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Growth of soil bacteria, on penicillin and neomycin, not previously exposed to these antibiotics

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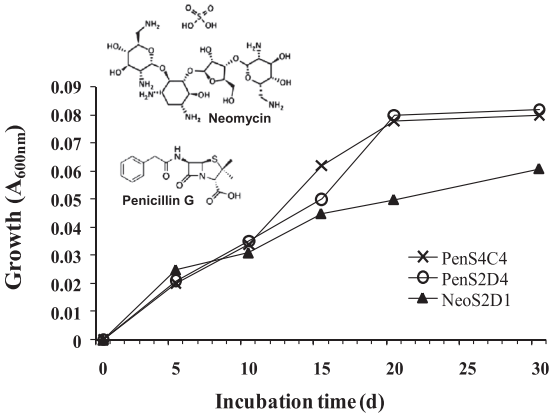
HIGHLIGHTS

- Nineteen bacterial isolates from four soils subsisted on penicillin or neomycin as sole C sources.
- Most of the isolates were resistant to six other antibiotics at concentrations as high as 1000 mg L^{-1}.
- The 19 isolates were assigned to diverse orders within the phyla *Proteobacteria* and *Bacteroidetes*.
- Some antibiotic resistance genes were found in soil but not in the bacterial isolates.
- Soils are an under-appreciated reservoir of antibiotic resistance/degradation machinery.

GRAPHICAL ABSTRACT

Three bacterial isolates (PenS4C4, PenS2D4 and NeoS2D1) were obtained from soil not previously treated with the antibiotics penicillin and neomycin. Not only were they resistant to the presence of these antibiotics, but could use the nutrients and energy contained in these antibiotic molecules (at 1000 mg L^{-1}) to support growth (see growth curves below). Soils contain a potentially large and diverse pool of antibiotic resistant and degradation genes. This suggests potential ecological and health impacts yet to be explored and fully understood.



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ABSTRACT

There is growing evidence that bacteria, in the natural environment (e.g. the soil), can exhibit naturally occurring resistance/degradation against synthetic antibiotics. Our aim was to assess whether soils, not previously exposed to synthetic antibiotics, contained bacterial strains that were not only antibiotic resistant, but could actually utilize the antibiotics for energy and nutrients. We isolated 19 bacteria from four diverse soils that had the capability of growing on penicillin and neomycin as sole carbon sources up to concentrations of 1000 mg L^{-1}. The 19 bacterial isolates represent a diverse set of species in the phyla *Proteobacteria* (84%) and *Bacteroidetes* (16%). Nine antibiotic resistant genes were detected in the four soils but some of these genes (i.e. *tetM*, *ermB*, and *sull*) were not detected in the soil isolates indicating the presence of unculturable antibiotic resistant bacteria. Most isolates that could subsist on penicillin or neomycin as sole carbon sources were also resistant to the presence of these two antibiotics and six other antibiotics at concentrations of either 20 or 1000 mg L^{-1}. The potentially large and diverse pool of antibiotic resistant and degradation genes implies ecological and health impacts yet to be explored and fully understood.

Abbreviations: ARG, antibiotic resistant gene(s); Pen, penicillin; Neo, neomycin; SCS, single carbon source; PCR, polymerase chain reaction.

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1. Introduction

Antibiotics are synonymous with killing bacteria. They have revolutionized medicine and have saved countless lives. Furthermore, they have been used in large quantities for veterinary purposes or to promote the growth of animals. However, fifty to ninety percent of these antibiotics or their primary metabolites are excreted rapidly into the environment after administration to animals (Michael and Bester, 2006; Hammesfahr et al., 2008).

One problem associated with extensive use of antibiotics is that it is often accompanied by the rapid appearance of resistant strains. A recent database lists the existence of more than 20,000 potential resistance genes (r genes) of nearly 400 different types, and these are mostly found in the genomes of bacteria (Liu and Pop, 2009). Bacteria usually become resistant if they are exposed to sublethal doses of the antibiotic, but still high enough to select for resistant mutants. Control of antibiotic resistant bacteria is thought possible by limiting their use or by more sensible use.

Most known resistant bacteria have been discovered in clinical and veterinary settings. A number of recent studies have explored the fate of veterinary medicines from soils to surface waters and groundwater, and the development of multi-drug resistance (Nwosu, 2001; Popowska et al., 2012; Cytryn, 2013) via horizontal gene transfer mechanisms (Nelson and Cox, 2008). The concept of the soil as a source of antibiotic resistance, however, has actually been acknowledged for decades (Vanessa et al., 2007).

Soil contains a large and diverse microbial population. Bacteria can degrade and transform a vast array of organic compounds, including antibiotics, with the help of soil enzymes found either extracellularly or by enzymes associated with metabolizing and proliferating cells (Jenkinson and Ladd, 1981). Microbes thus serve a crucial role in determining the fate of compounds like antibiotics in soil. Native bacteria in soil generally do not contain naturally resistant genes against synthetic antibiotics unless they have been previously exposed to these antibiotics. This is not always the case and Dantas et al. (2008) showed that some soil bacteria can degrade and grow using carbon and nutrients from antibiotic substrates, even when not previously exposed to these antibiotics. These bacteria were surprisingly phylogenetically diverse and it is not known how widely distributed they are across the landscape.

