MICROBIAL COMMUNITY RESPONSE TO ANTHROPOGENIC POLLUTION: ANTIBIOTIC RESISTANCE GENES AND DIOXIN BIODEGRADATION

By

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A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

Crop and Soil Sciences – Environmental Toxicology – Doctor of Philosophy

2013

ABSTRACT

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The release of anthropogenic chemicals into the environment is vast and frequently hazardous. For instance, millions of kg of antibiotics are used each year in agriculture in the US and are released into the environment, and correlates with and likely contributes to antibiotic resistance in human pathogens, rendering some infections as untreatable. A second class of chemicals is persistent organic pollutants, such as dioxins, which are immune disruptors and are priority pollutants requiring remediation from the environment. Bacteria respond to chemical perturbation, in order to survive, in many ways: activation of antibiotic resistance genes (ARGs), horizontal gene transfer, transcription of degradative pathway genes, and other related systems such as toxic shock response. Often we use molecular methods to monitor the bacterial community responses and we have reviewed, analyzed, developed and validated hundreds of PCR primer sets specific to ARGs and aromatic carbon metabolism. We have found that in-feed antibiotics increase the abundance and diversity of ARGs both in individual swine, as well as farm-wide in manure, compost and soil amended with compost. Resistance gene abundance correlates with transposase abundance, indicating that the resistance genes may be genetically mobile and represents a potential risk to medical antibiotic treatment in humans. Using gene-targeted metagenomics we see that the diversity of dioxygenases which degrade dioxins exist in a greater extent than we currently have characterized. We went on to isolate a novel dibenzofuran-degrading consortium consisting of *Agromyces*

sp., Bacillales sp. and Comamonadaceae sp, which completely degrade dibenzofuran. This consortium can also cometabolize chlorinated dioxins including 2,3-dichlorodibenzo-*p*-dioxin. Microorganisms respond to anthropogenic pollution in order to survive; however, the response may have positive or negative human implications, both threatening human health (in the case of antibiotic resistance genes) or aid in the removal of toxic chemicals (in the case of dioxins).

ACKNOWLEDGEMENTS

The work presented in this dissertation would not have been possible without the support, encouragement and contribution from a great number of people. First of all I would like to thank my advisors, Dr. James Tiedje and Dr. Syed Hashsham, for their guidance, encouragement, instruction, and for being an example of life-long learners and innovators. I would also like to thank the other members of my advisory committee: Dr. Brian Teppen and Dr. Gerben Zylstra for their input, help and instruction. Next I would like to thank all the collaborators whose names are listed within this work for their contribution to successful science. I am sincerely grateful to my peers at Michigan State University (in no particular order): Drs. Tamara Cole, Shoko Iwai, Bob Stedtfeld, Ryan Penton, Woo Jun Sul, Erick Cardenas, Patrick Chain, Marius Vital, Adina Howe, Jim Cole and Benli Chai, as well as Qiong Wang, Jordan Fish, Tiffany Stedtfeld, Aaron Garoutte, Jiarong Guo, and James Kremer, for their friendship, ideas, conversations, and encouragement. All were so kind to me; they taught me needed methods, answered endless questions, and provided a helping hand. I especially want to thank Amanda Geaslin, Evan Ballard, and Weijia Wang who contributed much of the work to produce the data that is shown here.

Finally I would like to thank my family and friends. We have so dearly enjoyed our time in Michigan and owe much of that to our friends and neighbors, the Seedall, Phillips, Overson, Firth, Oliver, Hutchinson and Landon families. I would like to thank my parents Martin and Leslie Johnson, and Angela's parents Mark and Diane Soffe for their support and love as well as making many long trips to Michigan to visit us. I thank my sons Zach, Grant and Bennett for being so precious to their mother, loving soil, and trying to learn about bacteria. Finally, I thank

my wife, Angela, for the patience, sacrifice, and love she has shown to me. She has truly made this possible.

Financial support was provided by the Superfund Research Program grant P42 ES004911-20 from the U. S. National Institute of Environmental Health Sciences.

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with no cells or autoclaved cells were sacrificed at the end timepoint and showed the same concentration of the substrate as at 0 h (data not shown).

CHAPTER I

TWO CASES OF MICROBIAL COMMUNITY RESPONSE TO ANTHROPOGENIC POLLUTION: AGRICULTURAL ANTIBIOTICS AND DIOXINS

ABSTRACT

The response of a microbial community to a perturbation is based in ecology and competition. A number of factors are involved in establishing a new stable state, and populations that can best respond to the perturbation will become more dominant than in the previous state. This microbial community response may have detrimental (in the case of antibiotic resistance) or beneficial (in the case of bioremediation) human consequences.

The number of pathogens that are antibiotic resistant and the number of antibiotics to which they are resistant is increasing at a troubling rate. The cost of antibiotic resistance in human medicine is billions of dollars. We need a thorough understanding of the mechanisms of the development of multidrug resistance in order to contain or stop it. We propose that antibiotic resistance genes (ARGs), independent of their bacterial host, be treated as pollutants that cause harm to humans. The characterization of ARGs as pollutants introduces novel risk factors that are not presented by any other risk source, and may urge a lower acceptable level of exposure compared to other sources of risk such as cancer-causing chemicals. We provide the framework for the risk assessment of antibiotic resistance genes from animal agriculture. Antibiotic use in agriculture increases the abundance and diversity of ARGs in native soil bacteria and mammalian-associated bacteria in the manure and soil. Farmers are infected at very high rates of antibiotic resistance genes, which may be considered an occupational hazard. ARGs contamination can be spread in the environment, e.g. though water, dust, soil, and other vectors, which shows potential risk to human. ARGs are a significant source of risk and the knowledge gaps required to complete a full risk assessment from agriculture

should be completed. Animal farmers and meat produce from farms that use antibiotics are the major sources of risk. The environmental reservoir of ARGs provides novel genotypes of resistance to the general human population environment. Several critical control points are identified to reduce the spread of ARG pollution.

Environmental PCDD contamination jeopardizes human health and requires economically feasible remediation that detoxifies the environment. Recent studies have shown the bacterial degradation of toxic PCDDs, indicating that PCDD contamination could be addressed via bioremediation. In order to implement successful bioremediation of PCDDs, three areas of research need to be developed. First, the functions of putative dechlorinases need to be defined, beginning with reductive-dehalogenase- homologous genes. Second, the search for novel PCDD RDases and angular dioxygenases needs to continue with emphasis on molecular isolation techniques. Third, an effective implementation procedure should be developed for soil, water, and flyash environments.

INTRODUCTION

Microbial communities, and their membership, are largely shaped by general physical factors including, environmental factors (oxygen availability, temperature, moisture content, pH, carbon and nutrient content), and spatial distance (historical disturbances, access to other environments, etc) (Ramette et al. 2007). The manner in which microbial communities respond to changes in the physical environment is best understood in an ecological context. In environments with constant conditions, for example the animal gastrointestinal (GI) tract, microbial communities show a fairly high level of background stability (Durbán et al. 2012), while microbial communities in a less

consistent environment, like soil, the background community stability is low (Lauber et al. 2013). In the long term in any environment, pulse and/or press perturbations (short- or long-term changes in environmental conditions) are likely to occur. The community will respond to the perturbation, resulting in a new and stable state community composition (Schloss et al. 2012) or return to a previous state (Fries et al. 1997, Yagi et al. 2009). A number of organism (e.g. dormancy and stress response), population (e.g. growth rate) and community (e.g. diversity and turnover) level factors influence the resistance and resilience of populations within the community and provide the biological mechanisms by which a stable state will again be achieved (Shade et al. 2012). Additionally, in the GI tract, the bacterial community has been reported to mature, or to age with the host in a form of microbial community succession (Chung et al. 2012, Kim et al. 2012), a process of progressing from one stable state to another.

Anthropogenic pollution can be a chemical perturbation to natural microbial communities. This pollution may cause growth promoting or toxic effects to the microbial community. If the chemical is organic, it could be used as a carbon source, and the substrate and its metabolites may promote the growth of community members with the required enzymes for the metabolism of the substrate or those with an association with the pollutant metabolizers. Other chemicals, such as antibiotics, display toxic effects on microbial communities, killing a portion of the population, and allowing the resistant portion to multiply and become more dominant. For the purpose of this dissertation, we consider antibiotics and dioxins specifically as pollutant perturbations. Antibiotic exposure could be either a pulse (e.g. field application with manure) or press (e.g. in-feed antibiotics) perturbation, and dioxin contamination, depending on its recalcitrance and

length of persistence, may be either. Depending on the nature of the chemical perturbation, microbial communities will have a unique combination of factors most important to resilience of the bacterial community specific to that chemical. We will briefly describe these factors in our two situations.

Antibiotic perturbations can broadly alter the bacterial community, and those communities might not return to the previous stable state (Dethlefsen et al. 2008, Dethlefsen et al. 2011, Jakobsson et al. 2010, Jernberg et al. 2007, Young et al. 2004), indicating the strength of the perturbation and the role of ecology in regaining community stability. Many ecological factors play into the resilience (Shade et al. 2012) of gut microbiomes due to antibiotic perturbations. The first is bacterial dormancy, including persister cells, which are susceptible to antibiotics but survive due to dormancy (Lewis 2007). Individual organism stress tolerance to antibiotics can come from antibiotic resistance genes, which is key to survive this perturbation (Benveniste et al. 1973). Population stress tolerance can be achieved if a portion (even a low number of cells) of the native population survives the antibiotic course and is able to recolonize the gut (Costello et al. 2012). Horizontal gene transfer can aid in the dispersal of antibiotic resistance genes (Salyers et al. 2004) and horizontal gene transfer has been observed after other types of perturbations as well (Lenski et al. 1993). The growth rate of individual resistant and susceptible populations is important in the establishment of a new stable state. A study in soil found that the respiration rate following antibiotic treatment resulted in an immediate drop in respiration (due to microbial death), followed by an increase in respiration rate higher than the no-antibiotic control (Butler et al. 2011). This elegant experiment shows that the native community was first disturbed and susceptible

populations were killed, followed by the recolonization by the fastest growers, which resulted in an elevated respiration rate. This change in proportions of bacterial populations results in a community turnover, which is important in community recovery (Shade et al. 2012) and has been observed in the gut in response to antibiotics (Antonopoulos et al. 2009, Dethlefsen et al. 2011). Thus, it is an interesting combination of ecological forces that shape the reestablishment of a stable community.

The addition of an anthropogenic organic carbon pollutant follows similar but different principles in resilience and resistance to change. A toxic stress response to organic pollutants (Chai, in preparation) and chlorinated organics (Fries et al. 1997) has been observed but is likely less pronounced than with antibiotics. Dormancy would likely play a role in pulse perturbations, in that the carbon utilizing population would bloom during the perturbation and then may subside when the carbon source is depleted. Horizontal gene transfer has been observed in the transfer of initial oxygenases (Wilson et al. 2003) and may allow for the enrichment of more degrader phylotypes. In terms of carbon utilization, microbial community networks (Zhou et al. 2010), the carbon substrate and its metabolites are likely dispersed broadly in the community. Perturbations with biphenyl showed a response, mostly dominated by *Proteobacteria*, *Actinobacteria*, *Acidobacteria*, in the microbial community able to metabolize the parent molecule or its metabolite compounds (Sul, in preparation). The winner among potential pollution degraders will depend heavily on their comparative growth rates (Lauro et al. 2009). A pulse enrichment of organic carbon may more often result in the bloom of degrader populations with a gradual return to the previous state when the organic carbon is no longer present (Fries et al. 1997, Yagi et al. 2009).

We will now consider the ecological principles in the response of farm and gut microbial communities to perturbations with antibiotics and the associated risk, as well as the response of dioxin metabolizing populations and how to best implement dioxin bioremediation research and application strategies.

Part I:

Antibiotic resistant genes as pollutants: Implications and risk assessment

INTRODUCTION

Antibiotic resistance, especially multidrug resistance, in pathogens is challenging the efficacy of infection treatment as we have known it since the discovery of penicillin (Arias et al. 2009). Antibiotic resistance is often manifested when a bacteria acquires an antibiotic resistance gene, which allows the cells to either degrade the drug, remove the drug from the cell, or alter the cellular target of the drug, so as to prevent its antibacterial action (Alekshun et al. 2007). Even more problematic is when pathogens acquire resistance genes to multiple antibiotics and doctors are required to prescribe one or more antibiotics with or without successful outcomes. Methicillin-resistant *Staphylococcus aureus* (MRSA) (Klein et al. 2007, Klevens et al. 2007), vancomycin-resistant enterococci (Moellering 1998, Soderblom et al. 2010), totally drug-resistant (TDR) *Mycobacterium tuberculosis* (Udwadia et al. 2012, Velayati et al. 2009), *Acintobacter baumanii* strain AYE (Fournier et al. 2006), and multidrug resistance (MDR) enterobacteriaceae (Kumarasamy et al. 2010) are known human pathogens that resist treatment by traditional antibiotic treatment regimes and increase U.S. health care costs billions of dollars each year and cause up to a two-fold increase in mortality (Cosgrove et al. 2003). In 2005, there were over quarter million MRSA-related hospitalizations, and its antibiotic resistance cost the healthcare system an extra $830 million – $9.7 billion (Klein et al. 2007). As pathogens assemble a larger and larger antibiotic resistance arsenal, new antibiotics are coming to market at rate lower than any time in history (Wright 2011). The problem of antibiotic misuse and resistance has been recognized for decades (Rosenblatt-Farrell 2009). Governments are beginning to try to more tightly regulate antibiotic use in

humans (Hvistendahl 2012) and animals (Gilbert 2012, Hvistendahl 2012) but progress is slow because their use is often necessary and corresponding proof of harm, especially from agricultural use, is hotly debated (Phillips et al. 2004).

Recently, it has been put forth that antibiotic resistance genes (ARGs) themselves are pollutants (Gillings et al. 2012, Pruden et al. 2006, Storteboom et al. 2007, Zhu et al. 2013). Risk assessment models are used to estimate the exposure and the known dose-response to calculate the estimated risk due to hazardous pollutants and helps governments in knowing appropriate standards for release and cleanup. Traditional risk assessment can be divided into three major categories: non-cancer causing agents, carcinogenic agents, and biological agents. Toxic chemical dose-response curves are characterized by a no observed adverse effect level (NOAEL) and lowest observed adverse effect level (LOAEL), which describe a certain threshold dose under which there is no toxicity. Carcinogenic agents are treated more stringently because they follow the "one-hit model", which describes that a single molecule could alter a single DNA molecule causing a mutation, transforming a normal cell to a cancer cell. Pathogens, in some cases, are treated more stringently still because their risk is characterized by the one-hit model, as well as additional biological factors, such as cellular replication and human-to-human (or secondary) transmission (Microbial Risk Assessment-Draft, 2011). We propose that harm-causing genes, like antibiotic resistance genes be added as a fourth category of risk, comparable to microbial risk. However, antibiotic resistance genes are unique because they can spread through microbial communities independent of their bacterial host via horizontal gene transfer, they can be promoted and enriched in a mammalian host during responsible and needed antibiotic therapy to treat infections, and

there is no known short-term method to eradicate resistance genes from a host. Considering these four categories of risk assessment (chemical, carcinogenic, biological or genetic), compiling layers of host sensitivity and outbreak potentials of the risk agents, progressively less risk should be allowed when considering the control of the risk agent.

Implications of considering ARGs as a novel category of hazard and risk. Risk is the product of harm and exposure. A low exposure, high harm scenario results in sporadic and potentially unpredictable incidents of high risk. Antibiotic resistance genes, may present a similar low exposure, high harm scenario, with the key difference that the resistance gene can replicate and grow exponentially in the larger host population by secondary transmission. A number of factors differentiate gene risk assessment from microbial risk assessment which may facilitate the outbreak of a gene in large, high density populations:

a) **Horizontal gene transfer** – Individual genes or clusters of genes can be transferred into a bacterial chromosome via transformation (uptake of naked DNA), transduction (phage-mediated) or conjugation (usually plasmid mediated) (Alekshun et al. 2007, Ochman et al. 2000). Other mobile genetic elements such as transposons (Mahillon et al. 1998) and integrons (Boucher et al. 2007, Mazel 2006) facilitate the transfer and/or assemblage of many resistance genes into a single genetic element (Stokes et al. 1989). Bacterial horizontal gene transfer is said to be promiscuous, freely sharing genes throughout bacterial communities (Caro-Quintero et al. 2011, Smillie et al. 2011). Horizontal gene transfer both increases the extent of ARG contamination and prolongs the residence time of ARGs in those communities.

b) **Silent selection for ARGs** – Antibiotic resistance genes in and of themselves are harmless. A person may carry an extensive resistance gene reservoir in harmless, non-pathogenic commensal bacteria and never manifest any disease symptom to the host (Levy 1978). Those same genes when integrated into a pathogenic bacterial host are then causing harm. In fact, normal responsible antibiotic therapies may eliminate the pathogen, but at the same time encourage bacterial "breeding" and promoting the growth and replication of the resistant populations or transfer of the gene to previously susceptible population, especially commensals (Dethlefsen et al. 2011, Salyers et al. 2004). For example, subtherapeutic agricultural use of antibiotics has been shown to increase the abundance of ARGs 50,000 fold in swine (Zhu et al. 2013) with no signs of harm to the host. The term "silent" selection indicates that the resistance genes are selected and enriched without any outward manifestation of that process. Pathogen infections are much the opposite: an individual with a severe bacterial infection displays obvious symptoms and can then be isolated, treated, and hazard is removed. Resistance genes may be residing in the normal commensal microflora subject to secondary transmission while the host interacts with large populations, increasing the chance for outbreak.

c) **Co-selection prolongs residence time –** When antibiotics are present, organisms that hold an antibiotic resistance genes have a fitness advantage over those that are susceptible to the antibiotic. Thus, they increase in abundance in the community. Co-selection is when non-antibiotic chemicals enrich ARGs. Metals (Baker-Austin et al. 2006, Berg et al. 2010) and disinfectants (Gaze et al. 2011)

are commonly implicated in co-selection because the mechanisms of resistance to metals, disinfectants and antibiotics can be the same efflux pump (Knapp et al. 2011) or all the specific resistance genes to all the chemicals are co-localized to the same genetic element (Petrova et al. 2011). If an environment is contaminated with ARGs and any of these co-selective chemicals, there is an ecological pressure for the ARGs to persist increasing the residence time of the ARGs, and the probability for horizontal transfer to a pathogen.

d) **Environmental reservoir of antibiotic resistance genes –** Antibiotics largely originated from environmental bacteria, especially *Actinomycetes*. These antibiotic-producing organisms are also resistant to the antibiotics (D'Costa et al. 2006) and are the likely source of antibiotic resistance genes as supported by some evidence (Benveniste et al. 1973, Price et al. 2012). Indeed, resistance genes evolved long ago (D'Costa et al. 2011) and have had millennia to disperse among environmental populations. The appearance of antibiotic resistance genes and MDR in human-associated pathogens is unlikely to be accomplished by point mutation of bacterial DNA, but through horizontal gene acquisition especially when considering that the phenotype emerged so rapidly (Rosenblatt-Farrell 2009). Additionally, resistance genes may have even been a contaminant in early antibiotic preparations (as discussed in (Chee-Sanford et al. 2009)). All combined, the environment can be thought to be the source of the arsenal of antibiotic resistance genes to the human-society microbiome. It is then in the collective human-society microbiome in which they are then selected and transferred to pathogens and multidrug resistance can develop. Increased environmental

contamination with antibiotic resistance genes increases their likelihood to have contact with humans, and human microbiome contamination with ARGs increases the likelihood that pathogens will develop resistance, or that the resistant pathogens will have an outbreak in the human population. Thus the environmental contamination and human use of antibiotics are both very important factors in the development of antibiotic resistance genes in humans.

e) **Diversity of resistance genes and antibiotics –** The number of known antibiotic resistance genes is over 500 unique types. There are 34 unique resistance genes to tetracycline alone (Liu et al. 2009). Add to this there are about 50 antibiotic chemicals used in agriculture (FDA 2009) and more are used in human medicine. This diversity provides a multiplicity of ARGs for bacteria to incorporate into their genomes to acquire antibiotic resistance. It also complicates monitoring and detection of genetic determinants of resistance.

All these important factors are unique to genetic elements of risk, like ARGs, and increase the exposure element of the risk equation. We now provide a basic framework for the risk assessment of antibiotic resistance genes from animal farms to the human gut.

Risk assessment of resistance genes from animal farms. Up to this point, only a few quantitative risk assessments for antibiotic resistance genes have been attempted (as discussed in (Marshall et al. 2011)), but all have worked with limited datasets and have relied on severe assumptions. These models generally consider one endpoint (deaths due to antibiotic resistant foodborne infections) and do not address the molecular and physiological principles related to antibiotic resistance. These models have been challenged by pointing out that they have failed to address exposure pathways other than

contaminated food, horizontal gene transfer, interrelationships within a complex bacterial community like that of the gut microbiome, the rise of coselection and coselecting agents, and the cumulative resistome rather than only considering resistance in response to the corresponding antibiotic (McEwen 2012).

For the purpose of our risk assessment, we consider the human gastrointestinal tract as the habitat, antibiotic resistance genes as the hazard, and a human pathogen as the response. In environmental settings, the response could be expanded to include any antibiotic resistant bacterium because it can act as a vector to transport the ARG downstream in the environment, or to transfer the ARG to a mammalian-associated bacterium. As a model example, we will examine the exposure pathways and dose-response relationships in the passage of resistance genes from the farm to the farmer.

Hazard Identification: Farm ARGs. Agricultural antibiotic use represents the largest user of antibiotics. In the U.S., up to 80% of antibiotics produced are used in agriculture (Mellon et al. 2001) for animal growth promotion, disease treatment, and disease prevention in the swine, chicken, fish, and beef industries. The prudence of agricultural use of antibiotics has been argued for decades. Farmers have long said that antibiotic use is indispensible to their industry and controls disease, while research continues to show the rise in resistance genes in arable soil (Heuer et al. 2011), livestock (Looft et al. 2012), farm workers (Levy et al. 1976), and the general population (Marshall et al. 2011) due to agricultural use. The hazard we identify for this risk assessment is antibiotic resistance genes of any type that are present on the farm. It is difficult to draw a definite link between farm antibiotic use and harm in human populations because the same antibiotics are often used in both humans and animals. However, recent work has begun to draw the

link between farm antibiotic use and human health. Methicillin susceptible *S. aureus* CC398 gained antibiotic resistance due to agricultural antibiotic use and was then conferred from animal to human (Price et al. 2012), a strong link between the farm and human disease. As DNA sequencing becomes more and more available and inexpensive for high throughput of sequencing entire bacterial genomes, as was done by Price et al. (2012), we will be able to more clearly link the source of the resistance gene and the practices that introduce problems. Additionally, human gut has been shown to be a hotspot for horizontal gene transfer (Salyers et al. 2004), thus if antibiotic resistance genes are introduced to the gut they could easily be transferred between commensals or to pathogens and from the farm to humans (Forsberg et al. 2012). The European Union, and Denmark in particular, has stopped the use of some growth promoting antibiotics and decreased resistance levels have resulted (Aarestrup et al. 2001). With the current antibiotic use policies in the U. S., resistant diseases steadily increase, for example MRSA (Klein et al. 2007). Thus any novel antibiotic resistant gene entering the human population is likely to remain in the individual (Jakobsson et al. 2010) and the population via secondary transmission and at some point be transferred to a pathogenic bacterial host.

Exposure Characterization: Exposure pathways. Opposed to the assumptions of previous risk assessments, there are many potential exposure routes to agricultural antibiotic resistance genes. The fate of ARGs and the total human exposure pathway includes the farm, environmental, and human biosphere (Fig. 1.1). Each biosphere can be a source of ARGs due to antibiotic use in animals and humans and natural antibiotic-producing soil bacteria (D'Costa et al. 2006). Antibiotic resistance genes, and the bacteria that carry them, have various routes between the biomes. Within the farm environment,

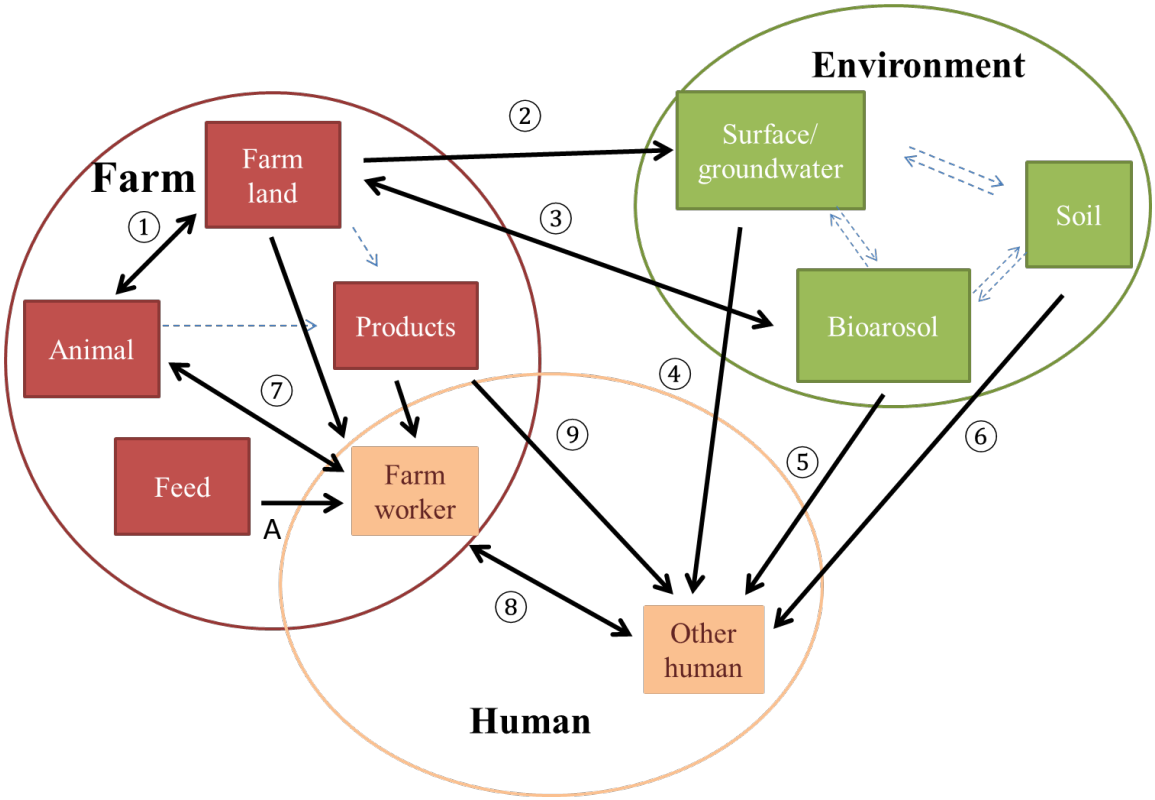

Fig. 1.1. Exposure pathways of antibiotic resistance genes among farm, natural environment and human biospheres. 1. Manure application, 2. surface runoff/leaching, 3. aerial dispersion, 4. water contact and recreation, 5. breathing bioaerosols, 6. soil contact and consumption, 7. farm animal, waste, and AB contact, 8. secondary transmission, and 9. consuming farm products. (A) represents selective pressure on resident resistant bacteria, not a bacterial source. For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation.

animals are the major source of ARGs, which are transported to the soil or crops through manure application or to the farm worker through physical contact with the animal or its waste (Akwar et al. 2007). Direct exposure to in-feed antibiotics may select for antibiotic resistance genes in the farmer directly (Fig. 1.1) and represent a secondary source of ARGs from the farm. Farm resistance genes can be transported to the human biosphere through manure-contaminated agricultural products (especially fresh meat) and by secondary transmission from the farmer to other humans. ARGs from the farm soil and manure can also be transported to the environment through water erosion to surface waters and ground water, or by wind erosion into the atmosphere for later deposition to surface soil, water or human populations. In the environmental biosphere, ARGs could be transported among the environment components, e.g., water, soil, and air, and then to the human biosphere by human ingestion of soil, water or dust (Fig. 1.1). As an initial risk assessment, we will focus on just one step of the entire pathway, that being the exposure to and fate of ARGs from the farm to the farmer GI tract (farm-to-gut model), with future works extending these principles to secondary transmission to the general human population.

In our estimation, the establishment of antibiotic resistant bacteria in the farm worker has been underemphasized in research and the discussion of transmission of antibiotic resistant bacteria. The adverse effects that could occur to the farm worker is the enrichment of antibiotic resistant bacteria in the microbiome of the farm worker, due to direct exposure to antibiotics by handling the chemicals themselves or feed amended with antibiotics, or exposure to antibiotic resistance genes from the animal, land, or farm products (meat or produce) (Akwar et al. 2007, Marshall et al. 2011). It has been written

that the primary route of ARG exposure to the general public is through the food chain and ARG contaminated meats (Phillips et al. 2004, Salyers et al. 2004). While this may be true, the farm worker is an important vector to carry ARGs from the farm to the general public. Levy et al. (1976) was fundamental in first finding that farm workers have a high load of antibiotic resistance in their gut microbiome. They found >80% of *E. coli* were antibiotic resistant in 31.3% of fecal samples from farm dwellers compared to 6.8% of fecal samples from farm neighbors (Levy et al. 1976). Another study showed a farm worker and his wife received a resistant plasmid from the animals (Hunter et al. 1994). At least nine other studies are summarized by Marshall and Levy (2011) showing that farm workers carry a high burden of antibiotic resistance genes in their gastrointestinal tract. A more recent study has shown that farm use of antibiotics, time spent in pig barns, contact with sick pigs, and intake of antimicrobials are all occupational risk factors associated with accumulating ARGs in the farm worker (Akwar et al. 2007). After workers establish resistance genes in their guts, the ARGs could be disseminated to the general public when they become sick and go to the hospital or by communicating disease to others (Hunter et al. 1994). These ARGs then enter the human community microbiome. This human-to-human secondary transmission of antibiotic resistance genes has been implicated (Church 2004, Smillie et al. 2011) and observed in the infection of others with antibiotic resistant diseases like totally drug-resistant tuberculosis (Velayati et al. 2009), New Delhi metallo-*ß*-lactamase-1 (NDM-1) containing Enterobacteriaceae (Kumarasamy et al. 2010), vancomycin resistance enterococcus (Soderblom et al. 2010), MRSA (Davis et al. 2012), and others. MRSA is a highly communicable infection because the pathogen commonly colonizes the skin. Farmers in the Netherlands were >760 time more likely to contract the

disease than the general public, and in addition, the disease was shown to be passed from farmer to family to nurse (Voss et al. 2005). MRSA is perhaps the best-documented and most abundant antibiotic resistant disease. There has been a steady increase in the number of MRSA cases in recent years. In the period of 2000–2005 the number of MRSA cases doubled and the percent of total *S. aureus* infections that were methicillin resistant increased by more than 25% (Klein et al. 2007).

It may be possible to identify the major paths of ARG exposure and first control those routes of exposure, also known as a critical control points. In the pathway from the farm back to the general human population, we see the farmer as an important vector for the transport of ARGs. For the occupational safety of farmers, their exposure to

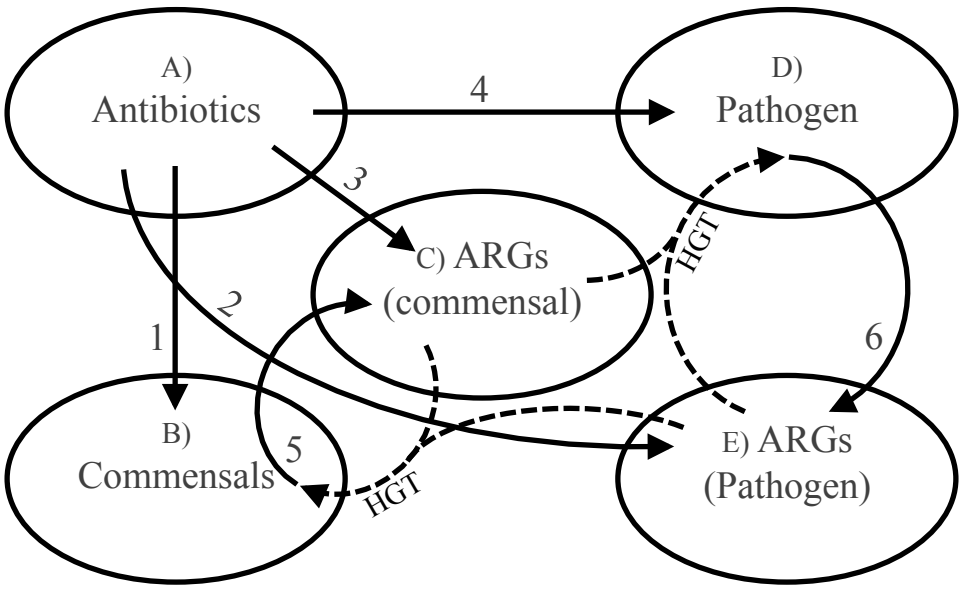

Fig. 1.2. Framework from which to design an antibiotic resistance gene centered risk assessment model for the human gut. Nodes (circles) indicate concentrations of entities and arrows indicate rates. Rates 1-4 are the selection pressure due to presence of antibiotics. Rates 5 and 6 are the rates of the commensals and pathogens, respectively, gaining antibiotic resistance genes via horizontal gene transfer (HGT). Dashed lines indicate the source of the resistance genes to be horizontally transferred.

antibiotics and ARGs should be as limited as possible, in an effort to decrease their odds of contracting an infection.

