Chapter 2
Detection of Antibiotics Produced by Soil and Rhizosphere Microbes In Situ

Linda S. Thomashow (✉), Robert F. Bonsall, and David M. Weller

2.1 Introduction

It has long been known that certain antibiotic-producing soil microorganisms are inhibitory to plant pathogens, both in the laboratory and in the field (Stallings 1954). The exploitation of these natural antagonistic interactions has been a driving force in research on the biological control of plant pathogens over the past century, but only in recent decades has pathogen control by antibiotics produced at biologically relevant levels in the environment been demonstrated conclusively. This progress, resulting from conceptual and technological advances made initially in the laboratory and then extended to the field, has set new standards for biocontrol research involving antibiotics. More generally, the approaches used in these studies may be useful in exploring the significance of other bioactive metabolites produced by microorganisms in their native habitats.

Among the conceptual advances underpinning progress towards understanding the role of antibiotics in the environment has been recognition that individual strains often are capable of producing more than one inhibitory compound. Detection methods based on the biochemical properties of a particular antibiotic therefore must be specific enough to distinguish among the repertoire of possible products (the number and optimal conditions for production of which usually must be determined empirically). In addition, assays based on biological activity must eliminate or compensate for the effects of metabolites other than the one of interest. This element of specificity generally was lacking in traditional studies in which activity against a target pathogen or indicator organism in vitro was taken as evidence of activity against that organism in situ. Perhaps more difficult is the need to

Linda S. Thomashow
USDA, ARS Washington State University, Pullman, WA, USA
e-mail: thomashow@wsu.edu

P. Karlovsky (ed.), *Secondary Metabolites in Soil Ecology. Soil Biology 14.*

compensate adequately for biological effects of metabolites other than the one under investigation, in part because the full range of products an organism is capable of producing seldom is known. A case in point is the well-known biocontrol strain *Pseudomonas fluorescens* Pf-5, in which DNA sequence analysis recently resulted in the discovery of a previously unknown group of bioactive cyclic lipopeptides (Gross et al. 2007).

Antibiotic detection in microbial habitats also has been facilitated by knowledge of the regulatory mechanisms by which microorganisms integrate antibiotic biosynthesis with growth and other metabolic processes, all of which require adequate nutrient supplies. Carbon and energy resources are scarce in bulk soil and, consequently, metabolic levels in microorganisms are low. Microbiological activity is more intensive in the spermosphere and rhizosphere of plants and in or around plant debris and fungal propagules where resources are comparatively abundant, and it is in these habitats that antibiotics are likely to be detected. The availability of metabolizable substrates places spatial constraints on the ability of microbial populations to produce antibiotics, and this influences the choice of method and sensitivity of detection of antibiotics, as well as the sample sizes that must be analyzed.

Technological advances in molecular biology and biochemistry, developed initially to dissect the genetics and regulation of antibiotic synthesis in vitro, have been indispensable in achieving the sensitivity and specificity needed to detect and assess the activity of antibiotics in situ. Biochemical approaches involving antibiotic extraction, fractionation, and characterization, usually on the basis of chemical and physical properties, provide direct and incontrovertible evidence of antibiotic production. However, direct approaches have limited sensitivity that depend not only on the physicochemical and biological properties of individual antibiotics, but also on the efficiency of recovery during extraction and the detection limit attainable with the instrumentation available. Amounts of antibiotic recovered usually are expressed relative to the size of the initial sample, but it must be remembered that these are average values, and localized antibiotic concentrations may be considerably higher in sites where microbial activity occurs. Thus, when biological aspects of antibiotic production are of interest, molecular approaches based on the detection of antibiotic activity or gene expression may be preferable to direct bioanalytical methods, assuming that the antibiotic biosynthesis genes themselves already have been identified. Molecular techniques enable the construction of mutant strains defective only in synthesis of the compound of interest, providing the specificity needed to assess the impact of particular antibiotics on other organisms in the soil environment. Alternatively, when the impact of physiological or edaphic conditions on antibiotic production is of concern, reporter strains can be constructed in which a readily monitored gene product, rather than the antibiotic itself, is assayed. Molecular approaches are fundamentally indirect and subject to limitations discussed more extensively in the following text, but they can provide a degree of sensitivity not achieved by direct analysis.

2.2 Direct Analysis: Sample Preparation and Chromatography

2.2.1 Collection and Storage

The detection of antibiotics in complex environmental matrices is influenced by the physical properties of the sample as well as the procedures used to process it prior to and during extraction. For rhizosphere samples, it is important to note whether specific portions of the root system have been harvested, how much plant tissue and how much soil are present, and how the tissue and soil have been separated. Our rhizosphere samples typically include the entire root systems of young seedlings as well as the soil particles that adhere to the roots after gentle shaking. Rhizosphere samples that cannot be extracted immediately are frozen and stored in the dark to prevent losses resulting from microbial degradation and sensitivity to heat or light. Bulk soil samples usually are collected to a known depth and broken up or milled, sieved, and stored frozen or dried prior to extraction, depending on the stability of the antibiotic.

