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Effects of fumigants on microbial diversity and persistence of *E. coli* O15:H7 in contrasting soil microcosms

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ABSTRACT

Persistence of *E. coli* O157 in the environment is a serious public health concern. However, little is known about the persistence of this pathogen after exposure to chemical compounds like fumigants in the environment. In this study, the persistence behavior of pathogenic *E. coli* O157:H7 was investigated after fumigation with methyl bromide (MeBr; CH_3Br) and methyl iodide (MeI, iodomethane; CH_3I) in soil microcosms under laboratory conditions. Our goal was to assess changes in soil microbial community structure and persistence of *E. coli* O157:H7 in microcosm soils after fumigation. PCR was used to amplify 16S rRNA genes from total bacterial community composition, and the products were subjected to denaturing gradient gel electrophoresis (DGGE). Microbial diversity as determined by DGGE was significantly higher in clay soil than sandy soil. Real-time PCR and plate counts were used to quantify the survival of *E. coli* O157:H7 in the two soils after fumigation with MeBr and MeI. The survival of the pathogen was higher in the non fumigated controls than the fumigated treatments when determined using plate counts. These results were confirmed by real time PCR analysis targeting the *stx*1, *stx*2, and the *eae* genes. *E. coli* O157:H7 survived for about 35 days when determined using the plate count method but continued to be detected at about the detection limit of 10^2 by real time PCR for more than 86 days. Our results showed that there was a fast inactivation of the pathogen during the first 35 days. After this period, a small proportion of the pathogen continued to survive in the soil microcosms. Subsequent enrichment of soil samples and immunomagnetic separation revealed the continuous presence of viable cells after 86 days of incubation. The data presented contribute to a better understanding of the behavior of *E. coli* O157:H7 in soil, and showed the need for more investigation of the role of dormant cells in soil that may be a source for recontamination of the environment.

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1. Introduction

Methyl bromide (MeBr; CH_3Br) is a broad spectrum, highly effective and relatively cheap fumigant used for pre-planting fumigation. It has been used extensively to control plant pathogens such as nematodes, soil-borne diseases, and weeds in economically important crops such as strawberries and nursery stock (Ferguson and Padula, 1994) in California and other parts of the world. Methyl bromide was scheduled for elimination in the United States and other developed countries by the year 2005 (USEPA., 2000) and in developing countries by 2015 because of its stratospheric ozone depletion potential. However, MeBr is still in use due to critical use exemptions for the cultivation of strawberries, tomatoes, and peppers in California and Florida. The proposed phase-out has resulted in an intensive search for alternative fumigants and the development of other integrated pest management strategies to replace MeBr. Methyl iodide (MeI, iodomethane, CH_3I) was reported as a potential alternative to the stratospheric ozone-depleting fumigant methyl bromide (MeBr) in the mid-1990s (Ohr et al., 1996; Sims et al., 1995). Methyl iodide is often referred to as the "drop-in replacement" because its fate, transport characteristics and effectiveness as a biocide are similar to those properties of MeBr (Ohr et al., 1996). MeI has a distinct advantage over MeBr in that its atmospheric lifetime is only <10 days, compared to 1.5 to 2 years for MeBr (Ruzo, 2006). Therefore, it is unlikely that MeI will reach the stratosphere and contribute to ozone depletion (Rassmussen et al., 1982; Solomon et al., 1994), although the volatilization of MeI may be similar to that of MeBr. As a preplant soil fumigant, MeI can be used alone, or in combination with chloropicrin (CP) to control plant pathogens, nematodes, insects and weeds on crops such as strawberries, tomatoes, peppers, ornamentals, turf, trees and vines (USEPA, 2010).