Conceptual model. We developed a conceptual model of the important biological interactions in the human gut that we would like to be able to describe in the future with a mathematical model (Fig. 1.2). The nodes represent the concentration of a biological factors and the arrows represent the rate of certain interactions between the nodes. Different exposure and response rates will apply to each state and data will be found in the literature to describe the exposure, persistence factors and interaction (Table 1.1) and inform a mathematical model that will predict the probability of acquiring a pathogen with an antibiotic resistance gene that originated from the farm. These risk estimates will be compared to actual studies that determined the risk of farmers acquiring certain observed levels of antibiotic resistance (e.g. (Levy et al. 1976)). Some assumptions will have to be made in the model, including: i) bacterial conjugation as the only form of horizontal resistance gene transfer, ii) an average conjugation rate, iii) nature of flow through the GI tract, and iv) the insignificance of the evolution of novel resistance determinants through genetic mutation, among others.

Bacterial conjugation has been highlighted as the major mechanism of horizontal resistance gene transfer (Gillings et al. 2012). Transformation and transduction are other mechanisms of horizontal resistance gene transfer, but they have been shown to be minor contributors. Transformation, or the uptake of DNA outside of a bacterial cell – so called naked DNA, is a pathway of horizontal gene transfer that is most easily eliminated from our model. (Nordgård et al. 2007) found no evidence or resistance gene transformation in an in vivo model system. Additionally concerns over genetically modified crops were

settled when it was shown that transformation of resistance genes does not occur in the human gut or in the environment (Ramessar et al. 2007). Transduction, or the incorporation of DNA inserted by bacteriophages, can be a mechanism of horizontal resistance gene transfer (Davies et al. 2010), and it may be significant due to the vast abundance and antibiotic activation of viruses in a bacterial population (Allen et al. 2011), but for simplicity we do not consider it here.

While a great deal of work has been done to describe conjugation rates between various organisms (Dionisio et al. 2002, Hunter et al. 2008), conjugation rates are too complex to be able to model at this point. For this purpose we chose strains for which we have data, that appear to not have extreme conjugation rates, and for which the acceptor is a opportunistic pathogen: *Escherichia coli* as the donor, and *Enterococcus* as the acceptor.

Genetic mutations are another mechanism for the introduction of resistance genes in a bacterial community. While they can arise in bacterial genomes and lead to beneficial mutations, such as antibiotic resistance (Dai et al. 2012), we are not considering their contribution in this particular risk assessment. Genetic mutations are very rare, occurring in only 1 of 10^7 to 10^{10} bacteria (Rosenblatt-Farrell 2009). Beneficial mutations are even more rare, so in this assessment, we consider the contribution to resistance from beneficial genetic mutations to be insignificant.

This mathematical risk assessment model is the first step in producing a biologically relevant model that describes the ingestion rate, selection, replication, horizontal transfer, death rate and persistence of antibiotic resistance genes in the gut of

the farmer. With validation and additional modifications, the model can then be applied to describe outbreak models in larger human populations.

Table 1.1. Description of parameters involved in the conceptual human gut resistance gene-centered risk assessment model

Node	Node description	Exposure	Persistence factors	Interactions
A	Concentration of antibiotics (ABs) in gut	ABs ingested from environmental sources, not therapeutic	Residence time and body absorption	none
B	Concentration of commensals	Commensal ingestion rate	Residence time, growth rate, and death rate	AB selection
C	Concentration of ARGs in commensal bacteria	ARG ingestion rate (from commensals)	Residence time, growth rate, and death rate of the host	AB selection and HGT
D	Concentration of pathogens	Pathogen ingestion rate	Residence time, growth rate, and death rate	AB selection
E	Concentration of ARGs in pathogens	ARG ingestion rate (from pathogens)	Residence time, growth rate, and death rate of the host	AB selection and HGT

Part II:

Bioremediation and detoxification of polychlorinated dioxin contaminated environments

The work in Chapter I: Part II has been published:

Johnson, Timothy Alan (2008). Bioremediation and detoxification of polychlorinated dioxin contaminated environments. MMG 445 Basic Biotechnology eJournal [Online], 4 (1)

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INTRODUCTION

Environmental contamination of polychlorinated dibenzo-p-dioxins (PCDDs), or dioxins, poses "one of the most challenging problems in environmental science and technology" (Yoshida et al. 2005) because of their toxicity, persistence, and bio-unavailability (Field et al. 2008). Dioxin contamination is important because the compound is carcinogenic (Mandal 2005). PCDDs are released into the environment from a variety of sources including: combustion, incineration, pulp and paper manufacturing, pesticides, and some natural sources (Kulkarni et al. 2008). Fig. 1.3 shows the structure of the compound, and many different chlorinated dibenzo-p-dioxin (CDD) congeners are defined by differing number of chlorine substituents and location of substitution (Chang 2008). Dioxin congeners will be abbreviated as listed above. The release of dioxins into the environment has resulted in contaminated soil that need treatment (Haglund 2007). Currently, PCDD contaminated sites are remediated only by physical and chemical processes that are very expensive (large remediation projects cost from $100 to $500 million) (Weber et al. 2008). Studies have demonstrated that these remediation practices are not sustainable, because of high cost and complex logistics related to containment or relocation of PCDDs as compared to the potential benefits of PCDD destruction (Weber et al. 2008). Bioremediation has been examined as a technique to degrade or detoxify dioxins at a lower cost.

Microbial degradation of dioxins has been studied extensively, and takes place through anaerobic reductive dechlorination (ARD) or through aerobic dioxygenation

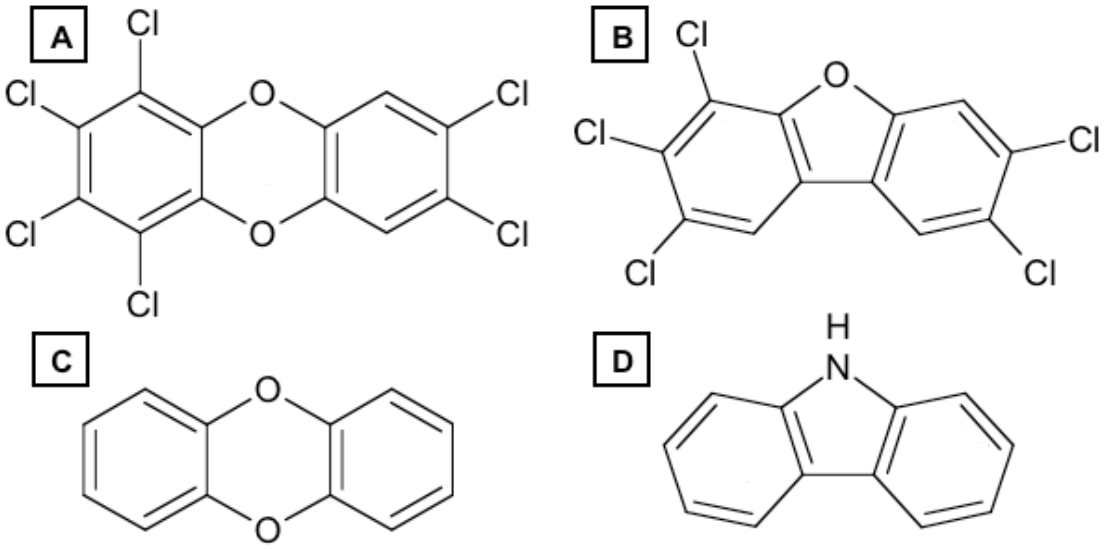

Fig. 1.3. Structures of compounds. A) 1,2,3,4,7,8-hexachlorodibenzo-p-dioxin (HCDD); B) 2,3,4,7,8-pentachlorodibenzofuran (PeCDF); C) Dibenzo-p-dioxin (DD); D) carbazole (CAR). Different congeners (species) of these compounds are named by listing the locations of the chlorine subsitutions. Adapted from: Chang YS, 2008, J Mol Microbiol Biotechnol, 15:152-171.

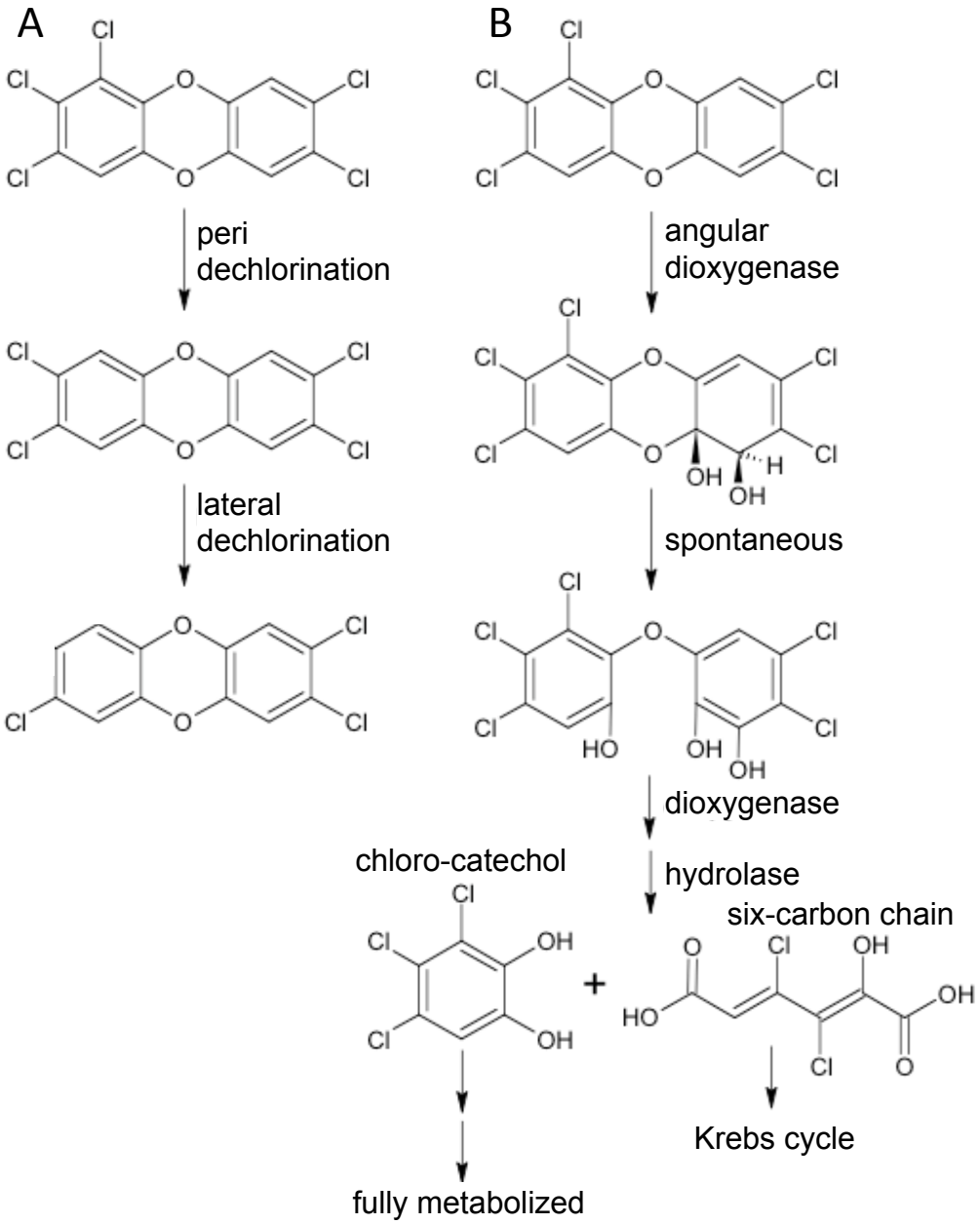

Fig. 1.4. Proposed PCDD dechlorination and oxidation pathways. Pathway A shows peri- and lateral-dechlorination of 1,2,3,7,8-PeCDD. Pathway B shows the oxidation of 1,2,3,7,8-PeCDD by the indicated enzymes. These pathways are shown for illustrative purposes, and are not confirmed. Adapted from (Field et al. 2008, Wittich 1998, and Nam et al. 2006).

(Chang 2008, Field et al. 2008, Hiraishi 2008, Wittich 1998). ARD takes place in anaerobic microbial environments when a chlorine atom is removed and replaced with a hydrogen atom (Mohn et al. 1992) (see Fig. 1.4A). The chlorinated compound acts as the terminal electron acceptor, and "Dehalococcoides" can save energy from the dechlorination process (Hiraishi 2008).

Table 1.2 presents a summary of observed microbial dechlorination of CDDs. Dechlorination rates decrease with increasing chlorine substitution, and complete dechlorination of the compound by bacteria has not been observed. Recently, it was shown that toxic 1,2,3,4,7,8-HCDD was dechlorinated by "Dehalococcoides" ethenogenes strain 195 to less toxic congeners (Liu et al. 2008). In aerobic environments, microbes can degrade the compound through aerobic oxidation and subsequent cleavage of the aromatic rings. The oxidation pathway in Fig. 1.4B shows that after both ether bridges are broken, a chloro-catechol and a six-carbon chain are formed. Table 1.3 presents a summary of observed aerobic oxidation of PCDDs by bacteria. Increased chlorine substitution decreases the rate of oxidation (Habe et al. 2002). Recently, toxic 1,2,3,4,7,8-HCDD was shown to be oxidized to less toxic compounds (Nam et al. 2006). Sphingomonas (especially strain RW1), and Pseudomonas are the most efficient PCDD oxidizers (Field et al. 2008).

It is critical that microbial degradation of PCDDs results in a less toxic product. 2,3,7,8-TCDD is the most toxic congener. Other congeners with all four lateral chlorines are also highly toxic. The compound decreases in toxicity when any lateral chlorine is removed (Mandal 2005), or when the aromatic structure is broken (Chang 2008). For this reason, lateral dechlorination is of great importance because it results in a detoxified

product (Liu et al. 2008). Anaerobic dechlorination and aerobic angular dioxygenation detoxify PCDDs, and the genes involved in these two reactions will be called dioxin detoxification genes (DDGs) for convenience in this paper.

Bioremediation of PCDD. PCDDs are subject to microbial degradation and detoxification as demonstrated in Tables 1.2 and 1.3, but much advancement is needed in order to develop a successful PCDD bioremediation strategy. First, putative, or alleged, dechlorinases need to be described. Second, the isolation of novel DDGs should continue with increased emphasis on using molecular techniques. These two steps will describe PCDD degrading enzymes more fully. Then, an effective implementation procedure should be designed for the contaminated environment (soil, water sediment, or flyash) with a plan to promote the expression of PCDD degrading enzymes. When these steps are taken, bioremediation of PCDDs could make the transition from the laboratory bench to the field.

Function of putative dechlorinases. "Dehalococcoides" sp. has been identified as the most successful PCDD dechlorinator (Hiraishi 2008), but we cannot implicate a specific gene or genes related to PCDD dechlorination (Hölscher et al. 2004). Progress is being made to describe these reductive dechlorinases (RDases). The entire genomes of two dioxin dechlorinators "Dehalococcoides" ethenogenes (Seshadri et al. 2005) and "Dehalococcoides" sp. strain CBDB1 (Bedard et al. 2007) were both published in 2005. Two tetrachloroethene RDases were characterized and named pceA and tceA. About 50 unique sequences that shared the characteristic features of these two genes were found among "Dehalococcoides" sp. strains CBDB1, FL2, BAV1 and 195 and were termed reductive-dehalogenase-homologous (RDH) genes. The function of these genes has not

Table 1.2. Summary of studies that have demonstrated the successful dechlorination of PCDDs. Many studies show dechlorination of dioxin-like compounds including PCDFs, but this table only concerns PCDDs, which are the most recalcitrant of all dioxin-like compounds. The table shows the compound, the organism found to degrade it, the degradation rate (based on the indicated reference), the genes involved, metabolites, and references. It is important to note that the specific gene(s) relative to PCDD dechlorination have not been isolated, and no study has shown the complete dechlorination of PCDD under anaerobic conditions.

PCDD Compound	Microbial Culture	Degrad. Rate (% remov / time)	Known Genes Involved	Products[d]	References of Interest
23-DCDD	*Dehalococcoides* sp. CBDB1	53% / 28 days	PDGs	2-CDD	Bunge et al. (2003)
123-TrCDD	*Dehalococcoides* sp. CBDB1, Anaerobic consortium[c]	60% / 57 days	PDGs	23-/ 13-DCDD, 2-MCDD	Bunge et al. (2003) Ballerstedt et al. (2004)
124-TrCDD	*Dehalococcoides* sp. CBDB1, Anaerobic consortium[c]	55% / 57 days	PDGs	13-DCDD, 2-MCDD	Bunge et al. (2003) Ballerstedt et al. (2004) Bunge et al. (2008)
1234-TCDD	Anaerobic, methogenic, and sulfate-reducing enrichments[c], Dehalococcoides sp. CBDB1, D. ethanogenes 195[c]	24% / 84 days	PDGs	124-TrCDD 13-/ 23-DCDD 2-MCDD	Bunge et al. (2003) Fennell et al. (2004)
12378-PeCDD	*Dehalococcoides* sp. CBDB1	75% / 84 days	PDGs	2378-TCDD, DCDDs, 237-TrCDD	Bunge et al. (2003)
123478-HCDD	Mixed culture containing *D. ethanogenes* 195	10% / 200 days	PDGs	1378-/ 1248-TCDD	Liu (2008)

[a] Adapted from Field and Alvarez (2008). [b] Putative dehalogenase genes. We cannot implicate a specific gene or genes to the dechlorination of PCDD/Fs. Only genes similar to tceA or pceA have been implicated. [c] The culture was first enriched on another clorinated electron acceptor. [d] General product list and not specific to one culture. Products are listed in decreasing order of rate of formation.

Table 1.3. A summary of important studies that have demonstrated the successful oxidation of PCDDs. The table shows the compound, the organism(s) found to degrade it, the degradation rate (an average of many studies), the genes involved, the formed products, references. The three genes listed here are angular dioxygenases. MCDF oxidation is listed to show its oxidation relative to MCDD.

PCDD Compound	Microbial Culture	Degrad. Rate (% remov/time)	Genes Involved	Products	References of Interest
1-/ 2-MCDD	*Rhodococcus*, *Pseudomonas,* Bacillus, *Klebsiella, Sphingomonas, Burkholderia, Terrabacter,* Klebsiella, others	75% / 2 days	CARDO[b], DFDO[c], DDDO[d]	chlorocatechol, chloro acids, hydroxyl-CDD	Habe et al. (2001a) Wittich et al. (1992)
2-/ 3-/ 4-MCDF	*Sphingomonas, Terrabacter, Klebsiella, Pseudomonas, Burkholderia, others*	88% / 1 day	DFDO	chlorosuccinyl acids, CBPs	
23-/ 27-/ 28-DCDD	*Sphingomonas, Pseudomonas, Rhodococcus, Terrabacter,* P450 enzyme	42% / 4 days	CARDO, DFDO, DDDO	chlorocatechols, chloroketone, CDDs	Habe et al. (2001a) Sulistyaningdhah et al. (2004) Hong et al. (2002)
123-/ 237-TrCDD	*Spingomonas wittichii* RW1, *Pseudomonas* sp. EE41, P450 enzyme	31% / 30 days	CARDO, DFDO, DDDO	polychlorocatechol, polychloroethers, hydroxy PCDD	Nam et al. (2006) Sulistyaningdhah et al. (2004) Habe et al. (2001)
1234-TCDD	*Sphingomonas wittichii* RW1, *Pseudomonas veronii* PH-03	15% / 5 days	DDDO DFDO	polychlorocatechol, polychloroguaiacol	Hong et al. (2002)
2378-TCDD	Pseudomonas testosteroni G1036, Bacillus magaterium	60% / 244 days	DFDO	hydroxy-TeCDD, unidentified	
123478-HCDD	Spingomonas wittichii RW1	10% / 5 days	DDDO	tetrachlorocatechol, others	Nam et al. (2006)

Table 1.3 (cont'd)

[a] Adapted from Field and Alvarez, 2008. All listed oxidations utilized another compound (DF, CAR, others) as the growth substrate. [b] carbazole 1,9a dioxygenase (Habe et al., 2001a) [c] dibenzofuran 4,4a dioxygenase (Habe et al., 2001a) [d] dibenzo-*p*-dioxygenase (Wittich et al., 1992)

been described (Hölscher et al. 2004). Determining if these genes encode for PCDD RDases would allow us to probe for these genes in PCDD contaminated environments by real-time PCR or microarray, to monitor their expression, and the extent of microbial PCDD dechlorination. This technique has been used to study polychlorinated biphenyl (PCB) biodegradation. "Dehalococcoides" was found to dechlorinate Aroclor 1260, a mixture of PCBs. Quantitative real-time PCR indicated that the "Dehalococcoides" population increased by nearly two orders of magnitude in the presence of Aroclor 1260 (Bedard et al. 2007).

Isolation of novel dioxin detoxification genes (DDGs). Notwithstanding recent observation of highly chlorinated dioxin detoxification (Liu et al. 2008, Nam et al. 2006, Nam et al. 2008), the known diversity of DDGs is narrow. Dehalogenating bacteria of all chloro-organic compounds are phylogenetically diverse (Hiraishi 2008), but efficient PCDD dechlorinators are limited to the "Dehalococcoides" group. Efficient PCDD oxidizers are mainly only Sphingomonas and Pseudomonas (see Tables 1.2 and 1.3). Sipilä et al. has claimed, "... we are unfortunately only in the beginning of grasping the overwhelming diversity of bacteria involved in biodegradation in soil" (Sipilä et al. 2008). PCDD contaminated environments likely harbor many other DDGs that remain unknown.

Multiple factors may contribute to why our knowledge of DDGs is limited. First, PCDD degradation is generally very slow in nature and yields little biomass production, which may contribute to the seemingly elusive nature of DDGs (Hölscher et al. 2004). Second, most studies have attempted to screen environmental samples to a pure culture that can detoxify PCDDs (Bunge et al. 2003). It might be that dioxin detoxifying

organisms rely on other organisms to provide substrates, and/or provide enzymes for reactions further down the pathway (biphenyl dioxygenase, hydrolase, chloro-catechol degrading genes), and thus screening to a pure culture may result in a loss of the PCDD degrading cultures. For these reasons it may be necessary to localize DDGs by way of molecular methods, before culturing and enrichment procedures. This type of procedure was followed in the description of RDH genes. Probing PCDD contaminated environments with broad primers from conserved regions of known angular dioxygenases may lead to the discovery of novel angular dioxygenases. Iwai et al. used a broad primer set targeting biphenyl/toluene dioxygenases together with pyrosequencing to obtain over 900 unique dioxygenase sequences in historically contaminated environmental samples, demonstrating large biodiversity of dioxygenases in the environment (Iwai et al. 2010).

Effective implementation procedure. PCDDs are found in surface soils, water sediments, and incinerator flyash, and each environment presents unique challenges in the implementation of PCDD bioremediation An effective implementation procedure will promote growth of microbes of interest and the expression of DDGs under the environmental conditions of the contaminated site. Biostimulation and bioaugmentation have been shown to promote the growth of microbes of interest and the expression of DDGs. Biostimulation is the practice of amending the environment with a necessary growth substrate or co-substrates, and bioaugmentation is the inoculation of the environment with a microorganism, or microbial consortium known to have the desired degrading ability. 1,2,3,4-tetrachlorobenzene has been shown to increase the dechlorination rate of 1,2,3,4-TCDD by native microbial communities compared to samples with only a growth substrate (Ahn et al. 2005). Ahn et al. tested the affect of

bioaugmentation (with “Dehalococcoides” ethenogenes strain 195) and biostimulation (with 1,2,3,4-tetrachlorobenzene, and 2’,3’,4’- trichloroacetophenone) on the dechlorination of PCDDs, and at the same time monitored community and gene dynamics. They found that heavily contaminated sites harbored more indigenous dechlorinators than less contaminated sites, by denaturing gradient gel electrophoresis (DGGE). Dechlorination at the heavily contaminated sites was not greatly enhanced by biostimulation and bioaugmentation, while at less contaminated sites dechlorination was enhanced by these methods (Ahn et al. 2008).

Surface soils. PCDDs are generally only found in the upper portions of the soil profile because the compound is extremely insoluble in water (water solubility equals 0.019 ppb) (Field et al. 2008), which prevents the compound from leeching or moving with ground water. Surface soils are generally aerobic and converting a large environment of this type to an anaerobic state to promote ARD would be difficult and expensive. Therefore, ARD in surface soils is likely to not occur at a significant rate, but dioxygenation would be the most common mode of dioxin detoxification by bacteria.

Biodegradation of PCDDs with dioxygenation as the only designed mode of detoxification would result in the persistence of a significant fraction of toxic dioxin congeners, because dioxygenation of HpCDD and OCDD has not been observed (Field et al. 2008). In this situation, it may be helpful to employ a chemical treatment simultaneously. Zero valence iron (ZVI) has been shown to rapidly dechlorinate highly chlorinated PCDDs, even OCDD. This process was carried out in an anaerobic environment in conjunction with PCDD dechlorinating bacteria (Chang 2008). It is not known if this process would function in an aerobic environment, but if it does, highly

chlorinated PCDDs could be dechlorinated by ZVI, and the products would be subject to dioxygenation, which may result in a highly detoxified environment.

Another promising technology in surface soils would be the use of phytoremediation and rhizoremediation. Phytoremediation is the removal and/or degradation of pollutants by plants, and rhizoremediation is the degradation of pollutants by soil microbes that grow very close to plant roots. As of 2007, there was no documentation of dioxin uptake by plants from the soil (Jou et al. 2007). A study by Jou et al. reported dioxin uptake by tappa (Boussonetia papyrifera) in highly contaminated soils, and by Physalis angulatal in low contaminated soils (Jou et al. 2007). Dioxins are taken up at lower levels in zucchini cultivars (Inui et al. 2008) and some other annual and perennial plants (Fan et al. 2009). The mechanism of PCDD accumulation was not stated. It is possible PCDDs are more bioavailable in the rhizosphere of these plants, which allows for its uptake. In a study by Sipilä et al. (2008), it was determined that the microbial community structure, as determined by terminal restriction fragment length polymorphism (TRFLP), changed with the addition of PAHs and the cultivation of birch trees. The diversity of dioxygenases was greater in the rhizosphere compared to the bulk soil. The PAH degrading microbial community in the bulk soil was 48.5% dissimilar from the PAH degrading microbial community in the rhizosphere. This study outlines an excellent method of determining the diversity of genes that execute the desired function (Sipilä et al. 2008). Similar studies should be carried out in regards to PCDD dioxygenases of the rhizosphere. Sipilä et al. also showed an increase in microbial diversity in the rhizosphere (Sipilä et al. 2008). This may be advantageous to PCDD oxidation, because as indicated in Fig. 1.4B, many different enzymes are required in

PCDD oxidation. These enzymes could be supplied by not just one key organism, but a variety of soil bacteria. The rhizosphere may provide a favorable environment for a diverse dioxin detoxifying microbial community.

Water sediments. PCDD contaminated soil particles can be eroded and deposited in rivers and lakes. These PCDD contaminated water sediments sink to the floor of the body of water and remain in an anaerobic environment. Such an environment would facilitate the ARD of PCDDs, but M/DCDD would remain (see Table 1.2 for the products of ARD reactions). There is no known mechanism to biodegrade M/DCDD further under anaerobic conditions. Thus, the detoxified M/DCDD product may be the best solution to PCDD contaminated sediments (Bunge et al. 2003).

Flyash. Flyash is a major source of environmental dioxin contamination (Kulkarni et al. 2008). PCDDs form chemically when organic matter is burned in the presence of chlorine and flyash is a residue after incineration. Nam et al. performed a series of experiments to test the degradability of PCDD in flyash by Sphingomonas wittichii strain RW1. The total organic carbon content of the flyash was 0.0014 ± 0.004%, which is very low compared to soil, indicating that the environment is nutrient depleted and a harse environment for microbial growth. In 2005, they observed 75.5% removal of all PCDD and 83.8% removal of 2,3,7,8-TCDD from flyash by way of degradation and adsorption onto live and dead cell biomass (Nam et al. 2005). In 2008, a mix of 4 bacterial and 5 fungal strains were combined to form a dioxin-degrading biocatalyst in flyash. This biocatalyst degraded 68.7% of all PCDD and 66.8% of 2,3,7,8-TCDD substituted congeners. In this study it was shown that fungal strains provided extra cellular non-specific oxidases to degrade highly chlorinated congeners. These fungal oxidases are

non-specific, meaning they are not specific to a single substrate, but can degrade an array of compounds, including very stable PCDDs and lignin (Nam et al. 2008). Flyash is the only environment that has shown fungi to be effective degraders of PCDDs in situ. This may be because the microbes were in a carbon-depleted environment and PCDDs were available for carbon and energy use. In soil, the total organic carbon content is higher, fungi are not effective PCDD degraders because they preferencially degrade higher energy yielding compounds (Field et al. 2008).

Conclusion. Current physical and chemical remediation of PCDD contaminated sites is not sustainable and bioremediation could be a more favorable alternative. There are two research initiatives that need to be completed before bioremediation is a viable option for PCDD clean-up. First, a higher percentage of toxic PCDD congeners need to be shown to be degraded by bacterial enzymes. Second, these laboratory methods must be successfully implemented in the field. The bacterial degradation of a few highly chlorinated congeners, and many mono-, and di-CDDs has been demonstrated. Recent studies have shown both the bacterial dechlorination and oxidation of 1,2,3,4,7,8-HCDD, which indicates that the bacterial enzymes may exist to degrade other toxic PCDD congeners, but have not yet been discovered, isolated, or characterized. Reductive-dehalogenase- homologous (RHD) genes are suspected to dechlorinate PCDDs. Their function has not been confirmed, but is a likely area of future research. Use of broad primers from conserved regions of known angular dioxygenases together with molecular methods (real-time PCR, microarrays, pyrosequencing, DGGE, and TRFLP) to detect the expression of DDGs may lead to easier isolation and characterization of novel DDGs, rather than isolation through culturing methods. Once we have isolated a sufficient

number of DDGs, in situ bioremediation strategies need to be developed. PCDDs exist in surface soils, water sediments and flyash. Aerobic surface soils would allow for aerobic dioxygenation, but would not allow for anaerobic reductive dechlorination (ARD). Rhizoremediation may encourage a greater dioxygenase diversity, and higher PCDD bioavailability to increase bioremediation rates. PCDDs in water sediments may be detoxified through ARD. PCDD contaminated flyash may best be detoxified by a bacterial and fungal biocatalyst, which has already been shown to be successful.

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CHAPTER II

COMPARISON OF THE SPECIFICITY AND EFFICACY OF PRIMERS FOR AROMATIC DIOXYGENASE GENE ANALYSIS OF ENVIRONMENTAL SAMPLES

The work presented in this chapter has been published:

Iwai S, Johnson TA, Chai B, Hashsham SA, Tiedje JM. 2011 Comparison of the specificity and efficacy of primers for aromatic dioxygenase gene analysis of environmental samples. Appl Environ Microbiol 77(11): 3551-3557

Author contributions:

S.I. and B. C. determined the in silico specificities of the published primer sets. T.A.J. reviewed previously published primer sets and their efficacy. S.I. and T.A.J. made recommendations to future primer use. S.I., T.A.J., S.A.H and J.M.T. wrote the paper.

ABSTRACT

Aromatic dioxygenase genes have long been of interest for bioremediation and aromatic carbon cycling studies. To date, 115 primers and probes have been designed and used to analyze dioxygenase gene diversities in environmental samples. Here we analyze those primers' specificity, coverage and PCR product length compared to known aromatic dioxygenase genes by using *in silico* analysis as well as summarize their differing advantages or effectiveness from over 50 reported experimental studies. We also provide some guidance for primer use in future studies.

