Antibiotic Production by Soil and Rhizosphere Microbes in situ

Linda S. Thomashow¹, Robert F. Bonsall², and David M. Weller¹

¹USDA-ARS, Root Disease and Biological Control Research Unit and
²Department of Plant Pathology, Washington State University
Pullman, WA 99164-6430

Telephone: (509) 335-0930
Fax: (509) 335-7674
e-mail:thomasho@mail.wsu.edu
Broadly defined, antibiotics include a chemically heterogeneous group of small organic molecules of microbial origin that, at low concentrations, are deleterious to the growth or metabolic activities of other microorganisms (48). That soil is rich in microorganisms capable of antibiotic synthesis is well accepted, but the frequency with which synthesis occurs at ecologically significant levels in nature has been much less clear. Over the past decade, however, genetic and molecular techniques, coupled with sensitive and bioanalytical assays and equipment, have been applied to demonstrate conclusively that microorganisms synthesize a variety of antibiotics, even under field conditions, in the rhizosphere (that portion of the soil enriched in carbon and energy resources released by plant roots). These antibiotics can contribute to microbial competitiveness and the suppression of plant root pathogens, and the bacteria that produce them are therefore of considerable interest as a practical means of plant disease control. More generally, the techniques used to understand the role of antibiotics in the rhizosphere are applicable to other habitats where mechanisms of microbial antagonism or the production of bioactive metabolites are of interest.

When used together, the bioanalytical and molecular approaches are complementary, allowing the detection and quantification of metabolites produced in situ as well as an evaluation of their activity, and hence their ecological significance. The direct detection of a metabolite provides irrefutable evidence that the genetic and physiological potentials for its synthesis have been met, and the amounts recovered are in part a function of the net rates of synthesis and turnover under particular experimental circumstances. Direct measurements are most informative when the identity and physical properties of the metabolite are known so that procedures for extraction can be optimized, and are of value in assessing the relative amounts present over a range of conditions or in monitoring persistence and dissemination in the environment. Even when the structure is known, sensitivity of detection is likely to be the single most limiting factor to the direct analysis of
Thomashow et al.

metabolites produced in situ. The comparatively large sample sizes from which metabolites are extracted generally preclude direct analyses of substances produced by localized populations in spatially restricted sites in soil or on plant surfaces.

Molecular approaches offer highly sensitive but indirect alternatives to the direct analysis of bioactive metabolites produced in situ. These techniques detect either the potential for synthesis as inferred from the presence or expression of biosynthetic genes, or an activity attributable to the presence of the metabolite itself. For example, introduced and indigenous antibiotic-producing strains can be detected and enumerated by using probes and primers based on unique DNA sequences within genes specific for antibiotic biosynthesis. Such sequences also have been applied to access novel biosynthetic genes directly from soil without the need for culturing (44). Reporter gene systems (described elsewhere in this volume) can be used to monitor the transcription of antibiotic biosynthesis genes expressed in situ. When the impact of metabolites on other organisms is of primary interest, as when antibiotic-producing agents are introduced for purposes of biological control, bioremediation, or biofertilization, antibiotic-nonproducing mutant derivatives are indispensable in distinguishing between effects due specifically to the antibiotic and those attributable to other activities of the introduced agents.

This chapter reviews factors known to affect the production, activity and detection of antibiotics in situ, discusses methods for extraction and quantification from soil and plant materials, and describes approaches to detecting biosynthetic genes, their expression, and the effects of synthesis in soil habitats. Research until now has focused mainly on the activities of a few bacterial genera producing compounds of known structure, but the techniques that have been developed may be applicable to diverse taxa producing structurally undefined bioactive metabolites as well.
FACTORS AFFECTING ANTIBIOTIC PRODUCTION, ACTIVITY, AND DETECTION

The quantity and quality of nutrients available and the ability to compete successfully for them are major determinants of microbial population size and metabolic activity, both of which are integrally linked to the regulation of antibiotic synthesis. Nutrients are not dispersed uniformly throughout soil, but rather, are localized in the spermosphere and rhizosphere of plants, and in and around plant debris, wounds, lesions, and fungal propagules. When antibiotics have been detected in nature it has been in samples enriched in these microhabitats, which are localized regions of intense microbial interaction (51, 52).

