4. Characterization of surfactant fractions separated by RP-HPLC.

Fraction	RP-HPLC retention time (min)	Halo on [a] SW medium	Drop-collapse [b] test	Foam [c] formation	Zoospore [d] Motility	Zoospore [d] Lysis
1	0-3	-	- [*]	-	-	-
2	3-15.5	-	+ [*]	-	-	-
3	15.5-17.2	-	+	-	-	-
4	17.2-19.7	-	+	-	+	+
5	19.7-21.6	-	+	+	+	+
6	21.6-24	-	+	-	+	+
7	24-26.4	-	+	-	+	+/- [e]
8	26.4-31.5	+	+	+	+	+
Water		-	-	-	-	-

[*] + positive reaction; - negative reaction.
[a] Evaluated on SW medium after 3 days at 25°C.
[b] Dissolved fractions were tested on an oily surface for their ability to cause the collapse of a drop of water.
[c] Dissolved fractions were observed for foam formation after vigorous shaking.
[d] Zoospore motility and lysis were observed microscopically after mixing the dissolved fractions with zoospore suspensions of *P. infestans*.
[e] Lysis was not characteristic; the effect resembled leakage at one point.

with fungal pathogens is to generate biosynthetic mutants defective in the production of biosurfactants and to compare their behaviour with the parental strain. As a first step toward determining the role of the surfactants produced by *P. fluorescens* SS101 in biocontrol, we generated mutants defective in surfactant production. By using surfactant-deficient mutant 10.24, we established the determinative role of biosurfactants produced by *P. fluorescens* SS101 in the control of Pythium root rot of hyacinth (Fig. 3). Further characterization of the surfactant-deficient mutants showed that they were not impaired in the production of several properties other than surfactants (Table 3), suggesting that apparently no global regulatory system such as the *gacS*/*gacA* pair (Heeb and Haas, 2001) was disrupted. Reduced colonization may have accounted for the poor performance of the mutants in the hyacinth bioassays. However, preliminary results indicate that the surfactant-deficient mutants and the wild type strain colonized the roots of hyacinth and wheat to the same extent (data not shown).

Sequencing of the flanking regions of the transposon in two surfactant-deficient mutants, 10.24 and 17.18, led to the localization of the Tn*5* elements in condensation domains of peptide synthetases (Figs. 4, 5). Peptide synthetases are multifunctional enzymes involved in the nonribosomal synthesis of diverse and often complex metabolites, including antibiotics, siderophores, and biosurfactants (Huang *et al.*, 2001; Marahiel *et al.*, 1997; Stachelhaus *et al.*, 1998). These enzymes are composed of modules, which contain all enzymatic activities to incorporate one constituent into the final product. These modules are in a co-linear arrangement with the primary structure of the product, and they can be divided

into domains responsible for single chemical reactions (Stachelhaus *et al.*, 1998). An initiation module consists of an adenylation and a thiolation domain. An elongation module contains a condensation, an adenylation, and a thiolation domain. Additional domains for substrate modification, such as epimerization may occur (Marahiel *et al.*, 1997). Condensation domains are found as a part of repetitive modules. They are about 450-aa in length and coincide in frequency with the number of peptide bonds that have to be formed for the linear peptide of final length (Stachelhaus *et al.*, 1998). Although in our study there is a very good correlation between the phenotype of the two mutants and the nature of the disrupted genes, complementation and expression studies should still be carried out to confirm and further characterize possible pleiotropic effects of the disruptions. However, peptide synthetases are normally very large multimodular proteins (Marahiel *et al.*, 1990), making complementation studies at this stage difficult to carry out.

The CMC value of the surface-active extract obtained from *P. fluorescens* SS101 was reached at concentrations between 25 and 50 μg ml^{-1} (Fig. 6). The CMC value, the minimum amount of surfactant required to cause the maximum decrease in surface tension, is an important measure of the surface activity and allows comparisons with other surfactants (Georgiou *et al.*, 1992). The surfactants produced by *P. fluorescens* SS101 can lower the surface tension of water to 29.7 mN m^{-1} (Table 2). The CMC and the minimum surface tension values obtained for the surfactants of *P. fluorescens* SS101 are comparable to the ones found for rhamnolipids. For example, rhamnolipids produced by *P. aeruginosa* UG2 lowered the surface tension to 31.4 mN m^{-1} and achieved a CMC at 30 μg ml^{-1} (Van Dyke *et al.*, 1993). A biosurfactant produced by *B. lycheniformis* JF-2, with properties similar to surfactin, lowers the surface tension to 27 mN m^{-1} and reaches the CMC at about 20 μg ml^{-1} (Javaheri *et al.*, 1985).

Our studies employing RP-HPLC and LC-MS techniques showed that the surface-active extracts from *P. fluorescens* SS101 contain eight different fractions (Table 4). Fraction 8 was the most abundant, accounting for approximately 95% of the extract. Preliminary analysis suggests that fraction 8 is a cyclic lipopeptide, which is consistent with the partial sequence obtained from the Tn*5* flanking regions of the two mutants 10.24 and 17.18. The conclusive identification of fractions 8 and fractions 1 to 7 is ongoing. Given the actvity of fractions 4 to 7 against zoospores, we expect these fractions to be precursors or derivatives of the dominant fraction 8. A larger scale isolation and separation will be needed to provide proof for this hypothesis. Also the activity of fractions 4 to 8 against zoospores correlates well with the proposed mode of action of peptide antibiotics, i.e., pore formation in the membrane and subsequent explosion (Hutchison and Johnstone, 1993). Fraction 7 caused leaking of the

contents of the zoospore through a point in the membrane, illustrating pore formation. The observation that effects of fraction 4 to 7 on zoospores were delayed in comparison to the effects of fraction 8, may be related to the relatively low concentration of these fractions. Further studies are necessary to determine the activity of all fractions at the same concentration against zoospores and mycelium of multiple oomycetes.

MATERIALS AND METHODS

Microorganisms and growth conditions. Naturally occurring *Pseudomonas* spp. were isolated from roots of wheat, grown in raw soils, on King's medium B [KMB] agar supplemented with chloramphenicol (13 µg ml^{-1}), ampicillin (40 µg ml^{-1}), and cycloheximide (100 µg ml^{-1}) [KMB^{+}] (Simon and Ridge, 1974). All *Pseudomonas* strains were grown on KMB. Spontaneous rifampicin resistant derivatives of strain SS101 were selected on KMB supplemented with rifampicin (100 µg ml^{-1}). Mutants disrupted in their ability to produce surfactants are resistant to rifampicin (100 µg ml^{-1}) and kanamycin (100 µg ml^{-1}). *Escherichia coli* strain S17 λ *pir* containing the mini-Tn*5lacZ* element in plasmid pUT (De Lorenzo *et al.*, 1990) was grown in liquid Luria Bertani (LB) amended with kanamycin (25 µg ml^{-1}). SW medium (Siegmund and Wagner, 1991), containing cetyltrimethylammoniumbromide (CTAB)-methylene blue, was used to select surfactant-producing *Pseudomonas* spp. Isolates that produce anionic surfactants are able to form a halo around the colony after 3-5 days of growth on SW medium. All bacterial strains were stored at -80°C in LB or KMB-broth supplemented with 40% (v/v) glycerol.

Pythium intermedium isolate P52, one of the causal agents of root rot in hyacinth, was obtained from the collection maintained at Applied Plant Research, Section Flower Bulbs (Lisse, The Netherlands) and used in the bioassays. *P. ultimum* var. *sporangiiferum* CBS 219.65 and *P. volutum* CBS 699.83 were obtained from the Dutch collection of microorganisms (CBS, Utrecht, The Netherlands). *Albugo candida* FLS 000 and *Phytophthora infestans* 88069 are from the collection maintained in the Laboratory of Phytopathology (Wageningen University, The Netherlands). All *Pythium* isolates were routinely grown on potato dextrose agar (PDA; Oxoid Ltd., Basingstoke, Hampshire, England). *P. infestans* was grown on rye agar medium (Caten and Jinks, 1968). *A. candida* was multiplied on brussels sprout plants grown in climate chambers at 18°C. For zoospore production, *P. ultimum* var. *sporangiiferum*, *P. intermedium*, and *P. infestans* were grown on V8-juice agar (V8; N.V.Campbell Foods, Puurs, Belgium) amended with 10 g l^{-1} $CaCO_3$ ($V8^{+}$) at 25°C. Mycelial plugs of all *Pythium* isolates and *P. infestans* were stored in sterile mineral oil at 15°C. Leaves infested with *A. candida* were stored in plastic bags at -20°C.