INTRODUCTION

Aromatic compounds such as polycyclic aromatic hydrocarbons (PAHs), biphenyls and dioxins are widespread contaminants due to human activities as well as from natural events (Field et al. 2008, Green et al. 2004). Aromatic dioxygenases, which initiate the aerobic degradation of these compounds, have been of considerable interest for bioremediation. Furthermore these aromatic hydrocarbons have basic structures similar to some plant and soil aromatic compounds, and hence these and similar dioxygenase genes are likely involved in the natural carbon cycle. Improved understanding of the microbial contribution to terrestrial carbon turnover, especially of its more resistant aromatic components, is of increasing importance for predicting and potentially influencing the carbon cycle, and hence affect climate change (Bardgett et al. 2008).

The α-subunits of these multi-component dioxygenases have a well-known, common structure central to its electron transfer and catalysis called the Rieske center, and mononuclear iron, and are called Rieske non-heme iron oxygenases (Gibson et al. 2000). These dioxygenases have been divided into four groups based on the substrates metabolized, which also correspond

to their phylogeny: toluene/biphenyl (T/B), naphthalene (or PAH), benzoate and phthalate dioxygenases (Gibson et al. 2000). The first two groups have been the most studied and 115 primers and probes have been reported for their study (see Table S1 in the supplemental material). As more recent studies have revealed much greater diversity of these genes in nature (Ding et al. 2010, Iwai et al. 2010, Kimura et al. 2009, Marcos et al. 2009, Yagi et al. 2009), an evaluation of the current primers is timely for informing future studies. Primer coverage and specificity, which are important factors in choosing a primer set appropriate for the experimental purpose, have never been compared and discussed. In this review, we compared the 115 primer sets in terms of specificity, coverage and length of the products by using *in silico* analysis, and by summarizing their performance from published studies.

Biphenyl and PAH dioxygenase genes. As reference sequences, we retrieved 464 Rieske non-heme iron dioxygenase genes from the Pfam protein family database (Finn et al. 2008), which are more than 350aa in length and have both the Rieske family domain (Pfam PF00355) (Rieske center) and the Ring_hydroxyl_A family domain (Pfam PF00848) (iron binding site). Retrieved protein sequences were aligned using MUSCLE (Edgar 2004), and dissimilarity matrices calculated and used in DOTUR (Schloss et al. 2005) for Complete Linkage Clustering. A distance cutoff of 0.2 produced 120 clusters. The middle distance sequence in each cluster was selected as a representative sequence for the cluster. The representative sequences were used for constructing a phylogenetic tree by the Neighbor-joining method using Phylip 3.67 software (Felsenstein 1989) (data not shown). Of the retrieved genes, the aromatic dioxygenase genes including well-characterized toluene/biphenyl and PAH dioxygenase genes, are located in red branches. The rest of the gene clusters located in bottom half of the tree are benzoate dioxygenases or not functionally characterized Rieske-type dioxygenases. Since aromatic

dioxygenase genes usually indicate genes in the red-branched clade and thus most of the primers were designed for those genes, we used the 44 clusters that represent 204 dioxygenase genes in this large clade as reference sequences in this study. We grouped those reference genes into five sub-clades: PAH dioxygenases from Gram-negative bacteria (PAH-GN, blue circles), toluene/biphenyl dioxygenases (T/B, green circles), other dioxygenases I and II (OT-I and -II, yellow circles) and PAH dioxygenases from Gram-positive bacteria (PAH-GP, orange circles).

Primer selection: Importance of primer coverage, specificity and PCR product length. In addition to basic primer design strategies written elsewhere (Robertson et al. 1998), three criteria should be considered for dioxygenase primer selection: coverage, specificity, and PCR product length. Primer coverage, which is the size of allele of the target gene that should be amplified by the primer set during PCR as estimated from known sequences, is a key parameter because it suggests the possible diversity of the sequences that will be recovered from natural samples. The coverage range depends on the conservation of the primer region, degeneracy of the primer, and PCR conditions that may allow some primer-template mismatches. Secondly, specificity, or to what extent the primers align with the correct genes, while not aligning with other undesired genes, is also critical. Specificity is counter to coverage. Higher degeneracy is often used to increase coverage to more alleles; but this may result in lower specificity. Primer coverage-specificity relationships are especially critical when using primers for deep sequencing methodologies such as pyrosequencing – less specificity increases the chances of obtaining many non-target sequences. Thus, an appropriate balance of coverage and specificity for each primer should be examined. If a highly degenerate primer is selected for one end of the amplicon, a low degenerate one could be chosen for the other end to increase the specificity of the primer set. Another important consideration is that different samples have different dioxygenase gene

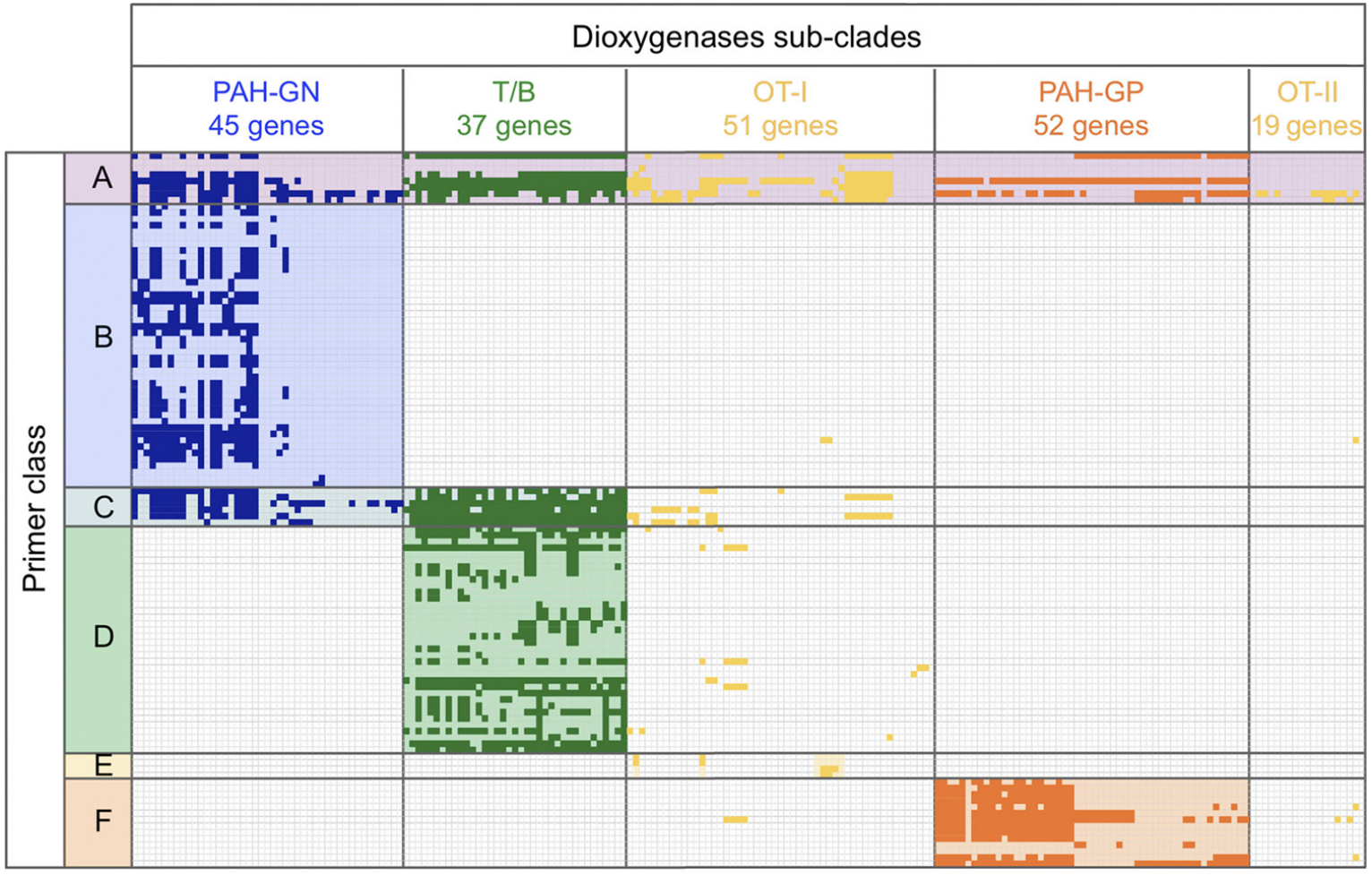

Fig. 2.1. Primer coverage pattern against 204 reference dioxygenase genes. Each box indicates perfect match between the gene and the primer. Primers can be classified into the six classes noted on the left, based on their coverages: class A, targets all five reference sub-clades; class B, targets PAH-GN; class C, targets PAH-GN and T/B; class D, targets T/B; class E, targets dioxin dioxygenases in OT-I; class F, targets PAH-GP.

contents. Therefore, it is always important to test multiple annealing temperatures on the particular samples to determine the specificity of the primer set. Third, PCR product length, or the distance between forward and reverse primers, is an important consideration since the currently available instruments provide sequence for only partial gene lengths. For traditional Sanger sequencing, up to around 700 bp can be sequenced. For pyrosequencing using the current Genome Sequencer FLX titanium system (454 Life Sciences, Branford, CT), sequences up to 400-500 bp can be obtained from PCR product sizes of 200 to 600 bp. Using Genome Analyzer (Illumina, San Diego, CA), 150 bp is currently the longest obtained sequence. Longer sequences of course lead to a better understanding of the gene, which is the ultimate goal, but the higher capacity of the 454 and Illumina technologies provide a much greater sampling of nature's gene diversity, also an important goal.

The Role of Primer Design: Lessons from Previous Studies. Considerable research has taken place in trying to understand the aromatic degradation potential of bacteria in terms of diversity of dioxygenases in the environment, and for the type of dioxygenase contained within bacterial isolate(s) with known aromatic degradation capacity. As noted, 115 primers have been reported for environmental studies (see Table S1 in the supplemental material). The question for future studies is 'which primers do I use, and are further improvements possible?' We considered the role of target gene specificity, clade coverage, amplicon length, and reference sequences used in primer design as the parameters for judging quality of primers and then relate this to the experimental outcome from use of these primer sets.

Coverage of previous primers. To summarize the coverage of previously reported primers, each primer was searched against 204 reference dioxygenase genes using BLAST+ (Camacho et al. 2009) and perfect match genes were mapped. From overall patterns that indicate the coverage

of amplified genes (shown by density of boxes in sub-clades in Fig. 2.1), primers were grouped into six classes. The first class (class A) covers all five reference sub-clades. [DP2 or Rieske_f (Ni Chadhain et al. 2006) and Nah-for (Zhou et al. 2006) primers showed especially high coverage of more than 100 genes (Fig. S1). Those high coverage primers have high degeneracy and were designed to target the highly conserved Rieske motifs.] The other classes are more specific to each target sub-clade(s): class B targets PAH-GN, class C targets PAH-GN and T/B, class D targets T/B, class E targets especially dioxin dioxygenases in OT-I, and class F targets PAH-GP. An expanded, readable map of target genes for each primer is shown as Fig. S1 in the supplemental material.

While the published studies used various techniques to accomplish different goals, in terms of coverage of primers, Lozada et al. (Lozada et al. 2008) highlighted a comparison of a broad coverage primer set with a narrow coverage primer set. The Ac114F/Ac596R primer set has been used frequently (Gomes et al. 2007, Jeon et al. 2003, Stach et al. 2002, Tuomi et al. 2004, Wilson et al. 1999) with fairly consistent success. In this study (Lozada et al. 2008), clone libraries were constructed using the Ac114F/Ac596R primer set yielding seven distinct sequence clusters, five of them novel (only 58% to 68% identical to known amino acid sequences). Using the narrow coverage Cyc372F/Cyc854R primer set, which targets *phnA1*, all sequences showed high amino acid identity (98.6 to 100%) to *Cycloclasticus* isolates. The authors were able to detect broad diversity with the Ac144F/Ac596R primer set and then find specific genes, possibly specific to *Cycloclasticus*, with the second primer set. Primers specific to other phylotypes were attempted for this second step, but they did not result in amplification.

Primer set P8073/P9047 is also an instructive example. This primer set was designed with one reference sequence, *phnAc* (Lloyd-Jones et al. 1999), and our BLASTn results also show that

it is the only sequence with a perfect match (see Fig. S1). In multiple studies, this primer set was more specific to apparent PAH dioxygenases contained in uncultured bacteria. In two studies, strains isolated by PAH enrichment did not produce amplicons using these primers, yet the primer did produce amplicons in soils and a biofilm contaminated with phenanthrene (Lloyd-Jones et al. 1999, Stach et al. 2002). In two other studies this primer set was able to produce amplicons from PAH-degrading isolates while primer sets targeting the *nahAc* gene were less successful (Widada et al. 2002, Wilson et al. 2003). In the later study, they postulate that the *phnAc* gene was horizontally transferred, because identical copies of the gene were detected in phylogenetically diverse isolates (Wilson et al. 2003). Apparently the primer set targets a broad set of genes that may be dissimilar to those currently known. These examples indicate that previously known genes are only part of nature's diverse gene pool and that *in silico* analysis based on previous sequence data is a helpful first step in estimating primer coverage but is not (and should not) be expected to be completely accurate.

Control of primer specificity. Perfect match sequences are not only the products of PCR amplification. Some primers showed a lesser number of perfect match genes or even no matches although the primers were designed to target a wide range of genes. For example, the pah-rhdα primers by Ding et al. (2010) have no perfect match sequence in reference genes. Those primers were designed to use a lower annealing temperature and allow several mismatches during PCR in order to amplify a broader set of target genes. This effect could not be reflected in Fig. 2.1 because we were not able to estimate the effect of PCR conditions on specificity. Thus, the summary of previous studies in Table S3 can help in understanding actual primer coverage including the effect of PCR conditions.

Targeting conserved motifs within the target gene enhances primer specificity to the target gene. Ní Chadhain et al. (2006), as have others, designed a primer set targeting the Rieske center of the dioxygenase, a highly conserved portion of the gene. They include degenerate bases to increase coverage, but relied on the conserved positions to maintain specificity. Clone libraries were constructed in two studies (Ni Chadhain et al. 2006, Yagi et al. 2009), and in both studies the amplicons formed many clusters. Some clusters were distantly related to reference sequences, indicating that the primer amplified a gene similar to those from both cultured and uncultured bacteria.

Another method of ensuring specificity is to use a large number of reference sequences with which to find consensus sequences within the gene to use in primer design. Recently we designed the BPHD-f3/BPHD-r1 primer set with 31 reference sequences to ensure the positions selected as primers were conserved between many strains. This allowed known conserved motifs to be contained within the amplicon to use as a quality filter during pyrosequencing data analysis. As a result we found that the *bphA1* genes in the environment were mainly novel, with only a few similar to known sequences (Iwai et al. 2010). This result suggests that the current knowledge severely under-represents *bphA1*sequences in nature, which may be the case for many organic carbon-degrading genes, and indicates new strategies are needed for assessing nature's gene repertoire.

PCR product length of dioxygenase primers. Since PCR product length is limited for certain sequencing technologies, we summarize the positions of each primer for easy calculation of estimated product length with different combinations of primers. Using MUSCLE alignment of 204 dioxygenase genes, primer annealing positions were calculated based on *bphA* from *Burkholderia xenovorans* LB400 (GenBank: M86348). The length of the PCR product in each

primer pair varies from less than 100-bp to the entire α-subunit. Although two primer sets in class A target genes from all sub-clades, the amplified product lengths of both are 78bp, which limits classification and diversity assessment of these sequenced PCR products. The primers could be tried in different combinations after consideration of similar annealing temperatures and the coverage of sub-clades depending on the purpose of the study or the location of conserved motifs within the gene.

Choosing appropriate primer sets. We recommend the following general steps in choosing primers for gene-targeted (amplicon) metagenomics: (i) Select the target sub-clade, e.g all, PAH-GN, T/B or PAH-GP. (ii) Collect a set of candidate primers from each of the groups based on success of past studies (Table S3 in the supplemental material), and their coverage against the desired genes (Fig. S1 in the supplemental material). (iii) The amplicon length should be as long as possible to gain maximum information, but short enough so that it can be sequenced through the reverse primer for quality control (the current 454 Titanium version of pyrosequencing is able to sequence up to 500bp). (iv) Include conserved regions within the amplicon so that those sequences can be used as a quality filter in processing. We considered all primers as candidates for forward or reverse primers; however, it is possible that a primer will not function as well for the reverse complement, especially due to low GC content in the 3' end (≤40% in last five bases). All primers should be tested empirically since predictions are not reality. Amplification conditions may also need further optimization and for the type of sample matrix.

From our evaluation of past work, we offer a few primer pair suggestions. For the most comprehensive targeting of dioxygenase clades (all, our category A), we suggest DP1/Rieske_f and ARHD2R. For other sub-clades such as PAH-GN, T/B and PAH-GP, we suggest Ac596R (use as a reverse complement) and NAPH-2R, adoB1 (use as a reverse complement) and BPHD-

r1, NidA-forward and pdo1-r, respectively. We also recommend DP1/Rieske_f and Nah-for (use as a reverse complement) that covers 92 of the reference genes from all classes as general probes to determine if a novel isolate or gene contains a Rieske dioxygenase motif, but are not generally recommended for amplicon sequencing due to their short length of 77 bp.

Conclusions. Many recent studies reveal a much higher diversity of aromatic dioxygenases in environmental samples than previously thought and hence suggest a need for a better approach. One key is the selection of primer sets. The primer coverage, specificity and length that we summarized as well as our summary of the experimental studies will help guide the experimenter in choosing the most appropriate primer sets for new studies. We also expect that the current expansion of environmental metagenome sequencing projects will provide an additional sequence resource for evaluating and improving primers as that data will not be limited by what is in GenBank, which is biased toward easily cultured strains. For example, about 100 Gbp of soil metagenomic data contains about 1,000 of biphenyl dioxygenase-like genes (personal communication from J. R. Cole). As we write there are already soil metagenome projects producing terabases of sequences. The next phase in primer design may entail how to detect and assess the dioxygenase-like genes in these resources for improving our knowledge of nature's aromatic degradation capacity.

Acknowledgements. This work was supported by the Superfund Basic Research Program grant P42 ES004911-20 from the U.S. National Institute of Environmental Health Sciences.

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CHAPTER III

IN-FEED ANTIBIOTIC EFFECTS ON THE SWINE INTESTINAL MICROBIOME

The work presented in this chapter has been published:

Looft T, Johnson TA, Allen HK, Bayles DO, Alt DP, Stedtfeld RD et al. 2012 In-feed antibiotic effects on the swine intestinal microbiome. Proc Natl Acad Sci USA 109(5): 1691-1696

and is reprinted here with the permission of the publisher. Supplemental material is provided with the published version of this chapter at the journal website.

Author contributions:

T.L., W.J.S., S.A.H., J.M.T., and T.B.S. designed research; T.L., T.A.J., H.K.A., W.J.S., T.M.S., and T.B.S. performed research; D.O.B., D.P.A., R.D.S., T.M.S., B.C., J.R.C., and S.A.H. contributed new reagents/analytic tools; T.L., T.A.J., and H.K.A. analyzed data; and T.L., T.A.J., H.K.A., J.M.T., and T.B.S. wrote the paper.

ABSTRACT

Antibiotics have been administered to agricultural animals for disease treatment, disease prevention, and growth promotion for over 50 years. The impact of such antibiotic use on the treatment of human diseases is hotly debated. We raised pigs in a highly controlled environment with a portion of the littermates receiving a diet containing performance-enhancing antibiotics (chlortetracycline, sulfamethazine, and penicillin [known as ASP250]) and the other portion receiving the same diet but without the antibiotics. We employed phylogenetic, metagenomic, and qPCR-based approaches to address the impact of antibiotics on the swine gut microbiota. Bacterial phylotypes shifted after 14 days of antibiotic treatment, with the medicated pigs showing an increase in *Proteobacteria* (1% to 11%) compared to non-medicated pigs at the same timepoint. This shift was driven by an increase in *Escherichia coli* populations. Analysis of the metagenomes showed that microbial functional genes relating to energy production and conversion were increased in the antibiotic-fed pigs. The results also indicate that antibiotic resistance genes increased in abundance and diversity in the medicated swine microbiome despite a high background of resistance genes in non-medicated swine. Some enriched genes, such as aminoglycoside O-phosphotransferases, confer resistance to antibiotics that were not administered in this study, demonstrating the potential for indirect selection of resistance to classes of antibiotics not fed. The collateral effects of feeding subtherapeutic doses of antibiotics to agricultural animals are apparent and must be considered in cost-benefit analyses.

INTRODUCTION

Antibiotics are the most cost-effective way to maintain or improve the health and feed efficiency of animals raised with conventional agricultural techniques (Cromwell 2002, Dibner et al. 2005). In addition to improving feed efficiency, antibiotics are commonly given to livestock, poultry, and fish for disease treatment and prevention. The sum of agricultural antibiotic use reportedly accounts for as much as half of all antibiotics produced in the U. S. (Lipsitch et al. 2002). Despite the clear benefits of antibiotics to agriculture, liberal antibiotic use combined with rapid and widespread emergence of both animal and human pathogens resistant to multiple antibiotics has led some to question the prudence of current antibiotic use (Aarestrup et al. 1999, Levy 1978). Studies of environmental and intestinal microbial communities reveal enormous diversity of antibiotic resistance genes (Allen et al. 2010, Martinez et al. 2009, Sommer et al. 2009). The addition of antibiotics to feed introduces a selective pressure that may lead to lasting changes in livestock commensal micro-organisms. Also, reservoirs of antibiotic resistance genes have been shown to be stable in bacterial communities, even in the absence of antibiotics (Gotz et al. 1996, Salyers et al. 1997, Stanton et al. 2011, Stanton et al. 2011). A central concern of increased abundance of antibiotic resistance is the transfer of resistance to pathogens (Martinez 2008). As a result, the FDA recently released a draft guidance recommending restrictions on the use of antibiotics in animal agriculture (Health et al. 2010). The Infectious Diseases Society of America testified before a congressional subcommittee in support of such limitations (2010).

Bacteria that inhabit the gastrointestinal tract of animals are important for the maintenance of host health. The intestinal microbiota assists the host in nutrient extraction, immune system and epithelium development, and are a natural defense against pathogens (Zoetendal et al. 2004). Contrary to these benefits, the gut microbiota may antagonize future

disease treatment by facilitating the dissemination of resistance alleles across distantly related organisms. For example, commensal bacteria of the human colon harbor antibiotic resistance genes and can transfer these genes to pathogens (Karami et al. 2007, Shoemaker et al. 2001). In fact, horizontal gene transfer is largely the cause of multidrug resistance in Gram-negative bacteria (Leverstein-van Hall et al. 2002). With the identification of antibiotic resistance genes in commensal bacteria in the human foodchain (Barbosa et al. 1999, Li et al. 2010, Stanton et al. 2003), the role of the gut microbiota as a reservoir of resistance genes for animal and foodborne pathogens needs to be explored.

Valuable insights have been gained by culture- and PCR-based approaches to study narrow groups of bacteria or genes, such as erythromycin resistance in swine isolates (Wang et al. 2005); however, the comprehensive effects of daily feeding of subtherapeutic doses of antibiotics on livestock microbiotas have not been studied. We therefore sought to extensively evaluate the effects of in-feed antibiotics on the entire gut microbiota. Phylotyping, metagenomic, and parallel quantitative PCR (qPCR) approaches were used to track changes in microbial membership and encoded functions, enabling the detection of so-called "collateral" effects of antibiotics, i.e. effects outside of the intended growth promotion and disease prevention. These collateral effects included increases in *Escherichia coli* populations and in the abundance of certain antibiotic resistance genes.

Piglets were birthed at the National Animal Disease Center, Ames IA, and housed together in highly-controlled, decontaminated rooms to avoid cross contamination among the medicated animals, non-medicated animals, and other resident barn animals. Neither the piglets nor the sow were exposed to antibiotics prior to the study. This design was to ensure that the inoculum for the piglets would come horizontally from their mother, minimizing variability so

that effects of antibiotic treatment could be detected. At 18 weeks of age, one group of littermates received ASP250 feed (medicated) and the other received the same but unamended feed (non-medicated) for three weeks. ASP250 is an antibiotic feed additive containing chlortetracycline, sulfamethazine, and penicillin that is commonly given to swine for the treatment of bacterial enteritis and for increased feed efficiency. Fecal samples were collected just before treatment (day 0), and after 3 days, 14 days, and 21 days of continued treatment. Day 0 samples were used to describe the swine intestinal microbiome prior to antibiotic treatment period.

RESULTS

Shifts in community membership with ASP250. We collected 133, 294 sequences of the V3 region of the 16S rRNA gene from a total of 12 fecal samples. Data from pigs of the same treatment and sampling date were grouped to appraise an antibiotic effect on community membership. As reported for a mammalian intestinal environment (Ley et al. 2008), and recently in a swine metagenome (Lamendella et al. 2011), the majority of classifiable sequences (75-86%) belonged to the *Bacteroidetes*, *Firmicutes*, and *Proteoba1cteria* phyla (Table S1). Of the *Bacteroidetes*, the *Prevotella* genus was consistently abundant, as was shown to be a feature of the swine microbiome (Lamendella et al. 2011). The Bray-Curtis index was calculated for all sample combinations and an analysis of similarities (ANOSIM) was performed. A multi-dimensional scaling (MDS) plot of these data indicated divergence of the day 14 samples from the day 0 samples ($p<0.01$), and the medicated microbiome diverged from the non-medicated ($p<0.05$) (Fig. 1A), demonstrating changes in microbial community membership over time and with treatment.

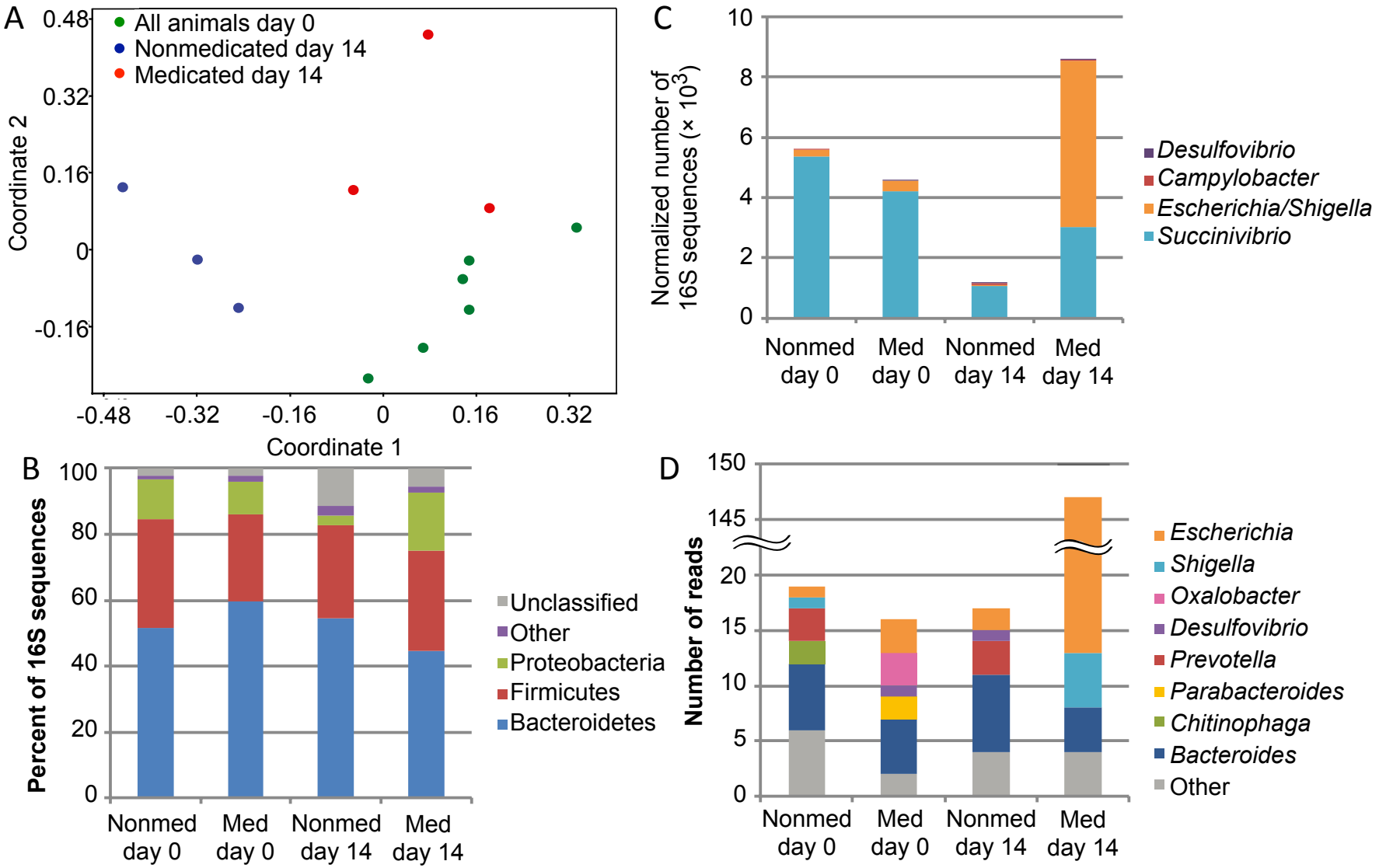

Fig. 3.1. Shifts in fecal bacterial community membership with antibiotic treatment. (A) A multi-dimensional scaling (MDS) analysis of Bray-Curtis similarity coefficients calculated from 16S rRNA gene sequence data from individual animals at days 0 and 14 shows the

Fig. 3.1 (cont'd)

similarity among replicate pig fecal samples. (B) Phylum-level composition of fecal microbial communities. Data were pooled for a given treatment and timepoint and are shown as percent abundance. (C) Genus-level composition of *Proteobacteria*, shown as the total number of sequences (normalized to 50,000 total reads). (D) Predicted genera of COG3188 homologues found in the swine metagenomes based on BLASTx analysis. COG3188 was overrepresented in the medicated metagenome versus the non-medicated metagenomes.

Specific changes in the microbial community associated with ASP250 treatment included a decrease in the abundance of *Bacteroidetes*, along with members of *Anaerobacter*, *Barnesiella*, *Papillibacter*, *Sporacetigenium* and *Sarcina* genera. Members of the *Deinococcus-Thermus* and *Proteobacteria* phyla increased with ASP250 treatment as well as *Succinivibrio* and *Ruminococcus* genera (Table S1). The increase in *Proteobacteria* abundance with in-feed ASP250 was particularly striking: from 1% of the population in non-medicated animals to 11% of the population with antibiotic treatment (Fig. 1B). Specifically, *E. coli* populations were the major difference between medicated and non-medicated animals, comprising 62% of the *Proteobacteria* in medicated animals (Fig. 1C). The increase in *E. coli* was confirmed in the metagenomic data (Fig. 1D) and by qPCR targeting the *uidA* gene of *E. coli* ($p<0.05$). A separate study using 12 pigs similarly treated but with analysis by culture-based techniques further established that swine fed ASP250 have an increased *E. coli* population at 14 days post treatment, showing a 20 to 100 fold greater *E. coli* abundance in medicated than non-medicated swine (Fig. S1).

Shifts in functional gene abundance with ASP250. DNA samples from the feces of non-medicated and medicated pigs at day 0 and 14 were isolated, and samples of like treatment and sampling date were pooled for pyrosequencing. Metagenome sequences (1, 202, 058 total) were analyzed in MG-RAST for SEED subsystems (Glass et al. 2010), and in-house for clusters of

orthologous groups (COGs). All metagenomes showed functional stability over time by both COG and subsystem analyses (Fig. S2). The most abundant SEED subsystem of known function was carbohydrate metabolism, mirroring what was previously reported for the swine metagenome (Lamendella et al. 2011). A statistical analysis of COGs revealed shifts in microbial community functions with ASP250: the medicated metagenome contained 169 COGs that were significantly more abundant than in the non-medicated metagenomes (Table S2). Three COGs (0477, permeases of the major facilitator superfamily; 1289, predicted membrane protein; 3570, streptomycin 6-kinase) contain swine metagenomic genes that are annotated as resistance genes in the antibiotic resistance gene database (ARDB). Three of the COGs with the lowest p-value (3188, 3539, and 3121) contained genes related to P pilus assembly, and additionally among the statistically significant COGs are transposases (0675, 1662, and 4644).

To identify themes among differentially represented COGs between the medicated and non-medicated metagenomes, COGs of Table S2 were clustered by their respective COG category. Only one COG functional category, energy production and conversion (C), was found more frequently ($p<0.05$) in the medicated metagenome than in the non-medicated metagenomes (Table S3).