The extraction efficiency and the sensitivity of detection are major factors in determining the sample size. Soil samples in the range 1–10 g (Jacobsen et al. 2004; Kim and Carlson 2006; Stoob et al. 2006; Thiele-Bruhn et al. 2004) and root systems of 50–200 seedlings or 25–30 g of roots with adhering soil (Bonsall et al 1997; Raaijmakers et al 1999; Thomashow et al. 2002) are representative. For quantitative determinations, the efficiency of recovery can be estimated from a standard curve in which the antibiotic has been spiked into control samples in amounts spanning the range expected in test samples. In addition, test samples can be amended with an internal standard having properties similar to those of the compound of interest, but which does not occur naturally in the sample matrix or interfere with subsequent analyses.

2.2.2 Sorption and Sample Preparation

The distribution of antibiotics between soil solids and water has gained scientific attention in recent years owing to concern over the environmental fate and consequences of the large amounts of veterinary pharmaceuticals used in animal husbandry. While focused on veterinary antibiotics, the information gained from these studies is largely consistent with earlier work describing the behavior of organic compounds in soil. In general, antibiotics adsorb rapidly to the surfaces of soil particles and dissolved organic matter, and recovery declines continuously over time (Blum et al. 1994; Chiou 1989; Weber and Miller 1989). Thus, recoveries of sulfonamide antibiotics spiked into aged agricultural soils were significantly reduced after 6–17 days compared with a contact time of just 90 min (Stoob et al. 2006). Adsorption does not necessarily inactivate antibiotics, however, as tetracycline and tylosin, two

widely used growth promoters in food animal production, remained biologically active even when tightly adsorbed to clay particles (Chander et al. 2005).

Sorption is a complex phenomenon influenced not only by the physicochemical properties of an antibiotic but also by the composition and structure of solid matrices. Most antibiotics are moderately soluble in water and many have pK_a values within the range of pH values found in soils, indicating that their ionic form, solubility, and sorptive properties will be strongly influenced by the pH and ionic composition of the soil (ter Laak et al. 2006a; Tolls 2001). Below pH 6.5, nonionic forms of organic acids and phenolic compounds are readily sorbed by soil organic matter (Chiou 1989), and at pH values above the pK_a, charge interactions occur with inorganic soil constituents. Veterinary pharmaceuticals were associated with dissolved organic matter and soil particles much more strongly than predicted simply on the basis of hydrophobic interactions, perhaps because of cation exchange, cation bridging at clay particle surfaces, surface complexation, and hydrogen bonding (Tolls 2001). Cation bridging is thought to account for association with clay minerals, with sorption strongly related to particle size and, hence, to surface area. Soils rich in aluminum and iron oxyhydroxides have a high sorptive capacity for carboxyl and phenolic hydroxyl groups, and some soils rich in Mn^{2+} have a high capacity for organic acids (Dalton et al. 1989; Lehmann et al. 1987). Considering the variability in composition among soils, it is not surprising that sorption coefficients (the ratio of the concentrations of a compound in the sorbent and aqueous phases at equilibrium) can differ by several orders of magnitude from one soil to another, and efforts to develop models predictive of sorption coefficients based on soil properties have had limited success. Up to 78% of the variation in sorption coefficients of three veterinary compounds among 11 soils could be explained when six soil properties (pH, organic carbon content, clay content, cation-exchange capacity, aluminum oxyhydroxide content, and iron oxyhydroxide content) were integrated, but not when they were considered separately. The remaining variability was related to concentration effects associated with pH-dependent antibiotic ionization (ter Laak et al. 2006b).

In an efficient extraction process, the distribution of an antibiotic in the soil or rhizosphere is shifted from the sorbed form to a solvent. This process is facilitated by stirring, shaking, sonication, or, more recently, pressurized liquid extraction. The last of these requires specialized equipment, but is thought to increase sample wetting, solvent penetration, and diffusion rates (Ramos et al. 2002) and to reduce solvent consumption and extraction time (Stoob et al. 2006). The composition of the liquid phase is determined largely by the solubility and charge properties of the antibiotic, and to a lesser extent by the need to minimize the coextraction of soil organic compounds likely to interfere with subsequent purification and analysis. The liquid phase for soil and rhizosphere antibiotics typically is a mixture consisting of a polar organic solvent in water, adjusted to a pH below the pK_a of the antibiotic to facilitate partitioning into the solvent. The extractant solution may also contain agents such as Na_2EDTA, citric acid, NaCl, and McIlvine buffer to improve the recovery of antibiotics that form strong complexes with divalent and trivalent metal ions present in the soil (Petrović et al. 2005).

Procedures suitable for the extraction of the most frequently identified antibiotics produced in the rhizosphere by *Pseudomonas* spp. have been published (Bonsall et al. 1997; Raaijmakers et al. 1999) and can be adapted for other substances by adjusting the amount of sample required and selecting appropriate solvents. This method can recover phenazine-l-carboxylic acid (PCA), its hydroxyphenazine derivatives, pyrrolnitrin, pyoluteorin, and 2,4-diacetylphloroglucinol (DAPG) from field samples with recoveries of 60% or better. Briefly, roots with adhering rhizosphere soil are shaken in 80% acetone acidified to pH 2.0 with 10% trifluoroacetic acid. The solids are removed by settling or centrifugation and the filtrate is collected and concentrated after passage through a solvent-compatible filter.