Production of zoospores. Zoospores of *P. ultimum* var. *sporangiiferum (Pus)* and *P. intermedium* were produced using a modification of a method described by Zhou and Paulitz (1993). *Pus* or *P. intermedium* were grown on V8+ for three days at 25°C. A fully-grown agar plate was cut into 2-cm wide strips and one half of the strips was transferred to another petri plate. Both plates were flooded with 20 ml of sterile water and kept at 25°C. After 1 h, the water was discarded and replaced with the same volume of water. Plates were incubated at 18°C for four days, water was removed and replaced with the same volume and incubated at 18°C for 2 h. Zoospores of *A. candida* were obtained by immersing infected leaves containing zoosporangia in sterile water followed by incubation for 2 h at 15°C. *P. infestans* zoospores were obtained by flooding 14-day old plates grown on $V8^{+}$ medium with 15 ml of sterile water followed by incubation at 4°C for 3h. Zoospore suspensions of 10^{3} to 10^{5} zoospores ml^{-1} were typically obtained.

Soils. Three soils were obtained from agricultural fields in the Netherlands. Soils CB, SV, and SSB were collected in December 1997 from the upper 50 cm of the soil profile, air dried for a week, and passed through a 0.5-cm mesh screen prior to use. Soils CB, SV, and SSB were collected from a polder in the South-West of the Netherlands, located 10 km from the city of Bergen op Zoom. These soils are physicochemically similar, containing on average, 27% clay, 10% silt, and 51% sand. The organic matter content is on average 4.3% and the pH is 7.5. Under field conditions, all soils have been cropped continuously to wheat, with exception of CB soil, which was cropped to wheat and sugar beet in a rotation scheme (1:2). The soil used in the biocontrol assays with hyacinth was collected from fields near the city of Lisse, The Netherlands. The soil was steamed and left in open

air for six months for recolonization. This sandy soil has an organic matter content of 1% and pH of 7.0.

Wheat cultivation. Pots containing 200 g of sieved soil were sown with 15 seeds of wheat (cv. Bussard). Plants were grown in a climate chamber at 15°C with a 12-h photoperiod. Plants received 50 ml of 1/3 strength Hoagland's solution (macroelements only) twice a week. After 30 days, the roots were harvested and the remaining soil plus excised roots were mixed, returned to the same pot and sown again with 15 wheat seeds. This process of successively growing wheat and harvesting roots was repeated seven times. For each soil, four replicates were used and the whole experiment was performed twice.

Isolation of surfactant-producing *Pseudomonas* spp. Population densities of total and surfactant-producing *Pseudomonas* spp. were determined on roots of wheat grown for seven successive cycles of 4 weeks each. For each replicate, roots of five randomly selected plants were harvested and loosely adhering soil was gently removed. One gram of roots plus adhering rhizosphere soil was suspended in 5.0 ml of sterile distilled water, vortexed for 1 min and sonicated for 1 min in an ultrasonic cleaner. For isolation and enumeration of pseudomonads, samples were dilution plated onto KMB^+ and plates were incubated at 25°C for 48 h. Approximately 10% of the total fluorescent *Pseudomonas* colonies obtained on KMB^+ were randomly selected and tested for halo formation on SW medium. The experiment had 4 replicates and was performed twice.

Selection of surfactant-producing *Pseudomonas* that lyse zoospores. Bacterial isolates able to form a halo arround the colony when grown on SW medium were screened for their ability to lyse zoospores. Bacterial suspensions containing approximately 10^9 CFU ml^{-1} were prepared from colonies grown on KMB medium for 48 h at 25°C. A 10-µl aliquot of the bacterial suspension was mixed on a glass slide with an equal volume of zoospore suspensions of *P. ultimum* var. *sporangiiferum*. The behavior of the zoospores was observed under a light microscope (Dialux 20 EB; Leitz Wetzlar, Germany) at 100X magnification for a period up to 5 min.

Biochemical characterization. Gas chromatography-fatty acid methyl ester (GC-FAME) analysis and API 20 NE test were performed to classify isolate SS101. API 20 NE tests were performed following recommendations of the supplier (BioMerieux, S.A., Lyon, France). For GC-FAME analysis, isolate SS101 was cultivated on tryptic soy broth agar (Becton Dickinson, Cockeysville, MD) and incubated for 24 h at 28°C. Cells were collected with a 4-mm-diameter transfer loop and processed for extraction of fatty acids following the procedures as outlined by the manufacturer (Microbial ID, Inc., Newark, DE). Fatty acid methyl esters were analyzed using a Microbial Identification System equipped with an HP5890 series II gas chromatograph, HP3365 Chem Station and version 3.9 of the aerobe library (Microbial ID, Inc.).

Tn*5* mutagenesis and characterization of the mutants. Surfactant-deficient mutants of a spontaneous rifampicin-resistant derivative of strain SS101 were obtained by biparental mating with *E. coli* strain S17 λ *pir* harbouring the mini-Tn*5lacZ-Km* element in plasmid pUT (De Lorenzo *et al.*, 1990) according to protocols described by Sambrook and Russel (2001). Transformants were selected on KMB supplemented with rifampicin and kanamycin and subsequently transferred to SW medium. Transformants unable to produce a halo arround the colony were selected after one week of growth on SW medium at 25°C. RAPD analysis with primers M13, M12, and D7, was performed to confirm strain integrity. Southern blot analysis with the KM probe was performed to determine the number of copies of the Tn*5lacZ-Km* element in the surfactant-deficient mutants. Surfactant-deficient mutants were characterized for their ability to cause lysis of zoospores of *P. ultimum* var. *sporangiiferum*, *P. intermedium*, *A. candida* and *P. infestans* as described above. Bacterial cell suspensions used for zoospore lysis tests were vortexed and checked for foam formation, a phenomenon correlated with surfactant production (Zajic and Seffens, 1984). The drop collapse test was performed as described by Jain *et al.* (1991) and Hildebrand (1989). Protease and phospholipase C activities were detected by growing the bacterial isolates on skim milk and egg yolk agar, respectively (Sacherer *et al.*, 1994). β-galactosidase activity was tested on KMB plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactosidase (X-gal) and isopropylthio-β-D-galactosidase (IPTG) according to standard protocols (Sambrook and Russel, 2001). Fluorescence was evaluated by growing the bacterial strains on Pseudomonas agar F plates for 48 h and subsequent observation under near UV light (360 nm).

DNA isolations and digestions. For RAPD-analysis, heat-lysed cell suspensions were used (Raaijmakers *et al.*, 1997). Total genomic DNA, used for Southern blot analysis and anchored PCR (aPCR) amplifications, was extracted from bacterial strains by a modified version of a CTAB-based protocol (Ausubel *et al.*, 1995). A 1.5-ml sample of overnight bacterial cultures was centrifuged for 3 min at 14,000 rpm, supernatant was discarded and the pellet was resuspended in 550 µl of TE buffer (Tris 10mM, EDTA 10mM, pH 8.0) amended with lysozyme (1.82 mg ml^{-1}), and incubated at 37°C for 30 min. Seventy-five µl of 10% SDS amended with proteinase K (0.86 mg ml^{-1}) was added to the bacterial suspension and thoroughly mixed. After

15 min of incubation at 65°C, 100 µl of 5 M NaCl and 80 µl CTAB/NaCl (0.3 M CTAB, 0.7 M NaCl) were added. After 10 min of incubation at 65°C, DNA was obtained by extraction with chloroform/isoamyl alcohol (24:1, vol/vol), isopropanol precipitation and subsequent washes with 70% ethanol. The extracted DNA was dissolved in 100 µl of 10 mM Tris pH 8.0 containing RNAase (20 µg ml^{-1}) and stored at –20°C.