Pervasive antibiotic resistance in the absence of antibiotic exposure. The discovery that resistance-related COGs fluctuated with antibiotic treatment led to further scrutiny of the metagenomes by BLAST against the ARDB (Liu et al. 2009). All metagenomes, regardless of antibiotic treatment, harbored sequences similar to diverse antibiotic resistance genes representing most mechanisms of antibiotic resistance: efflux pumps, antibiotic-modifying enzymes, and modified or protected targets of the antibiotic (Fig. 2A). This analysis detected 149 different resistance genes in the day 0 metagenomes.

The finding of diverse fecal antibiotic resistance genes in the non-medicated metagenomes was supported by parallel qPCR analysis. A rich array of 58 resistance genes was detected at least once in the swine fecal samples by qPCR. Samples from non-medicated animals showed a total of 51 different resistance genes, but few were shared between animals: only six (*erm*(A), *erm*(B), *cfiA*, *mefA*, *tet*(32), and *aadA*) were detected in 66% of the samples and none were found in more than 80% of the samples. No enrichment of these genes was observed in the medicated animals even though *tet*(32), a ribosomal protection protein, is known to confer resistance to an administered antibiotic (tetracycline). Samples from medicated animals yielded more homogenous resistance gene diversity: 39 genes were detected in at least one medicated sample, 20 were detected in 66% of samples, and 11 (*cfiA*, *mefA*, *erm*(A), *erm*(B), *tet*(32), *tet*(O), *aadA*, *aph(3')-ib*, *bcr*, *acrA*, and *bacA)* were detected in at least 8/9 of the samples.

qPCR and metagenomic analyses reveal shifts in resistance gene richness and abundance in medicated pigs. Statistical analysis of the ARDB results showed 23 genes to be differentially represented in the medicated and non-medicated metagenomes (Table 1). The 20 genes that were more abundant in the medicated metagenome were associated with efflux, sulfonamide resistance, and aminoglycoside resistance, the latter of which represents resistance to a class of antibiotics not present in ASP250 (Table 1).

The qPCR results mirrored the metagenomic analysis, revealing six resistance-gene types with statistically significantly greater abundance in the medicated animals than in the non-medicated animals ($p<0.05$): tetracycline efflux pumps, class A beta-lactamases, sulfonamide resistance genes, aminoglycoside phosphotransferases, and two types of multi-drug efflux (Fig. 2B, Table 1). No statistical difference in abundance was found for these six resistance gene types

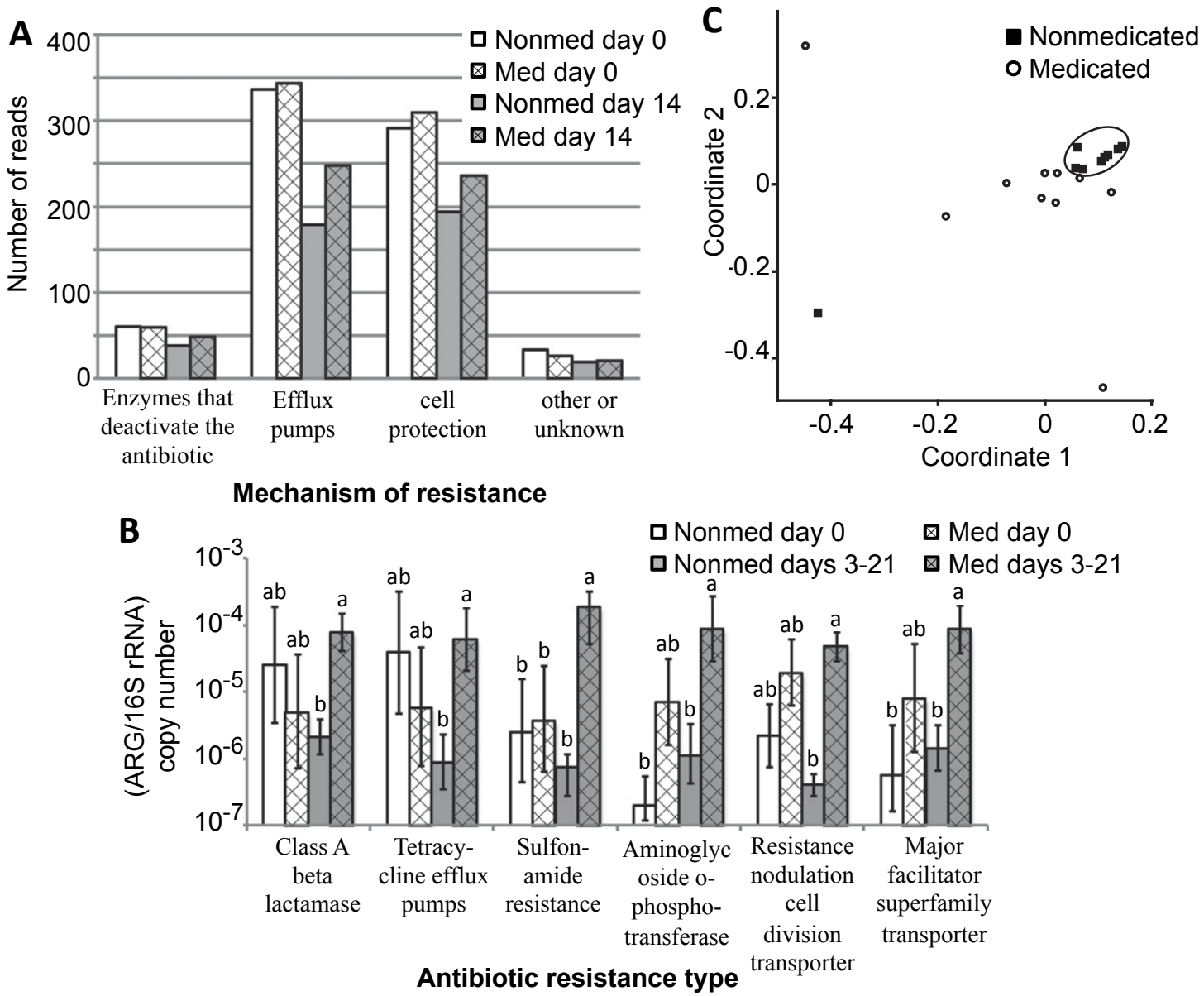

Fig. 3.2. Changes in diversity and abundance of antibiotic resistance genes in swine feces with antibiotic treatment.

Fig. 3.2 (cont'd)

(A) Metagenomes were analyzed by BLASTx against the ARDB, and the number of reads were normalized to 100,000 total reads per metagenome. (B) Differences in the abundance of resistance genes were assessed by calculating the ratio of resistance gene copy number (ARG) to 16S rRNA gene copy number per sample as detected by qPCR. Columns denoted by the same letter are not statistically significant ($p>0.05$) within each resistance type. Error bars represent the standard error of the mean. (C) Bray-Curtis similarity coefficients were calculated from qPCR-derived resistance gene abundance data and plotted in a multidimensional scaling graph. The distance between points indicates the degree of difference in the diversity of resistance genes between samples. The medicated sample outlier (square) is from one medicated pig on day 21. Measures for day 0 samples are not shown.

between the medicated and non-medicated microbiomes on day 0 (Fig. 2B) suggesting that in-feed ASP250 caused the effect. Resistance-gene abundance increased most dramatically in the 3- and 14-day samples (Fig. S3), indicating that antibiotic treatment induced a rapid shift in the abundance of resistance genes.

ASP250 treatment increased the diversity of resistance gene types as detected by qPCR (Shannon indices 1.4 [medicated] and 0.8 [non-medicated]; $p = 0.04$). A t-test comparing the mean number of resistance genes in the metagenomes at day 14 to the corresponding non-medicated metagenome confirms this result ($p<0.05$). Additionally, the structure of the resistance gene communities (beta diversity) was altered by antibiotic treatment, as determined by a two-way ANOSIM ($p<0.01$) of Bray-Curtis measures; however, the comparison *R*-value was 0.25, indicating that the degree of separation is limited. Nevertheless, resistance gene diversity converges with ASP250 treatment, presumably due to the selective pressure of the antibiotics (Fig. 2C). Taken together, these results show that feeding antibiotics increases the diversity of resistance genes within an individual sample and homogenizes that diversity between treated samples.

DISCUSSION

We assessed the effect of ASP250 on the swine antibiotic resistome using phylotype, metagenomic, and qPCR approaches. The results show that the swine microbiome harbors diverse resistance genes even in the absence of selective pressure. Six genes in particular were detected at high frequency in both the medicated and non-medicated microbiomes. These genes could represent a core antibiotic resistome for this cohort of swine. Indeed, it was suggested that *tet*(32) is abundant in farm animals (Melville et al. 2001), and our data support that conclusion for swine. Unfortunately, the core swine resistome also includes resistance of clinical importance: *cfiA* confers resistance to carbapenems, a class of broad-spectrum beta-lactam antibiotics of last resort (Maltezou 2009). The constant selective pressure of 50 years of in-feed antibiotics appears to have established a high background level of resistance in the swine microbiome.

Antibiotic treatment caused a detectable increase in the abundance of resistance genes even above the high background of resistance, and many of these were likely enriched due direct interaction with the antibiotics in ASP250. For example, sulfamethazine presumably selected for the sulfonamide resistance genes *sul2* or *sul1* present in 8 of the 9 medicated samples. Additionally, class A beta-lactamases were overrepresented in the medicated animals and confer resistance by cleaving such beta-lactam antibiotics as penicillin. Many of the other enriched resistance genes function by exporting chemicals. Such efflux includes but is not limited to antibiotics and may allow bacteria that lack specific resistance genes to survive antibiotic pressure. Multidrug efflux is frequently associated with the medically alarming issue of multiple-drug resistance and can be found on mobile genetic elements (Martinez 2009). In addition to the effects on specific gene families, in-feed antibiotics homogenized the richness of resistance

Table 3.1. Antibiotic resistance genes differentially represented ($p < 0.05$) in the medicated versus non-medicated pig fecal samples as detected by metagenomics (number of sequences in the medicated (n=1) vs. non-medicated (n=3) metagenomes per resistance gene) and qPCR (gene copy number / 16S rRNA gene copy number) during the treatment period.

	Genes(s) detected by		
Mechanism of resistance	Metagenomics	qPCR	Confers resistance to
More prevalent in the treated metagenome			
ABC transporter system. Macrolide-lincosamide-streptogramin B efflux pump.	*lmrA*		Lincomycin
Aminoglycoside O-phosphotransferase. Modifies aminoglycosides by phosphorylation.	*aph(3′′)-Ib*, *aph(6′)-Ic*, *aph(6′)-Id*	*aph(3′′)-Ib*	Streptomycin
Class A β-lactamase. Cleaves the β-lactam ring.		bla_{TEM-1} bla_{SHV-2}	β-Lactams
Major facilitator superfamily transporter, tetracycline efflux pump. Multidrug resistance efflux pump.	*emrD*, *mdfA*, *mdtH*, *mdt*L, *rosA*, *tet*(B)	*tet*(B), *bcr*	Chloramphenicol, tetracycline, deoxycholate, fosfomycin, Florfenicol, sulfathiazole
Resistance-nodulation-cell division transporter system. Multidrug resistance efflux pump.	*adeA*, *amrB*, *mdtF*, *mdtN*, *mdtO*, *mdtP*, *oprA*, *tolC*	*acrA*	Fluoramphenicol, aminoglycoside, macrolide, acriflavine, doxorubicin, erythromycin, puromycin, β-lactams
Ribosomal protection protein. Protects ribosome from inhibition by tetracycline.	*tet*(M)	*tet*(O)	Tetracycline
Sulfonamide-resistant dihydropteroate synthase. Cannot be inhibited by sulfonamide.	*sul2*	*sul2*	Sulfonamide
More prevalent in the control metagenomes			
Resistance-nodulation-cell division transporter system. Multidrug resistance efflux pump.	*mexF*		Chloramphenicol, fluoroquinolone
Ribosomal protection protein. Protects ribosome from inhibition by tetracycline.	*tetB*(P), *tet*(Q)		Tetracycline

genes among individuals over time. The breadth of the current study enabled the visualization of this intriguing phenomenon despite the tremendous resistance gene heterogeneity across samples.

One type of resistance, the aminoglycoside O-phosphotransferases, increased in abundance with in-feed ASP250 although they do no confer resistance to the antibiotics therein. This suggests an indirect mechanism of selection, perhaps by co-occurrence on mobile elements conferring resistance to ASP250 antibiotics. Ten of the thirteen phosphotransferases identified in the medicated swine metagenome are homologous (7/10 have 100% amino acid identity) with the streptomycin phosphotransferase on the pO86A1 plasmid in *E. coli* O86:H- (accession number YP_788126). Resistance genes aggregate on plasmids in response to selective pressure (Barlow 2009), and pO86A1 carries at least two other resistance genes (accession number NC_008460). This congregation of resistance genes on mobile genetic elements could offer a fitness advantage to a bacterium living in the constant presence of antibiotics. However, this would be an undesirable collateral effect of in-feed antibiotics because these resistance gene clusters could be transferred to *E. coli* or other potential human pathogens in the swine gut or in the agriculture environment. Regardless of the mechanisms of selection, the results show that antibiotic use increased the abundance of resistance genes specific to and beyond the administered antibiotics from a diverse pool of background resistance genes in the swine microbiome, and that this increase was detectable even above a high background of resistance gene diversity.

The collateral effects of antibiotics extend beyond influencing resistance genes. Statistical analysis of COGs in the swine metagenomes showed that genes encoding virulence, gene-transfer, and energy production and conversion functions are selected by in-feed

antibiotics. Specifically overrepresented COGs included some relating to P pilus assembly; the P pilus has been described for attachment and virulence in *E. coli* (Sauer et al. 2000). Additional COGs of interest in the medicated metagenome included transposases, which are known to participate in the transfer of antibiotic resistance genes (Lupski 1987). These functions could enhance the stability and spread of resistance genes in microbial communities. Additionally, an increase in the abundance of genes encoding energy production and conversion functions could be a factor in growth-promoting properties of at least some antibiotics, but further experiments are required to test this. Antibiotics are thought to improve feed efficiency in agricultural animals primarily by decreasing the bacterial load, which is beneficial to the host by reducing competition for nutrients and decreasing the host's cost of responding to the microbes (Dibner et al. 2005). Analysis of the swine metabolome after antibiotic treatment showed an effect on various biosynthetic pathways including sugar, fatty acid, bile acid, and steroid hormone synthesis (Antunes et al. 2011). COGs may therefore be useful signposts for identifying microbes and functions important to the performance-enhancing effects of antibiotics like ASP250.

Changes in microbial functions result from changes in microbial membership, and interesting membership shifts were detected. The decrease in *Bacteroidetes* in the treated animals may relate to the growth-promoting benefits obtained from feeding swine ASP250 as part of their diets. Obese mice have lower levels of *Bacteroidetes* relative to *Firmicutes* in their feces when compared to lean mice (Turnbaugh et al. 2006). The obese mice have improved energy harvesting capacity, presumably due to this shift, and perhaps this shift is related to improved feed conversion in swine. Also, an increase in *E. coli* prevalence in response to oral antibiotic treatment has been reported for amoxicillin, metronidazole, and bismuth, (Antonopoulos et al.

2009); metronidazole, (Pelissier et al. 2010); and vancomycin and imipenem, (Manichanh et al. 2010) in the mammalian gut microbiota. However, amoxicillin plus the beta-lactamase inhibitor clavulanic acid administered both in the feed and intramuscularly resulted in decreased *E. coli* in pigs (Thymann et al. 2007), and oral ciprofloxacin yielded decreased *Proteobacteria* populations in humans during treatment (Dethlefsen et al. 2008). These results are an important reminder of the varying collateral effects of different antibiotics. *E. coli* are both commensal and pathogenic inhabitants of mammalian gastrointestinal tracts; an increase in *E. coli* could be beneficial or harmful, either to the host or to the food chain. Additionally, increased *E. coli* populations associated with excessive weight gain in pregnant women (Santacruz et al. 2010) is an unfavorable result in this host but parallels a potential growth-promoting role for this bacterium in livestock. The cost and benefit of a given antibiotic for a desired outcome must therefore be carefully weighed.

Differences among the rarer members of the microbial communities between treatment and control animals are less understood, and invite further investigation. Of those that increased with treatment, members of the *Deinococcus-Thermus* phylum are known for being resistant to environmental stress; these organisms have only recently been identified in the human gut [22]. Also, *Ruminococcus* spp. are common in ruminants and are frequent found in the hindgut of pigs (Rincon et al. 2007). Adept at degrading cellulose, an increase in *Ruminococcus* spp. after antibiotic treatment may aid in feed conversion in swine. Taken together, the data suggest numerous possibilities for how the swine gut microbiota might be involved with the improved feed efficiency afforded by certain in-feed antibiotics.

CONCLUSIONS

The results show that even a low, short-term dose of in-feed antibiotics increases the abundance and diversity of antibiotic resistance genes, including resistance to antibiotics not administered, and increases the abundance of *E. coli*, a potential human pathogen. Additionally, analysis of the metagenomes implicated functions potentially involved with improved feed efficiency. The study design featured environmental control in a single uniform inoculum source (the mother); control of the host genetics; no exposure of the sow or piglets to antibiotics except for the treatment; and identical diet except for the inclusion of ASP250 in one group. Future studies should include other in-feed antibiotics, multiple litters of swine with robust replication, and the identification of the antibiotic-induced mechanisms that lead to increased feed efficiency. Implications of antibiotic resistance on human and animal health need to be taken into account when discussing agricultural management policies and evaluating alternatives to traditional antibiotics. With the use of antibiotics in animal agriculture at a crossroads, studies like this and others that highlight the collateral effects of antibiotic use are needed.

MATERIALS AND METHODS

Swine. Six pigs (siblings) were used in this study and were split into two groups of three: a group to receive antibiotics and a group to receive no antibiotics. Animals were raised in accordance with National Animal Disease Center Animal Care and Use Committee guidelines. The rooms housing the pigs were decontaminated prior to the beginning of the study. A pregnant sow was obtained from a hog farm at which she had no prior exposure to antibiotics. The piglets shared a pen with the sow for three weeks after birth; her feces were therefore the primary bacterial inocula for the piglets. After weaning, all pigs were fed the same diet (TechStart® 17-

25, Kent Feeds, Muscatine, IA) until the start of the study, at which point the medicated pigs were moved to a new clean room and given the above diet but containing ASP250 (chlortetracycline 100 g/ton, sulfmethazine 100 g/ton, penicillin 50 g/ton). Freshly voided feces was collected from non-medicated and medicated animals just before treatment (medicated and non-medicated day 0) and 3, 14 and 21 days after treatment.

DNA Extractions. Feces were processed as follows for phylotype and metagenomic analysis. Ten g of fresh feces per sample were collected and blended in 300 ml sterile PBS. After suspension, the feces were centrifuged at 250 X G for 5 min to remove the large particles (such as insoluble food) from the sample. The supernatant was retained and centrifuged at 10,000 X G for 30 min at 4°C to pellet the bacterial cells. The supernatant was poured off and the pellet was washed by suspending it in PBS and spinning it again at 10,000 X G for 30 min at 4°C. Two g of the washed pellet was used for DNA extractions using the Power Max Soil DNA Isolation Kit following the manufacturer's protocol (MO BIO Laboratories, Carlsbad, CA, USA). DNA samples were quantified on a Nanodrop ND-1000 UV-Vis spectrophotometer (Nanodrop Technologies, Wilmington, DE). DNA integrity was determined by gel electrophoresis. Extracted DNA was stored at -20°C.

16S rRNA gene amplification. Amplification of the V1-V3 region of bacterial 16S rRNA genes was carried out with the conserved primers 8F (5′-AGAGTTTGATCCTGGCTCAG (Wilmotte et al. 1993)) and 518R (5′-ATTACCGCGGCTGCTGG (Don et al. 1991)) with attached unique eight-nucleotide sequence barcodes (Hamady et al. 2008). The V3 region of the 16S rRNA gene was amplified and sequenced as it was shown to be highly informative (Baker et al. 2003). We designed PCR primers with the following PCR reactions contained 200 μM of each deoxyribonucleotide triphosphate, 2.0 μM of each primer, 2.0 U Ampligold *Taq* polymerase

(Applied Biosystems, Foster City, CA), 2.5 mM $MgCl_2$, 50 ng template DNA, Ampligold *Taq* buffer (Applied Biosystems), and water to 50 µl. PCRs were performed in a PTC-225 thermal cycler (MJ Research, Watertown, MA, USA) with the following protocol: 3 min at 95°C, 21 cycles of [1 min at 95°C, 30 s at 56°C, 45 s 72°C], and a final elongation step for 3 min at 72°C. PCR products were separated by gel electrophoresis and purified using MinElute kit (Qiagen Inc., Valencia, CA)

DNA sequencing. PCR products were sequenced on a 454 Genome Sequencer FLX, using the manufacturer's protocol for FLX chemistry (Roche Diagnostics, Branford, CT). For sequencing the metagenome, DNA from the feces was pooled by treatment group (non-medicated, medicated) for each time point (day 0, day 14). Day 14 samples were sequenced using FLX chemistry and day 0 samples were sequenced using Titanium chemistry (Roche Diagnostics).

Phylotype analysis. Only sequences longer than 50 bp were used for phylotype analysis (phylotyping), which totaled 133,294 sequences (70,667 unique sequences) from 12 fecal samples. After binning the samples by barcode, phylogenetic analysis and taxonomic assignments of the V3 portion of the 16S rRNA gene were made using the Ribosomal Database project (RDP) web tools (Cole et al. 2009). Additional phylotype comparisons and hypothesis testing were performed with the software package mothur (Schloss et al. 2009). Bray-Curtis similarity coefficients were calculated from 16S rRNA gene sequence data from individual animals at 0 and 14 days and plotted in a multi-dimensional scaling (MDS) graph to show the similarity among samples. MDS plots and analysis of similarities statistical tests were done in PAST.

Metagenomic analysis. Sequence replicate artifacts were removed using a local version of the 454 Replicate Filter (Gomez-Alvarez et al. 2009) and specifying a sequence identity cutoff of

0.9, a length difference requirement of 0, and a check for a 3-base identical sequence at the beginning of each cluster. The clustered sequences were assigned to COGs by using BLASTx to compare the nucleic acid sequences to the database of proteins that was originally used to identify COGs. The BLAST reports were parsed to extract COG information, and COG frequencies were calculated and tabulated using SAS (SAS Institute Inc., Cary, NC). COG frequencies were subsequently analyzed in ShotgunFunctionalizeR (Kristiansson et al. 2009) using the testGeneFamilies.dircomp function and Poisson group statistics to perform gene-centric analysis between two groups (non-medicated [n=3] and medicated [n=1] swine metagenomes). Differences with $p<0.05$ were significant, and the significant COGs were labeled with their respective COG category to visualize trends. Metagenomic sequences belonging to select significantly different COGs were analyzed to infer phylogeny. Phylogeny assignments were made by extracting sequences belonging to the COGs of interest, BLASTx comparison of those sequences to the GenBank nonredundant protein database, extraction of the top hit accession, and retrieval of the phylogeny for that accession. COG counts were also corrected for differences in the estimated average genome size of each metagenome and reanalyzed as above, invoking the eff.nseq adjustment using the testGeneFamilies2.dircomp function (Beszteri et al. 2010). Because different methods of average genome size calculations could affect the outcome, COG counts were also corrected with the average genome sizes that were calculated by GAAS (Angly et al. 2009). These adjustments did not dramatically affect the results, and therefore only the results of the original ShotgunFunctionalizeR calculations are reported.

Swine metagenomes were also examined for the presence of known antibiotic resistance genes. Sequences were locally analyzed by BLASTx comparison of the sequences against the Antibiotic Resistance Gene Database (ARDB) (Liu et al. 2009), which was kindly provided by

the ARDB authors. The BLASTx parameters were optimized for short reads and diversity by using a bitscore cutoff of ≥60 and an identity cutoff of 35%. Antibiotic resistance gene-centric analysis was carried out in R using the testGeneFamilies function as described above. Differences with $p<0.05$ were significant. For ecological analyses, the number of hits was normalized to 100,000 submitted reads and analyzed using MDS and cluster analyses with the Bray-Curtis similarity measurement in PAST (Hammer et al. 2001).

Quantitative PCR (qPCR). Quantitative PCR primers, reagents and DNA samples were loaded into six subarrays of OpenArray plates (Applied Biosystems) as described previously (Stedtfeld et al. 2008). For each 33 nl qPCR reaction, 1 ng of extracted DNA was added as template. Quantitative PCR reagents and conditions were preformed as previously described (Stedtfeld et al. 2008). Relative gene copy numbers were calculated as follows: gene copy number = $10^{(26-C_T)/(10/3)}$, where C_T equals the threshold cycle (Table S6). Amplification curves were manually inspected using quality control measures. The abundance of the 16S rRNA gene was determined as previously described (Stedtfeld et al. 2008). *E. coli* was quantified by using a *uidA* primer set as described previously (Leigh et al. 2007). Copy numbers of the *uidA* and 16S rRNA genes were calculated in relation to a standard curve, which was generated by using 10-fold dilutions of 10^8 to 10^0 copies as template, in triplicate reactions. Those reactions targeting 16S rRNA and *uidA* were preformed separately from the OpenArray platform.

Statistical analysis of qPCR results: Abundance and diversity. All qPCR data were normalized between samples by dividing the gene copy number by 16S rRNA copy number, and subsequently natural log transformed in order to achieve normal distribution. A repeated measures ANOVA model was used to determine if treatment or time was significantly related to the abundance of antibiotic resistance genes and Shannon diversity in different samples. The best

covariance structure of the residuals for each response variable was determined and used for repeated measures ANOVA testing (SAS ver. 9.2, SAS Institute, Cary, NC, 2008). A Bonferroni adjustment was not used in the comparison of resistance genes or resistance gene types due to excessive reduction in power of tests; therefore, the reported p-values were not corrected for multiple comparisons.

Shannon diversity was calculated using PAST ver. 1.87 (Hammer et al. 2001) using data normalized between samples (resistance gene copy number / 16S rRNA gene copy number). Bray-Curtis coefficients were calculated for each of the samples using the natural log transformed data, as recommended (Anderson et al. 2006). A two-way ANOSIM was calculated using these data, considering treatment and time as the two factors. Two-way ANOSIM analysis and MDS plots were completed using the Bray-Curtis measure for beta diversity in PAST ver. 1.87 (Hammer et al. 2001).

Culturing *Escherichia coli*. The antibiotic feed trial was repeated with an independent set of pigs. Twelve pigs (offspring from three sows) were housed and maintained as described above. Six pigs received antibiotics (ASP250) and six receive no antibiotics continuously for 21 days before being sampled.

E. coli was cultured from fresh pig intestinal contents at necropsy, from both medicated and non-medicated animals after 21 days of feed. Serial dilutions were plated on MacConkey plates with lactose and incubated overnight at 39°C. Colony forming units were enumerated for each animal (Fig. S2).

Acknowledgments. The authors thank Sam Humphrey, Uri Levine and Lea Ann Hobbs for technical support; Vince Young for helpful conversations; the MSU Crop and Soil Science statistical consultation center for statistical advice; and Rich Zuerner and Tom Casey for

comments on the manuscript. The MSU research was supported by the Pharmaceuticals in the Environment Initiative. Sequences SRS151798.1-SRS151809.1, SRX038404.4, SRX038406.4, SRX038407.3, and SRX038408.2 were deposited in GenBank. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture. USDA is an equal opportunity provider and employer.

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CHAPTER VI

DIVERSE AND ABUNDANT ANTIBIOTIC RESISTANCE GENES IN CHINESE SWINE FARMS

The work presented in this chapter has been published:

Zhu Y-G, Johnson TA, Su J-Q, Qiao M, Guo G-X, Stedtfeld RD et al. 2013 Diverse and abundant antibiotic resistance genes in chinese swine farms. Proc Natl Acad Sci USA 110(9): 3435-3440

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Author contributions:

Y.-G.Z. and J.M.T. designed research; T.A.J. preformed the qPCR reactions and analysis; J.-Q.S. extracted DNA, M.Q., and G.-X.G. preformed the chemical analysis; T.A.J., R.D.S., and S.A.H designed new primers; T.A.J. validated and characterized the primer sets; J.-Q.S., made contributions in the antibiotic concentration determination method; T.A.J., J.-Q.S., and M.Q. analyzed data; and Y.-G.Z., T.A.J., J.-Q.S., and J.M.T. wrote the paper.

ABSTRACT

Antibiotic resistance genes (ARGs) are emerging contaminants posing a potential worldwide human health risk. Intensive animal husbandry is believed to be a major contributor to the increased environmental burden of ARGs. Despite the volume of antibiotics used in China, little information is available regarding the corresponding ARGs associated with animal farms. We assessed type and concentrations of ARGs at three stages of manure processing to land disposal at three large-scale (10,000 animals per year) commercial swine farms in China. In-feed or therapeutic antibiotics used on these farms include all major classes of antibiotics except vancomycins. High capacity quantitative PCR arrays detected 149 unique resistance genes among all the farm samples with the top 63 ARGs being enriched 192-fold (median) up to 28,000-fold (maximum) compared to their respective antibiotic-free manure or soil controls. Both antibiotics and heavy metals used as feed supplements were elevated in the manures suggesting the potential for co-selection of resistance traits. The potential for horizontal transfer of ARGs because of transposon-specific ARGs is implicated by the enrichment of transposases—the top six alleles being enriched 189-fold (median) up to 90,000-fold in manure as well as the high correlation ($r^2 = 0.96$) between ARG and transposase abundance. In addition, ARG abundance correlated directly with antibiotic and metal concentrations indicating their importance in selection of resistance genes. Diverse, abundant, and potentially mobile ARGs in farm samples suggest that unmonitored use of antibiotics and metals is causing the emergence and release of ARGs to the environment.

INTRODUCTION

The spread and aggregation of antibiotic resistant genes into multidrug resistant pathogens is challenging life-saving antibiotic therapies (Arias et al. 2009, Udwadia et al. 2012). Indeed, the expansion of the antibiotic resistance gene reservoir in the environment has been caused by antibiotic use in humans and animals (Knapp et al. 2010). Furthermore, a growing body of direct and indirect evidence from the past 35 years (Marshall et al. 2011), establishes that farm antibiotic use correlates repeatedly with the rise and spread of associated resistance genes in human pathogens, as well as the direct transfer of antibiotic resistant bacteria from animals to humans (Forsberg et al. 2012, Levy 1978, Price et al. 2012, Smillie et al. 2011). Antibiotic use has increased the frequency of horizontal gene transfer and resistance gene fixation in genomes leading to the development of diverse resistance genes in genomic islands (Gillings et al. 2012). *Acinetobacter baumannii* is a case in point. In 30 years, it evolved from being completely antibiotic susceptible to being multidrug resistant by expanding a genomic island by 66-kb, including 45 resistance genes, which were horizontally transferred from various genera of bacteria, some of which likely originated from the environment (Fournier et al. 2006). Antibiotic resistant strains can then be distributed worldwide, aided by a number of human factors, but especially international travel due to commerce, immigration and recreation (Church 2004). Antibiotic resistance genes (ARGs) are now recognized as environmental pollutants and action is being sought to preserve the efficacy of antibiotics. The World Organisation for Animal Health, together with the U. S. Food and Drug Administration and the World Health Organization, urge improved regulation of veterinary antibiotic use in over 100 developing countries (Gilbert 2012).

China is the largest antibiotics producer and consumer in the world. In a 2007 survey, the estimated annual antibiotics production in China was 210 million kg, and 46.1% were used in livestock industries (Hvistendahl 2012), at least four times the amount used in the U.S. livestock industry in 1999 (Chee-Sanford et al. 2009). In China, the use of antibiotics both for animal disease treatment and growth promotion is unmonitored, which often leads to high usage, reflected by the high concentrations of antibiotic residues (hundreds of mg tetracycline kg^{-1}) that are commonly detected in animal manures (Pan et al. 2011, Qiao et al. 2012). Manure is a major source of antibiotic pollution in the environment, and China produces an estimated 618 billion kg of swine manure annually (Wang et al. 2006). Most veterinary antibiotics are poorly absorbed by the animal, hence excreted (Alcock et al. 1999), and dispersed to soil when the manure is spread as fertilizer, the desired practice for recycling nutrients. Furthermore, the use of subtherapeutic levels of antibiotics in animal feeds causes an increase in antibiotic resistance traits in manure (Binh et al. 2008, Looft et al. 2012) and manure-amended soils (Ghosh et al. 2007) and downstream river waters and sediments (Pruden et al. 2012). In addition, metals are added to swine feed for growth promotion and disease control, and may provide a long-term co-selective pressure for antibiotic resistance (Baker-Austin et al. 2006). The scale of the livestock industry in China and the volume of antibiotics usage provide an opportunity to assess the impact of large-scale animal farm practices on antibiotic resistance genes in the environment. Previously, tetracycline resistance (*tet*) genes in soils adjacent to representative Chinese swine feedlots were positively correlated to concentrations of tetracycline residues (Wu et al. 2010) raising the question of whether the diversity and abundance of the antibiotic resistance reservoir extends beyond tetracycline resistance genes due to the use of additional antibiotics, possible co-selection for other resistance genes, and/or recruitment of multidrug efflux pump genes.