Whether an antibiotic will reach an ecologically significant or chemically detectable level in situ depends not only on the rates at which it is synthesized and degraded, but also on the fractions available in a biologically active state or for extraction and analysis. Soil particles and colloids, plant tissues, and microbial decomposition all act as sinks for biologically active molecules. The relative importance of physicochemical and biological processes in determining the fate of an antibiotic in situ depends on the level of microbial activity and the rate and reversibility of sorption to soil surfaces, which in turn vary according to the chemistry of the compound and the conditions in the immediate environment. In the upper soil horizons, microbial catabolism and perhaps plant uptake are thought to have greater impact on antibiotic availability than does soil quality (2, 3).

DIRECT ANALYSIS: CHROMATOGRAPHIC TECHNIQUES

Extraction from soil or plant material: physical and chemical considerations
The efficiency of recovery of antibiotics from natural sources is influenced by their stability, chemical and physical interactions with the sample matrix and the extraction solvent, and the handling of the sample before and during extraction. Properties such as solubility, thermolability, photosensitivity, and susceptibility to oxidation can be deduced from the behavior of the compound when produced in vitro. When the chemical structure is known, insight can be gained into solubility, stability, and potential interactions with soil components. For example, methoxy groups are ring activators of phenolic compounds, increasing their susceptibility to oxidation, whereas carboxyl groups bonded directly to the ring reduce susceptibility to oxidation (3). Sorption of phenolic acids by soils is increased by the presence of methoxy groups or acrylic side chains (11).

Antibiotics produced in situ adsorb rapidly to organic matter and charged groups on the surface of soil particles, and recovery declines continuously over time (3, 8, 50). The pH of soil and extractant solutions determines the charge of ionizable antibiotics, which in turn influences their solubility, affinity for soil colloids and organic matter, and uptake or diffusion into microorganisms and plants. Between pH 4.5 and 6.5, nonionic forms of organic acids and phenolic compounds are readily and irreversibly sorbed by soil organic matter (8, 17) or polymerized into humic substances (10-12). At pH values above the pKₐ (approximately pH 4.5 for phenolic acids), important charge interactions occur with components of the inorganic soil fraction. Interactions may be direct, as when hydroxy acid anions bind to positively charged metal oxides, or indirect, as when such acids bind to negatively charged surfaces through divalent cationic bridge molecules. Soils rich in hydroxy-Al and -Fe compounds have a high adsorption rate and capacity for carboxyl and phenolic hydroxyl groups, and some Mn²⁺-rich soils also have a high sorptive capacity for organic acids (11, 30). Clays are much less reactive (19), with adsorption related more to available surface area than to retention mechanism (11). However, adsorption through exchange ions can result in irreversible
oxidation coupled with a reduction of Fe or Mn oxides (30), or bring organic molecules into proximity with each other, favoring polymerization and irreversible retention (10).

**Sample collection and preparation**

The processing of soil and plant tissue samples, including collecting, storing, and sieving, can affect antibiotic recovery. For plant-associated samples, it is important to note whether specific portions of the plant or root system are sampled, how much plant tissue and soil are present, and how they are separated. Quantitative variation can occur among replicate samples depending on whether macroscopic organic matter is distributed uniformly or removed by sieving.

Of particular importance are the measures taken to minimize microbial degradation of bioactive substances prior to and during extraction. If samples cannot be extracted immediately, freezing is preferable to air- or oven-drying because losses resulting from degradation, thermolability, and irreversible sorption can occur during the drying process.