Additional concentration and fractionation steps are required prior to chromatographic analysis in order to remove organic matter typically present in large amounts in soil extracts. Dark-colored organic contaminants can be removed by centrifuging solutions of some antibiotics frozen at −20 °C in acidified 35% acetonitrile (Bonsall et al. 1997), but other antibiotics may not remain soluble under these conditions. Antibiotics with ionizable residues can be separated from many contaminants by exploiting the pH-dependent differential solubility of the neutral and charged forms in organic and aqueous solvents. Thus, isolation procedures in the past routinely included at least one liquid–liquid extraction step to partition antibiotics away from salt residues and impurities, and into organic solvents from which they could be concentrated readily (Thomashow et al. 1990). More recently, however, solid-phase cartridges that exploit pH-dependent ionic speciation and polarity differences among antibiotics have largely replaced liquid–liquid extraction procedures. Solid-phase extraction (SPE) offers many advantages over liquid–liquid partitioning: less solvent waste and reduced operator exposure to solvents, and more rapid and efficient isolation and concentration of analytes. Early applications of SPE from soil samples include the preparative enrichment of DAPG on octadecyl silica (Shanahan et al. 1992) and the trapping of macrocyclic xanthobaccin compounds produced by *Stenotrophomonas* sp. SB-K-88 in the rhizosphere of sugar beet by growing the seedlings in a mixture of sand and Amberlite XAD-2 (Nakayama et al. 1999). A variety of SPE sorbents have been evaluated for antibiotic cleanup and recovery in studies addressing the environmental impact of human and veterinary pharmaceuticals. Among the most frequently employed are polymeric Oasis HLB (lipophilic divinylbenzene plus hydrophilic *N*-vinyl pyrrolidone) cartridges because they tolerate a broad range of pH, have greater capacity than alkyl-bonded silicas, and enable good recovery of both polar and nonpolar compounds (Díaz-Cruz and Barceló 2005; Petrović et al. 2005), and Oasis MCX cartridges containing a mixed-mode sorbent with cation-exchange and reversed-phase characteristics effective for polar to medium-polar compounds (Petrović et al. 2005). Tandem SPE employing sequential strong anion exchange and HLB cartridges has been used successfully to simultaneously reduce interfering organic matter and extract veterinary antibiotics (Jacobsen et al. 2004; Renew and Huang 2004).

2.2.3 Chromatography and Detection

Among the chromatographic methods available to fractionate and detect antibiotics from soil, the simplest but most limited in analytical capability is thin layer chromatography (TLC). TLC does not require sophisticated instrumentation, nor do samples generally require extensive cleanup prior to analysis. Compounds can be separated with good resolution and methods are readily adaptable for applications ranging from high throughput to preparative-scale work. Both normal and reversed-phase adsorbents have been used with a variety of mobile-phase solvent systems (Thomashow et al. 2002). Substances are visualized by UV absorption, chromogenic reaction with spray reagents, or bioautography, in which indicator organisms suspended in agar or broth are overlaid on chromatograms to detect bioactive spots. Antibiotics are identified on the basis of the appearance, distance traveled relative to the solvent front (R_f value), and cochromatography with authentic standards in more than one adsorbent or solvent system. Quantities are estimated from spot size and intensity, or size of the inhibition zone for bioautography, at various dilutions relative to known amounts of standards run on the same plate. The need for standards cannot be overemphasized, as variation in preparative methods and the sources and specifications of adsorbents and support media can result in significant differences between observed and published R_f values. Because many of the antibiotics produced by soil microorganisms and rhizosphere microorganisms are not commercially available, well-characterized strains capable of producing these substances in vitro often are the most convenient source of standards.

High performance liquid chromatography (HPLC), coupled with UV spectroscopy or various forms of mass spectrometry (MS), now is used routinely to fractionate, detect, and identify antibiotics produced in the rhizosphere (Bakker et al. 2002; Chin-A-Woeng et al. 1998; Glandorf et al. 2001; Huang et al. 2004; Nielsen and Sørensen 2003; Raaijmakers et al. 1999; Thomashow et al. 2002). These techniques are adaptable to a wide variety of analytes and offer a high degree of reproducibility, resolving capability, sensitivity, and quantitative accuracy. Considerations in developing and optimizing a chromatographic system include selection of the column, the mobile phase, the elution profile, and the mode of detection. Reversed-phase columns with octadecyl (C_{18}) or octyl (C_8) bonded silica packing, and gradient elution with acidified acetonitrile–water or methanol–water are commonly used. For high-throughput applications, isocratic elution avoids time- and solvent-consuming column reequilibration between samples if satisfactory resolution can be achieved. Shorter columns also may speed up analysis, albeit with the risk of reduced separation. Retention time and peak shape typically are optimized via the solvent composition and elution profile.