While antibiotic resistant bacteria have been isolated and characterized from farm soils (D'Costa et al. 2006, Ghosh et al. 2007), this method only samples microbes that are culturable and express their ARGs under those conditions. ARGs of non-cultured populations, as well as "silent" or not expressed ARGs (Enne et al. 2008), are sources of risk as they contribute to the resistance reservoir, and could be horizontally transferred, or expressed under other conditions. We used high capacity quantitative PCR (qPCR) (Looft et al. 2012) with 313 validated primer sets, which target 244 ARGs (Table S1) from all major classes of ARGs, to extensively sample the antibiotic resistance reservoir. We sampled three large-scale commercial swine farms each from different regions of China at three stages of manure management: manure (M), manure compost (C) and soil receiving manure compost (S). Manure from pigs never fed antibiotics and soil from a pristine forest soil in Putian, China were used as experimental controls.

RESULTS

Antibiotics and metal concentrations. Antibiotics and their use as reported by the farmers are listed in Table S2. Total tetracycline concentrations in these manure and soil samples were as high as 15.2 mg kg^{-1} and 0.78 mg kg^{-1}, respectively, as was determined previously (Qiao et al. 2012). Of the sulfonamides analyzed in this study, sulfamethoxazole had the highest concentrations for all samples ranging from 1.08-3.02 μg kg^{-1} (Fig. S1). Sulfadiazine was also detected in all samples in a range of 0.50-4.81 μg kg^{-1}. Of the fluoroquinolones analyzed in this study, only ofloxacin and enrofloxacin were observed in most samples. The highest mean concentration of ofloxacin (335 μg kg^{-1}) and enrofloxacin (96.0 μg kg^{-1}) were observed in

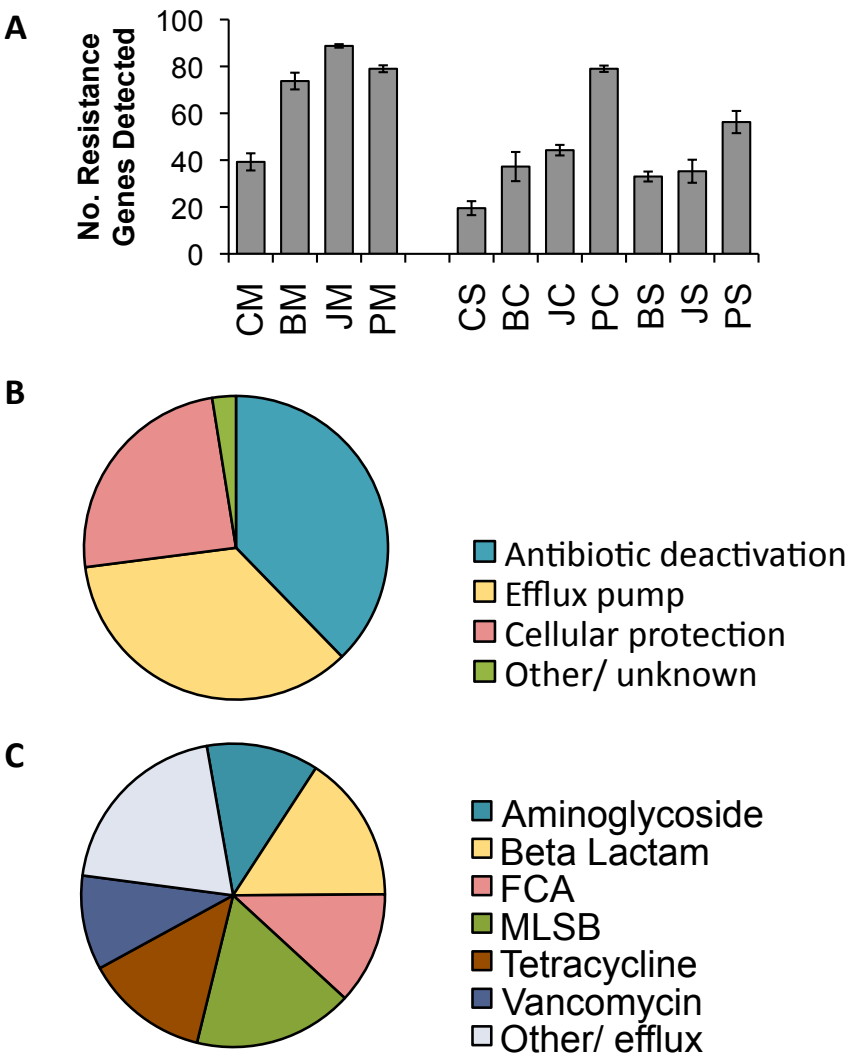


Fig. 4.1. Antibiotic resistance gene detection statistics. Sample names are abbreviated with two letters representing location and sample type: first C, B, J, and P (control, Beijing, Jiaxing, and Putian, respectively) and second M, C, and S (manure, compost, and soil [with compost amendment], respectively). Since many resistance genes were targeted with multiple primers, if multiple primer sets detected the same gene, this was only counted as detection of a single unique resistance gene. A) Average number of unique resistance genes detected in each sample. Error bars represent SEM of four field replicates. The resistance genes detected in all samples were classified based on B) the mechanism of resistance, and C) the antibiotic to which they confer resistance. FCA = fluoroquinolone, quinolone, florfenicol, chloramphenicol, and amphenicol resistance genes. MLSB = Macrolide-Lincosamide-Streptogramin B resistance.

Putian compost and soil samples, respectively (Fig. S1). Zinc, copper, and arsenic, used as feed additives were also elevated above background concentrations. The highest mean concentrations of copper, zinc and arsenic were detected in Putian manure, Jiaxing compost and Beijing manure, respectively, with copper up to 1,700 mg kg^{-1} manure (Fig. S2). The concentration of copper, zinc and arsenic were much higher in manure than in compost and soil samples, with the exception of the Jiaxing compost, where copper and zinc had the highest concentrations of all the samples.

Diversity of antibiotic resistance genes. We detected 149 unique antibiotic resistance genes among all of the samples, which is three times more ARGs types than were found in the control samples (Fig. 1A). The ARGs detected in these farms encompass the three major resistance mechanisms: efflux pumps, antibiotic deactivation, and cellular protection (Fig. 1B), and potentially confer resistance to all major classes of antibiotics (Fig. 1C) including vancomycin. Resistance gene profiles indicate the patterns and degrees of ARGs enrichment for each site (Fig. 2), and that manure samples are much different from the other samples with the exception of the Putian compost (key differences between the Putian manure and compost from the other manure and compost samples are outlined in black, Fig. 2). The compost and soil samples also cluster separately with the exception of one of the Beijing compost replicates, which grouped with the soil samples. Furthermore, Shannon diversity (indicating richness and abundance) of ARGs from farm samples was significantly higher than the control samples (Fig. S3).

Abundance of antibiotic resistance genes. Antibiotic resistance genes were highly enriched in the farm samples. We used the sum of the enrichment of all unique ARGs in a sample to approximate total enrichment in the farms. Maximum enrichment occurred in the manure samples at Beijing (121,000-fold) and Jiaxing (39,000-fold) farms, and in the compost at the

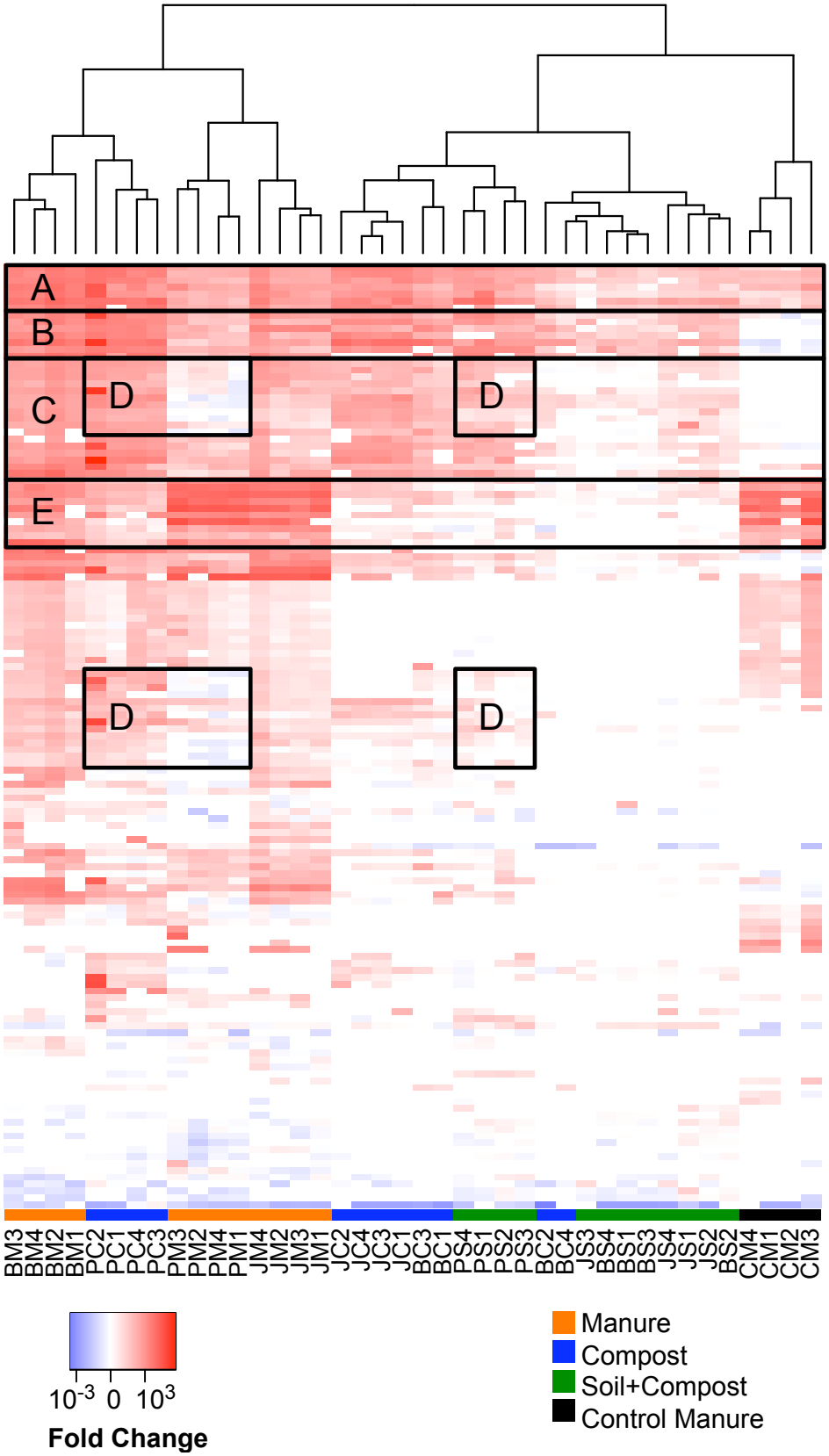


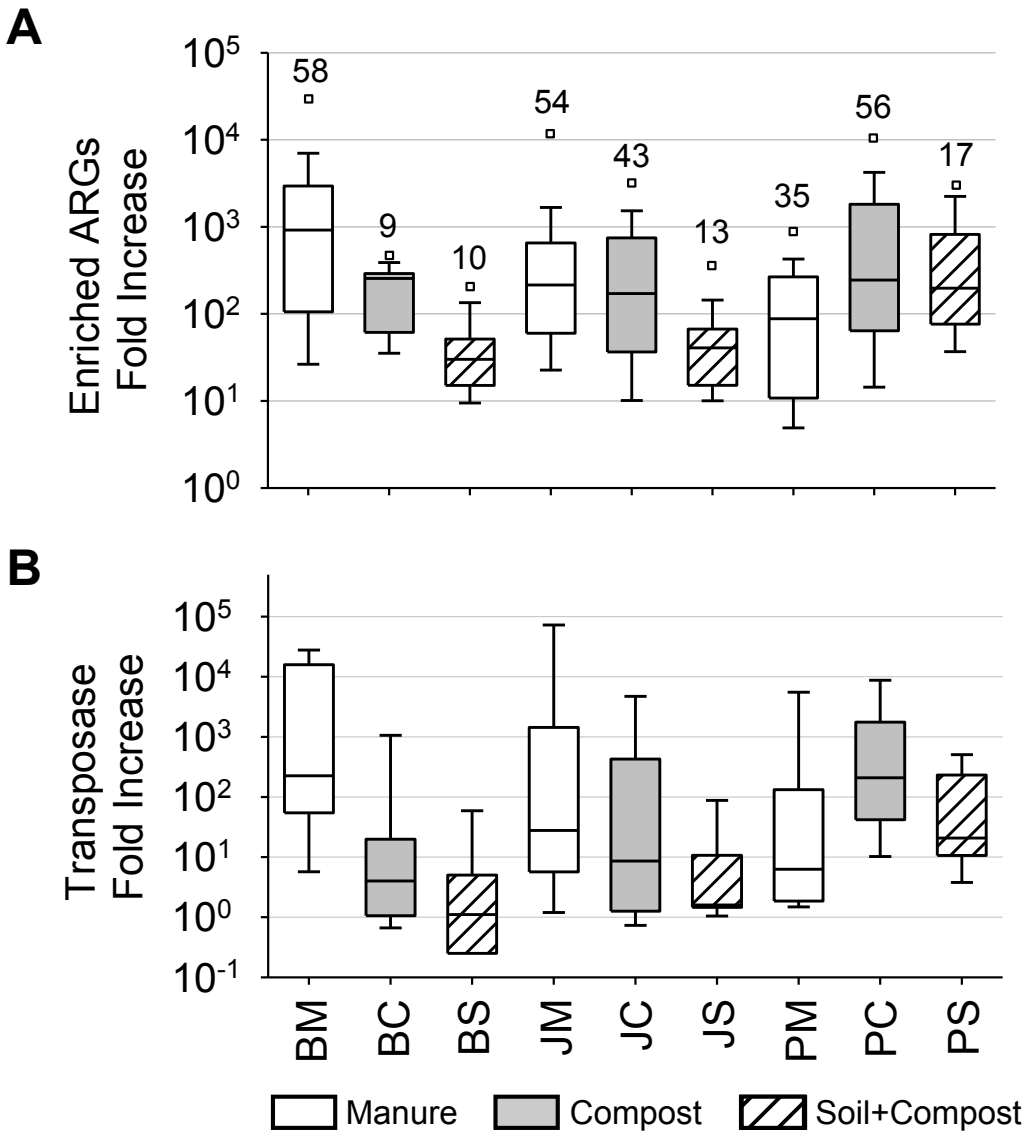
Fig 4.2. Resistance gene profile from the farm sites. Each column is labeled with the sample name (same abbreviation scheme as Fig. 1, with numbers representing field replicates), and

Fig 4.2 (cont'd)

each row is the results from a single primer set. Values plotted are the $\Delta\Delta C_T$ with the control soil being the reference sample for all samples. The legend denotes corresponding fold change values, which is a log scale. All primer sets (223) that showed amplification in at least one sample are shown. Columns were clustered based on Bray-Curtis diversity measures. Black boxes delineate resistance profiles: A) Enriched in all samples, including control manure (CM); B) Enriched in all farm samples, but not the CM; C) Widely enriched in most of the farm samples but not the CM; D) Genes that were enriched in the Putian compost but not the Putian manure; and E) Strongly enriched in CM and farm manures.

Putian farm (57,000-fold enrichment), demonstrating the large expansion of the antibiotic resistance reservoir in these farms, including the enrichment of up to 19 unique *tet* genes in a single site (see Table S4 for enrichment details). A total of 63 unique ARGs were significantly enriched in at least one sample compared to controls at an overall median enrichment of 192-fold for all samples. The maximum enrichment of a single ARG was over 28,000-fold in the Beijing manure (Fig. 3A). In terms of absolute abundance, an aminoglycoside phosphorylation gene *aphA3* is found 43% as frequently as the 16S rRNA gene in the manure samples, based on a 0.58 average value of the delta threshold cycle (ΔC_T) values (Table S3); meaning, this single gene would be found in nearly one in every second bacterium, assuming a single copy of each gene in single genomes. In general, enrichment of individual ARGs decreased in soil samples, but is still elevated with average enrichment of nearly 100-fold, while some genes were enriched over 1,000-fold compared to the soil control. The Putian soil had more unique resistance genes enriched at a higher level than the other two farm soils, which was likely aided by conditions that allowed for positive enrichment of ARGs from the manure to compost stages at Putian. When combining the data from all farms, 56, 44 and 17 unique ARGs were statistically elevated in the manure, compost and soil samples, respectively.

Fig. 4.3


A
Enriched ARGs Fold Increase
10^5
10^4
10^3
10^2
10^1
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58
9
10
54
43
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Fig. 4.3 (con't)

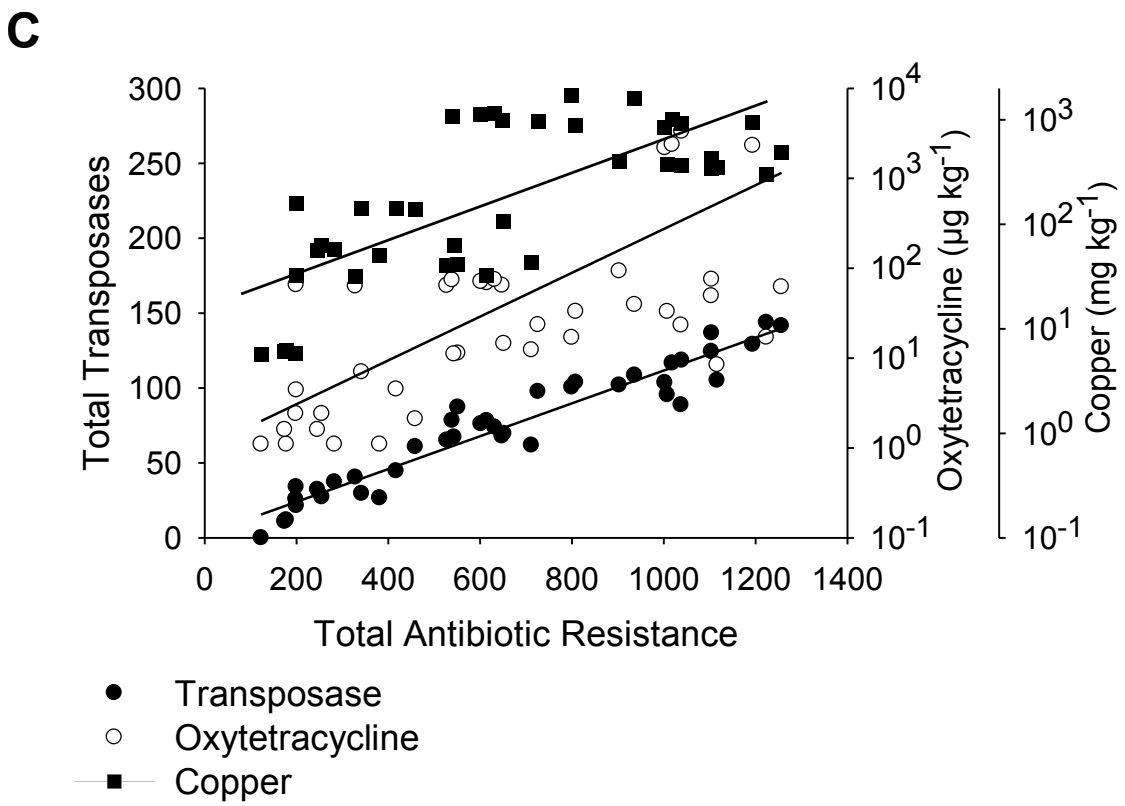

Fig. 4.3. Abundance of resistance genes and transposases. In the box plots, the symbols indicate: box, 25th to 75th percentile; horizontal line, median; whiskers, 10th and 90thpercentile; and square, maximum value. The y-axis is a log scale of fold increase: farm manure compared to the control manure, and farm compost or soil compared to the control soil. A) Only statistically enriched resistance genes are represented. The number above each site indicates the number of primer sets that yielded statistically significant results. B) Summary of all nine primer sets used to target different transposase alleles (in B, top whisker represents the maximum value). C) Correlation of total resistance and transposase abundances, oxytetracycline concentration, and copper concentration. Total antibiotic resistance and total transposases values are the sum of $\Delta\Delta C_T$ values of all assays of that type in each sample. The sample identifiers below panel (B) apply to both panel (A) and (B).

Transposase enrichment. Transposases, in parallel to ARGs, were highly enriched (Fig. 3B). Transposases were found in all samples (Fig. 2, subgroups A and B), and were enriched up to 90,000-fold in the manure samples and up to 1,000-fold in the soil samples. The abundance of ARGs are highly correlated to the levels of transposases in these farm samples (Fig. 3C), e.g. as

high as 0.970 for correlation between the abundance of tetracycline resistance genes and transposase genes (Table S5).

DISCUSSION

Feed additive usage. These swine farms use a complex mixture of growth promoting chemicals, including antibiotics and metals. However, the individual dosage of each chemical, when considered alone, on these farms is not excessive when compared to other farms globally. Total tetracyclines in manure and soil samples were as high as 15.2 mg kg^{-1} and 0.78 mg kg^{-1}, respectively (Qiao et al. 2012), which is within the range reported for some European soils between 2002 and 2005 (Chee-Sanford et al. 2009). However, other farms in China use higher concentrations of antibiotics; for example, tetracycline and sulfonamide concentrations in manure reported previously (Pan et al. 2011) were as high as 764 mg kg^{-1} and 20 mg kg^{-1}, respectively, while in this study their maximum concentrations were only 15 mg kg^{-1} and 5 µg kg^{-1} manure, respectively. On the other hand, the Jiaxing and Putian farms used 13 types of antibiotics, which is close to the estimate of the number of antibiotics used in fisheries along the entire Thai coastline. In addition to antibiotics, metals used as feed additives likely contributed to the complex mixture of resistance determinants in these farms. The metal feed additives zinc, copper, and arsenic were elevated above background concentrations, at levels typical in Chinese swine farms (Shi et al. 2011) and only slightly higher than concentrations reported in the U.S. and Europe (maximum values reported as 1300 – 1550 mg copper kg^{-1} manure (Bolan et al. 2003)). While the concentration of no single growth promotion additive is excessive in these farms, it is the number of additives used that is striking. The effect of mixtures of resistance

determinants is unknown, but presumably increases the likelihood of co-resistance in genetic elements (Gillings et al. 2012).

Enlarged diversity and abundance of the environmental resistance reservoir. This study documents the breadth and extent of the antibiotic resistance reservoir in large-scale animal production facilities. Furthermore, we provide measures to estimate the field-scale response to composting and subsequent soil application representing typical manure management practices in China as a case study. The diverse set of resistance genes detected (Fig. 1) potentially confer resistance to all major classes of antibiotics, including antibiotics critically important for human medicine (WHO, 2009), e.g. macrolides (*mphA, erm* genes), cephalosporins (*blaTEM*, *blaCTX-M*), aminoglycosides (*aph* and *aad* genes), tetracycline (*tet genes*). While a number of vancomycin resistance genes were detected in these farm samples (Fig. 1C), we do not expect significant phenotypic resistance to vancomycin because detection levels were low and resistance is dependant on multigene *van* operons (Arias et al. 2009, D'Costa et al. 2006). However, our detection of individual *van* genes may be an indication that enrichment for *van* operons is possible under alternative conditions. In general, genes potentially conferring resistance to aminoglycosides, tetracyclines, sulfonamide, florfenicol, and quaternary ammonium compounds were enriched most broadly in all farm samples. Beta lactam and macrolide resistance genes were enriched primarily in manure samples, although they may still be present but at levels below detection in the downstream samples. A previous study using a similar qPCR method, sampling only a few individual pigs, detected 57 resistance genes, but only eight were enriched (Looft et al. 2012). D'Costa *et al*. (D'Costa et al. 2006) found resistance to a broad range of antimicrobials, but only considered cultured actinomycete strains. One specific type of resistance studied broadly is that for tetracyclines. In a survey of 14 *tet* genes among hundreds of

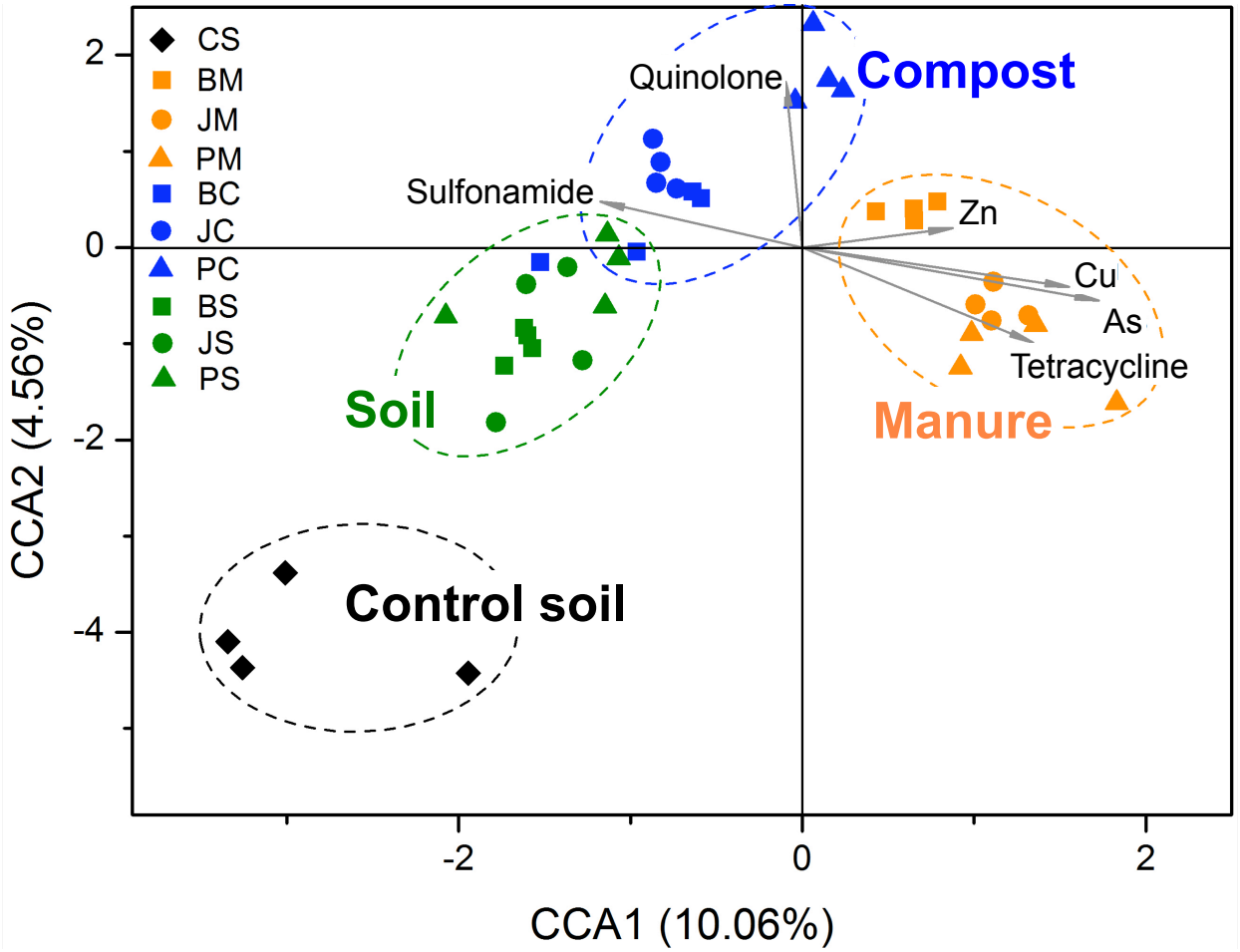

Fig. 4.4. Canonical correspondence analysis (CCA) compares the abundance of detected resistance genes (symbols) and the concentration of heavy metals and antibiotics (arrows). The results showed that pig manure samples were positively correlated to the concentrations of copper, zinc, arsenic, and total tetracyclines. Environmental variables were chosen based on significance calculated from individual CCA results and variance inflation factors (VIFs) calculated during CCA. The percentage of variation explained by each axis is shown, and the relationship is significant (p=0.005). CCA analyses were performed in R 2.13.0 with vegan package 1.17-9.

tetracycline resistant soil isolates, Ghosh and LaPara (2007) found that the most common genes were: *tetL*, *tetA*, *tetM*, and *tetG* (*tetW* was not included in their survey). We detected 22 of the 28 tetracycline resistance genes targeted on our array. The most abundant *tet* genes (based on ΔC_T values, Table S3) in the manure were *tetQ*, *tetW*, *tetX*, *tet*(*32*), *tetO*, *tetM*, *tetL*, *tetG* while in the soil they were *tetG*, *tetL*, *tetA*, *tetW*, the latter set being similar to those found in soil by Ghosh and LaPara (2007). The increased number of resistance genes we detected compared to previous studies reflects our sampling at the herd and field levels and the use of a high-throughput qPCR method of detection.

The resistance genes found in our samples were not limited to the antibiotics administered. Aminoglycosides were not used in the Putian farm, but more than ten aminoglycoside resistance genes were enriched in that farm up to more than 10,000-fold. Similarly, *floR* was enriched 500-fold in the Jiaxing compost but amphenicols were not used at that farm. Co-enrichment of these genes is most likely due to aggregation of resistance genes on mobile genetic elements (Barlow 2009, Heuer et al. 2009, Levy et al. 1976, Looft et al. 2012, Varga et al. 2009), as has been observed directly (Heuer et al. 2012). In addition, the abundance of ARGs in these samples is correlated with the concentrations of antibiotics, as well as with copper, zinc, and arsenic (Fig. 3 and Table S5). Heavy metal presence provides another co-selective pressure for antibiotic resistance (Baker-Austin et al. 2006), and may aid in long-term persistence of ARGs during manure management and disposal (Berg et al. 2010). Only a few multidrug efflux pumps (*qacE*Δ*1* and *dfrA1*) were broadly enriched (Table S6). It seems that genes with specific mechanisms of resistance were preferably enriched in these high selection pressure environments. The diversity of resistance genes enriched and co-enriched at the farm

level is concerning because this broad set of ARGs or a subset thereof could be (co-) enriched or transferred to pathogens under future selection conditions.

Swine farms are known hotspots for pervasive and abundant antibiotic resistance both in antibiotic-free animals (Jackson et al. 2004, Looft et al. 2012) and especially in antibiotic treated animals (Enne et al. 2008, Heuer et al. 2011). The level of enrichment of individual resistance genes is on par with previous field scale studies. Tetracycline resistance genes were enriched 10^2–10^4-fold in cattle waste lagoons (Peak et al. 2007), while the median enrichment of ARGs in this manure was about 10^4-fold. Of bacterial isolates from swine and chicken manure, 92% and >80%, respectively, were found resistant to at least one antibiotic (Enne et al. 2008, Levy et al. 1976). We estimate about 43% of bacteria possess at least the *aphA3* gene, hence it is feasible that upwards of 90% of the entire community would carry one of the other 148 resistance genes detected. Considering all antibiotic resistance genes combined in the manure or compost samples, we estimate a total of 50,000-fold enrichment (Table S4). While enrichment of individual resistance genes is similar to previous studies, we were able to capture a more complete picture of the total level of the antibiotic resistance reservoir.

Potential for horizontal gene transfer of ARGs. This study highlights that ARGs in swine farms are not only diverse, but are also remarkably abundant, which together offers a higher statistical probability of dispersal, further selection and/or horizontal transfer in the environment. The emergence and spread of ARGs are closely associated with mobile genetic elements such as plasmids, integrases and transposases (Binh et al. 2008, Heuer et al. 2012, Zhang et al. 2011). The high degree of transposase enrichment and correlation with ARG abundance suggests that horizontal gene transfer may have aided the enrichment of ARGs. The transposases detected most frequently belong to the IS6 family of insertion sequences, which are typically found

flanking an array of genes, often resistance genes (Mahillon et al. 1998). The most abundant member of the IS6 family in these samples, IS26 has been isolated, along with integrons in multi-drug resistant plasmids in enterobacteria (Miriagou et al. 2005). Integrons most commonly contain resistance cassettes encoding *aadA* genes (Singh et al. 2005), as well as *qacEΔ1* and *sul2*, which were among the most enriched genes in this study. The Putian farm ARGs that are more enriched in the compost than the manure (Fig. 3, "D" boxes, Table S6) are predominately *aadA* and other aminoglycoside resistance genes and their enrichment may be due to their presence in integrons which also hold a resistance gene cassette relevant to the drugs used on the farm (Binh et al. 2009, Heuer et al. 2009). Additionally, the combination of antibiotics and metals may provide a stronger selection for realized horizontal gene transfer within the microbial community than either alone (Baker-Austin et al. 2006, Gillings et al. 2012, Heuer et al. 2009, Petrova et al. 2011). It appears that a number of factors in swine farms could contribute to elevated rates of horizontal gene transfer, including elevated concentrations of antibiotics, metals, ARGs, and mobile genetic elements, making subsequent dispersal, (co-) enrichment, or horizontal transfer, including to human-associated bacteria, more probable.