Methyl iodide use in US agriculture is receiving significant focus due to its recent registration (USEPA, 2010). California recently announced its decision to become the forty-eighth US state to register MeI (California Department of Pesticide Regulation, 2010). Concern over the use of MeI as an agricultural fumigant is based on its potential to cause serious health effects to humans after emission and inhalation. Recently, our laboratory has tested different methods of reducing emissions of methyl iodide from agricultural soils (Ashworth et al.,

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2011; Luo et al., 2010), but little or no studies have been done on the long term impact on soil bacterial composition. Changes in the soil microbial population can be observed following fumigation with MeBr and other fumigants (Dungan et al., 2003; Ibekwe et al., 2001; Martin, 2003). Ibekwe et al. (2010) observed decline in *E. coli* O157:H7 population in soils cultivated with lettuce and fumigated with MeBr and MeI in a growth chamber. This study was conducted for 60 days in two walk-in growth chambers. However, *E. coli* O157:H7 survived longer in non-fumigated soil than in fumigated soil. A subsequent study showed that the effect of these fumigants on rhizosphere and phyllosphere microbial composition was insignificant (Ibekwe et al., 2009). Due to the increased focus on food safety related to fresh produce, there are several other studies of *E. coli* O157:H7 survival in different environments such as soil, manure and water (Jiang et al., 2002; Kudva et al., 1998; Vital et al., 2008). These studies showed that the availability of nutritional resources and key abiotic conditions are critical to *E. coli* O157:H7 population survival and even growth in such environments. However, under fluctuating environmental conditions, such as those present in many soil environments, growth may be differential and gross bacterial death may ensue if the death rate exceeds the growth rate.

Our goal was to assess changes in soil microbial community structure and persistence of *E. coli* O157:H7 in soil microcosms after fumigation with MeBr and MeI. PCR was used to amplify 16S rRNA from total bacterial community composition, and the products were subjected to denaturing gradient gel electrophoresis (DGGE). The Shannon-Weaver index of diversity (*H*) was used to determine the effects of both fumigants on soil microbial community structure. For our main objectives, plate count and real-time PCR approaches were used to determine the survival of *E. coli* O157:H7 in the two soil microcosms. The survival data were fitted to a biphasic model as proposed by Coroller et al. (2006) with the Geeraerd and Van Impe inactivation model-fitting tool (GInaFiT) (Geeraerd et al., 2005) as described by Franz et al. (2008).

2. Materials and methods

2.1. Soils and chemicals

Clay soil (Willows silty clay, saline–alkaline) and sandy soil (Dello sand) were collected from Mystic Lake dry bed and the Santa Ana River bed, respectively, in Riverside County, California. The clay soil has a bulk density of 1.51 Mg m^{-3} with 3.7% sand, 49.1% silt, and 47.2% clay. The sandy soil has a bulk density of 1.67 Mg m^{-3} with 99.1% sand, 0.20% silt, and 0.70% clay. The soils were sieved through a 4 mm sieve before planting, and the high salt content (electrical conductivity = 15 ds m^{-1}) in the clay soil was reclaimed as described by Ibekwe and Grieve (2004). These soils were chosen because these are the two main soil types supporting cattle production in the area and cattle are the main source of *E. coli* O157:H7 in the environment.

Methyl iodide (>99% purity) was purchased from Chem Service (West Chester, PA) and methyl bromide (>99% purity) was obtained from Great Lakes Chemical Company (West Lafayette, IN).

2.2. Bacterial strain and growth conditions

E. coli O157:H7 strain 72 pGFP was kindly provided by Dr. Pina Fratamico (Fratamico et al., 1997). This strain contains Shiga-like toxin genes 1 and 11 (*stx*1, *stx*2) and the pGFP expressing the green fluorescent protein (GFP) containing an ampicillin resistance gene and was used for pathogen enumeration from soil. *E. coli* O157:H7 was cultured at 37 °C overnight in modified Tryptic Soy broth (mTSB) (Difco Laboratories Inc., Cockeysville, MD) supplemented with 100 μg of ampicillin ml^{-1} (Sigma, St Louis, MO). Cells were harvested by centrifugation at 5000 xg for 10 min and resuspended in phosphate buffered saline (PBS) (Fisher Scientific, Pittsburgh, PA) to a concentration of ~10^8 CFU ml^{-1}.