Detection commonly is by UV absorption, and because photodiode-array detectors concurrently monitor a range of wavelengths, they offer important advantages over fixed-wavelength detectors. Individual components within a mixture can be monitored simultaneously, each at its own absorption maximum, and subsequent

spectral analyses can provide insight into peak purity and identity. Alternatively, amperometric detection may provide greater sensitivity and selectivity for some phenolics, and fluorometric detection may offer similar advantages for compounds such as indoles and some phenazines. Detection and quantification are further improved by coupling HPLC with MS, which enables confirmation of the identity of compounds on the basis of their molecular structure. Because samples from complex matrices such as soil typically include organic material that reduces detection sensitivity and interferes with quantification, soil extracts typically are analyzed by MS/MS, time-of-flight (TOF) MS, or triple-quadrupole TOF (Q-TOF) MS. The technical differences and relative merits among these mass analyzers, which increase detection sensitivity by providing an additional degree of chemical separation of analytes from interfering compounds in the matrix, have been considered in several recent reviews (Díaz-Cruz and Barceló 2005; Kim and Carlson 2005; Petrović et al. 2005). Q-TOF-MS, in particular, has become an important analytical tool because it can provide high mass-accuracy data and full MS/MS spectra, enabling both screening and confirmation of analytes in a single run. In our hands, the detection limits of DAPG and phenazine-1-carboxylic acid, produced in the rhizosphere of wheat, by Q-TOF-MS are 15 ng and 800 pg, amounts about 20-fold and 500-fold greater, respectively, than can be detected by photodiode-array spectroscopy. DAPG, produced by indigenous populations of *P. fluorescens* on the roots of field-grown wheat, was present at about 20 ng per gram of root fresh weight (Raaijmakers et al. 1999) and a wide variety of other antibiotics produced in situ have been reported at levels ranging from 5 to 5,000 ng per seed or gram of dry soil or root fresh weight (Thomashow et al. 1997).

2.3 Indirect Evidence of Antibiotic Production

2.3.1 Detection of Antibiotic Biosynthesis Genes

Because the detection of antibiotics by direct methods requires knowledge of their biophysical properties and can be laborious and costly, it often is more convenient to monitor the potential for, or consequences of, antibiotic production in the environment. The detection of antibiotic biosynthesis genes, whether or not expressed, provides insight into the distribution of antibiotic producers in nature and serves as a first indication that antibiotics may be present at biologically relevant levels. For biological phenomena such as the suppression of plant pathogens that are mediated by antibiotic production in situ, the detection of biosynthesis genes is a convenient means of monitoring the frequency, diversity, and dynamics of introduced or indigenous antibiotic-producing populations.

Molecular methods have been published (De La Fuente et al. 2006; Delaney et al. 2001; Mavrodi et al. 2001, 2007; McSpadden-Gardener et al. 2001; Thomashow et al. 2002) for the detection of key biosynthesis genes of the most frequently studied antibiotics produced in the rhizosphere, as well as for some antibiotics (Gross

et al. 2007) with emerging roles in microbe–plant interactions. For example, a 745-bp internal fragment from the highly conserved *phlD* gene of the DAPG biosynthesis pathway has been used to enumerate DAPG producers from the roots of wheat (Raaijmakers et al. 1997, 1999) and maize (Picard et al. 2002) by colony hybridization and PCR, and a rapid PCR-based dilution-end-point assay for DAPG producers with a detection limit of approximately 10^3 colony-forming units per rhizosphere also has been developed (McSpadden et al. 2001).

Stringency is a critical determinant of sensitivity and specificity in all hybridization- and PCR-based detection strategies and is modulated by the selection of appropriate probe or primer sequences and by rigorous control of experimental conditions. Primers used to screen for antibiotic genes in environmental isolates or to detect the total population capable of producing a particular antibiotic must be *nonspecific* enough to accommodate templates with minor sequence heterogeneity due to sequence polymorphisms or codon degeneracy. On the other hand, it may be necessary to quantify specific subfractions (genotypes) of an antibiotic-producing population, as is the case for DAPG producers in which *phlD* polymorphisms are indicative of the affinity of a strain for particular host crops (De La Fuente et al. 2006). The typical approach to the design of such primers is to first align the DNA sequences from several homologues of the target gene. The alignment will reveal suitably spaced blocks of conserved or unique sequences from which primer pairs can be selected and PCR conditions optimized to meet the required specificity criteria. An alternative approach, which has been applied to detect genes involved in the synthesis of polyketide antibiotics, involves back-translating the amino acid sequence of a conserved region from related strains according to the preferred codon usage of the target species, and then synthesizing degenerate primers (Metsä-Ketalä et al. 1999; Seow et al. 1997).

Whereas colony hybridization and PCR require culturing of environmental isolates prior to gene detection, real-time PCR provides a culture-independent means of detecting antibiotic biosynthesis genes in DNA isolated directly from environmental sources. Eliminating the requirement for bacterial growth shortens assay turnaround time and avoids questions about the suitability of the culture conditions employed, whether isolates capable of antibiotic production are present in a viable-but-nonculturable condition, and if the populations detected after enrichment are skewed owing to inhibitory interactions among strains during growth (Validov et al. 2005). A culture-independent quantitative real-time PCR method for detection of the *phlD* gene has been developed that has a detection limit comparable to those of culture-based approaches, can detect both introduced and indigenous populations, and is capable of distinguishing among strain genotypes (Mavrodi et al. 2007).