The role of manure management in controlling ARGs. The long-term goal of manure management is to remove environmental contaminants, while disposing of this high volume waste product and capturing its value to improve soil fertility. The goal in the case of ARGs is to identify practices that decrease their concentration to a greater degree than by simple dilution (Chen et al. 2007). Manure composting decreased the abundance of ARGs at Beijing, but abundance remained nearly the same in the Jiaxing manure, and in Putian, composting actually increased the abundance of ARGs. Composting concentrated sulfonamides (Fig. S1), sulfonamide resistance genes and some metals (Fig. S2), consistent with the observation that

sulfonamide resistance genes are more recalcitrant than tetracycline genes (Dolliver et al. 2008, McKinney et al. 2010, Pruden et al. 2012). The common practice of spreading compost on soil was not sufficient to reduce ARG abundance to background levels, and the Putian soil showed up to 3,000-fold enrichment. However, the practice decreased ARG concentrations substantially below compost levels. The relatively high enrichment of ARGs in Putian soil may be due to higher manure/soil ratio and/or shorter time before sampling after amendment compared to other farms. These observations highlight the need to determine adequate composting time to reduce resistance levels before release to the more uncontrolled environment (Storteboom et al. 2007), as well as to define soil and landscape features that would minimize dispersal to the human food chain.

Resistance gene diversity and abundance patterns specific to each management type indicate the influence of the antibiotics as a selective pressure. These profiles show that generally samples of the same management type clustered together (Fig. 4). The relationships between the structure of detected ARGs and antibiotic and heavy metal concentrations were assessed with canonical correspondence analysis (CCA). Manure samples grouped separately by the first axis and were strongly affected by arsenic, copper and tetracycline concentrations, which are likely among the dominant factors driving the changes in ARG structures on these farms (Fig. 4). Although only three farms are included in this study, regardless of their location (a separation of over 2000 km), composting technique, or antibiotic dosage, the ARGs resistance profiles are similar, indicating that similar ARG reservoirs are likely common across China and in other countries where management practices are similar.

The diversity and abundance of ARGs reported in this study is alarming, and clearly indicates that unmonitored use of antibiotics and metals on swine farms has expanded the

diversity and abundance of the antibiotic resistance reservoir in the farm environment. The co-enrichment of ARGs and transposases further exacerbates the risks of ARG transfer from livestock animals to human-associated bacteria, and then spread among human populations (Marshall et al. 2011, Smillie et al. 2011). Policies and management tools to facilitate prudent use of antibiotics and heavy metals, including their combined use, in animal industries and animal waste management are needed. Decreased resistance levels have been observed in Europe after the disuse of agricultural antibiotics (Aarestrup et al. 2001). Pig manure, with its abundant and diverse ARGs and sheer volume, is a major source of resistance genes and as such a public health hazard. Microbes from manure, compost, or soil containing the ARGs are subject to dispersal via runoff into rivers (Pruden et al. 2012), leaching to subsurface waters, air dispersal via dust, human travel, and distribution of agricultural products, including compost for gardening, which could expand a local contamination to regional and even global scales (Church 2004, Smillie et al. 2011).

MATERIALS AND METHODS

Sampling. A total of 36 samples were collected in 2010 from three Chinese provinces including (from north to south): Beijing (Beijing farm), Zhejiang (Jiaxing farm), and Fujian (Putian farm). The manure and compost samples were obtained from representative swine farms with an animal intensity of 10,000 market hogs or more per year. Soil samples were collected from a nearby agronomic field to which manure-based compost had been applied. Four replicates were taken from each sample type and farm, and all the samples were kept on dry ice during transportation and stored at -80°C before DNA extraction and chemical analysis.

These are typical large-scale swine farms. Pigs are continuously housed on concrete. The manure was sampled within one day after excretion in all cases. In Beijing, compost was managed in outdoor windrows with aeration, for 2 weeks. In Jiaxing, pile composting was used with regular stir (1-2 times per day), for about 10 days. In Putian they used pile composting with limited aeration, for 2-4 weeks. In Jiaxing and Putian, compost products are packed and sold as commercial organic fertilizer for local farmers. For soil amendment, the composted manure spreading rate varies, but is approximately 10 tons / hectare, applied once per year. At the Beijing and Jiaxing farms, the soil had been receiving manure compost for more than 2 years, and the most recent application was 2 months before sampling. At the Putian farm, the soil had been receiving manure compost for more than 3 years, and the most recent application was one week before sampling.

Control samples received no known antibiotic input. The control soil is from a pristine forest in Putian, China. This soil has had no anthropogenic antibiotic input, and has a similar ARG abundance and diversity profile as another temperate region, antibiotic-free grassland soil we studied. The control pig manure samples were mixtures of DNA extracted from feces from pigs birthed from a mother with no antibiotic exposure and grown in facilities with no antibiotic exposure but fed a normal grower diet (see Looft et al. 2012 for further details). Sample CM1 was taken from six 84 day-old pigs not feed antibiotics. Samples CM2-4 were each taken from a single animal at three time points between 86 and 104 days old. The control manure was used as a comparison against the farm manures, while the control soil was used as a comparison against both the farm compost and farm soil.

Antibiotic and metal quantitation. Sulfonamides (SAs) and quinolones (FQs) concentrations were analyzed in this study, including sulfadiazine (SD), sulfamerazine (SM1),

sulfamethoxydiazine (SMT), sulfamethazine (SM2), sulfamethoxazole (SMZ), norfloxacin (NOF), ofloxacin (OF), enrofloxacin (ENR) and ciprofloxacin (CIP). Previously, five target tetracyclines, and ten degradation products were analyzed (Qiao et al. 2012).

Metals were analysed in air-dried, milled samples after oxidative digestion in sealed tubes by inductively coupled plasma-mass spectrometry (Agilent 7500cx, USA). Quantities were determined relative to reference standards. Sample extraction and analysis procedures for both antibiotics and metals are described in supplemental materials.

DNA extraction. High molecular weight community DNA was extracted by the freeze-grinding, SDS-based method (Zhou et al. 1996) and was purified using a low melting agarose gel followed by phenol extraction. DNA concentration and quality was determined with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE).

Primer design. A majority of the primer sets (247) were designed, used, and validated in a previous study (Looft et al. 2012). For this study, 89 new primer sets were designed for categories of resistance genes not previously targeted as thoroughly. The same design parameters were used as before (Looft et al. 2012). Reference sequences were harvested from the ARDB (http://ardb.cbcb.umd.edu/). Additional validation of the primer sets was performed and is described in the supplemental materials.

Quantitative PCR. All quantitative PCR reactions were performed using the Applied Biosystems OpenArray® platform, as described previously (Looft et al. 2012), except that a threshold cycle (C_T) of 27 was used as the detection limit. Generally the technical triplicates were tested during separate testing occasions (plate and day of testing) as a method of quality control. The $\Delta\Delta C_T$ method of comparison (Livak et al. 2001) was used to compare relative abundance between samples:

$$\Delta C_T = C_{T,(ARG)} - C_{T,(16S)} \quad [1]$$

$$\Delta\Delta C_T = \Delta C_{T,(Target)} - \Delta C_{T,(Ref)}, \quad [2]$$

where: C_T is the threshold cycle, ARG is one of the 313 antibiotic resistance gene assays, 16S is the 16S rRNA gene assay, Target is the experimental sample, and Ref is the reference sample. The reference sample used as a comparison depended on the purpose of the analysis. When the purpose was to reveal changes among all farm types and the dynamics or ARGs because of manure management, the control soil was the reference sample for all farm samples, as was the case in Fig. 2. Average C_T values were calculated by averaging the four field replicates. If there was no amplification in one of the four field replicates, it was considered a false negative and discarded. In calculation of the ΔC_T of the reference sample, if there was no amplification, the detection limit C_T (27.0) was used. Genes were considered statistically enriched if the range created by three standard deviations of the mean fold change was entirely > 1.

Acknowledgments. We thank Benli Chai and Amanda Geaslin for technical assistance. Funding for Y.G.Z. was provided by the Ministry of Science & Technology of China (2009DFB90120 & 2011DFB91710) and at Michigan State University by their Pharmaceuticals in the Environment Initiative.

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CHAPTER V

GENE-TARGETED METAGENOMICS OF PUTATIVE DIBENZO-*P*-DIOXIN DEGRADING ANGULAR DIOXYGENASES

ABSTRACT

Novel primer sets were designed to target angular dioxygenases with potential to degrade dioxins, the most toxic and persistent pollutants. Dibenzo-*p*-dioxin, dibenzofuran and carbazole dioxygenase targeted metagenomics elucidated that current understanding of these gene families underestimates actual diversity in nature, both in human-contaminated and pristine environments.

INTRODUCTION

Aromatic hydrocarbons comprise a chemically diverse class of organic compounds that include monoaromatics (e.g. benzoate), biphenyls, polycyclic aromatics (e.g. naphthalene), and more toxic oxygen-linked aromatics (e.g. dibenzofuran, and dibenzo-*p*-dioxin). Polychlorinated dibenzo-*p*-dioxins/furans (PCDD/Fs) are released into the environment from a variety of sources including: combustion, incineration, pulp and paper manufacturing, pesticides, and some natural sources (Kulkarni et al. 2008). Anthropogenic release of dioxins has resulted in contaminated soil that need treatment (U.S. Congress 1991). However, even pristine rural soils with low human impact have detectable levels of dioxins at low concentrations (1 - 1000 $\mu g\ kg^{-1}$) (Schaum et al. 2007).

Gene-targeted amplicon sequencing offers an important alternative to culturing individual isolate strains to identify novel biodiversity, as was done with biphenyl dioxygenase (Iwai et al. 2010). We have progressed to characterize dioxygenases with activity toward problematic dioxin chemicals. Microbial degradation of dioxins has been studied, but there are few tangible results, especially in terms of isolated degraders. Other than the well characterized (chloro)dioxin degrading strain *Sphingomonas wittichii* RW1 (Wittich et al. 1992), only one other strain has been reported to use dibenzo-*p*-dioxin as a sole carbon and energy source: *Pseudomonas veronii*

PH-03 (Hong et al. 2004). Angular dioxygenation adds molecular oxygen to the two carbons adjacent to the ether oxygen and results in catechol and a six-carbon aliphatic acid (Field et al. 2008). Three angular dioxygenases are known to catalyze the first step of this pathway: dioxin dioxygenase (*dxnA1*), dibenzofuran dioxygenase (*dbfA1* or *dfdA1*), and carbazole dioxygenase (*carAa*) (Field et al. 2008). A significant number of PCR primers exist to probe samples for aromatic ring-hydroxylating dioxygenase genes (ARDHs), including a quantitative PCR primer specific to only *Sphingomonas* sp. RW1 *dxnA1* (Hartmann et al. 2012), but no previously published primer set suitable for amplicon sequencing targets dioxygenases active toward dioxins specifically, without targeting other dioxygenases that do not have activity against dioxins (Iwai et al. 2011).

MATERIALS AND METHODS

A manual search of the GenBank database was performed to harvest reference sequences related to angular or dioxin or dibenzofuran or carbazole and dioxygenase. An initial analysis based on amino acid similarity of these reference sequences showed that these genes formed three groups within the superfamily of Rieske dioxygenases. These three groups are represented by the following genes (Table 1): group 1: dioxin 1,10a dioxygenase (*dxnA1*) and dibenzofuran 4,4a dioxygenase (*dfdA1*), group 2: dibenzofuran 4,4a dioxygenase (*dbfA1*), and group 3: carbazole 1,9a dioxygenase (*carAa*). The DDBJ/EMBL/GenBank non-redundant protein database was searched using Hidden Markov Models built from all reference sequences from each cluster with HMMER (Eddy 2009). The results were obtained from the December 2010 release of FunGene (http://fungene.cme.msu.edu). An HMM bits saved score cutoff of 700 was used, and no

Table 5.1: Reference sequences used in primer design, PCR validation of primer specificity and designation of reference sequences in clusters with obtained environmental sequences. Positive control column indicates if the strain DNA was used in PCR validation of primer specificity. PCR validation column indicated which primer set produced an amplicon with that strain. References listed detail the activity of the strain toward dioxins. * *Rhodococcus* sp. RHA1 produced only a faint band with the dbfA1 primer set.

Cluster no.	Organism (Protein ID)	Primer group	Positive control	PCR validation	Reference
d1	*S. wittichii* str. RW1 (CAA51365)	1	Y	dxnA1	(Wittich et al. 1992)
d2	*Terrabacter sp.* YK3 (BAC06602)	1			(Iida et al. 2002)a
d2	*Nocardioides* sp. DF412 (BAG06223)	1	Y	dxnA1	(Miyauchi et al. 2008)
d2	*Rhodococcus* sp. HA01 (ACC85677)	1			(Aly et al. 2008)
d2	*Terrabacter* sp. YK1 (BAG80728)	1			(Iida et al. 2002)a
d2	*Rhodococcus* sp. YK2 (BAG80733)	1	Y	dxnA1	(Iida et al. 2002)b
d3	*Bacillus tusciae* DSM 2912 (YP_003590130)				
d3	*Terrabacter* sp. DBF63 (BAB55886)	2	Y	dbfA1	(Kasuga et al. 2001)
d3	*Rhodococcus* sp. HA01 (ACC85681)	2			(Aly et al. 2008)
d3	*Sphingomonas* sp. LB126 (ABV68886)	2			(Schuler et al. 2008)
d3	*Paenibacillus* sp. YK5 (BAE53401)	2	Y	dbfA1	(Iida et al. 2006)
d3	*Rhodococcus* sp. YK2 (BAC00802)	2	Y	dbfA1	(Iida et al. 2002)b
d3	Rhodococcus sp. DFA3 (BAD51811)		Y	dbfA1	(Noumura et al. 2004)
d4	*Sphingomonas* sp. CB3 (AAC38616)	1			(Shepherd et al. 1998)
NA	*Sphingomonas* sp. KA1 (YP_718182)	2			(Urata et al. 2006)
c1	*Sphingomonas* sp. KA1 (YP_717942)	3	Y	carAa	(Urata et al. 2006)
c1	*Sphingomonas* sp. JS1 (ACH98389)	3			
c1	*Sphingomonas* sp. KA1 (YP_717981)		Y	carAa	
c1	*Sphingomonas* sp. XLDN2-5 (ADC31794)				
c4	*Pseudomonas stutzeri* OM1 (BAA31266)	3	Y	carAa	Ouchiyama et al. 1998)
c4	*P. resinovorans sp.* CA10 (NP_758566)	3	Y	carAa	Ouchiyama et al. 1993)
c4	*Janthinobacterium* sp. J3 (BAC56742)	3	Y	carAa	(Nojiri et al. 2005)
c4	*Pseudomonas* sp. XLDN4-9 (AAY56339)	3			(Li et al. 2004)
c4	carbazole-degrading bacterium CAR-SF (BAG30826)	3			(Fuse et al. 2003)
c4	*Pseudomonas* sp. K23 (BAC56726)				
c5	*Nocardioides* sp. IC177 (BAD95466)	3			(Inoue et al. 2005)
NA	*Burkholderia xenovorans* LB400 *bphA1*	none		none	
NA	*Rhodococcus* sp. RHA1 *bphA1*	none		dbfA1*	

additional sequences were obtained through this search, which reinforces the low number of sequenced (or cultured) strains with activity toward dibenzo-*p*-dioxin. Degenerate primers were designed from amino acid consensus regions (Table 2). No non-specific microbial genes are targeted by the primer sets (when allowing no mismatches), as determined by an NCBI nucleotide BLAST.

The specificity of the primer sets was determined experimentally. DNA was obtained from positive control cultured organisms. An average of four positive controls were used for each primer set and the correct length amplicon was observed with 100% frequency. Positive controls from one cluster were then used as template with primers targeting other clusters as negative controls for those clusters. Two organisms with biphenyl/toluene dioxygenases (*Burkholderia xenovorans* str. LB400, and *Rhodococcus* sp. RHA1) were also used as negative controls. With the exception of a single weak amplification out of 42 total validation reactions, the primers were specific to only the gene cluster for which they were designed and did not produce amplicons from the closely neighboring gene clusters (Table 1). These results indicate that these primers are extremely specific to the targeted reference sequences.

The above primer sets were used in gene-targeted metagenomics as has been done previously targeting other genes (Iwai et al. 2011a, Iwai et al. 2010, Vital et al. 2013). Two environmental samples were chosen as template DNA: a well characterized polychlorinated biphenyl (PCB)-contaminated rhizosphere (Leigh et al. 2007), and a pristine Kansas prairie soil (KS), from the Konza Prairie (39°05'N, 96°35'W). We suspect that both these soils are contaminated with polyaromatic hydrocarbons, and likely low levels of dioxins, due to industrial activity (in the case of the PCB-rhizosphere sample) or with sequencing adapters, 8 base oligo multiplex sequencing barcodes, together with the gene specific sequence. PCR conditions with

Table 5.2. Primer sequences and PCR conditions of the three primer sets. These PCR conditions were optimized for the soil samples described. The target positions described are for reference amino acid sequences: * position based on *Sphingomonas wittichii* RW1, *dxnA1*, and † position based on *Sphingomonas* sp. KA1, *carAa*. ‡ annealing temperature.

Primer Set	Target position	Sequence (5' - 3')	Ta‡ (°C)	Primer conc. (µM)	Mg^{2+} conc. (mM)
dxnA1/dfdA1	145-150*	TACAAVGGGCTGRTTTTCGG	51	1.2	4
	312-307*	GARAAVTTVGGGAACAC			
dbfA1	205-210*	GGCGACGACTAYCACGTGCT	51	0.8	3.5
	373-368*	TCGAAGTTCTCGCCRTCRTC			
carAa	69-74†	TGCCTNCAYCGHGGBGT	63	0.8	2.5
	268-263†	TTSAGHACRCCBGGSAGCCA			

Table 5.3. Obtained sequences statistics. A mock community (MC) was composed of the strains used in validation of primer specificity (Table 1) and yielded the correct sequences and are not described further.

Primer set	Sample name	Match barcode	Passed initial processing	Contains frame-shift	Passed Frame-Bot	Avg. length
dxnA1/ *dfdA1*	Rhizosphere	2319	2095	456	641	389
	KS	2844	2521	451	690	375
	Environmental sample total	5163	4616	907	1331	
	MC	1247	1204	626	1204	450
carAa	Rhizosphere	720	543	128	193	460
	KS	673	594	194	339	465
	Environmental sample total	1393	1137	322	532	
	MC	612	501	340	500	476

sequencing adapters, 8 base oligo multiplex sequencing barcodes, together with the gene specific sequence. PCR conditions were optimized (Table 2). PCR products were prepared as described previously (Iwai et al. 2010) and mixed with other samples for pyrosequencing (Roche 454 GSFLX Titanium Sequencer). No amplicons were obtained using the *dbfA1* primer set. Raw reads were filtered through barcode matching and quality filtered using the Ribosomal Database Project II (RDP-II) Pyro Initial Process tool (Cole et al. 2009) using the following parameters: forward primer maximum mismatches: 2, and minimum length: 300. Because many reads were not read through the reverse primer, the reverse primer filter and quality score filter were not applied at the initial filter stage. Reads passing the initial filters were aligned, frameshift corrected, and translated into protein sequences using the RDP FrameBot tool. The FrameBot reference set was obtained from the FunGene repository using a manual selection for genes related to polyaromatic dioxygenases in general. A broad diversity of reference genes was selected to assure that the correct reading frame to the obtained sequences would be found even if the obtained sequence was outside the intended target range. Protein reads with a length greater than 100 amino acids and ≥ 30% identity to the nearest reference sequence were for further analysis (Table 3). Quality filtered protein sequences and corresponding reference amino acid sequences were aligned by HMMER and trimmed to same length. The combined sequences were clustered using a 0.5 identity cut-off, using the RDP mcClust tool on the FunGene site. Clusters not containing a reference sequence were considered novel clusters (Iwai et al. 2010). The 0.5 identity cutoff was chosen because it is about at this distance that reference sequences were clustered to determine primer design groups. One representative sequence from each cluster was selected using the representative sequence tool on the RDP FunGene pipeline. These sequences were used to construct a nearest neighbor-joining tree using MEGA 5.1 software.

RESULTS AND DISCUSSION

The obtained sequences (1863 total) revealed the *in situ* diversity of putative dioxygenases, similar to those that have been shown to degrade dioxins (Fig. 1). The majority of *dxnA1*/*dfdA1* sequences formed novel clusters. Many clusters were shared in both the KS soil and the rhizosphere soil. However, the dominant *dxnA1*/*dfdA1* clusters differ between sites. Two clusters (clusters d1, d5; Fig 1A) comprise 68% of sequences in the KS sample while these same clusters only represent 4% of the rhizosphere sequences. Notably cluster d1 contains the reference sequence from (chloro)dioxin oxidizing *Sphingomonas wittichii* str. RW1 and represents nearly 10% of the KS soil angular dioxygenase gene community. This indicates that genes similar to this important dioxygenase may be present in this prairie soil. The rhizosphere sample was dominated (49% of sequences) by two clusters (d6 and d7), while these same clusters only

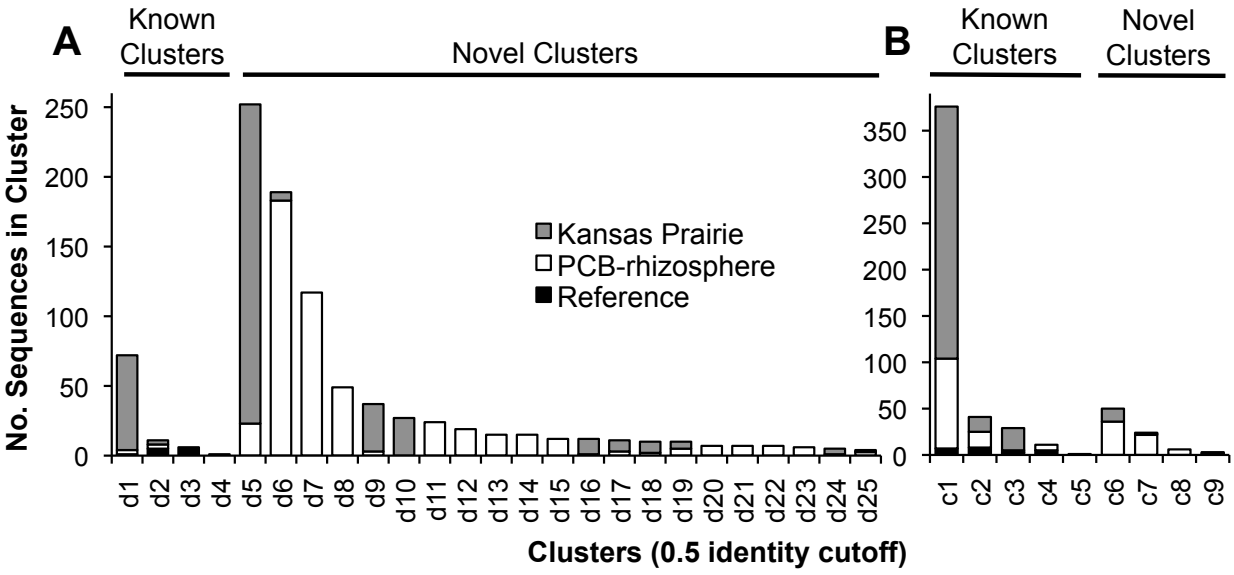

Fig. 5.1. Results of clustering obtained sequences with the reference sequences. (A) Results using the *dxnA1*/*dfdA1* primer set. (B) Results using the *carAa* primer set. Clusters are only shown that contained at least four sequences. There were an additional 12 clusters that contained two or three sequences.

represent 1% of the KS sequences. These data show site-specific populations composed of novel dioxygenases. The specificity of the primer sets was again confirmed because reference sequences used to design the *dbfA1* primer set did not cluster with any of the obtained sequences using the *dxnA1*/*dfdA1* primer set (using our 0.5 similarity cutoff). Similarly, many of the obtained sequences, including the most abundant novel cluster, formed a clade on the same branch as *dxnA1* and *dfdA1* (Fig. 2). Other sequences were more distantly related to this clade and their function is less predictable. While a majority of *carAa* sequences clustered with reference sequences at 50% similarity as is shown (Fig. 1B, cluster c1), similar ecological trends, including site specific populations, were also observed with the *carAa* obtained sequences. The *carAa* obtained sequences cluster separately from the reference sequences at 70% similarity cutoff.

A consensus amino acid sequence of obtained sequences was compared to an aligned consensus sequence of reference sequences, and each searched for conserved amino acids. A known iron-binding motif ($DX_2HX_{3\text{-}4}H$ (Nojiri et al. 2005), where X is any given amino acid) was observed in >95% of sequences of both *dxnA1*/*dfdA1* and *carAa* obtained sequences (Fig. 3). In addition, another conserved motif (>95%), NWK or NWR, was observed and is shown in Fig. 3. Although no associated function could be found in the literature regarding a role of the NW(K/R) motif, it appears essential to the protein due to its high conservation. It is possible that the NW(R/K) motif plays a role in positioning of the substrate binding amino acid. In the case of *carAa*, Gly-178 is implicated to hydrogen bond to carbazole and the NWR motif is situated on the same alpha helix as Gly-178 (Nojiri et al. 2005). The identity of the third amino acid of this motif is specific to each group

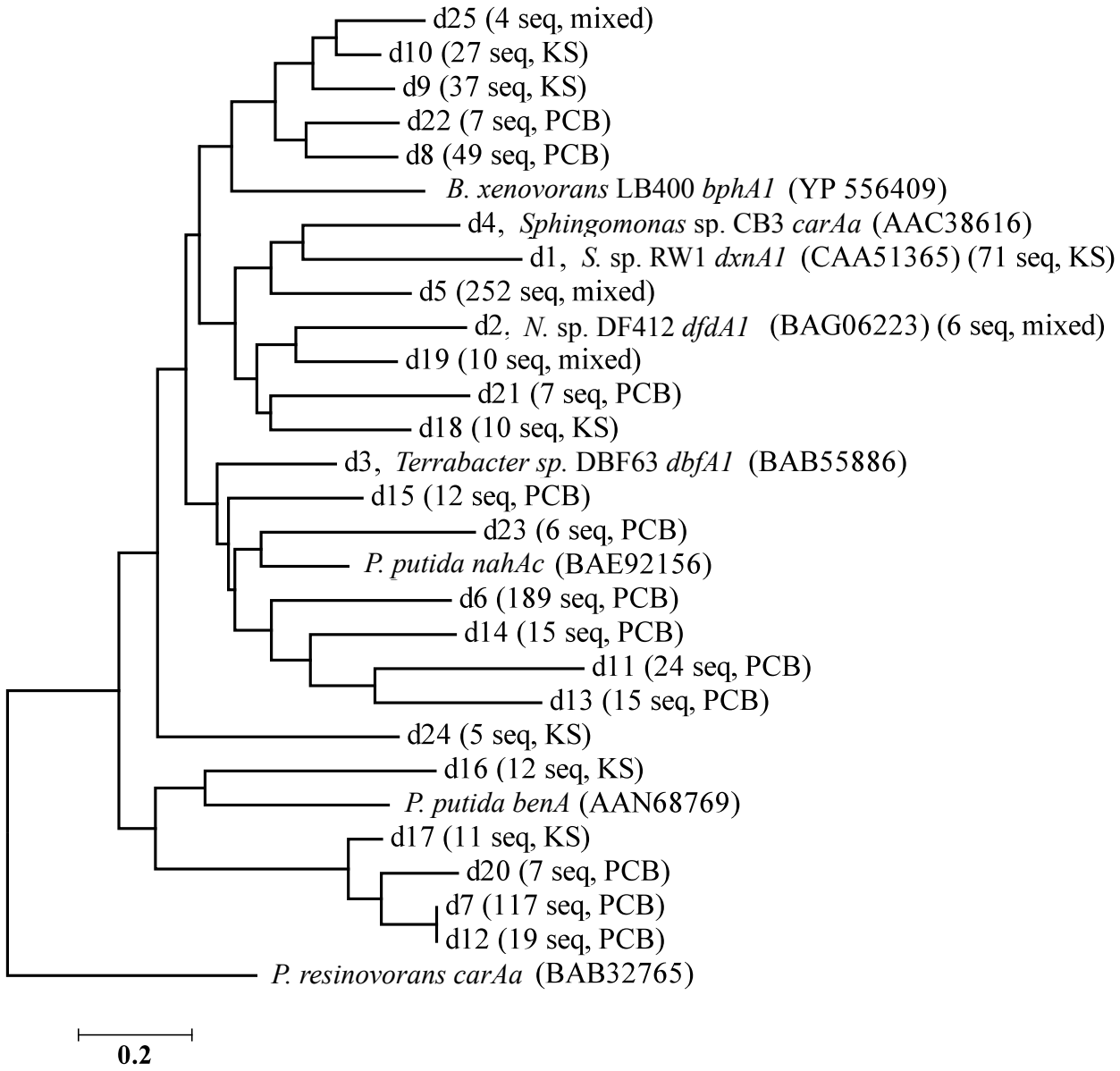

Fig. 5.2. Nearest neighbor-joining tree of the representative sequences of each cluster shown in Fig. 1. Branch names designate: cluster name (from Fig. 5.1), name and accession number of reference sequence in that cluster (if applicable), number of obtained sequences from pyrosequencing, and the predominate sample from which the sequences originated.

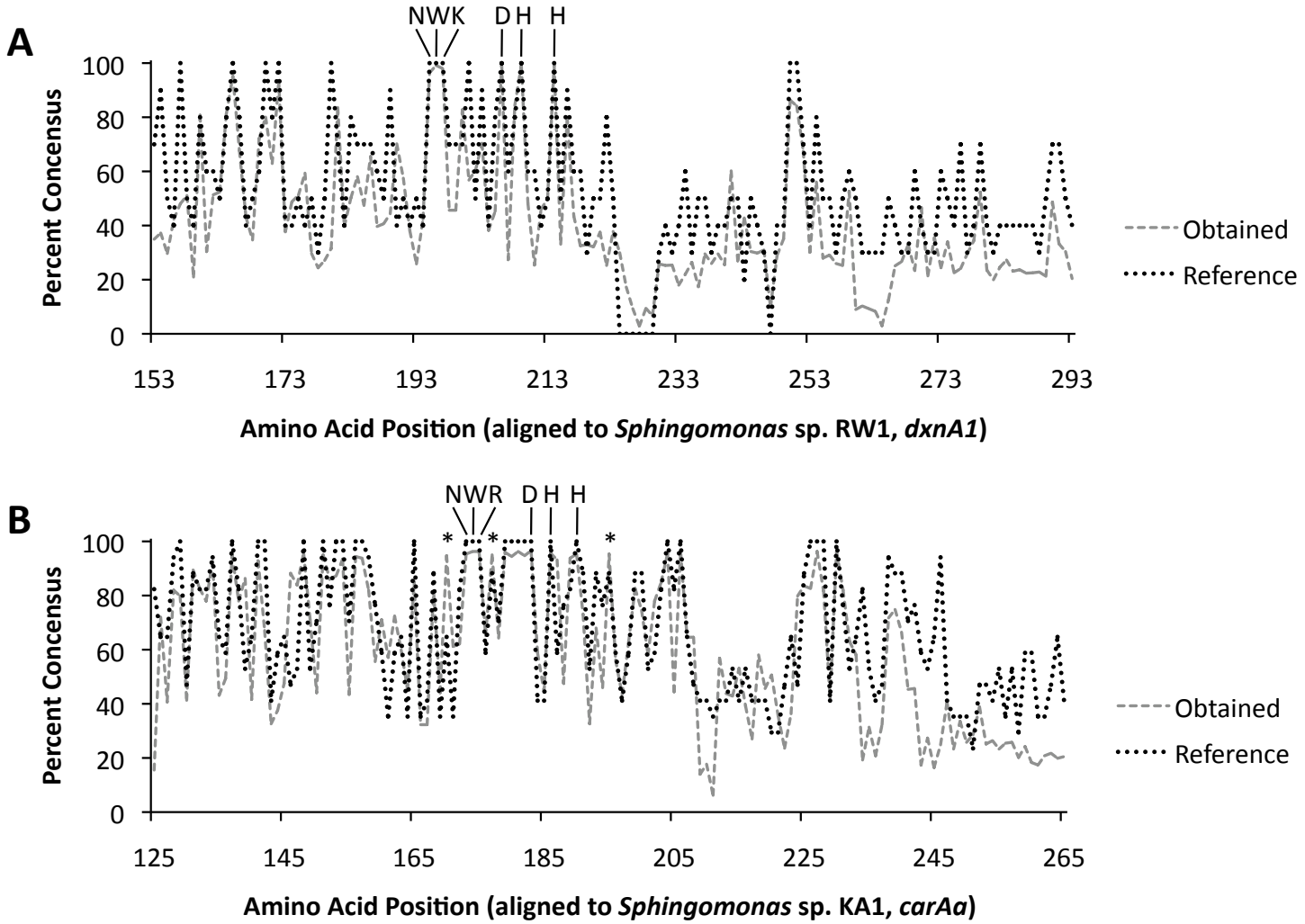

Fig. 5.3. Percent conservation of translated obtained nucleotide sequences to protein sequences. (A) Results using the *dxnA1*/*dfdA1* primer set. (B) Results using the *carAa* primer set. Key conserved amino acid positions are indicated. The $DX_2HX_{3\text{-}4}H$ iron-binding site is indicated as well as the uncharacterized, yet highly conserved NW(K/R) motif. The asterisk (*) indicates positions for which obtained sequences were conserved at a higher rate than reference sequences.