For Southern hybridizations, genomic DNA was digested with 5U of *EcoR*I or *Kpn*I (Promega), which are enzymes without restriction sites in the Tn*5lacZ-Km* element (De Lorenzo *et al.*, 1990). Digestions were performed in a total volume of 100 µl containing 2.0 µg of DNA. For aPCR, the size of the fragments between the restriction sites (*Apo*I or *Sma*I) and the Tn*5lacZ-Km* inserts in the different mutants was determined by Southern hybridization with the KM probe. Genomic DNA (1 µg) was digested with *Apo*I or *Sma*I for 12 h using 10U of each enzyme at 50 and 25°C, respectively. Digested DNA was precipitated with 4M LiCl, washed with 70% ethanol, dissolved in 15 µl of sterile distilled water and separated on 1% agarose gels in 1X TBE.

Southern hybridization. DNA transfer from agarose gels to Hybond N^+ nylon membranes (Amersham Pharmacia Biotech) were performed according to standard methods (Sambrook and Russel, 2001). High-stringent conditions comprised prehybridization for 1.5 h at 65°C, hybridization for 12 h at 65°C, membrane washings twice each for 5 min with 2X SSC (1X SSC is 0.15 M NaCl plus 0.015 M sodium citrate)-0.1% SDS at room temperature, and membrane washings twice each for 30 min with 0.1X SSC-0.1% SDS at 65°C. The 575-bp KM probe, specific for the kanamycin resistance gene contained within the Tn*5LacZ-Km* element, was obtained by direct PCR DIG-labelling (Roche Corp., Basel, Switzerland) of the fragment using primers KM1 (5'-CCCGATGCGCCAGAGTTGTT) and KM2 (5'-TCACCGAGGCAGTTCCATAGG). Hybridized probes were immunodetected according to the protocols provided by the supplier.

PCR and RAPD analyses. PCR amplifications were performed in a 25-µl reaction mixture containing 3 µl of the diluted heat-lysed cell suspension, or 10 ng of genomic DNA, 1X PCR buffer (Perkin-Elmer, Nieuwerkerk aan de IJssel, The Netherlands), 1.5 mM $MgCl_2$ (Perkin Elmer), 200 µM of each dATP, dCTP, dGTP, and dTTP (Promega, Leiden, The Netherlands), 20 pmol of each primer, and 2U of AmpliTaq polymerase (Perkin-Elmer). Anchored PCR (aPCR) was performed in a 50-µl reaction mixture with the high fidelity PCR system (Roche, Roche Diagnostics, Mannheim, Germany) following the recommendations of the supplier. For random amplified polymorphic DNA (RAPD) analysis, 40 pmol of each primer were used. Amplifications were carried out with a MJ Research PTC-200 thermocycler. The PCR program for aPCR consisted of an initial denaturation at 95°C for 5 min followed by 30 cycles at 94°C for 30 sec, annealing beginning at 72°C for 40 sec and afterwards decreasing by 0.5°C per cycle, and extension at 72°C for 2 min. The PCR program for RAPD analysis was described previously (Keel *et al.*, 1996). RAPD analysis was performed with primers M13, M12, and D7 (Keel *et al.*, 1996) and repeated three times for each primer. Samples (10-15 µl) of the PCR products were separated on a 1% agarose gel or on 2% agarose gels for RAPD analysis in 1X TBE buffer (90 mM Tris-borate, 2 mM EDTA [pH 8.0]) at 60-80 V for 1-3 h. The GIBCO-BRL 100-bp ladder (Life Technologies B.V., Breda, The Netherlands) was used as size marker. PCR products were visualized with a UV transilluminator.

Sequencing Tn*5* flanking regions. DNA flanking the Tn*5lacZ-Km* insertions in different mutants was sequenced by aPCR (Stuurman *et al.*, 1996). aPCR was performed in three steps, where the first consisted of ligating synthetic anchors to genomic DNA digested with the enzymes *Apo*I or *Sma*I. For *Apo*I (with 5'-AATT overhang) the anchor was *EcoR*I-16 (5'-AATTGGCGGTGAGTCC) and for *Sma*I (with blunt end) the anchor was An-B (5'-TGCGGACT). The second step was a linear PCR with primers EOF (5'-ACTTGTGTATAAGAGTCAG) or EIR (5'-AGATCTGATCAAGAGACAG) targeting the inverted repeats of the Tn*5* transposon. The third step consisted of performing a nested PCR with primer RH24 (5'-AGCACTCTCCAGCCTCTCACCGCA) for DNA digested with *Apo*I or primer 5'-VECT24 (5'-AGCACTCTCCAGCCTCTCACCGCC) for DNA digested with *Sma*I, and primers EOF or EIR. Amplified fragments were purified from agarose gels by using the QIAEX II kit (QIAGEN GmbH, Hilden, Germany) and cloned into pCRT7/CT-TOPO (Invitrogen, Breda, The Netherlands). Cloned aPCR products were sequenced with primers T7 (5'-TAATACGACTCACTATAGGG) and V5 (5'-ATCCCTAACCCTCTCCTCGGT). Sequences were trimed and assembled by using the DNAstar program (DNAstar Inc., Madison, WI, USA). BLASTN and BLASTP were conducted on the sequences. Sequences were aligned by using the CLUSTAL W 1.81 program (Thompson *et al.*, 1994).

Bioassays. Inoculum of *P. intermedium* P52 was prepared by growing the oomycete for 3 weeks at room temperature in a sterile mixture containing 50% sandy soil, 50% river sand, and oatmeal (6 g l^{-1}). *Pythium* inoculum was mixed with the sandy soil at a 1% rate (v/v). Hyacinth bulbs, cv. Pink

Pearl, were disinfested with 1% formaline and stored at 9°C in a climate chamber. Four weeks prior to the bioassays, the bulbs were transfered to a 17°C climate chamber. Suspensions of *P. fluorescens* isolate SS101 or its surfactant-deficient mutant 10.24 (10^7 CFU ml^{-1}) were used to treat the bulbs. In soil treatments, initial bacterial concentrations were 10^7 CFU g^{-1} soil fresh weight. Hyacinth bulbs were transferred to plastic pots filled with approximately 2,75 kg of soil which was treated or not with *Pythium* and/or *P. fluorescens* strains. After 8 weeks of growth at 9°C in the dark, roots were excised from the bulbs, fresh weight was determined and/or disease caused by *P. intermedium* P52 was scored on a 0 to 5 scale. In this disease index, 0 indicates no disease, 1 indicates 1 to 20% loss of root biomass relative to the control, 2 indicates 20 to 40% loss of root biomass, 3 presents 40 to 60% less roots, 4 is 60 to 80% less roots, and 5 is 80 to 100% less roots. The disease index was scored visually.

Partial purification of surfactants. Bacterial cells obtained from cultures of strain SS101 grown on KMB agar for 48 h at 25°C were suspended in sterile demineralized water and centrifuged twice (Sorvall RC5C; Sorvall instruments) at 6000 rpm for 20 min at 4°C. Suspensions of the surfactant-deficient mutant 10.24 were also included. The supernatant containing the surfactants was filter-sterilized (0.2 µm), acidified to pH 2.0 with 9% HCl and kept on ice for 1 h (Javaheri *et al.*, 1985). The precipitate was collected by centrifugation (6000 rpm, 30 min, 4°C) and washed with acidic demineralized water (pH 2.0) twice. The precipitate was resuspended in sterile demineralized water, adjusted to pH 8.0 with 0.2 M NaOH and lyophilized (Labconco Corp., Kansas City, USA). The lyophilized extract was stored at –20°C.

Surface tension measurements. Bacterial cell suspensions were obtained from cultures grown for 48 h on KMB medium at 25°C. Concentrations were adjusted to OD_{600}=1 and therefrom dilutions were made. For determining the critical micelle concentration (CMC-value), the partially purified extract was dissolved in sterile water (pH 8.0) at concentrations of 0, 0.01, 0.1, 1, 10, 25, 50, 100, and 1000 µg ml^{-1}. Surface tension measurements were carried out with a Krüss K6 tensiometer (Krüss GmbH, Germany). Measurements were performed at 25°C and sterile distilled water was used to calibrate the tensiometer.