2.3. Microcosm experimental design

The microcosm experiment was set up in 1.0 l Mason Kerr self sealing wide mouth glass containers (Lima, OH). The soil (1.5 kg) was adjusted to a moisture content of about 12% (for equal distribution of fumigant in soil) by adding *E. coli* O157:H7 inoculants or water and mixing in a larger container before transfer to each microcosm. This was to maintain the same level of pathogen concentrations and moisture content. After the inoculation, fumigants were added. The experimental design consisted of two fumigants at three different concentrations in duplicates (0.5x, 1x, and 5x, where 1x is 48 kg ha^{-1} for MeBr and 40 kg h^{-1} for MeI). These resulted in spiking approximately 1.73 g ml^{-1} of MeBr and 2.28 g ml^{-1} of MeI per gram of soil into each microcosm for the 1x treatments. Microcosms were sealed for 24 h after fumigant application, and vented continuously through a small opening in the cover for the remainder of the experiment as previously described (Ibekwe et al., 2001). Soil samples were taken from individual microcosm (with a sterile spatula) for heterotrophic bacteria, *E. coli* O157:H7 concentration, and total bacterial DNA before fumigation and at days 1, 3, 7, 14, 21, 28, 35, 49, 56, and 86 after fumigant treatment. Bacterial concentrations were determined by plating soil on Tryptic soy agar (TSA; Becton Dickinson) plates containing 100 μg of ampicillin ml^{-1} (TSA-A). The GFP-labeled *E. coli* O157:H7 colonies were counted under an UV light. Total bacterial DNA was extracted from samples, and heterotrophic bacteria were counted on TSA.

2.4. DNA extraction, PCR amplification, and DGGE analysis

Total bacterial community DNA was extracted from soil samples (0.5 g) with the Power Soil DNA Kit (MoBio Laboratories, Solana Beach, CA) and stored at −20 °C. A 236-bp DNA fragment in the V3 region of the small subunit ribosomal RNA genes of eubacteria was amplified by using primer set PRBA338f and PRUN518r (Øverås et al., 1997). Ready-To-Go PCR beads (GC Healthcare Biotech, Piscataway, NJ) and 5 pmol of primers in a total volume of 25 μl were used in the PCR reaction. PCR amplifications were done under the following conditions: 92 °C for 2 min; 30 cycles of 92 °C for 1 min, 55 °C for 30 s, 72 °C for 1 min followed by a final extension at 72 °C for 6 min.

DGGE was performed with 8% (wt/vol) acrylamide gels containing a linear chemical gradient ranging from 30 to 70% denaturant with 100% defined as 7 M urea and 40% formamide. Gels were run for 3.5 h at 200 V with the DCode™ Universal Mutation System (Bio-Rad Laboratories, Hercules, CA). DNA was visualized after ethidium bromide staining by UV transillumination and photographed with a Polaroid camera. Major bands were excised for identification of bacterial species. Bands were placed into sterilized vials with 20 μl of sterilized, distilled water and stored overnight at 4 °C to allow the DNA to diffuse out of the gel strips. Ten microliter of eluted DNA was used as the DNA template with the bacteria primers above but without the GC-clamp. DNA was cloned into the TOPO TA cloning kit (Invitrogen, Carlsbad, CA). Isolation of plasmids from *E. coli* was performed using the Qiagen plasmid mini kit (Valencia, CA). Four plasmids from each band were sequenced to check for purity of clones. The purified plasmids were sequenced with the ABI PRISM Dye Terminator Cycle Sequencing Kit with AmpliTaq DNA Polymerase, FS (Applied Biosystems, Foster City, CA) with forward and reversed primer M13. Sequence identification was performed by using the BLAST database (National Center for Biotechnology Information: www.ncbi.nlm.nih.gov) to identify the major bands excised from DGGE.