While some recent advancements have been made in dioxin degradation with previously isolated strains, especially *S. wittichii* str. RW1 (Nam et al. 2006), progress in isolating novel dioxin degrading strains has been slow for several decades (Field et al. 2008). In the neighboring biphenyl dioxygenase clade, despite having many more degrader strains isolated, gene-targeted metagenomics still revealed extensive novel diversity (Iwai et al. 2010). This study reveals a number of novel dioxygenase sequence clusters of intermediate sequence similarity between the *dxnA1* and *dfdA1* genotypes. This reveals that there is likely a continuum of genetic diversity between these two relatively distinct but functionally similar groups. According to obtained dioxygenase sequences, the majority of putative dioxin degraders in these communities, have no cultured representatives and their diversity, in terms of number of clusters, far exceeds that of known degraders, as was previously found for *bphA1* diversity (Iwai et al. 2010).

Acknowledgements. We thank Dr. Gerben Zylstra for his suggestions and guidance, Dr. Hideaki Nojiri and Dr. Keisuke Miyauchi for providing reference strains of previously isolated dibenzofuran degraders, and Hyung-Inn and Jihee Lee for technical assistance. This work was supported by Superfund Research Program grant P42 ES004911-20 from the U. S. National Institute of Environmental Health Sciences.

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CHAPTER VI

ISOLATION AND CHARACTERIZATION OF A NOVEL DIBENZOFURAN DEGRADING CONSORTIUM FROM A PRISTINE PRAIRIE SOIL

ABSTRACT

Dioxin chemicals, including furans, are U.S. Environmental Protection Agency priority pollutants to be removed from the environment. Only a few dioxin-degrading microorganisms, enzymes and pathways have been reported. We isolated a novel dibenzofuran-degrading consortium from a native prairie soil. Growth of the consortium is accompanied simultaneous disappearance of the substrate, indicating mineralization of the latter (6 h doubling time). The minimum required membership of the consortium is currently being defined, but there is some evidence to indicate that it is composed of three phyla: Firmicutes (*Paenibacilles* sp.), Proteobacteria (unclassified *Comamonadaceae* sp.), and Actinobacteria (*Agromyces* sp.). The consortium has grown together stably during several serial transfers. When the members were cultured separately with dibenzofuran, no growth was observed. Electron microscopy confirmed the presence of three cell types surrounding dibenzofuran crystals. The consortium degrades dibenzofuran by the traditional degradation pathway described elsewhere. Whole genome sequencing of the unclassified *Comanomadaceae* strain revealed genes potentially involved in initial dioxygenation of dibenzofuran, and salicylate degradation, but lacks genes required for 2-oxopent-4-enoate, which gene is provided by *Agromyces* sp. from the consortium. The consortium cometabolizes dibenzo-p-dioxin, 2-monochlorodibenzo-p-dioxin, as well as 2,3-dichlorodibenzo-p-dioxin. A number of studies imply that degradation of environmental pollutants occurs via consortia action, and this is direct evidence of this mechanism for degradation of the difficult to biodegrade angular polyaromatic ether hydrocarbons.

INTRODUCTION

Polyaromatic hydrocarbons are a class of organic compounds that include polychlorinated dibenzo-*p*-dioxins (DD) and dibenzofurans (DF). Environmental contamination of polyaromatic hydrocarbons poses a challenging problem (Yoshida et al. 2005) because of their toxicity and persistence (Field et al. 2008). Furthermore, certain members of this class of chemicals are included on the EPA priority list as potentially dangerous or carcinogenic to humans (Mandal 2005), and bioaccumulate in the food chain (Van den Berg et al. 1998). Polychlorinated dibenzo-*p*-dioxins/furans (PCDD/Fs) are released into the environment by: combustion of chlorine containing organic materials, pulp and paper manufacturing, pesticides, and natural sources (Chang 2008, Kulkarni et al. 2008). While soils contaminated with dioxins are mandated to be remediated (U. S. Congress, 1991), even rural soils have detectable levels of dioxins at low concentrations (1 – 1000 ppt) (Schaum et al. 2007). Currently, only physical and chemical processes are practiced to remediate contaminated soils and sediments (Field et al. 2008). Bioremediation is under consideration as a technique to sustainably remove or detoxify dioxins.

The fate of dioxins in the environment is not well understood, and there are large discrepancies in the estimated atmospheric deposition, and the amount measured in soil (Baker et al. 2000). Additionally, an EPA survey of dioxin levels in air and soil samples from 29 sites showed a slight correlation between total organic soil carbon and some PCDD/F congeners (r^2 = 0.51), and that octachlorodibenzo-*p*-dioxin is the most abundant PCDD/F congener at all 29 sites. The relative proportion of octochlorodioxin compared to other PCDD congeners increases in soils compared to air samples, while the relative proportion of all other congeners decreases (Schaum et al. 2007). It appears some processes occur within the soil matrix that causes a shift in

the relative proportion of PCDD/F congeners, which likely includes biological metabolism. Thus, we sought to isolate novel dioxin degrading organisms from natural soils with a history of burning of organic residues.

Microbial degradation of dioxins has been studied, and a few strains have been isolated capable of degrading DD or DF as the sole carbon and energy source (Miyauchi et al. 2008, Wittich et al. 1992). These strains were generally isolated from industrial areas with human contamination of polyaromatic hydrocarbons. However, the diversity of the initial enzymes of the degradation pathways from previous isolates is low, comprising three tight clusters, represented by: *Sphingomonas wittichii* str. RW1 (*dxnA1*), *Nocardioides* sp. DF412 (*dfdA1*), and *Paenibacillus* sp. YK5 (*dbfA1*) (Field et al. 2008, Iwai et al. 2011). To dibenzofuran, these angular dioxygenases add oxygen to the two carbons adjacent to the ether oxygen, which results in the cleavage of the ether bridge (Chang 2008, Field et al. 2008) and the formation of 2,2',3-trihydroxybiphenyl, which is later cleaved forming salicylic acid and 2-oxopent-4-enoate. Here, we report the isolation and characterization of a novel dibenzofuran-degrading consortium. It is able to cometabolize mono- and dichlorodibenzo-*p*-dioxins as well as unchlorinated dibenzo-*p*-dioxin.

MATERIALS AND METHODS

Chemicals and media. All chemicals used in this study were of the highest purity available. Growth substrates were purchased from the following manufactures: dibenzofuran (Sigma-Aldrich; St. Louis, MO, USA), dibenzo-*p*-dioxin (Wako Chemicals; Richmond, VA, USA), 2-MCDD and 2,3-DCDD (AccuStandard, Inc.; New Haven, CT, USA). Two mineral media used which have been described previously, K2 (Zaitsev et al. 1991) but substituting 20 ml l^{-1} Hunter's Mix in place of yeast extract, and MM4Y (Iida et al. 2006). Dilute undefined media,

10% Luria-Bertani (AccuMedia; Neogen, Lansing, MI, USA) and 20% R2A (Himedia, Mumbai, India), were prepared according to manufacture's instructions except for the dilution factor. Solid media was prepared with 15 g L^{-1} Agar Select (AccuMedia; Neogen, Lansing, MI, USA).

Enrichment and isolation of DF, DD and 2-MCDD degrading bacteria. Soil samples were taken from protected pristine prairie sites in Morris Prairie, Jasper County, Iowa, USA (41.768, -92.963) and Konza Prairie Kansas, USA, as well as an Amazonian black earth soil, or terra preta. All soils were enriched for degraders by using either K2 or MM4Y media, either DF, DD or 2-MCDD in duplicate serial transfers for every condition. The first step of enrichment was a 20% (by weight) soil slurry in fresh medium and carbon source, and subsequently 10% culture transfer into fresh medium and carbon source. All enrichment cultures were in bottles (Wheaton; Millville, NJ, USA) with Teflon-lined lids allowing for at least 10X headspace volume and were opened every 48 hours for 10 min in a laminar flow hood to refresh O_2 content. The timing of the transfer depended on observation of depletion of the substrate in separate bottles, but generally was at least 30 d. The Iowa soil was incubated at 22 °C, while the Kansas and black earth soils were incubated at 30 °C, all with continuous shaking (200 rpm). Pure cultures were obtained by plating onto the corresponding solid medium with DF crystals on the lid of the dish as the carbon source. Individual colonies were picked, serially transferred from solid to liquid medium, and tested for their ability to deplete the carbon source in liquid culture.

Degradation experiments. Degradation experiments of DF were carried out in 25 ml vials with an aluminum sealed lid containing 2.5 ml of K2 medium. Liquid cultures with DF as the sole carbon source were prepared by adding the growth substrate dissolved in acetone (50 or 100 mg ml^{-1}) to empty flasks, allowing the acetone to evaporate in a laminar flow hood for 5 h, adding the mineral media, and sonicating the flask for 30 s using a FS20 mechanical ultrasonic cleaner

(Fisher Scientific, USA) in order to disperse substrate crystals. Tubes were incubated at 30 °C on a reciprocal shaker at 200 rpm. Bacterial growth on using DF as a sole carbon source was confirmed by measuring optical density, cell concentration (CFU ml^{-1}), and substrate depletion. Three vials were dedicated to more frequent optical density measurements, while a series of tubes were dedicated to measure optical density, cell concentration, and the vial was sacrificed to determine substrate depletion. A resting cell assay was used to test for the range of substrates that could be degraded #3-21. Cells were grown, concentrated to an optical density of 1.5 with fresh media, and aliquoted to a series of 7.5 ml vials with Teflon lined lids. The substrates (DF, DD, 2-monochlorodibenzo-*p*-dioxin, and 2,3-dichlorodibenzo-*p*-dioxin), dissolved in acetone, were added to the cells directly, in a volume of 1 or 2 µl, without removal of acetone. In parallel, cultures with autoclaved cells were established in the same manner. Each condition was tested in triplicate and data are shown as the mean of the experimental triplicates with error bars indicating the standard error.

Chemical extraction and analysis. Vials were sacrificed in order to extract residual substrate. The substrates were extracted using a method modified from Halden et al. (Halden et al. 1999) by first acidifying the culture, addition of an internal control polyaromatic (biphenyl or 2-monochlorodibenzo-*p*-dioxin), then extraction with acetonitrile, and agitation (60 min, 200 rpm). Particles were allowed to settle (10 min), and the supernatant was passed though a PTFE (0.2 µm). Aliquotes were analyzed by high-pressure liquid chromatography using a Perkin Elmer 200 Series equipped with a Supelco Discovery C-18 column (4.6 by 150 mm; particle size, 5µm) and a UV/Vis detector set to 220 nm. Mobile phase was 85% acetonitrile, 15% deionized water. An injection of 50 µl was by autosampler. Obtained peak areas and retention times were compared to known concentrations of standard chemicals.

To isolate DF metabolites, #3 cultures were harvested at different growth phases, acidified with HCl to pH 1, extracted with 1 ml of ethyl acetate, similar to that done previously (Peng et al. 2012). The organic phase was dried in 1.8 ml polypropylene tubes. The residue was dissolved in 100 µL of acetonitrile. The sample was derivatized with 50 µL of MSTFA (N-methyl-N-(trimethylsilyl) trifluoroacetamide), inverted, vortexed, sonicated (5 min), and immersed in a 60 °C water bath for 1 hour. A positive control sample (gentisic acid) and solvent blanks of acetonitrile and acetonitrile/MSTFA underwent this process as well. The reacted samples were then transferred into autosampler vials as above and submitted to analysis by GC-MS. Samples were analyzed on an Agilent 6890N GC coupled to an Agilent 5973 mass spectrometer. A DB5-MS capillary column was installed in the GC (30 m length x 0.250 mm ID x 0.250 µm film thickness). The GC inlet temperature was 250 °C, the He carrier flow rate was 1.0 mL/min, the m/z range was 30-600, and the oven ramp was as follows: 70 °C (hold for 1 minute), then 7 °C/min to 300 °C.

DNA extraction and 16S rRNA sequencing. Consortium and individual strain DNA was extracted using the MoBio Utraclean Soil DNA isolation kit following the manufacture's instructions, with the addition of a incubation for 5 min at 65 °C prior to bead beating. The 16S rRNA gene was amplified in 25 µl reactions containing 2mM $MgCl_2$, 100 µg/ml bovine serum albumin, 2 U *Taq* DNA high fidelity polymerase (Roche), 200 µM deoxynucleoside triphosphates, 400 µM forward and reverse primers (8F: 5'-AGA GTT TGA TCC TGG CTC AG-3' (Ley et al. 2006) and 1522R: 5'-AAG GAG GTG ATC CAR CCG CA-3' (Zhang et al. 2002)). Amplicons were extracted from a 1% agarose gel (QIAquick Gel Extraction Kit; Qiagen, Valencia, CA, USA) and purified (QIAquick PCR Purification Kit; Qiagen). Purified amplicons

Table 6.1. Enrichments of interest that showed some level of substrate depletion.

Starting soil	Medium	Substrate	% removal	Time
Iowa prairie	MM4Y	DF	74%	30 d
Terra preta	K2	DF	50%	30 d
Terra preta	MM4Y	DF	70%	30 d
Iowa prairie	MM4Y	DD	24%	90 d
Terra preta	MM4Y	DD	70%	50 d
Kansas prairie	K2	DD	22%	45 d
Terra preta	MM4Y	2CDD	20%	60 d

Table 6.2. Genome assembly results.

	Raw sequences			Assembled Sequences			
Strain	Yield (Mbp)	% ≥ Q30 R1	R2	# Contigs	Size (Mbp)	ORFs	Avg. coverage
Sphingomonas sp. RW1		NA*	NA		5.93	5345	
Sphingomonas sp. RW1	357.47	0.945	0.901	282	7.18	6748	49.8
Y3	346.12	0.959	0.926	66	4.93	4656	70.2
W2	376.60	0.943	0.900	352	5.15	4818	73.1
IA1 I1 #3	815.19	0.952	0.913	365	9.82	9327	83.0
Y3 + W2					10.08	9474	

were directly sequenced using a ABI 3730 Genetic Analyzer from both ends to obtain the complete 16S rRNA sequence.

Whole genome sequencing, assembly and analysis. Cell pellets from the IA1I1#3 consorium grown with DF as the sole carbon source, as well as the *Commamonas* sp. W2 and *Agromyces* sp. Y3 strains (designated W2 and Y3 because they are from the white and yellow colony morphotypes, respectively) grown in 10% LB, were resuspended in 400 µl TES (10mM Tris-HCl (pH 7.5), 100 mM NaCl and 1 mM EDTA). Lytic enzymes were added to aid in cell lysis (400 µg Lysozyme, 25 U/ml lysostaphin, 1000 U/ml achromopeptidase, and 100 µg/ml

proteinase K) followed by incubation at 55 °C for up to 4 h. DNA was isolated from the cell lysates by phenol chloroform extraction and ethanol precipitation. Genomic DNA of multiple strains was multiplexed and sequenced using the Illumina HiSeq^{TM} 2000 (Illumina, Inc. USA). Sequences were assembled using Velvet (Zerbino et al. 2008) with various kmer lengths (19-51) using digitally normalized kmers (Brown et al. 2012). The assemblies that resulted in the most bases assembled, the longest contig and the fewest contigs were then merged to create a multi-k assembly. Assembled contigs were uploaded to RAST (http://rast.nmpdr.org/) for automatic annotation including Enzyme Commission number (EC number). EC numbers relevant to the known dibenzofuran degradation pathway were obtained from the KEGG database (http://www.genome.jp/kegg/) and searched all sequences with the same EC numbers were extracted from the assembled sequences. Known dioxin, dibenzofuran and carbazole dioxygenases were used as query in a BLAST analysis of the assembled sequences.

Morphological characterization. Electron microscopy was used both to determine the morphology of cells and to characterize the membership of the bacterial consortium. Cultures were grown in K2 medium with DF as the sole carbon source. Samples were prepared for electron microscopy as has been described previously, (Takeuchi et al. 2001), but the mounted samples were coated with osmium and imaged at the Center for Advanced Microscopy, Michigan State University, using a JEOL 7500F scanning electron microscope (Japan Electron Optics Laboratories, Ltd; Tokyo, Japan).

RESULTS

Enrichment for DF and DD degraders. Of the three soils, three substrates and two media used to create active enrichment cultures which degrade the supplied dioxin compound, only one

enrichment using the Iowa prairie soil, K2 medium and dibenzofuran (enrichment was named IA1) was concluded to be active. In fact, this enrichment culture completely removed dibenzofuran in four consecutive transfers. Other enrichments that showed varying degrees of substrate removal (Table 1). Our primary interest, when the enrichments were initiated, was to isolate bacterial strains that could utilize 2-MCDD or DD as a sole carbon and energy source, but despite screening hundreds of individual strains picked from agar plates, we were unable to isolate any strain proven to utilize 2-MCDD or DD as a growth substrate. Many strains were able to grow on solid medium with DD or 2-MCDD vapors, but we suspect that many of these strains were utilizing agar as a carbon source.

Isolation of the DF degrading consortium: IA1I1#3. When the IA1 enrichment culture was grown on solid K2 media, a white morphotype CFU dominated the plate, as well as a translucent spreading morphotype. At the time of transfer this spreading member was largely overlooked, and what was thought to be about 40 single white colonies were transferred from solid to liquid medium. Two cultures, IA1I1 and IA1I4, showed optical density of about 0.4. These cultures were preserved and we sought to purify the culture. Two separate attempts to transfer 10 single colonies from IA1I1 and IA1I4 into liquid culture failed to produce any dibenzofuran degrading strains. Another attempt was made to transfer 200 single colonies for growth on DD and this also resulted in no growth in all instances. In a third attempt, 20 single colonies were transferred into liquid culture with DF as the growth substrate, accidentally incubated at 30 °C, and one of the IA1I1 cultures became turbid and turned yellow, typical of ring *meta*-cleavage. This culture, IA1I1#3 (hereafter called #3), was proven to completely degrade dibenzofuran with a 9.8 h doubling time. Growth of the culture is accompanied by simultaneous disappearance of the substrate (Fig. 1A). Initially, it was believed that #3 was a pure culture, but when the culture was

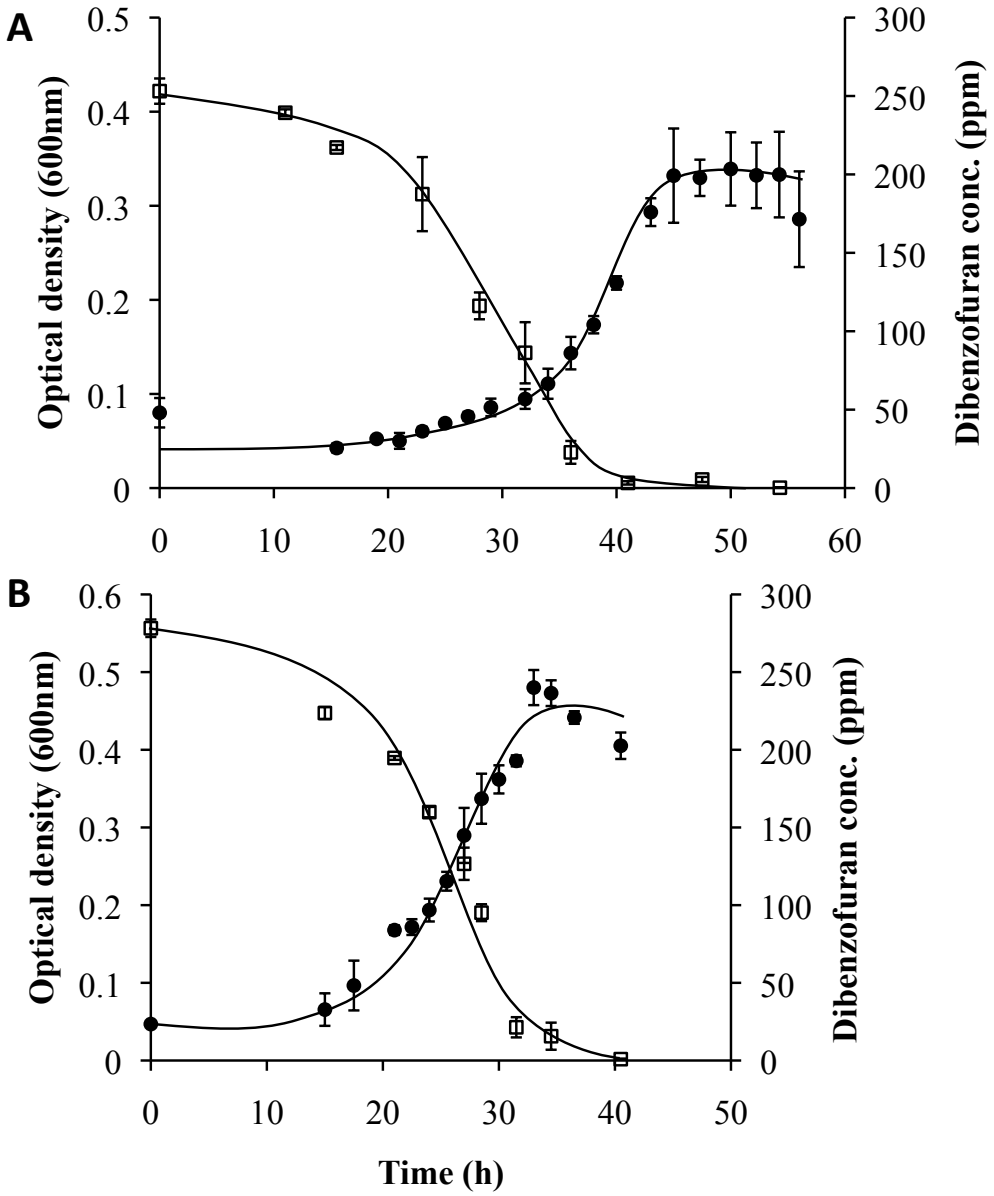

Fig. 6.1. DF degradation curves of a) IA1I1#3 and b) IA1#3-21. Optical density measurements were taken sequentially from the same cultures and DF concentrations were taken from individual sacrificed cultures. Points shown are the means of triplicate cultures and error bars indicate ± SEM. Vials with no cells or autoclaved cells were sacrificed at the end timepoint and showed the same concentration of the substrate as at 0 h (data not shown).

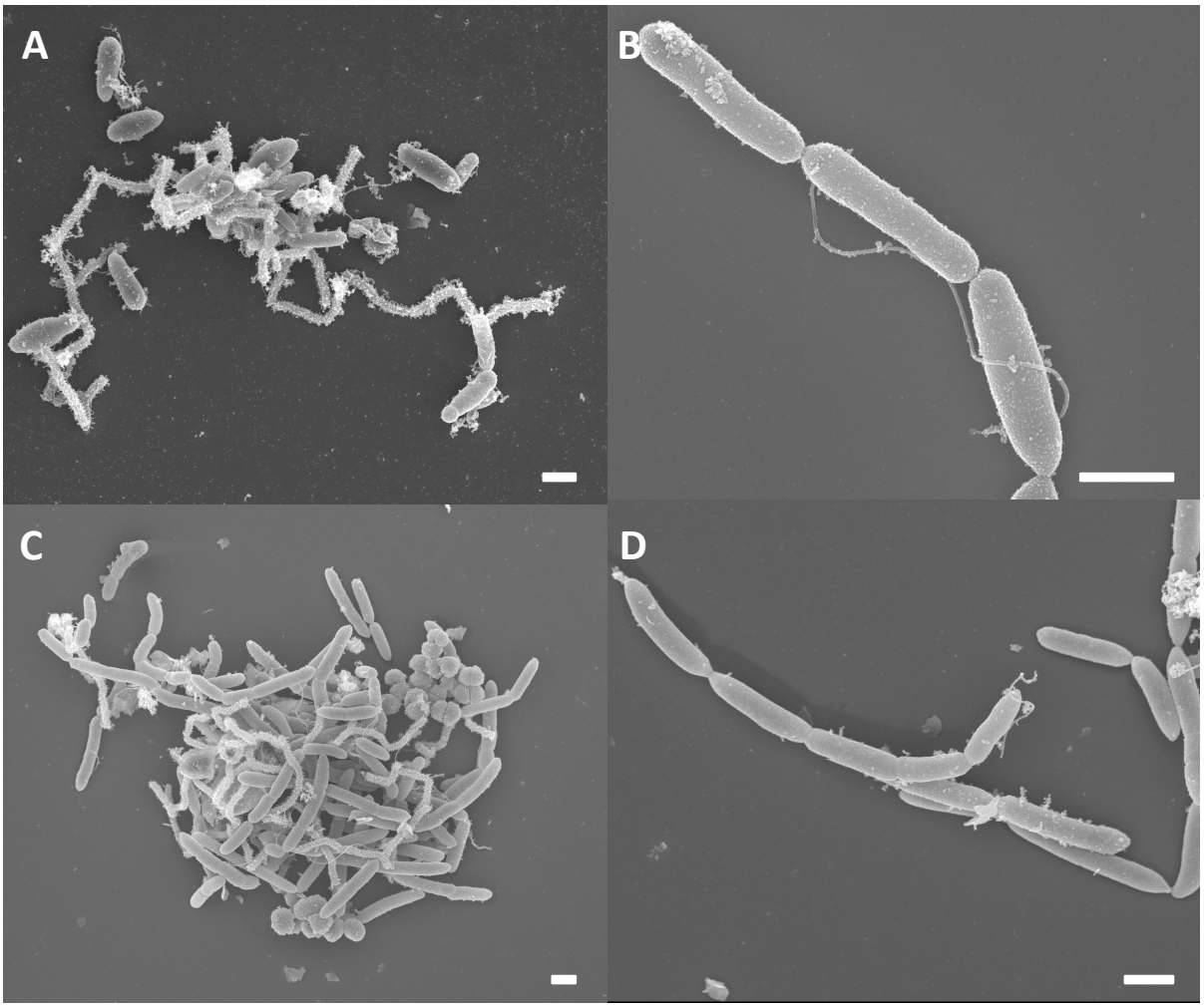

Fig 6.2. Electron micrographs of IA1I1#3 (A-B) and IA1I1#3-21 (C-D). In panel A three cell morphotypes are visible, short rod, hairy rod and a club shaped cell. Panel B is a detailed image of the short rods. In panel C, three cell morphotypes are visible from #3-21, short rod, hairy rod and a coccus. Panel D is a detailed image of the short rods. White bars are all 1µm.

grown on 20% R2A medium, two morphotypes were observed, a yellow and white types. These two morphotypes multiply in unison in a single growth rate phase. In fact, pyrosequencing of 16S rRNA amplicons from this consortium revealed three members are from three phyla: Firmicutes (unclassified *Bacillales* sp., accounting for 90% of sequences), Proteobacteria (unclassified *Comamonadaceae* sp., 10% of sequences), and Actinobacteria (*Agromyces* sp., 0.5% of sequences). Additionally, full-length 16S rRNA sequencing was preformed with white and yellow colonies, prepared for whole genome sequencing (called W2 and Y3, respectively). W2 was identified as *Comamonadaceae* sp. strain W2 (designated W2 because it is from the white colony morphotype) and the same 16S rRNA sequence was found in the whole genome sequencing. The 16S rRNA full-length amplicon sequencing of Y3 was not successful, but the whole genome sequence contained the same 16S rRNA sequence from pyrosequencing, which identified it as *Agromyces* sp. Y3 (designated strain Y3 because it came from the yellow colony morphotype). According to RDP SeqMatch tool, *Agromyces* sp. Y3 was most closely related (98%) to *Agromyces humatus*, and *Comamonadaceae* sp. W2 sequence was most closely related (95%) to *Acidovorax avenae* subsp. Avenae. The three members grow together stably during at least 9 serial transfers and the population ratio of 30-20% white colonies remained consistant. Electron microscopy confirmed the presence of three cell morphotypes (rod, long hairy rod, and club shaped) surrounding dibenzofuran crystals (Fig. 2A). From electron microscopy, it seems that the rod and hairy rod cell morphotypes are in approximate equal abundance, and the club morphotype is present at about 15%. The individual yellow and white morphotypes could be grown in pure culture in 10% LB (but not in undiluted LB), but when the two cultures were used as a combined inoculum into a single DF culture, no growth resulted.

Attempts to purify #3 to a pure culture or reconstitute from pure culture inputs. Multiple attempts were made to combine white and yellow colony types from #3 into a DF culture with the purpose of understanding the minimum membership requirements for growth on DF. However, transfer of the #3 group from solid to liquid medium is painstakingly unpredictable, resulting in growth in the liquid medium only three times in over 100 attempts. However, liquid-to-liquid transfers result in growth in every case. When combining 10 random colonies from 20% R2A plates were combined into DF cultures, 10 out of 22 attempts resulted in growth. When using 1 up to 5 colonies as the inoculum, a total of 190 colonies were distributed among 67 individual cultures, of which 3 resulted in growth. These three cultures were inoculated with 4, 2 and 1 colonies. The culture that grew from a single colony, called IA1I1#3-21 (hereafter referred to as #3-21), was then studied intensively. Initially it was thought that #3-21 was a pure culture of the white morphotype, but in subsequent transfers contaminating yellow colonies were observed on 20% R2A. Electron microscopy revealed 3 cell morphotypes: short rod, hairy rod and coccus. The rod shaped morphotype clearly dominated the culture with the hairy rod present at about 10%. There was a third minor member, a coccus shaped cell, which might make up 1% of the cells. These observations do not agree completely with colony morphology abundances, as white colonies make up about 99% of the colonies and yellow colonies are present at about 1%. The identity of one single white colony, which was grown in 10% LB, by 16S rRNA sequencing was *Agromyces* sp., while the identity of the culture grown with DF as the substrate was *Paenibacillus* sp. in two instances. Therefore, the #3-21 culture is not a pure strain and it may be the same consortium as #3, with the loss of the club shaped morphotype and contamination by the coccus. The #3-21 consortium grows faster than #3 (Fig. 1B) with a doubling time of 6.1 h.

Specificity of the degradative activity. Resting cell assays were used to determine if similar dioxin compounds could be degraded by #3-21. In comparison to the autoclaved cell controls, #3-21 showed considerable activity toward the compounds tested within the 24 h time period allowed (Fig. 3). DF (200ppm) was nearly completely degraded in 5 h (Fig. 3A). Dibenzo-*p*-dioxin was the least well degraded of the four substrates tested and 48% of 50 ppm was removed in 24 h. Chlorinated dioxins were degraded to a higher degree than unchlorinated dioxin – 79% of 50 ppm of 2-MCDD and 82% of 20 ppm of 2,3-DCDD were removed in 24 h. Little to no loss of the substrates was observed in the control vials, except the DF 24 h samples, which may be due to evaporation.