Soil microorganisms living in complex community structures, especially the actinomycetes, naturally produce antibiotics. These natural antibiotics degrade in soil because otherwise they would persist and build up to high levels. If the natural antibiotics are closely related chemically to synthetic antibiotics, they can support a reservoir of diverse antibiotic resistant/degradative genes in soil microbial populations. In addition, spontaneous mutations can occur even in the absence of antibiotics (Rodríguez-Verdugo et al., 2013). These genes can provide a soil microorganism with the ability to subsist on an antibiotic as a source of nutrients and energy. If these antibiotic resistant bacteria in soil are closely related to human pathogens, a human health problem is potentially created. The lack of basic knowledge of the role soil and soil bacterial communities play in creating antibiotic resistant bacterial strains has hampered effective prevention and control of resistance.

This study was conducted to assess whether 1) we could isolate and identify bacteria that could degrade and grow on penicillin and neomycin, even though they had not previously been exposed to these antibiotics, 2) the occurrence of antibiotic resistance genes (ARG) in diverse soils and bacterial isolates from these soils, and 3) the degree of multiple antibiotic resistance in the bacterial isolates. Penicillin and neomycin were selected because they belong to structural classes that are widely used in both clinical and livestock production situations. We were particularly interested in identifying the native bacterial isolates capable of utilizing these antibiotics at high concentrations as sole carbon and energy sources.

2. Materials and methods

2.1. Soil samples

Topsoils (0–15 cm) were sampled from a compost site where waste compost had been mixed into the soil, near the barn at an organic dairy, a pristine forest soil and a 20-year old no-tillage corn field. These sites were all located near the Ohio Agriculture Research and Development Center of The Ohio State University, America (Table 1). Preliminary investigations had indicated that none of these four sites had ever been treated with penicillin or neomycin, although manure at the compost site may have contributed some level of these antibiotics to this soil. At each site, a total of 10–12 soil samples were collected using a sterile spade, mixed thoroughly and then placed into a plastic bag to obtain one composite sample. The composite samples were transported to the laboratory on ice (2–6 h) and stored at −20 °C. Selected soil characteristics (Table 1) were analyzed according to the methods described in Sparks et al. (1996).

2.2. Chemicals

Two antibiotics, the β-lactam antibiotic penicillin G (Pen) and the aminoglycoside antibiotic neomycin sulfate (Neo), with purity of >99% were purchased from Sigma. Their chemical structures are displayed in Fig. 1.

2.2.1. Isolation of soil bacteria capable of subsisting on penicillin or neomycin

All liquid media used for isolating bacteria capable of subsisting on antibiotic were made by dissolving 20 mg L^{-1} of the penicillin or neomycin, as a single carbon source (SCS), into media containing 5 g $(NH_4)_2SO_4$, 3 g KH_2PO_4, 0.5 g $MgSO_4.7H2O$, 15 mg EDTA, 4.5 mg $ZnSO_4.7H_2O$, 4.5 mg $CaCl_2.2H_2O$, 3 mg $FeSO_4.7H_2O$, 1 mg $MnCl_2.4H_2O$, 1 mg H_3BO_3, 0.4 mg $Na_2MoO_4.2H_2O$, 0.3 mg $CuSO_4.5H_2O$, 0.3 mg $CoCl_2.6H_2O$ and 0.1 mg KI per liter water. The pH was adjusted between 6.2 and 6.5 using NaOH, and the media were sterilized through a 0.22 μm filter. Solid medium was prepared by adding 15 g agar per liter of liquid SCS media followed by autoclaving before adding antibiotics.

Culturing of soil bacteria capable of growth on antibiotics was initially accomplished using the method described by Dantas et al. (2008) with some modification. Soil (1 g dry weight) was suspended in 50 mL phosphate buffered saline solution (Dulbecco and Marguerite, 1954) that did not contain any carbon and placed on a shaker (150 oscillations per minute) for 30 min. Then 1 mL of this suspension was inoculated into 9 mL SCS-antibiotic media. To significantly reduce the transfer of residual alternative carbon sources present in the original soil suspension, samples were passaged (5.0 μL) into fresh SCS-antibiotic media (5 mL) two additional times after 7 days of growth, resulting in a 1.0×10^3 dilution at each passage. Isolates from the liquid cultures were obtained by plating on SCS-antibiotic agar medium at 30 °C. At such a low concentration of antibiotic as SCS, growth was slow but noticeable. Single colonies were then picked and re-streaked on corresponding plates to obtain pure colonies.

2.2.2. Identification of cultivable bacteria

The predominant colony types growing on the SCS-antibiotic agar medium were subcultured and identified by 16S rRNA gene sequencing. The 16S rRNA gene (rDNA) of each of the isolates identified in this study was amplified using the universal bacterial 16S primer pairs 27F (5′-AGA GTT TGA TCC TGG CTC AG-3′) and 1492R (5′-GTT TAC CTT GTT ACG ACT T-3′) to produce an amplicon of approximately 1499 base. The PCR mixture (total volume, 25 μl) consisted of 12.5 μl of 2X GoTaq Geen Master Mix, 0.5 μl of each primer (20 pM) and 2 μl of the bacteria isolates DNA template, and sufficient sterile double-distilled water to reach the desired final volume. PCR amplification was performed with the following program: 94 °C for 5 min; 34 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 45 s, and extension at 72 °C for 1 min; and a single final extension at 72 °C for 7 min. The PCR products were cleaned