Real-time PCR shares the same principles governing sensitivity, specificity, and primer design as standard PCR, but data collection and analysis in real-time PCR occur as the reaction proceeds in the instrument, making the technique much faster and less prone to contamination than standard PCR. Amplification is detected as an increase in fluorescence emitted by a dye after it has been incorporated into a double-stranded DNA product, the specificity of which is evaluated by melting

temperature and melting curve analysis after each reaction. Fluorescence is monitored at each PCR cycle, and because the cycle in which the first significant increase in fluorescence above the background is correlated with the initial amount of target template, the method is inherently quantitative. For measurements to be meaningful, however, reactions must be highly optimized with regard to amplification conditions, amplification efficiency, and primer concentration and specificity. Standard curves must be developed over a range of DNA concentrations and in order to relate template DNA concentration to bacterial population size, the size of the genome and the copy number of the template gene must be known. Procedures for recovering DNA from environmental samples also must be optimized, and because recoveries may differ among matrices differing in their physicochemical properties, recovery values should be determined separately for each sample matrix. Whereas real-time PCR efficiencies for DNA extracted from DAPG-producing bacteria applied to the roots of wheat ranged from 80 to 98%, only about 10% of the DNA present in those bacteria actually was recovered (Mavrodi et al. 2007), suggesting that the sensitivity with which antibiotic producers are detected by real-time PCR will improve as better DNA extraction techniques are developed.

2.3.2 Antibiotic Gene Expression In Situ

Transcriptional analyses of antibiotic gene expression provide a sensitive and convenient alternative to direct antibiotic isolation, especially when biosynthesis over time or in response to environmental conditions is of interest. Such studies typically employ strains in which a reporter gene, the product of which is easily monitored and not naturally present in the environment, is placed under the transcriptional control of a promoter regulating the expression of the antibiotic biosynthesis genes. The speed and sensitivity with which reporters such as the green fluorescent protein gene *gfp* or the ice nucleation gene *inaZ* can be assayed facilitate the use of samples as small as single seeds or seedlings, allowing sufficient replication that significant differences among treatments can be detected despite the sample-to-sample variation inherent in such studies (Loper and Lindow 2002; Thomashow et al. 2002).

Reporter gene expression provides evidence that antibiotic synthesis can occur under prevailing environmental conditions, but the expression level need not be indicative of the actual amount of antibiotic present in a biologically active state. This is partially because transcriptional activity is measured relative to the total population size even though that population is physiologically heterogeneous, having been recovered from a variety of different microhabitats on the roots. A further confounding factor arises from the differential turnover rates of antibiotics and reporter gene products. Antibiotics in soil can lose activity over time owing to adsorption (Chander et al. 2005) or degradation, either by the producer strain itself (Bottiglieri and Keel 2006) or by the indigenous microflora. On the other hand, some reporters, and especially green fluorescent protein, are relatively

stable and may more accurately reflect cumulative gene expression than instantaneous transcription rates. Finally, the complex regulatory circuitry involved in antibiotic synthesis (Haas and Keel 2003; Haas et al. 2000) and the structure of the reporter construct itself (Pessi et al. 2002) can influence the relationship between reporter gene expression and the amount of antibiotic actually produced.

2.3.3 Biological Activity In Situ: The Value of Mutants

Because antibiotics in the environment can reach biologically significant concentrations in localized sites while remaining at subthreshold or undetectable levels overall, their presence often is inferred from effects on other organisms that act as indicators of antibiotic activity. Such indirect estimates of antibiotic activity are of particular value when the measured effect can be attributed with certainty to the antibiotic of interest, and are greatly facilitated if wild-type strains can be compared directly with genetically defined, antibiotic-deficient mutants. The use of mutants is a preferred option when the activity of a producer strain is of interest over a range of environmental conditions that may impact on both the synthesis of an antibiotic and its biological availability (Ownley et al. 2003).

Antibiotic-deficient mutants may arise spontaneously or be induced chemically, by UV irradiation, or with molecular genetic techniques. The latter are used almost exclusively nowadays because the site of mutation can be localized, providing insight into the biochemical basis and specificity of the mutant phenotype. Mutants should be complemented with wild-type DNA to restore antibiotic synthesis and to help rule out the involvement of undetected second-site mutations. Thorough phenotypic characterization also is essential, especially when genes other than those involved directly in synthesis of the target antibiotic have been mutated. Because many bacteria produce more than one antibiotic, indirect assays based on inhibitory activity in vitro must eliminate or compensate for the effects of metabolites other than the one of interest.

2.3.4 Nontarget Effects of Antibiotic Production In Situ

Although most studies of antibiotic activity in situ have focused on the suppression of plant pathogens, the broader environmental consequences of antibiotic production in the environment also have received attention, particularly in relation to the introduction of recombinant strains of *P. fluorescens* engineered to produce multiple antibiotics (Bakker et al. 2002; Blouin-Bankhead et al. 2004; De Leij et al. 1995; Glandorf et al. 2001; Iavicoli et al. 2003; Leeflang et al. 2002; Moënne-Loccoz et al. 2001. Natsch et al. 1998; Thirip et al. 2001; Timms-Wilson et al. 2004;

Viebahn et al. 2003, 2005a, b; Winding et al. 2004). These studies have addressed effects on soil enzyme activities and available nutrients as well as impacts on the abundance and community structure of microorganisms that are closely related or unrelated to the introduced bacteria, and on protozoa, nematodes, and plants. Especially noteworthy is a 6-year field study of the effects on the soil microbial community of *P. putida* WCS358r, an antibiotic nonproducer modified to produce either phenazine-1-carboxylic acid or DAPG (Bakker et al. 2002; Glandorf et al. 2001; Leeflang et al. 2002; Viebahn et al. 2003, 2005a, b). The study revealed that the wild-type and recombinant strains both had transient effects on the composition of the fungal and bacterial rhizosphere microflora of wheat, with the effects of the recombinant strains sometimes lasting longer. The impact of the introduced strains differed from year to year, revealing no consistent pattern. The results are typical of those of other studies conducted under a variety of controlled or field conditions, and are consistent with the fact that populations of introduced rhizobacteria generally establish large populations immediately after inoculation onto the seed or into soil and then the densities decline over time and distance from the inoculum source. Collectively, the data indicate that the presence of antibiotic-producing bacteria affects rhizosphere microbial community structure, but the effects vary from study to study, often are less than those associated with routine agronomic practices, and are transient.