Composition of the surface active extract. All solvents used were liquid chromatography grade (LC grade). Acetonitrile (MeCN) and methanol were obtained from LAB-SCAN Analytical Sciences (Dublin, Ireland). Ultra pure water was obtained from a combined Seradest LFM 20 Serapur Pro 90 C apparatus (Seral, Ransbach, Germany). All LC solvents were degassed by vacuum filtration over a 0.45 µm membrane filter (Type RC, Schleicher & Schuell) prior to use. All high-performance liquid chromatography (HPLC) separations were carried out on an Alltech end-capped C18 columns of 250 mm length, particle size 5 µm. For fractionation, 4.6 mm diameter columns were used. Freeze drying was carried out on a Christ Alpha 1-2 freeze drier. Prior to fractionation, 10 mg of the partially purified extract was dissolved in 1 ml MeCN - H_2O (6:4). After membrane filtration, fractionation was performed by reverse phase-HPLC (RP-HPLC). The solvent was MeCN - H_2O (7:3) amended with 0.01% formic acid at a flow rate of 1.0 ml min^{-1}. Detection took place by UV at 210 nm. Eighteen injections of 50 µl were made and the fractions were collected manually in round bottom flasks. After removal of the MeCN with a rotary evaporator ($B_{\ddot{u}}chi$) *in vacuo*, the aqueous solution was lyophilised. All fractions were dissolved in 1.25 ml sterile water pH 8.0. Drop-collapse test, foam formation and activity against zoospores of *P. infestans* were conducted to determine the biological activity of the separated fractions.

Statistical analysis. Disease index data were ranked. After certifying normal distribution and homogeneity of variances, root weight data and ranked disease index data were analysed by ANOVA followed by Tukey's studentized range test (SAS Institute, Inc., Cary, N.C.).

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CHAPTER 7

SUMMARISING DISCUSSION

Because of the hazardous effects that pesticides may have on the environment and human health, there is a need for more environmentally safe methods to control plant diseases. Over the past decades, there has been an increasing interest in biological control of plant diseases by application of beneficial microorganisms. Among the potential bacterial biocontrol agents, the genus *Pseudomonas* is recognized as one of the most promising groups (Weller, 1988; Handelsman and Stabb, 1996; O'Sullivan and O'Gara, 1992). *Pseudomonas* biocontrol agents suppress plant diseases by several different mechanisms, including antibiosis, competition, and induction of resistance in the host plant (O'Sullivan and O'Gara, 1992; Handelsman and Stabb, 1996; Van Loon *et al*. 1998; Whipps, 2001).

Our studies focused on fluorescent *Pseudomonas* species that produce antibiotics that are inhibitory to the growth of various plant pathogenic fungi and oomycetes. Several antibiotics produced by *Pseudomonas* species play a key role in biological control of plant pathogens, including 2,4-diacetylphloroglucinol (2,4-DAPG), phenazines (PHZ), pyrrolnitrin (PRN), pyoluteorin (PLT), and surface-active antibiotics (Vincent *et al.*, 1991; Hill *et al.*, 1994; Maurhoffer *et al.*, 1992; Thomashow and Weller, 1988; Thrane *et al.*, 1999). To date, however, most of the information available on antibiotic-producing *Pseudomonas* spp. is derived from studies on model strains and little is known about the distribution, diversity and activity of indigenous populations harbouring these traits. A better understanding of the ecology of naturally occurring strains that harbour specific biocontrol traits will contribute to improve the efficacy of existing biocontrol strains and may help to identify new strains that are better adapted to specific soils or host-pathogen systems. Our **main objectives** in this thesis were to study 1) the distribution, 2) the diversity and 3) the activity of indigenous *Pseudomonas* strains that produce 2,4-DAPG, PHZ, PRN, PLT, or surface-active antibiotics.

DISTRIBUTION

Antibiotic-producing *Pseudomonas* species have been isolated from different soils and plant species. The natural ability of certain soils to suppress plant diseases is often correlated with the presence of relatively high densities of antibiotic-producing *Pseudomonas* spp. (Cook and Rovira, 1976; Stutz *et al.*, 1986; Raaijmakers *et al.*, 1997). The exploitation of natural disease-suppressive soils as a source of antagonistic microorganisms has proven to be a successfull approach to develop biocontrol strategies (Whipps, 1997; Weller, 1988). However, the selection procedures used to date have a random nature, are labour-intensive and do not make full use of existing knowledge of biocontrol mechanisms. The increasing availability of

cloned and sequenced genes involved in the biosynthesis of biocontrol traits has facilitated the development of specific primers and probes for targeted isolation. The conservation of sequences within the biosynthetic loci for 2,4-DAPG and PHZ antibiotics allowed the development of primers and probes to specifically isolate and monitor *Pseudomonas* strains, producing these antibiotics, in soil and rhizosphere environments (Raaijmakers *et al.*, 1997; Picard *et al.*, 2000).

We have used molecular probes and primers available for 2,4-DAPG and PHZ antibiotics (Raaijmakers *et al.*, 1997; Mavrodi *et al.*, unpublished) and developed new ones for PRN and PLT, two antibiotics with broad-spectrum activity. Colony hybridization followed by PCR was used to determine the frequencies of antibiotic-producing *Pseudomonas* and *Burkholderia* species in soils suppressive to *Geaumannomyces graminis* var. *tritici*, the causal agent of take-all disease of wheat (Chapters 2 and 4). Soils suppressive to take-all are referred to as take-all decline (TAD) soils. We demonstrated that *Pseudomonas* and *Burkholderia* species harbouring PRN and PLT genes were not present at detectable densities (< 10^4 CFU g^{-1} root) on roots of wheat grown in any of the Dutch TAD soils investigated. These results suggest that *Pseudomonas* and *Burkholderia* species that produce PRN and/or PLT do not play a significant role in the suppressiveness that operates in at least two Dutch TAD soils. This suggestion is based on the observation that threshold densities of at least 10^5 CFU g^{-1} root are required for significant suppression of several soil-borne diseases by *Pseudomonas* strains (Raajmakers *et al.*, 1995; Raaijmakers and Weller, 1998). Further studies will be necessary to establish whether these threshold population densities also apply for PRN- and PLT-producing *Pseudomonas* and *Burkholderia* spp. Almost all PRN- and PLT-producing reference strains reported to date were isolated from dicotelydon plant species rather than monocotelydon (Howell and Stipanovic, 1979; Levy *et al.*, 1992; Keel *et al.*, 1996). The fact that also in our studies no PRN and PLT producers were found in the rhizosphere of wheat may point to a preference of *Pseudomonas* spp. harboring these traits for the rhizosphere of dicotelydon plants. Recent studies demonstrated that wheat plants enrich for specific genotypes of 2,4-DAPG-producing *Pseudomonas* species (Wang *et al.*, 2001). This specific enrichment could be responsible for the displacement of PRN- and PLT-producing strains from the wheat rhizosphere. Since only a limited number of soils and only one moncotelydon plant species was included in our studies, a detailed analysis of multiple and diverse soils and host plants will be required to support this hypothesis. Understanding the host preference of antagonistic microorganisms harboring particular traits may be crucial in predicting their potential biocontrol efficacy in multiple host-pathogen systems.

Pseudomonas strains producing PHZ antibiotics were detected at densities of approximately 1.0 X 10^5 CFU g^{-1} root on roots of wheat grown in SL soil, one of the soils with history of long-term wheat cultivation. The PHZ-producing *Pseudomonas* spp. occurred together with 2,4-DAPG-producing *Pseudomonas* spp. (Chapter 2). The co-occurrence of indigenous 2,4-DAPG and PHZ-producers on roots of wheat allows the study of interactions and compatibility between both groups as well as their potentially synergistic role in disease suppressiveness. In this context, it is interesting to note that several studies have shown that simultaneous application of two or more antagonistic strains can lead to improved disease suppression (Dunne *et al.*, 1998; De Boer *et al.*, 1999). 2,4-DAPG-producing *Pseudomonas* species occurred on roots of wheat grown in most of the soils included in our studies. Their densities were not detectable or significantly lower on roots of wheat grown in the take-all conducive CB soil than on roots of wheat grown in at least two take-all suppressive soils (10^5 – 10^6 CFU g^{-1} root or higher). Their densities on roots of wheat grown in these TAD soils were above the threshold populations reported to be required for significant suppression of take-all of wheat (Raaijmakers and Weller, 1998). Subsequent studies with 2,4-DAPG-deficient mutants of representative strains of dominant genotypic groups recovered from Dutch and US TAD soils, indicated that 2,4-DAPG production is a determinant factor in the suppression of take-all of wheat (Chapter 4). These findings provide substantial support for a broader role of 2,4-DAPG-producing *Pseudomonas* in the natural suppressiveness of soils to take-all, previously demonstrated to occur only in TAD soils of Washington State, USA (Raaijmakers *et al.*, 1997; Raaijmakers and Weller, 1998). Further studies will be necessary to determine the role of 2,4-DAPG producers in TAD soils from other geographic regions. Also their occurrence at relatively high densities in the rhizosphere of crops other than wheat and in soils with no apparent relation with the TAD phenomenon (Picard *et al.*, 2000), deserves further investigations into their biological role in these environments. The specific enrichment of 2,4-DAPG-producing *Pseudomonas* in TAD soils is thought to occur, at least in part, by selective stimulation of nutrients released from the lesions caused by the take-all pathogen during the initial severe outbreaks of the disease. Our initial studies on *G. graminis*-induced selection of 2,4-DAPG-populations, however, indicated that the quantitative enrichment is not *G. graminis* specific, since similar increases in population densities of both total fluorescent and 2,4-DAPG-producing *Pseudomonas* populations were observed upon infection of wheat roots by *Rhizoctonia solani* (Chapter 4).