The lower limit of sample size is determined by the efficiency of extraction and the sensitivity of detection, whereas the upper limit is set by how much material can conveniently be processed. Soil sample sizes typically are one gram or larger (3, 12, 31, 45), and plant tissue samples of 80 seeds with glumes (26), root systems or root and crown segments from 50 to 200 seedlings (14, 23, 25, 26, 49), 25 to 30 g of roots with adhering soil (4, 41), or up to 17 g of potato tuber tissue (5) are representative. For quantitative determinations, it is necessary to generate a standard curve and to calculate the efficiency of recovery from control samples to which the compound of interest has been added in amounts spanning the range of concentrations expected in test samples. Samples must be replicated to assure that significant quantitative differences can be distinguished from sample-to-sample variation and to verify the detection response with respect to
concentration. Control samples should be of the same soil type and moisture content as the test samples, and should be incubated and extracted under the same conditions. It also may be advantageous to add an internal standard to samples to assess the efficiency of recovery (24). Such standards should have chemical properties similar to those of the analyte, should not occur naturally in the sample matrix, and must not interfere with subsequent analyses.

**Solvent considerations and extraction**

The choice of extractant will depend on the solubility and charge properties of the antibiotic, which can be predetermined empirically by processing cultures grown in vitro. Compounds in the soil solution can be extracted with water, but water alone does not provide meaningful estimates of biologically available pool sizes because the reversibly bound fraction is not recovered and many antibiotics are sparingly soluble at ambient temperatures. Aqueous solutions of salts (e.g., 0.5 M sodium acetate) and ethylenediamine tetraacetic acid (EDTA, 0.05 to 0.5 M, pH 7.0 to 8.0) are useful in recovering phenolic acids reversibly bound to mineral surfaces (3, 10, 12, 22), and sodium hydroxide solutions may recover at least part of the fraction sorbed irreversibly to clay or organic matter and plant debris (12, 24). In our experience, however, these aqueous extractants are much less efficient than organic solvents. For example, we recovered over 60% of a 4-µg sample of 2,4-diacetylphloroglucinol (2,4-DAPG) added to roots of wheat after two extractions with 80% acetone, whereas less than 10% was recovered by extraction with 0.5 M sodium acetate, 0.5 M NaOH, or 0.25 M EDTA, pH 7.0 (4). We know of no studies in which the distribution of antibiotics between biologically available and unavailable fractions has been evaluated. For one typical agricultural soil, however, the amount of 2,4-DAPG recovered was proportional to the rhizosphere population size of the producer strain (41), suggesting that the irreversibly sorbed fraction was constant over the range of concentrations detected.
A procedure suitable for the extraction of many of the antibiotics produced by fluorescent *Pseudomonas* spp. has been published (4), and can be adapted for other substances by adjusting the amount of sample required and selecting appropriate solvents. This method can recover phenazine-1-carboxylic acid, its hydroxyphenazine derivatives, pyrrolnitrin, pyoluteorin, and 2,4-DAPG, but not the phenazine compound pyocyanin, which has different solubility properties. In general, samples (either hydrated or dry) are dispersed in the extractant; the solid residues are removed by settling or centrifugation; and the filtrate is collected and concentrated after passage through a solvent-compatible filter. A wide range of ratios of sample mass to solvent volume has been reported, but values of 1:1 to 1:5 are typical. Repeat extractions with smaller solvent volumes are more efficient than single extractions, but also more time-consuming and can prolong user exposure to solvents. Of 40 µg of 2,4-DAPG mixed with 15 g of roots plus rhizosphere soil, 53.0-66.5% and 5.6-6.9% were recovered in two sequential extractions with 30 ml of 80% acetone in water (4).

Samples to be fractionated by high performance liquid chromatography (HPLC) often require additional processing to remove soil residues that can foul chromatographic columns and interfere with UV detection. Humic acids usually are sedimented at acidic pH, although this results in salt accumulation and sample dilution and may cause significant loss of yield (24). We have found that dark-colored organic contaminants can be removed by centrifugation after freezing solutions of 2,4-DAPG in acidified 35% acetonitrile at -20°C (4), but other antibiotics may not remain soluble under these conditions. Antibiotics with ionizable residues can be separated from some contaminants by exploiting the pH-dependent, differential solubility of the neutral and charged forms in organic and aqueous solvents (32, 49). Most isolation procedures therefore include at least one liquid-liquid extraction step to partition antibiotics away from salt residues and impurities, and into organic solvents from which they can be concentrated readily.
Solid-phase extraction to recover bioactive compounds from natural sources offers many advantages over liquid-liquid solvent partitioning. Less solvent waste is generated, isolation is rapid and efficient, and sensitivity of detection may be improved because trace substances in large volumes of solvent can be enriched. Waters Sep-Pak C<sub>18</sub> cartridges are convenient for analytical-scale samples (45, 46, 49), and an octadecylsilica column was effective for preparative enrichment of 2,4-DAPG (28). Aminooindoles and carboxylic indoles were recovered with high efficiency on Amberlite XAD-2 and C<sub>18</sub> columns, respectively (29), and macrocyclic xanthobaccin compounds produced by *Stenotrophomonas* sp. strain SB-K-88 in the rhizosphere of sugar beet were trapped by growing the seedlings in a 1:1 mixture of sand and Amberlite XAD-2 resin (37).