References

Bakker PA, Glandorf DC, Viebahn M, Ouwens TW, Smit E, Leeflang P, Wernars K, Thomashow LS, Thomas-Oates JE, van Loon LC (2002) Effects of *Pseudomonas putida* modified to produce phenazine-1-carboxylic acid and 2,4-diacetylphloroglucinol on the microflora of field grown wheat. Antonie Van Leeuwenhoek 81:617–624

Blouin-Bankhead S, Landa B, Lutton E, Weller DM, McSpadden Gardener B (2004) Minimal changes in rhizobacterial population structure following root colonization by wild type and transgenic biocontrol strains. FEMS Microbiol Ecol 49:307–318

Blum U, Worsham AD, King LD, Gerig TM (1994) Use of water and EDTA extractions to estimate available (free and reversibly bound) phenolic acids in Cecil soils. J Chem Ecol 20:341–359

Bonsall RF, Weller DM Thomashow LS (1997) Quantification of 2,4-diacetylphloroglucinol produced by fluorescent *Pseudomonas* spp. in vitro and in the rhizosphere of wheat. Appl Environ Microbiol 63:951–955

Bottiglieri M, Keel C (2006) Characterization of PhlG, a hydrolase that specifically degrades the antifungal compound 2,4-diacetylphloroglucinol. Appl Environ Microbiol 72:418–427

Chander Y, Kumar K, Goyal SM, Gupta SC (2005) Antibacterial activity of soil-bound antibiotics. J Environ Qual 34:1952–1957

Chin-A-Woeng TFC, Bloemberg GV, van der Bij AJ, van der Drift KMGM, Schripsema J, Kroon B, Scheffer RJ, Keel C, Bakker PAHM, Tichy J-V, de Bruijn FJ, Thomas-Oates JE, Lugtenberg BJJ (1998) Biocontrol by phenazine-1-carboxamide-producing *Pseudomonas chlororaphis* PCL 1391 of tomato root rot caused by *Fusarium oxysporum* f. sp. radicis-lycopersici. Mol Plant Microbe Interact 11:1069–1077

Chiou CT (1989) Theoretical considerations of the partition uptake of nonionic organic compounds by soil organic matter. In: Sawhney BL, Brown K (eds) Reactions and movement of

organic chemicals in soils. SSSA special publication 22. Soil Science Society of America, Madison, pp 1–29

Dalton BR, Weed SB, Blum U (1989) Differential sorption of exogenously applied ferulic, *p*-coumaric, *p*-hydroxybenzoic, and vanillic acids in soil. Soil Sci Soc Am J 51:1515–1521

De La Fuente L, Mavrodi DV, Landa BB, Thomashow LS, Weller DM (2006) *phlD*-based genetic diversity and detection of genotypes of 2,4-diacetylphloroglucinol-producing *Pseudomonas fluorescens*. FEMS Microbiol Ecol 56:64–78

De Leij FAAM, Sutton EJ, Whipps JM, Fenton JS, Lynch JM (1995) Impact of a field release of genetically modified *Pseudomonas fluorescens* on indigenous microbial populations of wheat. Appl Environ Microbiol 61:3443–3453

Delaney SM, Mavrodi DV, Bonsall RF, Thomashow LS (2001) *phzO*, a gene for biosynthesis of 2-hydroxylated phenazine compounds in *Pseudomonas aureofaciens* 30-84. J Bacteriol 183:318–327

Díaz-Cruz MS, Barceló, D (2005) LC-MS^2 trace analysis of antimicrobials in water, sediment and soil. Trends Anal Chem 24:645–657

Glandorf DC, Verheggen P, Jansen T, Jorritsma JW, Smit E, Leeflang P, Wernars K, Thomashow LS, Laureijs E, Thomas-Oates JE, Bakker PA, van Loon LC (2001) Effect of genetically modified *Pseudomonas putida* WCS358r on the fungal rhizosphere microflora of field-grown wheat. Appl Environ Microbiol 67:3371–3378

Gross H, Stockwell VO, Henkels MD, Nowak-Thompson B, Loper JE, Gerwick, WH (2007) The genomisotopic approach: A systematic method to isolate products of orphan biosynthetic gene clusters. Chem Biol 14:53–63

Haas D, Keel C (2003) Regulation of antibiotic production in root-colonizing *Pseudomonas* spp. and relevance for biological control of plant disease. Annu Rev Phytopathol 41:117–153

Haas D, Blumer C, Keel C (2000) Biocontrol ability of fluorescent pseudomonads genetically dissected: importance of positive feedback regulation. Curr Opin Biotechnol 11:290–297