Another approach often used to select for potential biocontrol agents consists of initially establishing their activity against plant pathogens and later identifying the

mechanism(s) responsible for this activity (Handelsman and Stabb, 1996). In fact, most model strains known to date were identified by this approach. With this approach, we identified *Pseudomonas* isolates that produce surface-active antibiotics with activity against oomycete pathogens (Chapter 6). Surfactant-producing *Pseudomonas* spp. occurred on roots of wheat at densities of approximately 10^5 CFU g^{-1} root, representing on average 3% of the population of culturable *Pseudomonas* spp. present in the rhizosphere of wheat. Subsequent characterization of 375 random isolates demonstrated that 6 isolates were able to cause cessation of motility and subsequent lysis of zoospores of at least three oomycete pathogens. Studies with probes and primers specific for biosynthetic genes involved in the production of this surfactant are now being conducted to obtain more information on the distribution of *Pseudomonas* harbouring this particular trait in other soil, rhizosphere and phyllosphere environments. Although in this thesis we focused only on one of the strains that adversely affected motility and caused lysis of zoospores, the remaining 369 isolates may produce surface-active compounds that have other and possibly stronger antagonistic properties than observed for the surfactant(s) produced by *P. fluorescens* strain SS101 (Chapter 6).

DIVERSITY

Several studies have shown that there is considerable diversity in antibiotic-producing *Pseudomonas* species (Keel *et al.*, 1996; Sharifi-Tehrani *et al.*, 1998; McSpadden-Gardener *et al.*, 2000). Most of these studies are based on polymorphisms in the 16S-rDNA. Work by Ramette *et al.* (2001) and Mavrodi *et al.* (2001) demonstrated that also polymorphisms within *phlD*, a key gene in the biosynthesis of 2,4-DAPG, can be used for diversity and phylogenetic studies on 2,4-DAPG-producing *Pseudomonas* strains. In Chapter 2, Random Fragment Length Polymorphism (RFLP) analysis of amplified fragments of *pltC* showed no polymorphisms, indicating that at least part of this biosynthetic gene is highly conserved among known PLT-producing *Pseudomonas* species. In contrast, RFLP analysis of *prnD*-fragments allowed discrimination between multiple PRN-producing *Pseudomonas* and *Burkholderia* species to a similar level as obtained by Random Amplified Polymorphic DNA (RAPD) analysis. Interestingly, *B. pyrrocinia* strain DSM10685 was clustered closer to *Pseudomonas* than to other *Burkholderia* strains. Subsequent phylogenetic analysis with the four pyrrolnitrin biosynthetic genes from three other strains, including one *B. cepacia*, one *P. fluorescens*, and one *Myxococcus fulvus* confirmed that *prn* genes of *B. pyrrocinia* are more

similar to those of *P. fluorescens* than to those of *B. cepacia*. 16S-rDNA sequence analysis confirmed the taxonomic status of the species included in the analysis. Collectively, these results suggested that the PRN gene cluster may have been transferred from *Pseudomonas* to *B. pyrrocinia* or vice versa. Although, gene transfer between different microbial genera and between microbes and plants seem to be a very widespread phenomenon (Rivera *et al.*, 1998; Jain *et al.*, 1999), sequence analysis of *prn* genes of multiple *Burkholderia* and *Pseudomonas* strains will be required to further support this hypothesis.

In addition to specific antibiotic-biosynthetic genes, polymorphisms within the response regulator gene *gacA* proved to be a powerful complementary genetic marker for the genus *Pseudomonas* (Chapter 3). It is remarkable that a relatively small-sized gene like *gacA* has such strong resolving capacity for phylogenetic purposes. Comparable results were obtained with the 249-bp *oprI* gene, which allowed molecular taxonomic studies in pseudomonads belonging to the rRNA group I, a group that contains the genuine *Pseudomonas* species (De Vos *et al.*, 1998). Polymorphisms within *gacA* homologs also allowed distinction of five subclusters in the group of enteric bacteria, including *Escherichia coli*, *Salmonella, Klebsiella pneumoniae, Pectobacterium caratovorum*, and *Yersinia* species. These results indicated that *gacA* homologs also may provide a complementary genetic marker for phylogenetic studies of Gram-negative bacteria other than *Pseudomonas*.

ACTIVITY

The activity of two antibiotics, 2,4-DAPG and the surfactant produced by *P. fluorescens* strain SS101, was investigated in more detail in Chapters 5 and 6. We found that different infectious propagules of *Pythium* differ considerably in their sensitivity to 2,4-DAPG (Chapter 5). We observed variation in sensitivity to 2,4-DAPG among isolates of various *Pythium* species (Chapter 5) and also initial studies with the surfactants indicate variation in sensitivity among oomycetes, in particular *Pythium* species (Chapter 6). Protective mechanisms responsible for these differential responses are currently being studied in detail. Variation in sensitivity within pathogen populations to metabolites produced by antagonistic microorganisms is probably one of the major factors responsible for inconsistenty and, in some cases lack of efficacy, of biological control (Mazzola *et al.*, 1995). In addition to biotic factors, we also showed that the activity of 2,4-DAPG against *Pythium* species is influenced by pH (Chapter 5). Although this conclusion is based on *in vitro* experiments, one may envisage the

impact of these findings on interactions between 2,4-DAPG producers and pathogenic fungi in the plant rhizosphere. Despite the well-documented role of 2,4-DAPG in biocontrol of several plant diseases (Vincent *et al.*, 1991, Fenton *et al.*, 1992, Keel *et al.*, 1992), it appears that this antibiotic has no determinative role in the competitiveness of 2,4-DAPG-producing strains in the rhizosphere (Carrol *et al.*, 1995, Chapter 4). It is difficult to imagine that these genes remained in numerous plant-associated *Pseudomonas* strains in the course of evolution without an apparent advantage in rhizosphere competence. The role of 2,4-DAPG in rhizosphere competence should be investigated in more detail with other plant species and plant-growth stages. Also the role of surfactant production in rhizosphere competence will be addressed in the near future.

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SUMMARY

Bacteria of the genus *Pseudomonas* are potential biocontrol agents of plant diseases caused by various fungi and oomycetes. Antibiotic production is an important trait responsible for the activity of several *Pseudomonas* strains against plant pathogens. Despite the amount of information obtained during the past decades on biosynthesis and regulation of antibiotics, little is known about the distribution and diversity of antibiotic-producing *Pseudomonas* spp. in natural environments. Knowledge about the diversity of naturally occurring populations of antibiotic-producing *Pseudomonas* spp. could contribute to improve biological control by the identification of new strains that are ecologically more competent. The main objectives in this thesis were to study the distribution, diversity, and activity of antibiotic-producing *Pseudomonas* spp. in rhizosphere environments.

In Chapter 1, an overview is given on detection, distribution and diversity of antibiotic-producing *Pseudomonas* spp. Special attention is paid to the biosynthesis and regulation of the antibiotics 2,4-diacetylphloroglucinol (2,4-DAPG), phenazines (PHZ), pyrrolnitrin (PRN), pyoluteorin (PLT), and biosurfactant antibiotics, which are subject of the experiments described in this thesis.