**Chromatography and detection**

Thin layer chromatography (TLC) is widely used to fractionate antibiotics recovered from natural materials (Table 1). TLC does not require expensive instrumentation, nor do samples generally need 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. Tissue extracts do not require extensive purification and many samples can be run simultaneously. Both normal and reversed-phase adsorbents have been used with a variety of mobile-phase solvent systems (Table 1). Substances are visualized by UV absorption, chromogenic reaction with spray reagents, or bioautography, in which suspensions of indicator organisms in agar or broth are overlaid on chromatograms to detect bioactive spots (18). Antibiotic identity is confirmed by appearance, distance traveled relative to the solvent front (R<sub>f</sub> value), and cochromatography with standards in at least two different solvent systems. 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 (15). Differences of up to 40%
between observed and published $R_f$ values may be encountered unless the same adsorbents (preferably from the same vendors) and preparative methods are used (see, e.g., reference 5).

The versatility, resolving capability, and quantitative accuracy offered by HPLC make it the method of choice for most analyses of antibiotics produced in situ. HPLC is readily coupled with techniques such as mass spectrometry or NMR to further resolve mixtures of related compound and to provide insight into chemical structure (7, 41, 47). Considerations in optimizing or developing a chromatographic system include selection of the column, the mobile phase, the elution profile, and the detector to be used. Reversed-phase columns have been used almost exclusively for antibiotics produced in situ, and a variety of liquid phase systems and elution profiles have been described (Table 2 and Figure 1). For high-throughput applications, isocratic elution avoids time- and solvent-consuming column re-equilibration between samples and is preferable to gradient elution if satisfactory resolution can be achieved.

Detection most frequently is by UV absorbance, 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 (49). Greater sensitivity and selectivity of detection can be obtained for some phenolic compounds by amperometric detection (45), and fluorometric detection may offer similar advantages for compounds such as indoles (29) and some phenazines.

DETECTION AND ACTIVITY OF ANTIBIOTIC GENES: MOLECULAR APPROACHES

Detection in situ of antibiotic biosynthesis genes

When the DNA sequences encoding genes for antibiotic synthesis are known, molecular
probes and primers can be designed to detect and enumerate microorganisms capable of antibiotic synthesis or to evaluate and exploit the biosynthetic diversity in these populations even without cultivating them. These molecular techniques target conserved DNA sequences within well-defined biosynthetic gene clusters. The sensitivity and specificity of detection depend on the selection or design of appropriate targets, probes, or primers, and on control of the stringency of PCR amplification or DNA hybridization.