Huang Z, Bonsall RF, Mavrodi DV, Weller DM, Thomashow LS (2004) Transformation of *Pseudomonas fluorescens* with genes for biosynthesis of phenazine-1-carboxylic acid improves biocontrol of Rhizoctonia root rot and in situ antibiotic production. FEMS Microbiol Ecol 49:243–251

Iavicoli A, Boutet E, Buchala A, Métraux JP (2003) Induced systemic resistance in *Arabidopsis thaliana* in response to root inoculation with *Pseudomonas fluorescens* CHA0. Mol Plant Microbe Interact 16:851–858

Jacobsen AM, Halling-Sørensen B, Ingerslev F, Hansen SH (2004) Simultaneous extraction of tetracycline, macrolide and sulfonamide antibiotics from agricultural soils using pressurised liquid extraction, followed by solid-phase extraction and liquid chromatography-tandem mass spectrometry. J Chromatogr A 1038:157–170

Kim S-C, Carlson K (2005) LS-MS^2 for quantifying trace amounts of pharmaceutical compounds in soil and sediment matrices. Trends Anal Chem 24:635–644

Kim SC, Carlson K (2006) Occurrence of ionophore antibiotics in water and sediments of a mixed-landscape watershed. Water Res 40:2549–2560

Leeflang P, Smit E, Glandorf DCM, van Hannen EJ, Wernars K (2002) Effects of *Pseudomonas putida* WCS358r and its genetically modified phenazine producing derivative on the *Fusarium* population in a field experiment, as determined by 18S rDNA analysis. Soil Biol Biochem 34:1021–1025

Loper JE, Lindow SE (2002) Reporter gene systems useful in evaluating in situ gene expression by soil- and plant-associated bacteria. In: Hurst CJ, Crawford RL, Knudsen GR, McInerney MJ, Stetzenbach LD (eds) Manual of environmental microbiology, 2nd edn. American Society of Microbiology, Washington, pp 627–637

Lehmann RG, Cheng HH, Harsh JB (1987) Oxidation of phenolic acids by soil iron and manganese oxides. Soil Sci Soc Am J 51:352–356

Mavrodi OV, Mavrodi DV, Thomashow LS, Weller DM (2007) Quantification of 2,4-diacetylphloroglucinol-producing *Pseudomonas fluorescens* in the plant rhizosphere by real-time PCR. Appl Environ Microbiol 73:5531–5538

Mavrodi OV, McSpadden-Gardener BB, Mavrodi DV, Bonsall RF, Weller DM, Thomashow LS (2001). Genetic diversity of *phlD* from 2,4-diacetylphloroglucinol-producing fluorescent *Pseudomonas* spp. Phytopathology 91:35–43

McSpadden-Gardener BB, Mavrodi DV, Thomashow LS, Weller DM (2001) A rapid polymerase chain reaction-based assay characterizing rhizosphere populations of 2,4-diacetylphloroglucinol-producing bacteria. Phytopathology 91:44–54

Metsä-Ketalä M, Salo V, Halo L, Hautala A, Hakala J, Mäntsälä P, Ylihonko K (1999) An efficient approach for screening minimal PKS genes from *Streptomyces*. FEMS Microbiol Lett 180:1–6

Moënne-Loccoz Y, Tichy HV, O'Donnell A, Simon R, O'Gara F (2001) Impact of 2,4-diacetylphloroglucinol-producing biocontrol strain *Pseudomonas fluorescens* F113 on intraspecific diversity of resident culturable fluorescent pseudomonads associated with the roots of field-grown sugar beet seedlings. Appl Environ Microbiol 67:3418–3425

Nakayama T, Homma Y, Hashidoko Y, Mizutani J, Tahara S (1999) Possible role of xanthobaccins produced by *Stenotrophomonas* sp. strain SB-K88 in suppression of sugar beet damping-off disease. Appl Environ Microbiol 65:4334–4339

Natsch A, Keel C, Hebecker N, Laasik E, Défago G (1998) Impact of *Pseudomonas fluorescens* strain CHA0 and a derivative with improved biocontrol activity on the culturable resident bacterial community on cucumber roots. FEMS Microbiol Ecol 27:365–380

Nielsen TH, Sørensen J (2003) Production of cyclic lipopeptides by *Pseudomonas fluorescens* strains in bulk soil and in the sugar beet rhizosphere. Appl Environ Microbiol 69:861–868

Ownley BH, Duffy BK, Weller DM (2003) Identification and manipulation of soil properties to improve the biological control performance of phenazine-producing *Pseudomonas fluorescens*. Appl Environ Microbiol 69:3333–3343

Pessi C, Blumer C, Haas D (2002) *lacZ* fusions report gene expression, don't they? Microbiology 147:1993–1995

Petrović M, Hernando MD, Díaz-Cruz MS, Barceló D (2005) Liquid chromatography-tandem mass spectrometry for the analysis of pharmaceutical residues in environmental samples: a review. J Chromatogr A 1067:1–14

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

Raaijmakers JM, Weller DM, Thomashow LS (1997) Frequency of antibiotic-producing *Pseudomonas* spp. in natural environments. Appl Environ Microbiol 63:881–887