Whole genome sequencing of IA1I1#3. *Sphingomonas wittichii* str. RW1 was sequenced as a control genome. The combined assembly resulted in a size of 7.18 Mbp, while the actual genome is 5.93 Mbp. The cause behind the difference is unknown. Choice of kmer length had little influence on the total size of the assembly – the merged assembly of W2 was 0.2Mbp larger than the smallest assembly of all individual kmers (19-mer) assembled (Table 6.2). Genes of interest (*dxnA1A2*) were found by BLAST within the RAST web interface, but not by EC number search. The *Comamonadaceae* sp. strain W2 genome holds genes that could be responsible for the upper pathway and one half of the lower pathway. Three candidate genes for initial dioxygenation (with corresponding beta subunits identified) were distantly related to the two known clusters of dibenzofuran dioxygenases (Fig. 5) and were annotated as 3-phenylproprionate dioxygenase (PP), benzoate 1,2-dioxygenase (ben), and ring hydroxylating dioxygenase (RHD). No gene was annotated as 2,2',3-trihydroxybiphenyl dioxygenase (*dbfB*), but there are a number of candidate genes based on EC number (data not shown). 2-Hydroxy-6-oxo-6-phenyl-2,4-hexadienoic acid

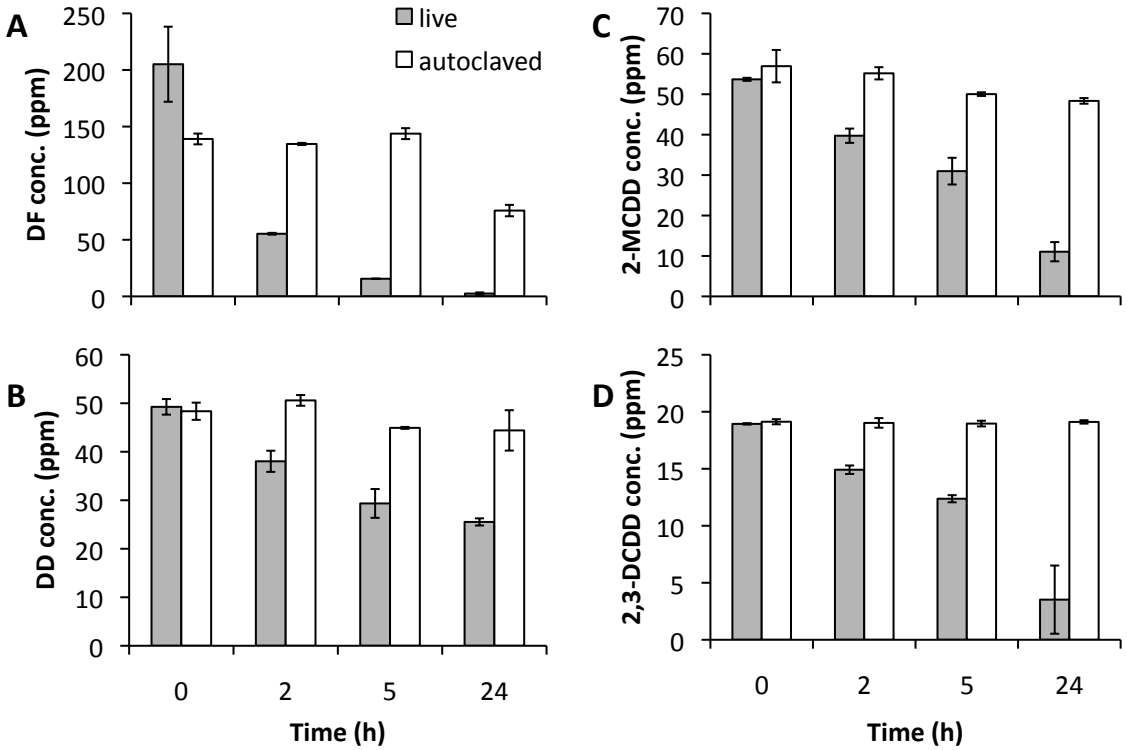

Fig. 6.3. Range of substrate degradation by IA1I1#3-21. The key shown in panel A applies to all panels. Bars indicate the mean of triplicate vials and error bars indicate ± SEM. 2-MCDD = 2-monochlorodibenzo-*p*-dioxin, 2,3-DCDD = 2,3-dichlorodibenzo-*p*-dioxin.

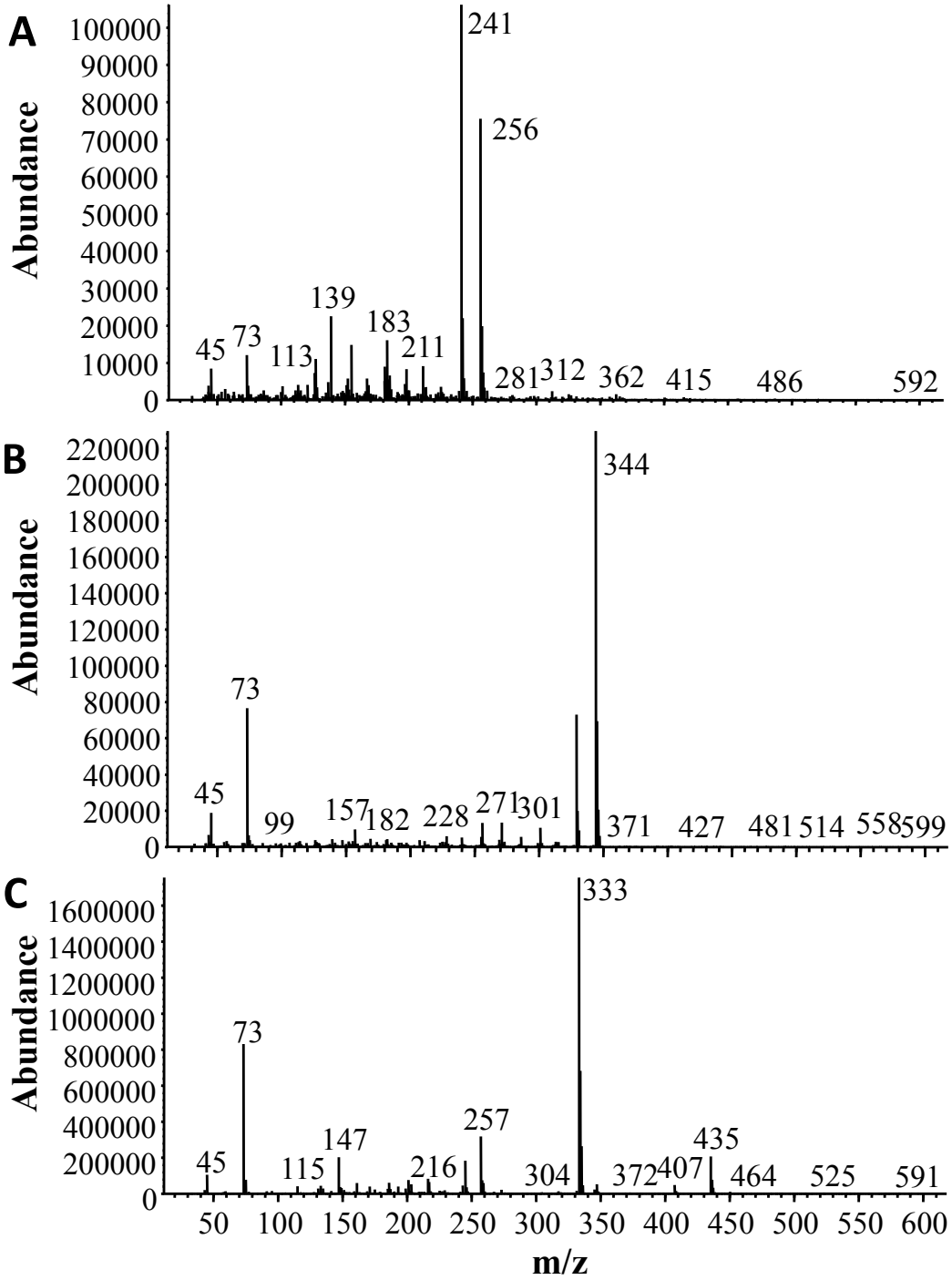

Fig. 6.4. Mass spectra from three peaks specific to the IA1I1#3 culture. Two peaks at 19.618 and 19.915 min had the same mass spectra as shown in panel A and was identified as hydroxydibenzofuran. The metabolite shown in panel B eluted at 24.783 min and was identified as dihydroxydibenzofuran. The metabolite in panel C was identified as 2-hydroxy-6-(2'-hydroxyphenyl)-6-oxo-2,4-hexadienoate (HOHPDA) and eluted at 25.768 min. Mass spectra could not be found which indicated the presence of 2,2',3-trihydroxybiphenyl, salicylate, or 2-oxopent-4-enoate.

hydrolase (*dbfC*), as well as salicylate hydroxylase, were identified in *Comamonadaceae* sp. strain W2. These genes were identified in all different contigs within the *Comamonadaceae* sp. strain W2 genome. The only gene in the pathway of any significance that was not seen in the *Comamonadaceae* sp. strain W2 genome was 2-keto-4-pentenoate hydratase, which was found in the *Agromyces* sp. strain Y3 genome. The #3 consortium metagenome sequences did not result in the sequencing of any *Bacillus* strain, but was composed completely of the W2 and Y3 genotypes (Table 6.2).

Degradation pathway of IA1I1#3. During early growth of both #3 and #3-21, the medium becomes turbid and yellow-orange simultaneously. This is traditionally known as build up of products from *meta*-cleavage of the ring. The metabolites that accumulated during incubation of #3 were determined by mass spectrometry. The metabolite in Fig 4A represents two peaks of significant size found in the gas chromatogram and was identified as hydroxydibenzofuran. The two peaks showed the same mass spectra profile and are likely different hydroxydibenzofuran congeners. Dihydroxydibenzofuran and 2-hydroxy-6-(2'-hydroxyphenyl)-6-oxo-2,4-hexadienoate (HOHPDA) were also identified (Fig. 3B,C). No other compounds of significance were identified, including salicylate or potentially novel degradation pathway intermediates.

DISCUSSION

Despite extensive effort, and clear depletion of DF and DD (Table 1), it appears that bacterial dioxin degradation is generally a function of a community rather than an individual strain. Generally we transferred 50-100 colonies from each enrichment dilution plating back into liquid culture and with the exception of #3, we never saw reproducible growth and simultaneous substrate depletion. Even in the case of #3, we still see that degradation requires a consortium

Fig. 6.5

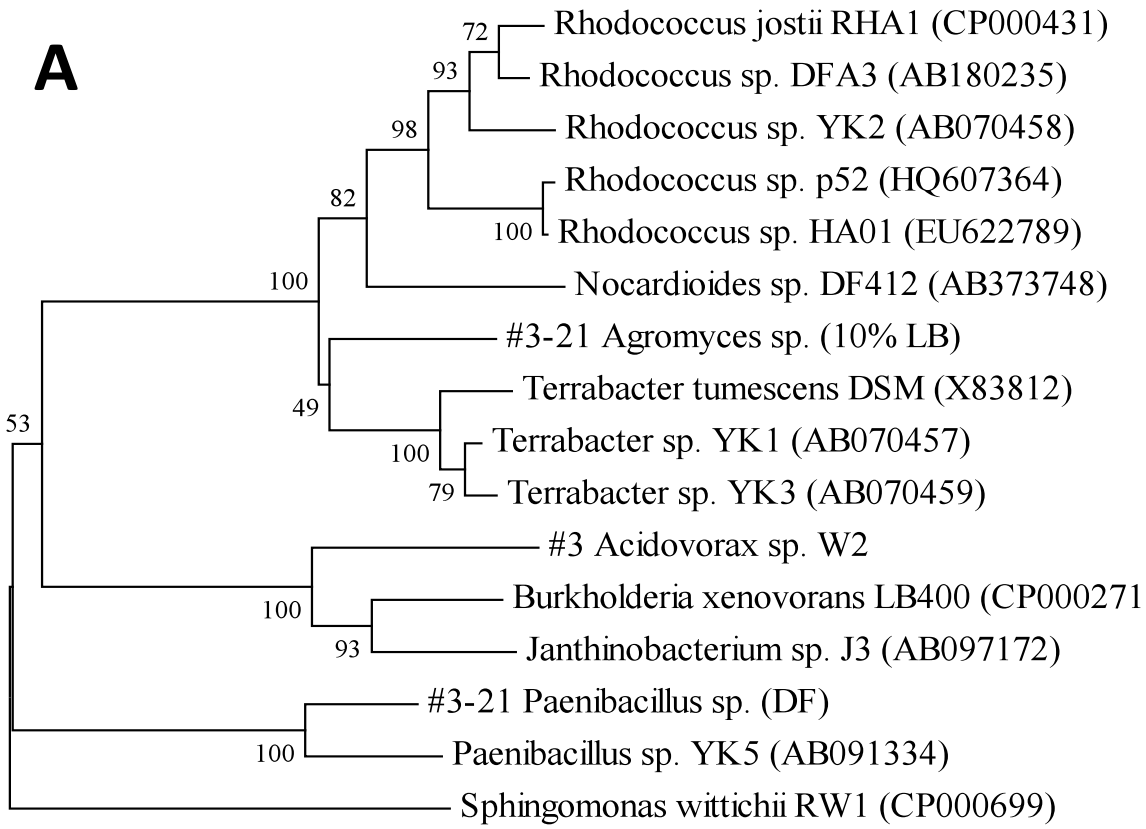
A
Rhodococcus jostii RHA1 (CP000431)
Rhodococcus sp. DFA3 (AB180235)
Rhodococcus sp. YK2 (AB070458)
Rhodococcus sp. p52 (HQ607364)
Rhodococcus sp. HA01 (EU622789)
Nocardioides sp. DF412 (AB373748)
#3-21 Agromyces sp. (10% LB)
Terrabacter tumescens DSM (X83812)
Terrabacter sp. YK1 (AB070457)
Terrabacter sp. YK3 (AB070459)
#3 Acidovorax sp. W2
Burkholderia xenovorans LB400 (CP000271
Janthinobacterium sp. J3 (AB097172)
#3-21 Paenibacillus sp. (DF)
Paenibacillus sp. YK5 (AB091334)
Sphingomonas wittichii RW1 (CP000699)
72
93
98
82
100
100
53
49
100
79
100
93
100

Fig. 6.5 (cont'd)

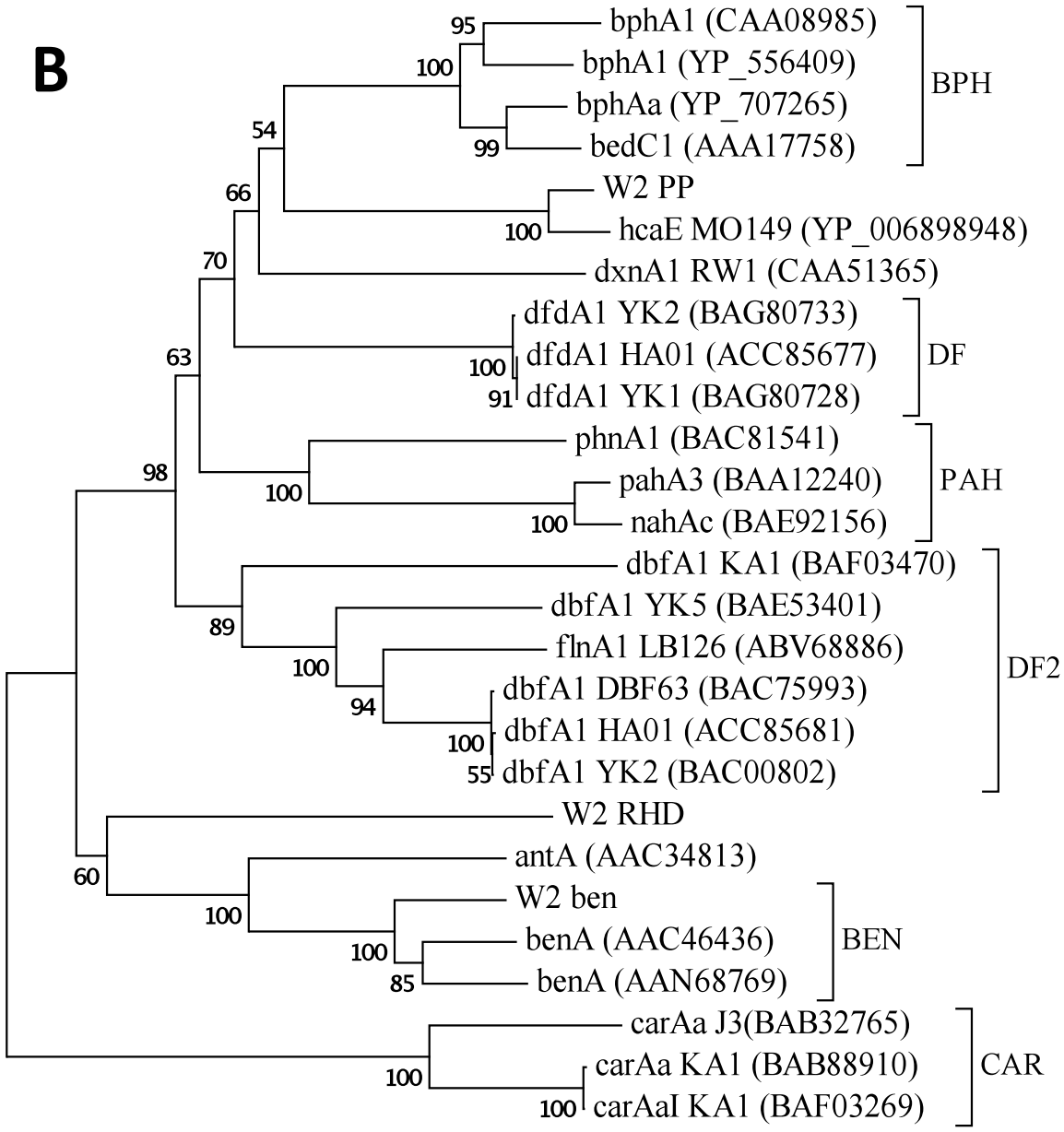

Fig. 6.5. Phylogenetic relationships of A. the 16S rRNA sequence of members of the IA1I1#3 consortium compared to known DF degraders and B. ring hydroxylating dioxygenases from *Acidovorax* sp. W2 compared to known Rieske dioxygenases. In panel B clades with activity toward a specific substrate are indicated: BPH biphenyl, DF dibenzofuran, PAH polyaromatic hydrocarbons, BEN benzoate, CAR carbazole.

and not a simple pure strain. This indicates that dioxin degradation in these microcosms may generally be due to microbial community or consortia action rather than being due to a single strain. Multiple strains may be required to provide enzymes and or compounds not present in a single strain, e.g., upper or lower degradation pathway genes, or extracellular compounds that increase the solubility of the substrate. Other research teams have indicated that attempts to isolate dioxin-degrading bacteria more often results in the isolation of bacterial consortia rather than individual strains is common (Wittich et al. 1999), and carbon sharing in a bacterial community, in the case of biphenyl, was shown to be extensive (Sul, in preparation). Bacterial consortia may provide required services in the degradation of recalcitrant organic pollutants by providing necessary enzymes (Arfmann et al. 1997) or rapidly remove toxic metabolites and increase consortium resilience with parallel carbon utilization (Pelz et al. 1999). Indeed, it was reported that the majority of 3-chlorobenzoate (3CBA) degraders were not efficient 3CBA degraders, suggesting that xenobiotic compounds are generally degraded by cross-feeding generalists, rather high efficiency specialists (Rhodes, in preparation). In our case, one possible explanation for the requirement of the consortium is that *Paenibacillus* sp. completes initial dioxygenation (based on similarity to known dibenzofuran degraders, abundance in #3-21, and lack of dibenzofuran dioxygenases in the other sequenced strains) *Comamonadaceae* sp. W2 provides salicylate mineralization (based on identification of salicylate hydroxylase in genome sequence), and *Agromyces* sp. Y3 provides 2-oxopent-4-enoate mineralization (based on identification of 2-keto-4-pentenoate hydratase in genome sequence).

The members the #3-21 consortium are phylogenetically diverse, each being a member of separate phyla, *Bacillus*, *Actinobacteria*, and *Proteobacteria*. The *Bacillus* member was more specifically identified as *Paenibacillus* sp. and its 16S rRNA sequence is 95% similar to the

dibenzofuran-degrading *Paenibacillus* sp. YK5 (Iida et al. 2006). The other two strains are more distantly related to know dibenzofuran degrading strains (Fig. 6.5). Other *Paenibacillus* strains have been characterized as gram-positive rods that form colonies that are yellow, glossy, and circular with complete margins (Xie et al. 2012). Unfortunately, the *Paenibacillus* strain was not sequenced in the shotgun metagenome of #3. We are not certain the cause for certain why no sequences arose from this metagenome assignable to *Paenibacillus*. It is possible the cells did not lyse, or were so overly dominated by the other two strains. While *Acidovorax* strains have been shown to degrade polyaromatic hydrocarbons (Singleton et al. 2009) the closest 16S rRNA match (95%) to the *Acidovorax* sp. W2 sequence was the phytopathogen *Acidovorax avenae* subsp. Avenae (Willems et al. 1992). While *Agromyces* sp. Y3 was most closely related (98%) to *Agromyces humatus*, isolated from Roman catacombs (Jurado et al. 2005) another species *Agromyces bauzanensis* was found to degrade phenol, but not polyaromatic hydrocarbons (Zhang et al. 2012).

From the evidence that we now have, it appears that the dioxygenase that will be found responsible for initial dioxygenation will be unique from those previously found. Ring hydroxylating dioxygenases, found in the *Acidovorax* sp. strain W2 genome, were distantly related to known dibenzofuran dioxygenases. One of these dioxygenases was most similar to 3-phenylproprionate dioxygenase from *Bordetella bronchiseptica*, a human pathogen. Because the #3 16S rRNA pyrosequencing showed a dominance of the *Paenibacillus* strain, as well as obtaining the *Paenibacillus* sequence from 16S rRNA PCR amplification from a #3-21 DF culture, this strain may indeed carry out initial dibenzofuran dioxygenation of its relation to other dibenzofuran degraders. This will have to be determined in the future.

The Rieske-type family of dioxygenases (to which dibenzofuran dioxygenases belong) generally has a broad substrate range and can cometabolize a variety of related compounds (Gibson et al. 2000). For example, a recently isolated dibenzofuran degrader was able to cometabolize 14 related polyaromatics including dibenzo-*p*-dioxin (Peng et al. 2012). Our findings are no different, in that #3-21 cometabolizes dibenzo-*p*-dioxin, 2-monochlorodibenzo-*p*-dioxin (2-MCDD) and 2,3-dichlorodibenzo-*p*-dioxin (2,3-DCDD). The most toxic polychlorinated dioxin congener is 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. Thus 2,3-DCDD is an important chemical to cometabolize because it is dichlorinated in two of four of these toxic lateral positions. According to a review by Field et al. (2008), there are 7 known strains that have been reported to cometabolize 2,3-DCDD with varying degrees of efficiency. *Sphingomonas wittichii* str. RW1 was able to remove 28.4% (18ppm) of an initial concentration of 63 ppm 2,3-DCDD in 0.7 d (Wilkes et al. 1996). Strain IA1I1#3-21 was able to remove 15 ppm in 24 h which makes it nearly as efficient as *S. wittichii* str. RW1 at cometabolism of 2,3-DCDD and is as or more efficient than all the other 6 strains (Field et al. 2008).

Overall, we have isolated a novel phylogenetically diverse dibenzofuran-degrading bacterial consortium with activity toward polychlorinated dioxin. This is an uncommon metabolic range as polychlorinated dioxins are generally unmetabolized. Much remains to be uncovered in regards to the carbon network within this consortium and their biological interplay and may serve as an interesting model system for symbiosis, community carbon metabolism, simple metagenomics and assembly method development, as well as the bioremediation of dioxins.

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CHAPTER VII

CONCLUSIONS AND FUTURE RESEARCH DIRECTIONS

CONCLUSIONS

Human society has a vast impact on the environment especially in terms of chemical disposal in the environment. Microbial communities respond to these chemical perturbations following general ecological principles, which may result in altered composition of the microbial community. In some instances the microbial response could have negative human impacts, such as enrichment of antibiotic resistance in response to antibiotic use and/or disposal. In other instances, changes to the bacterial community helps human society goals, such as the degradation of polyaromatic hydrocarbons and restoration of a natural chemical profile. The goal of this work was two-fold: (i) to describe the antibiotic resistance gene reservoir in response to agricultural antibiotic use and (ii) to identify and isolate novel dioxin dioxygenases/ degraders in response to dioxin enrichment. The key accomplishments and findings of this work include:

1. Developed and validated highly parallel quantitative PCR. This technology will help fill critical knowledge gaps for the determination of the antibiotic resistance gene reservoir in environmental samples. We are already using the qPCR approach to determine the abundance of resistance genes in many important scenarios: water treatment plants, fisheries, human disease diagnostics, public parks, and ancient mammoth gut samples.
2. Resistance genes are enriched in individual swine guts in response to subtherapeutic antibiotics. Co-selection for resistance to antibiotics not in the feed is also observed. This finding highlights the importance of characterizing the complete antibiotic resistance gene reservoir because there may be enrichment of ARGs beyond what might be expected. Taking this a step farther, in active swine farms, resistance genes are highly enriched in manure, compost and soil receiving the manures. Thus management strategies

need to be discussed with the goal of reducing antibiotic resistance gene release and accumulation in the environment.

3. Enrichment of resistance genes due to horizontal gene transfer appears to be common on swine farms. We observed a high correlation between resistance gene and transposase allele abundance. This correlation should be investigated further by determining the genetic organization of resistance genes and mobile genetic elements, including plasmids.
4. In general, antibiotics impose a strong selective pressure both on the microbiome of individual swine and in the farm manure, compost and soil ecosystems. Resistance genes are enriched due to vertical and horizontal transfer. Agricultural antibiotic use is creating a large resistance gene reservoir and may pose a risk to human health, hence appropriate disposal and remediation strategies are needed to remove resistance genes from this environment and contain their dispersal. Risk assessment models may play an important role in determining the critical control points in limiting the dispersal of ARGs.
5. Developed and validated novel dioxygenase primers. These primers are important new tools to detect dioxygenases with potential activity toward dioxins.
6. Dioxygenase sequences obtained from the environment are generally unique from known dioxin/ dibenzofuran dioxygenases. This is a crucial finding because it indicates that potentially many novel dioxin or dibenzofuran degrading strains could be isolated from the environment to increase our known arsenal of dioxin biodegraders.
7. Dioxin degraders are present in “pristine” soils. The newly isolated #3-21 originated from a pristine prairie soil and can utilize dibenzofuran as a sole carbon and energy source. It can cometabolize chlorinated dioxins with similar efficiency to RW1. Isolation of novel chlorinated dioxin degraders was one of our primary goals and #3-21 meets this goal.

8. Recalcitrant hydrocarbon degradation may commonly be the action of consortia. Traditional isolation of a pure culture that utilizes a substrate as its sole carbon and energy source may limit the number of degraders that could be isolated if mixed consortia were considered. With metagenomic sequencing the genomes of all the members of the #3-21 consortium were determined and the role of each can be determined and confirmed with additional strain characterization.

FUTURE DIRECTIONS

We have shown that antibiotic resistance genes are enriched in swine farms due to the use of subtherapeutic in-feed antibiotic use and we have provided important data on the abundance of different classes of resistance genes. However, much of this story remains to be told. Following the risk assessment framework, there are many pieces of the story that remain to be uncovered:

1. Extent of horizontal antibiotic resistance gene transfer ($HG_{AR}T$) in the gut microbiome. We provide indirect evidence for horizontal gene transfer by associating the abundance of resistance genes and transposase alleles. Direct observation of $HG_{AR}T$ is difficult because it would require a known recipient and a known donor and subsequent observation of the recipient with a resistance gene from the donor. A good deal of past work on this topic has been completed either in vivo or in culture (Hunter et al. 2008) but these studies do not attempt to determine a community level transfer rate.
2. Also important for risk assessment is the concept of dose-response. This concept has largely been ignored in the field of agricultural antibiotic use and resistance, but is a foundation principle of risk assessment. These could be very simple experiments. For

example, antibiotic resistance genes could be the dose and the change in abundance is the response. This could be determined by feeding animals decreasing concentrations of commensal bacteria with known antibiotic resistance genes, and then monitoring the abundance of the resistance gene with qPCR in the gut through time. Dose-response relationships should be described for resistance genes, antibiotic use and/or supplemental metals use, or mixtures of the three with a comparison to the response: resulting resistance gene levels. This is a sort of black box approach to describing what happens to resistance genes in the gut, because it does not describe bacterial growth rate, $HG_{AR}T$ rates, or transduction rates individually, but it does give a total quantitative value for all these process occurring simultaneously in vivo. There could also be interesting co-selection studies done in this way -- feed the animal a resistance gene and a non-corresponding antibiotic and see what happens. These experiments could be done in any mammalian host so that results could be applicable to human processes also. The key here is that the dose of the resistance gene is the independent variable and the dose response relationship can be determined.

3. Another very interesting part of this story is secondary transmission, or animal-animal or human-human bacterial transfer. This can be determined by introducing an antibiotic free animal to conventional antibiotic-fed animals a few hours a day (or multiple animals with different contact times for dose-response) but the diet of the test individual remains antibiotic free. The response is the concentration of resistance genes changes in their gut. This could be a model for a farmer's contact with animals, population modeling, etc.
4. In regards to perturbation, are antibiotics a pulse or press perturbation and does this depend on the recalcitrance of the chemical? For example, tetracycline was degraded

significantly in the Chinese manure and composting stages, yet sulfonamides and quinolones were not. Would a one-time perturbation with sulfonamides provide a press perturbation manifested by long-term community changes and a new stable state, whereas a pulse perturbation with tetracycline would only cause temporary changes and a return to the previous stable state? Would repeated use of tetracyclines provide a press perturbation and the establishment of a new stable state? With the limited data that I have, I would hypothesize in the affirmative to these questions in regard to the resistance gene composition, but I do not have a good estimation in regards to the phylogeny of the community, which could be resolved with phylotyping.

5. Additionally, in general, I think gene-targeted metagenomics of resistance genes has not been studied in much detail. This oversight could stem from the fact that there are hundreds of resistance genes already identified, and because of that interest in diversity has waned, but the ecology of ARGs is still critically important. Additionally using technology like the Access Array from Fluidigm, one could amplify many resistance genes and sequence them all simultaneously. This can contribute to questions regarding horizontal and vertical gene transfer and the fate of genes in an environment.
6. The study of antibiotic resistance also would be a good avenue to develop other metagenomic analysis tools, such as nucleating assembly and synthetic biology of genes of unknown function. Antibiotic resistance genes can be quite abundant, depending on the source of the sample. Therefore it may make it an easier case to find resistance genes in a shotgun metagenome and from there begin assemblies. When full-length resistance genes are assembled, these open reading frames could be synthesized and inserted into an appropriate bacterial host to test the function of the gene. Often metagenomes are only

annotated in terms of similarity to genes of known function. If we could use resistance genes as a model gene category and determine the genetic divergence that maintains the same function, it would provide some range of confidence to homology-based pipeline metagenomic annotations.

We have isolated a dibenzofuran-degrading consortium. We are working on isolating all the members of the consortium in pure culture and being able to reconstruct the consortium by mixing the members back together from their pure cultures. While the role of each member of the consortium is yet to be defined, there are two foreseeable outcomes: a) one of the members (possibly *Paenibacillus*) can degrade dibenzofuran in pure culture and the other members of the consortium are community cheaters (especially *Micrococcus* since it was not even detectable in the #3 lineage of the consortium) or b) all the members are required in a true symbiotic consortium relationship. Additionally, perhaps a combination of the two situations is possible, with *Micrococcus* as a cheater and *Agromyces* as a symbiont. In either case, interesting ecological relationships could be examined. Community cooperation and cheaters are an interesting topic in ecology and can lead to the determination of community stability or collapse simply by altering the percent composition of the cheater population (Dai et al. 2013, Gore et al. 2009). Microbial cooperation, evolution (Lawrence et al. 2012) and community metabolism networks (Pelz et al. 1999) could also be studied in this simple microbial consortium:

1. Determine the dynamics of the cheater population of 3-21 and if there is a stable cheater concentration density and if cheater concentration affects the degrader growth rate.
2. Determine the interplay of carbon compounds between symbionts.
3. Carry out an evolution experiment with the consortium, similar to what was done in Lawrence et al. The consortium could evolve in polyculture, and the individuals could

evolve in monoculture being provided the carbon source they require in monoculture that is relevant to dibenzofuran degradation. Then test fitness characteristics of all the lineages after the evolutionary period.

4. In regards to perturbation: does a truly recalcitrant chemical, one that the community cannot degrade, actually provide a perturbation to the community, or does it simply pass through with the chemical and the community unchanged? This could be attempted with perhaps a chlorinated dioxin.
5. Can more dioxin degrading consortia be isolated if the culture strain purification process is omitted and it were simply a serial liquid transfer?
6. Can the complete degradative potential of a community, such as the soil community that gave rise to 3-21, be uncovered through metagenomics and metatranscriptomics of these soils or soil microcosms?

This is an exciting time to study microbial ecology. The ideas laid out here for future investigation would advance both basic science and ecology and aid in discoveries, with useful human application both to limit the spread of antibiotic resistance and to further dioxin bioremediation. In research, it is critical to balance and address all the following primary goals: basic science, scientific theory, method technology, application and looking for solutions to human problems.

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