Target selection is determined mainly by the experimental application, and to a lesser extent by technical considerations related to the sequences of interest. Some applications require amplification of full-length genes (44), requiring knowledge of the flanking gene sequences as well as that of the target, whereas for others, amplification of a well-conserved internal fragment is sufficient. Amplified fragments commonly range in size from about 600 to 1,000 bp or more and can be analyzed for DNA sequence or restriction fragment length polymorphisms to confirm identity or evaluate genetic diversity within target populations. Examples of target genes and antibiotic-producing species or strains known to harbor them are shown in Table 3. All of these genes are highly conserved within their respective biosynthetic pathways, and while some have homologues that function other than in antibiotic biosynthesis, the target genes themselves have proven sufficiently specific to detect antibiotic producers from natural sources. For example, the 745-bp internal fragment from the *phlD* gene of *Pseudomonas fluorescens* has been used extensively to enumerate producers of 2,4-DAPG among fluorescent pseudomonads from the rhizosphere of wheat (41, 42) and maize (38). Phloroglucinol producers were detected and confirmed by PCR at a limit of $10^4$ CFU per g of root when the fragment was used as a probe in colony hybridization (42). A rapid PCR-based assay for 2,4-DAPG producers also has been developed with a detection limit of approximately $10^3$ CFU per rhizosphere (35). Like the colony
hybridization protocol, the PCR method is culture-dependent, but is more efficient and can readily be coupled with random fragment length polymorphism analysis of the PCR product (33) to permit further genetic analysis of the most abundant 2,4-DAPG producers within a population. A similar culture-dependent method targeting a fragment of about 600 bp from the β-ketosynthase KSα subunit of the type II polyketide synthase (PKS) gene cluster was used for rapid preliminary phylogenetic analysis of unidentified Streptomyces spp. enriched from soil samples (36). In culture-independent applications, the strA gene was quantitated with a detection limit of 10^6 spores per g of soil by most-probable-number PCR in total community DNA isolated from bulk and rhizosphere soils (20), and intact KSβ subunit genes cloned from soil community DNA were used to investigate determinants of polyketide antibiotic chain length (44).

Stringency is a critical determinant of sensitivity and specificity in all hybridization- and PCR-based gene detection strategies, as are the structural considerations (reviewed elsewhere in this volume) that must be considered in the design of all PCR primers. In addition to meeting standard primer design criteria, primers for the detection of antibiotic genes in environmental isolates or in community DNA must be sufficiently nonspecific to accommodate templates that may exhibit sequence heterogeneity because of codon degeneracy. One approach to the design of such primers is to first align the DNA sequences from several homologues of the potential target. These sequences can be recovered from publicly accessible databases or they can be determined empirically, from PCR products amplified from known producer strains with primers designed from the sequence of one of the homologues (see, for example, reference 35). The alignment will reveal suitably spaced blocks of conserved sequence from which pairs of consensus primers can be developed that meet the structural criteria for primer design. These candidate primers should then be tested in various combinations to identify pairs that amplify products of the expected size.
from diverse positive control strains but not from negative control strains indigenous to the same habitats. An alternative approach to primer design, which has been applied successfully to conserved sequences at the C termini of known KSα subunits (36, 44) and in the active site of the acyl carrier protein (44) of type II PKSs, involves back-translating the amino acid sequence of the conserved region from related strains according to the preferred codon usage of the target species, and then synthesizing degenerate primers. Regardless of the method of primer development, additional control experiments should be conducted (for example, by Southern hybridization) to confirm that the amplified fragment is unique to producers of the antibiotic and specific to the antibiotic biosynthesis gene cluster. This is particularly important if the PCR template is obtained from uncharacterized environmental isolates or total community DNA. In one study (20), the presence of either or both strA (a streptomycin biosynthesis gene) and strB1 (a resistance gene) did not always correlate with streptomycin production by isolates from Brazilian soils, and in another (36), clones containing fragments amplified from KSα genes were found to contain sequences involved in spore pigment synthesis, rather than antibiotic production. Some *Streptomyces* species have two type II PKS gene clusters, one of which is involved in the biosynthesis of antibiotics and the other, in the synthesis of spore pigments; the clones in question apparently originated from the latter.

**Antibiotic gene transcription in situ**

Measurements of antibiotic gene transcription can provide a sensitive and convenient alternative to the direct recovery and quantification of metabolites produced in situ. Such measurements usually employ mutant strains in which a reporter gene, the product of which is readily monitored, is placed under transcriptional control of a promoter of antibiotic gene expression. The reader is referred to Chapter __ (Loper and Lindow) of this section for a general
description of the utility of reporter genes in situ. Certain additional considerations may require attention when reporter genes are used to study antibiotic gene expression because of the complex regulatory circuitry involved in the control of antibiotic production. For example, antibiotics such as 2,4-DAPG exert a regulatory effect on their own synthesis (43), and reporter genes that disrupt the biosynthetic operons for these substances can provide underestimates of transcriptional potential unless an additional, intact biosynthetic operon is present or the antibiotic is supplied exogenously. In contrast, phenazine synthesis is governed by a quorum-sensing mechanism in which an acyl-homoserine lactone autoinducer activates the promoter of the biosynthetic operon. Underestimates of phenazine gene transcriptional potential can result if the autoinducer is titrated by additional, plasmid-borne copies of the promoter. Finally, it must be kept in mind that while reporter genes provide a measure of transcriptional activity, the activity of antibiotics in situ also will be influenced posttranscriptionally by factors such as stability and biological availability that will vary according to environmental conditions.