In Chapter 2, the diversity, phylogenetic relationships, and frequency of *Pseudomonas* and *Burkholderia* species able to produce the antibiotics PRN or PLT were studied. Primers were developed from conserved sequences within the biosynthetic loci for each of the two antibiotics and allowed detection of multiple *Pseudomonas* and *Burkholderia* spp. that either produce PRN or PLT or both. Subsequent RFLP (Restriction Fragment Length Polymorphisms) analysis of the amplified *pltC* fragment showed no polymorphisms among PLT-producing *Pseudomonas* strains. Polymorphisms within the amplified *prnD* fragment, however, allowed the assessment of the diversity among PRN-producing *Pseudomonas* and *Burkholderia* spp. to a level similar to that obtained by RAPD (Random Amplified Polymorphic DNA) analysis. Phylogenetic analyses further revealed that the *prn* genes of *B. pyrrocinia* DSM10685 were more closely related to those of PRN-producing *Pseudomonas* strains, suggesting that lateral gene transfer may have occurred. PRN- and PLT-producing *Pseudomonas* and *Burkholderia* spp. were not detected on roots of wheat grown in five agricultural soils collected in the Netherlands, two of them being naturally suppressive to the take-all pathogen *Gaeumannomyces gramminis* var. *tritici*. These results suggested that these bacteria do not contribute to the natural suppressiveness found in Dutch take-all suppressive soils.

Chapter 3 focused on the response regulator gene *gacA* in *Pseudomonas* species. This gene influences the production of several antibiotics in antagonistic *Pseudomonas* spp. We developed primers and a probe for the *gacA* gene of *Pseudomonas* species and sequenced *gacA* from ten *Pseudomonas* strains isolated from different plant-associated environments. PCR analysis and Southern hybridization showed that *gacA* is highly conserved within the genus *Pseudomonas* and indicated that *gacA* can be used as a complementary genetic marker for detection of *Pseudomonas* spp. in environmental samples. Phylogenetic relationships inferred from the newly sequenced *gacA* genes and *gacA* homologs present in the databases, showed that *gacA* may also serve as a marker for phylogenetic studies of *Pseudomonas* spp. and Gram-negative bacteria other than *Pseudomonas*. Estimations of nonsynonymous to synonymous substitution rates (Ka/Ks ratios) showed that purifying selection is acting on *gacA*, indicating that there is a selective pressure to avoid substitutions leading to functional changes in the GacA protein.

Chapter 4 focused on the role of antibiotic-producing *Pseudomonas* spp. in Dutch take-all suppressive soils. Natural suppressiveness of soils to take-all disease of wheat, referred to as take-all decline (TAD), occurs worldwide. It has been postulated that different microbial genera and mechanisms are responsible for TAD in soils from different geographical regions. Based on population density studies and the use of antibiotic-deficient mutants, we demonstrated that fluorescent *Pseudomonas* spp. that produce 2,4-DAPG play a key role in the natural suppressiveness of two Dutch TAD soils. Our results showed that in addition to the physicochemically different TAD soils from Washington State (USA), 2,4-DAPG-producing fluorescent *Pseudomonas* spp. are also a key component of the natural suppressiveness found in Dutch TAD soils. Furthermore, it is the first time since the description of Dutch TAD soils, that at least part of the mechanisms and microorganisms operating in their suppressiveness are identified. In spite of similarities in population densitis and activity, 2,4-DAPG-producing *Pseudomonas* spp. found in the Dutch TAD soils are genotypically different from those found in TAD soils from Washington State.

In Chapter 5 we concentrated on the activity of the antibiotic 2,4-DAPG against *Pythium* species. The results of this study showed that within and among *Pythium* species there is variation in sensitivity to 2,4-DAPG. Also various propagules of *P. ultimum* var. *sporangiiferum* (*Pus*), that are part of the asexual stage of the life cycle, differ considerably in their sensitivity to 2,4-DAPG. Mycelium was the most resistant structure, followed by zoosporangia, zoospore cysts and zoospores. Transmission electron microscopy (TEM) studies revealed that 2,4-DAPG causes different stages of disorganisation in hyphal tips of *Pus*,

including alteration (proliferation, retraction and disruption) of the plasma membrane, vacuolisation and cell content disintegration. Additionally, we report, for the first time, that pH has a significant effect on the activity of 2,4-DAPG, with a higher activity at low pH. Furthermore, the level of acetylation of phloroglucinols also is a major determinant of their activity.

Chapter 6 focused on surface-active antibiotics produced by *Pseudomonas* spp. and their activity against oomycete pathogens. We report the discovery of *Pseudomonas* isolates that produce biosurfactants with lytic activity against zoospores of multiple oomycete pathogens. Application of cell suspensions of strain *P. fluorescens* SS101 to soil or hyacinth bulbs provided protection against root rot caused by *Pythium intermedium*. Five mutants of SS101, obtained by random Tn*5* mutagenesis, lacked the ability to cause lysis of zoospores. Subsequent genetic characterization of the Tn*5*-flanking regions of two surfactant-deficient mutants, 10.24 and 17.18, showed that the transposons had integrated in condensation domains of peptide synthetases. When applied to soil, mutant 10.24 was not able to protect hyacinth against Pythium root rot, whereas wildtype strain SS101 gave significant control. Reverse-phase high performance liquid chromatography (RP-HPLC) and liquid chromatography–mass spectrometry (LC-MS) yielded eight different fractions of which five had surface activity and caused cessation of motility and lysis of zoospores. Collectively, the results indicate that biosurfactants produced by *P. fluorescens* SS101 play an important role in interactions with zoosporic pathogens and in control of Pythium root rot of hyacinth.

In Chapter 7 the most important findings are summarised and discussed in the context of the existing knowledge.

In conclusion, the results presented in this thesis provide information on the distribution, diversity and activity of antibiotic-producing *Pseudomonas* spp. and describe the discovery of surface-active antibiotics that have great potential for controlling plant diseases caused by oomycete pathogens.

RESUMO

Bacterias do gênero *Pseudomonas* são agentes potenciais de controle biológico de doenças de plantas causadas por vários fungos e oomicetos. A produção de antibióticos é um importante atributo responsável pela atividade antagônica de diversos isolados de *Pseudomonas* contra patógenos de plantas. Apesar da quantidade de informação obtida durante as últimas décadas sobre a síntese e regulação de antibióticos, pouco se sabe sobre a distribuição e diversidade de bactérias produtoras de antibióticos do gênero *Pseudomonas* spp. na natureza. Dados sobre a diversidade de populações indígenas de bactérias produtoras de antibióticos do gênero *Pseudomonas* spp. pode contribuir para melhorar o controle biológico pela identificação de novos isolados que sejam ecologicamente mais adaptados. Os principais objetivos nesta tese foram estudar a distribuição, diversidade e atividade de bactérias do gênero *Pseudomonas* spp. produtoras de antibióticos.

No **Capítulo 1**, apresentamos uma revisão sobre detecção, distribuição e diversidade de bactérias do gênero *Pseudomonas* produtoras de antibióticos. Atenção especial é dada à síntese e regulação dos antibióticos 2,4-diacetilfloroglucinol (2,4-DAPG), fenazinas (PHZ), pirrolnitrina (PRN), pioluteorina (PLT), e antibióticos biosurfactantes, que em conjunto são o tema central dos experimentos descritos nesta tese.

No **Capítulo 2**, estudou-se a diversidade, relações filogenéticas e a frequência das bactérias dos gêneros *Pseudomonas* e *Burkholderia* capazes de produzir os antibióticos PRN ou PLT. Primers foram desenvolvidos de sequências conservadas dentro das regiões que codificam a síntese desses antibióticos e permitiram a detecção de múltiplos isolados dos gêneros *Pseudomonas* e *Burkholderia* spp. que produzem PRN, PLT ou ambos. Análises de RFLP (Restriction Fragment Length Polymorphisms) de fragmentos amplificados do gene *pltC* não mostraram nenhum polimorfismo entre isolados de *Pseudomonas* spp. capazes de produzir PLT. Por outro lado, os polimorfismos dentro do fragmento amplificado do gene *prnD* permitiram a avaliação da diversidade entre *Pseudomonas* e *Burkholderia* spp. capazes de produzir PRN em um nível semelhante ao obtido por análise de RAPD (Random Amplified Polymorphic DNA). Análises filogenéticas adicionalmente revelaram que os genes *prn* de *B. pyrrocinia* DSM10685 são mais relacionados aqueles provenientes de espécies de *Pseudomonas*, sugerindo uma possível transferência lateral desses genes. *Pseudomonas* e *Burkholderia* spp. produtoras de PRN e PLT não foram detectadas em raízes de trigo cultivado em cinco solos coletados na Holanda, sendo dois deles naturalmente supressivos ao patógeno do mal-do-pé do trigo, *Gaeumannomyces gramminis* var. *tritici*. Esses resultados sugeriram que essas bactérias

não contribuem para a supressividade natural que ocorre nos solos Holandeses ao mal-do-pé do trigo.