Raaijmakers JM, Bonsall RF, Weller DM (1999) Effect of population density of *Pseudomonas fluorescens* on production of 2,4-diacetylphloroglucinol in the rhizosphere of wheat. Phytopathology 89:470–475

Ramos L, Kristenson EM, Brinkman UAT (2002) Current use of pressurised liquid extraction and subcritical water extraction in environmental analysis. J Chromatogr A 975:3–29

Renew JE, Huang CH (2004) Simultaneous determination of fluoroquinolone, sulfonamide, and trimethoprim antibiotics in wastewater using tandem solid phase extraction and liquid chromatography-electrospray mass spectrometry. J Chromatogr A 1042:113–121

Seow KT, Meurer G, Gerlitz M, Wendt-Pienkowski E, Hutchinson CR, Davies J (1997) A study of iterative type II polyketide synthases, using bacterial genes cloned from soil DNA: a means to access and use genes from uncultured microorganisms. J Bacteriol 179:7360–7368

Shanahan P, Borro A, O'Gara F, Glennon JD (1992) Isolation, trace enrichment and liquid chromatographic analysis of diacetylphloroglucinol in culture and soil samples using UV and amperometric detection. J Chromatogr 606:171–177

Stallings JH (1954) Soil produced antibiotics-plant disease and insect control. Bacteriol Rev 18:131–146

Stoob K, Singer HP, Stettler S, Hartmann N, Mueller S, Stamm CH (2006) Exhaustive extraction of sulfonamide antibiotics from aged agricultural soils using pressurized liquid extraction. J Chromatogr A 1128:1–9

ter Laak TL, Gebbink WA, Tolls J (2006a) The effect of pH and ionic strength on the sorption of sulfachloropyridazine, tylosin, and oxytetracycline to soil. Environ Toxicol Chem 25:904–911

ter Laak TL, Gebbink WA, Tolls J (2006b) Estimation of soil sorption coefficients of veterinary pharmaceuticals from soil properties. Environ Toxicol Chem 25:933–941

Thiele-Bruhn S, Seibicke T, Schulten HR, Leinweber P (2004) Sorption of sulfonamide pharmaceutical antibiotics on whole soils and particle-size fractions. J Environ Qual 33:1331–1342

Thirup L, Johnsen K, Winding A (2001) Succession of indigenous *Pseudomonas* spp. and actinomycetes on barley roots affected by the antagonistic strain *Pseudomonas fluorescens* DR54 and the fungicide imazalil. Appl Environ Microbiol 67:1147–1153

Thomashow LS, Weller DM, Bonsall RF, Pierson LS (1990) Production of the antibiotic phenazine-1-carboxylic acid by fluorescent *Pseudomonas* species in the rhizosphere of wheat. Appl Environ Microbiol 56:908–912

Thomashow LS, Bonsall RF, Weller DM (1997) Antibiotic production by soil and rhizosphere microbes in situ. In: Hurst CJ, Knudsen GR, McInerney MJ, Stetzenbach LD, Walter MV (eds) Manual of environmental microbiology, 1st edn. American Society of Microbiology, Washington, pp 493–499

Thomashow LS, Bonsall RF, Weller DM (2002) Antibiotic production by soil and rhizosphere microbes in situ. In: Hurst CJ, Crawford RL, Knudsen, GR, McInerney MJ, Stetzenbach LD (eds) Manual of environmental microbiology, 2nd edn. American Society of Microbiology, Washington, DC, pp 636–647

Timms-Wilson TM, Kilshaw K, Bailey MJ (2004) Risk assessment for engineered bacteria used in biocontrol of fungal disease in agricultural crops. Plant Soil 266:57–67

Tolls J (2001) Sorption of veterinary pharmaceuticals in soils: a review. Environ Sci Technol 35:3397–3406

Validov S, Mavrodi O, De La Fuente, L, Boronin A, Weller D, Thomashow L, Mavrodi D (2005) Antagonistic activity among 2,4-diacetylphloroglucinol-producing fluorescent *Pseudomonas* spp. FEMS Microbiol Lett 242:249–256

Viebahn M, Glandorf DCM, Ouwens TWM, Smit E, Leeflang P, Wernars K, Thomashow L, van Loon LC, Bakker PAHM (2003) Repeated introduction of genetically modified *Pseudomonas putida* WCS358r without intensified effects on the indigenous microflora of field-grown wheat. Appl Environ Microbiol 69:3110–3118

Viebahn M, Veenman C, Wernars K, van Loon LC, Smit E, Bakker PAHM (2005a) Assessment of differences in ascomycete communities in the rhizosphere of field-grown wheat and potato. FEMS Microbiol Ecol 53:245–253

Viebahn M, Doornbos R, Wernars K, van Loon LC, Smit E, Bakker PAHM (2005b) Ascomycete communities in the rhizosphere of field-grown wheat are not affected by introductions of genetically modified *Pseudomonas putida* WCS358r. Environ Microbiol 7:1775–1785

Weber JB, Miller CT (1989) Organic chemical movement over and through soil. In: Sawhney BL, Brown K (eds) Reactions and movement of organic chemicals in soils. SSSA special publication 22. Soil Science Society of America, Madison, pp 305–354

Winding A, Binnerup SJ, Pritchard H (2004) Non-target effects of bacterial biological control agents suppressing root pathogenic fungi. FEMS Microbiol Ecol 47:129–141