Provided that reporter activity is correlated with antibiotic concentration, reporter genes offer significant advantages over direct isolation techniques when the objective is to evaluate metabolite synthesis in relation to time or over a range of edaphic or physiological conditions. The sensitivity with which reporters such as the green fluorescent protein or the ice nucleation gene inaZ can be detected allows for the use of smaller sample sizes (i.e., bacterial populations from single seeds or seedlings), so experiments can be highly replicated, facilitating the detection of differences between treatments despite the sample-to-sample variation typically encountered in such studies. The inaZ reporter has been used to detect differences in the level and timing of expression of phenazine (16) and pyoluteorin (28) biosynthetic genes in strains of Pseudomonas introduced onto individual seeds of various crop plants. In other studies, inaZ reported the
expression of the phenazine biosynthetic locus of *P. aureofaciens* on roots as influenced by the presence of heterologous rhizosphere isolates, providing evidence of interpopulation signaling in situ (39). A limitation of most of the reporter genes currently available is that transcriptional activity is expressed in relation to the total population size. Because the sampled population is physiologically heterogeneous, having been recovered from different microhabitats, only some of the cells are likely to be metabolically active, and neither the size of the active fraction nor its true level of activity can be determined. Reporters such as the green fluorescent protein that recently have provided insight into the spatial variability on plant surfaces as sensed by individual bacterial cells (21) undoubtedly will be useful in overcoming this limitation.

**Biological activity in situ: the value of mutants**

Because antibiotics can reach biologically significant threshold concentrations within microsites while remaining at low or undetectable levels overall, their presence often is inferred from effects on other organisms that act as indicators of antibiotic activity. The value of such indirect assays varies in proportion to the degree of certainty that the measured effect is due specifically to the antibiotic of interest, and is greatly enhanced when wild-type strains can be compared with genetically defined, antibiotic-deficient mutants. Such mutants have been used extensively to evaluate the importance of antibiotics in the antagonism of plant pathogens (14, 23, 27, 32, 40, 48). Less exploited is the use of mutants to evaluate the role of antibiotics in microbial survival and competitiveness (6, 34). 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 antibiotic synthesis and biological availability.

Antibiotic-deficient mutants may arise spontaneously or be induced chemically, by ultraviolet irradiation, or with molecular genetic techniques. The latter approach is much preferred
because the site of mutation can be localized to a specific biosynthetic gene. The strategy, which parallels Koch's postulates, consists of (1) mutagenesis; (2) phenotypic and/or genotypic characterization to identify antibiotic-deficient derivatives; (3) complementation with wild-type DNA to restore antibiotic synthesis; and (4) comparison of the activities of the mutant, wild type and restored phenotypes. A fifth step, in which the complementing gene is mutated, exchanged for the wild-type homologue, and shown to confer the mutant phenotype, is important to confirm the functional role of the mutated gene and to rule out the contribution of undetected second-site mutations, but often is overlooked.

Careful phenotypic screening is critical, particularly when the mutants are not genetically defined. Mutants should be indistinguishable from their parental strains in all traits other than the one of interest. Many bacterial isolates produce multiple bioactive metabolites with overlapping activity spectra, and phenotypic assays must be able to distinguish among them. Random mutagenesis procedures frequently give rise to regulatory mutants deficient in the antibiotic of interest as well as other co-regulated metabolites. Such mutants often exhibit altered growth characteristics or reduced environmental fitness and are unsuitable for in situ study. For genetically undefined mutants, in situ evaluations should include several independently derived isolates that are unlikely to have undetected or nonspecific mutations in common.
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