No Capítulo 3, estudou-se o gene regulador de respostas *gacA* em espécies de *Pseudomonas*. Este gene influencia a produção de antibióticos em bactérias antagonistas do gênero *Pseudomonas*. Primers e uma sonda para o gene *gacA* de espécies de *Pseudomonas* foram desenvolvidos. Sequenciou-se o gene *gacA* de dez espécies de *Pseudomonas* isoladas de diferentes combinações planta hospedeira-ambiente. Análise de PCR e Southern hybridization revelaram que o gene *gacA* é altamente conservado dentro do gênero *Pseudomonas* e indicou que *gacA* pode ser utilizado como um marcador complementar para o isolamento de *Pseudomonas* spp. de amostras ambientais. Relações filogenéticas inferidas das novas sequências do gene *gacA* e homólogos desse gene presentes nos bancos de dados genéticos mostraram que *gacA* pode também servir como um marcador para estudos filogenéticos não só de *Pseudomonas* spp. mas também de outras bactérias Gram-negativas. Estimações das taxas de substituição não-sinônima para sinônima de nucleotídeos (taxas Ka/Ks) revelaram que uma seleção purificadora está agindo sobre o gene *gacA*, indicando que existem pressões de seleção para evitar substituições de nucleotídeos que levem a mudanças na conformação estrutural e subsequente perda de função da proteína GacA.

No Capítulo 4, estudou-se o papel de *Pseudomonas* spp. produtoras de antibióticos em solos Holandeses supressivos ao mal-do-pé do trigo. Solos naturalmente suppressivos ao mal-do-pé do trigo, referidos como solos com declínio do mal-do-pé (DMP), ocorrem em todo o mundo. Postula-se que diferentes gêneros microbianos e mecanismos são responsáveis pelo DMP em solos de diferentes regiões. Baseado em estudos de densidade populacional e uso de mutantes deficientes na produção de antibióticos, demonstrou-se que *Pseudomonas* fluorescentes capazes de produzir o antibiótico 2,4-DAPG desempenham um papel chave na supressividade natural de dois solos Holandeses ao mal-do-pé do trigo. Nossos resultados mostraram que apesar dos solos do Estado de Washington (EUA) serem físico-quimicamente diferentes dos solos Holandeses, *Pseudomonas* produtoras do antibiótico 2,4-DAPG também são componentes chaves para a supressividade natural dos solos Holandeses ao mal-do-pé do trigo. Alem disso, essa é a primeira vez, desde a descrição dos solos Holandeses com DMP, que pelo menos parte dos mecanismos e microorganismos envolvidos na supressividade são identificados. Apesar das similaridades em termos de atividade e densidade populacional, as bacterias do gênero *Pseudomonas* produtoras de 2,4-DAPG encontradas nos solos Holandeses com DMP são geneticamente diferentes daquelas encontradas em solos com DMP do Estado de Washington.

No Capítulo 5, enfocou-se a atividade do antibiótico 2,4-DAPG contra espécies de *Pythium*. Os resultados desse estudo mostraram que existe variação na sensitividade ao antibiótico 2,4-DAPG dentro e entre espécies de *Pythium*. Vários propágulos de *P. ultimum* var. *sporangiiferum* (*Pus*), que são parte do ciclo assexual, diferiram consideravelmente quanto a sua sensitividade a 2,4-DAPG. Micélio foi a estrutura mais resistente, seguida de zoosporângios, cistos de zoosporos e zoosporos. Estudos de microscopia eletrônica de transmissão (MET) revelaram que 2,4-DAPG causa diferentes estágios de desorganização em pontas de hifas de *Pus*, incluindo alterações (proliferação, retração e ruptura) da membrana plasmática, vacuolisação e desintegração do conteúdo celular. Adicionalmente, registra-se, pela primeira vez, que o pH tem um efeito significativo sobre a atividade de 2,4-DAPG, com maior atividade em pHs mais baixos. Além disso, o número de grupos acetil presentes nas moléculas dos floroglucinois é também um importante determinante de sua atividade.

No Capítulo 6, concentrou-se no estudo de antibióticos produzidos por *Pseudomonas* spp. com atividade de superfície e sua ação contra oomicetos patogênicos. Encontraram-se isolados de *Pseudomonas* capazes de produzir biosurfactantes com atividade lítica contra zoosporos de vários oomicetos patogênicos. A aplicação de suspensões de células do isolado SS101 de *P. fluorescens* no solo ou em bulbos de jacinto (*Hyacinthus orientalis*) resultaram na proteção dessas plantas contra podridão radicular causada por *Pythium intermedium*. Cinco mutantes do isolado SS101, obtidos através de integração ao acaso do transposon Tn*5*, não apresentaram habilidade de causar a explosão de zoosporos. Subsequente caracterização genética das regiões vizinhas ao Tn*5* de dois mutantes, 10.24 e 17.18, mostraram que os transposons estavam inseridos em sequências codificadoras de domínios de condensação de enzimas responsáveis pela síntese de peptídeos. Quando aplicado em solo, o mutante 10.24 não foi capaz de proteger plantas de jacinto contra podridão radicular causada por *P. intermedium*, enquanto que o isolado tipo selvagem SS101 proporcionou um controle significativo. Cromatografia líquida de alta pressão e fase reversa (RP-HPLC) e cromatografia líquida conjugada com espectrometria de massa (LC-MS) permitiram a separação de oito frações diferentes do surfactante, das quais cinco apresentaram capacidade de diminuir a densidade superficial da água, causar a perda de motilidade e explosão de zoosporos. Coletivamente, estes resultados indicam que os biosurfactantes produzidos por *P. fluorescens* SS101 exercem um papel fundamental nas interações com patógenos capazes de produzir zoosporos e no controle da podridão radicular do jacinto causada por *P. intermedium*.

No Capítulo 7, os resultados mais importantes foram resumidos e discutidos no contexto do conhecimento existente.

Concluindo, os resultados apresentados nesta tese oferecem informações sobre a distribuição, diversidade e atividade de bactérias do gênero *Pseudomonas* produtoras de antibióticos e descrevem a descoberta de antibióticos com atividade de superfície que têm um grande potencial para o controle de oomicetos patogênicos.

SAMENVATTING

Bacteriën van het genus *Pseudomonas* zijn potentiële antagonisten van diverse plantenpathogene schimmels en oömyceten. De productie van antibiotica speelt een belangrijke rol in de activiteit van diverse *Pseudomonas* stammen tegen plantenpathogenen. Ondanks het feit dat er gedurende de laatste decennia veel informatie is verkregen over de biosynthese en regulatie van antibiotica, is er nog relatief weinig bekend over de verspreiding en diversiteit van antibiotica-producerende *Pseudomonas* spp. in natuurlijke milieus. Kennis over de diversiteit van natuurlijke populaties van antibiotica-producerende *Pseudomonas* spp. kan bijdragen aan een verbetering van biologische bestrijding middels de identificatie van stammen die ecologisch meer competent zijn. De doelstelling van het onderzoek beschreven in dit proefschrift is het bestuderen van de verspreiding, diversiteit en activiteit van antibiotica-producerende *Pseudomonas* spp. in de rhizosfeer.

In Hoofdstuk 1 wordt een overzicht gegeven van detectie, verspreiding en diversiteit van antibiotica-producerende *Pseudomonas* spp. Er wordt specifiek ingegaan op de biosynthese en regulatie van de antibiotica 2,4-diacetylphloroglucinol (2,4-DAPG), phenazines (PHZ), pyrrolnitrin (PRN), pyoluteorin (PLT), en oppervlakte-actieve antibiotica, welke onderwerp zijn van de experimenten beschreven in dit proefschrift.

In Hoofdstuk 2 is de diversiteit, phylogenie en frequentie van *Pseudomonas* en *Burkholderia* spp. die PRN of PLT produceren bestudeerd. Primers werden ontwikkeld op basis van geconserveerde sequenties in de biosynthetische loci van elk van beide antibiotica. Deze primers maakten detectie mogelijk van een grote collectie van *Pseudomonas* en *Burkholderia* spp. die PRN of PLT of beide antibiotica produceren. Met behulp van RFLP (Restriction Fragment Length Polymorphisms) analyse van het geamplificeerde *pltC* fragment konden geen polymorfismen worden aangetoond tussen PLT-producerende *Pseudomonas* stammen. De polymorfismen in het geamplificeerde *prnD* fragment maken het echter wel mogelijk om de diversiteit tussen PRN-producerende *Pseudomonas* en *Burkholderia* spp. te bepalen zelfs tot een vergelijkbaar niveau als verkregen met behulp van RAPD (Random Amplified Polymorphic DNA) analyse. Tevens tonen de phylogenetische analyses aan dat de *prn* genen van *B. pyrrocinia* DSM10685 meer verwant zijn met die van PRN-producerende *Pseudomonas* stammen dan met die van andere *Burkholderia* stammen, hetgeen mogelijk kan wijzen op uitwisseling van *prn* genen. PRN- en PLT-producerende *Pseudomonas* en *Burkholderia* spp. waren niet detecteerbaar op wortels van tarwe geteeld op vijf landbouwgronden verzameld in Nederland, waarvan twee gronden ziektewerend zijn tegen het

pathogeen *Gaeumannomyces gramminis* var. *tritici*. Deze resultaten suggereren dat deze bacteriën waarschijnlijk niet bijdragen aan de natuurlijke ziektewering van deze twee gronden.

Hoofdstuk 3 richt zich op het respons regulator gen *gacA* in *Pseudomonas* spp. Dit gen beïnvloedt de productie van verscheidene antibiotica in antagonistische *Pseudomonas* spp. Voor het *gacA* gen van *Pseudomonas* spp. werden primers en een probe ontwikkeld en van tien *Pseudomonas* stammen, geïsoleerd van verschillende planten, werd vervolgens het *gacA* gen geamplificeerd en gesequenced. De PCR analyses en Southern hybridisatie tonen aan dat *gacA* in hoge mate geconserveerd is in *Pseudomonas* en als complementaire genetische marker gebruikt kan worden voor detectie van *Pseudomonas* in rhizosfeer monsters. Phylogenetische verwantschappen, bepaald op basis van de nieuwe *gacA* sequenties en *gacA* homologen aanwezig in de database, toonden aan dat het *gacA* gen tevens dienst kan doen als phylogenetische marker voor *Pseudomonas* spp. en andere Gram-negatieve bacteriën. Zogenaamde Ka/Ks ratios tonen aan dat het *gacA* gen onder selective druk staat waardoor voorkomen wordt dat substituties leiden tot functionele veranderingen in GacA.

In Hoofdstuk 4 werd de rol van antibiotic-producerende *Pseudomonas* spp. in de ziektewerendheid van Nederlandse gronden tegen *G. gramminis* var. *tritici* bestudeerd. Ziektewering van bodems tegen *G. gramminis* var. *tritici* komt wereldwijd voor en algemeen wordt aangenomen dat verschillende microbiële genera verantwoordelijk zijn voor de ziektewering in bodems van diverse geografische oorsprong. Met behulp van populatiestudies, biotoetsen en het gebruik van antibiotica-deficiënte mutanten werd aangetoond dat *Pseudomonas* spp. die 2,4-DAPG produceren een sleutelrol spelen in de ziektewering van twee Nederlandse gronden. De resultaten laten zien dat ook in bodems die fysisch-chemisch verschillend zijn van de ziektewerende bodems van Washington State (USA), 2,4-DAPG-producing fluorescent *Pseudomonas* spp. een sleutelrol spelen. Het is tevens de eerste keer sinds de beschrijving van deze Nederlandse ziektewerende gronden dat tenminste een deel van de mechanismen en microorganismen die een rol spelen in de ziektewering zijn geïdentificeerd. Ondanks sterke overeenkomsten in populatiedichtheden en activiteit, zijn de populaties van 2,4-DAPG-producerende *Pseudomonas* spp. in de Nederlandse ziektewerende gronden genotypisch verschillend van de populaties gevonden in de ziektewerende gronden van Washington State.

De activiteit van het antibioticum 2,4-DAPG tegen *Pythium* spp. is in detail bestudeerd in Hoofdstuk 5. De resultaten laten zien dat tussen *Pythium* spp. variatie bestaat in gevoeligheid voor 2,4-DAPG. Ook diverse structuren van *P. ultimum* var. *sporangiiferum* (*Pus*), die deel uitmaken van de asexuale fase van de levenscyclus, verschillen aanzienlijk in hun

gevoeligheid voor 2,4-DAPG. Mycelium was de meest resistente structuur gevolgd door zoösporangia, geëncysteerde zoösporen en tot slot zoösporen. Transmissie electronen microscopie (TEM) toonde aan dat 2,4-DAPG verschillende stadia van desorganisatie veroorzaakt in hyphen van *Pus*, inclusief veranderingen (terugtrekking en disruptie) van de plasmamembraan, vacuolisatie en desintegratie van de inhoud van de cel. Daarnaast, wordt in dit proefschrift voor de eerste keer beschreven dat de pH een significant effect heeft op de activiteit van 2,4-DAPG, met een hogere activiteit bij lage pH. Tevens bleek dat de mate van acetylering van phloroglucinol bepalend is voor de activiteit.

Hoofdstuk 6 richt zich op oppervlakte-actieve antibiotica geproduceerd door *Pseudomonas* spp. en hun activiteit tegen oömyceten. We beschrijven de ontdekking van *Pseudomonas* isolaten die biosurfactants produceren met lytische activiteit tegen zoösporen van meerdere oömycete pathogenen. Toediening van celsuspensies van stam *P. fluorescens* SS101 aan grond en hyacintenbollen resulteerde in bescherming tegen wortelrot veroorzaakt door *Pythium intermedium*. Vijf mutanten van SS101, verkregen met behulp van Tn*5* mutagenese, hebben het vermogen om zoösporen te lyseren verloren. Genetische karakterisatie van de Tn*5*-flankerende regios van twee mutanten, 10.24 en 17.18, toonden aan dat de transposons geïntegreerd waren in condensatie domeinen van peptide synthases. Mutant 10.24 was niet in staat om hyacint te beschermen tegen Pythium wortelrot terwijl wildtype SS101 aanzienlijke bescherming bood. Met behulp van reverse-phase high performance liquid chromatography (RP-HPLC) en liquid chromatography–massa spectrometrie (LC-MS) werden acht fracties geïsoleerd waarvan vijf oppervlakte-actief waren en lysis van zoosporen veroorzaakten. Gezamenlijk geven deze resultaten sterke aanwijzingen dat oppervlakte-actieve metabolieten geproduceerd door *P. fluorescens* SS101 een belangrijke rol spelen in interacties met zoösporen van oömyceten en in bestrijding van Pythium wortelrot van hyacint.

In Hoofdstuk 7 worden de meest belangrijke resultaten beschreven in dit proefschrift samengevat en bediscussieerd in de context van bestaande kennis.

Samengevat bevat dit proefschrift nieuwe informatie over de verspreiding, diversiteit en activiteit van antibiotica-producerende *Pseudomonas* spp. en beschrijft het de ontdekking van oppervlakte-actieve antibiotica die van groot belang kunnen zijn voor de bestrijding van plantenziekten veroorzaakt door oömyceten.

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Jorge

ABOUT THE AUTHOR

Jorge Teodoro de Souza was born on 11th November 1970, in Rio Verde de Mato Grosso, Mato Grosso do Sul State (MS), Brazil. He obtained his BSc degree in Agronomy in January 1995 and his MSc degree in 1998 on biological control of nematodes, both at Lavras University, Minas Gerais State (MG), Brazil. In January 1998 he started his Ph.D. studies at the laboratory of Phytopathology of the Wageningen University, The Netherlands, on biological control of soil-borne pathogens with antibiotic-producing *Pseudomonas* spp.