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Detection Methods for the Genus *Lysobacter* and the Species *Lysobacter enzymogenes*

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Detection Methods for the Genus *Lysobacter*
and the Species *Lysobacter enzymogenes*

By

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Detection Methods for the Genus *Lysobacter* and the Species *Lysobacter enzymogenes*

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Strains of *Lysobacter enzymogenes*, a bacterial species with biocontrol activity, have been detected via 16S rDNA sequences in soil in different parts of the world. In most instances, however, their occurrence could not be confirmed by isolation, presumably because the species occurred in low numbers relative to faster-growing species of *Bacillus* or *Pseudomonas*. In this study, we developed DNA-based detection and enrichment culturing methods for *Lysobacter* spp. and *L. enzymogenes* specifically. In the DNA-based method, a region of 16S rDNA conserved among *Lysobacter* spp. (L4: GAG CCG ACG TCG GAT TAG CTA GTT), was used as the forward primer in PCR amplification. When L4 and universal bacterial primer 1525R were used to amplify DNA from various bacterial species, an 1100-bp product was found in *Lysobacter* spp. exclusively. The enrichment culturing method involved culturing soils for 3 days in a chitin-containing broth amended with antibiotics. Bacterial strains in the enrichment culture were isolated on yeast-cell agar and then identified by 16S rDNA sequence analysis. A strain of *L. enzymogenes* added to soils was detected at populations as low as 10^2 and 10^4 CFU/g soil by PCR amplification and enrichment culturing, respectively. In a survey of 58 soil samples, *Lysobacter* was detected in 41 samples by PCR and enrichment culture, out of which 6 yielded strains of *Lysobacter* spp. by enrichment culture. Among isolated strains, all were identified to be *L. enzymogenes*, with the

exception of a strain of *L. antibioticus*. Although neither method alone is completely effective at detecting *L. enzymogenes*, they are complementary when used together and may provide new information on the spatial distribution of the species in soil.

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Chapter 1 Literature Review

Taxonomy of *Lysobacter*

Christensen and Cook proposed genus *Lysobacter* in 1978. Before that, strains later classified as *Lysobacter* spp. were grouped in myxobacteria because they share some distinctive traits, including gliding motility and micropredatory behavior. Christensen and Cook (1978) considered *Lysobacter* to be related to other myxobacteria because of gliding motility but distinguished this genus from other myxobacteria by it being non-fruit forming and having high G+C content. *Lysobacter* is now grouped in γ -proteobacteria, and belonging to the family Xanthomonadaceae. *Lysobacter* is very closely related with the genera *Xanthomonas*, *Stenotrophomonas*, *Pseudoxanthomonas*, *Thermomonas* and *Xylella* by phylogenetic analysis (Bae *et al.* 2005). However, *Lysobacter* spp. also display a number of traits that distinguish them from other related bacterial genera including oxidase activity, 28°C optimum growing temperature, varying cell length (2 to 70 μm), high genomic G+C content (typically ranging between 65-72%) and the lack of flagella (Christensen and Cook 1978).

There are four species originally proposed by Christensen and Cook (1978) in the genus *Lysobacter*: *L. enzymogenes*, *L. antibioticus*, *L. brunescens*, and *L. gummosus*. Within these species, *L. enzymogenes* is the type species and the most commonly reported and studied. Within just the last four years, there were twelve new species reported: *L. concretionis* (Bae *et al.* 2005), *L. koreensis* (Lee *et al.* 2006), *L. defluvii*

(Yassin *et al.* 2007), *L. niabensis* (Weon *et al.* 2007), *L. niastensis* (Weon *et al.* 2007), *L. daejeonensis* (Weon *et al.* 2006), *L. yangpyeongensis* (Weon *et al.* 2006), *L. spongiicola* (Romanenko *et al.* 2008), *L. capsici* (Park *et al.* 2008), *L. oryzae* (Aslam *et al.* 2009), *L. daecheongensis* (Ten *et al.* 2008) and *L. ginsengisoli* (Jung *et al.* 2008). All new species were suddenly burst during recent 4 years, which is brought by advanced molecular biology techniques, including PCR amplification, molecular cloning, sequencing tool, and so on, to ignite the great revolution of species identification.

Physiology of *Lysobacter* spp. and *L. enzymogenes*

During years of research on *Lysobacter*, there were three main areas of extensive research: use as biological control agents for plant diseases, production of antibiotics for human medicine, and enzymes for commercial applications. Strains of *Lysobacter* spp. were reported to have broad spectrum antagonism in vitro against bacteria, fungi, unicellular algae and nematodes. The range of diseases controlled by biocontrol strains is extensive. *L. enzymogenes* has the most reported biocontrol effective strains than other species over time. For example, strain C3 of *L. enzymogenes* was reported to control diseases caused by fungal pathogens, including *Rhizoctonia solani* (Giesler and Yuen 1998), *Bipolaris sorokiniana* (Zhang and Yuen 1999 and 2000, Kilic-Ekici and Yuen 2004.), *Magnaporthe poae* (Kobayashi and Yuen, 2005), *Uromyces appendiculatus* (Yuen *et al.* 2001), and *Fusarium graminearum* (Yuen, *et al.* 2003). Strain C3 and others also were inhibitory to oomycetous pathogens in the genera *Aphanomyces* and *Pythium* (Kobayashi *et al.* 2005; Palumbo *et al.* 2005; Islam 2009;

Postma *et al.* 2009), to bacterial pathogens (Jiang *et al.* 2005) and also nematodes (Chen *et al.* 2006; Katznelson *et al.* 1964). This broad spectrum of biocontrol activity can be attributed to a wide variety of possible mechanisms: extracellular enzymes (Palumbo *et al.* 2003; Ahmed *et al.* 2003; Chohnan *et al.* 2004), secondary metabolites (Yuen *et al.* 2005), induced resistance (Kilic-Ekici and Yuen 2003), and hyperparasitism involving types III, IV and VI secretion systems (Reedy *et al.* 2003; Blackmoore *et al.* 2009; Patel *et al.* 2009)

The lytic activity by enzymes is the one of the systems contributing to broad spectrum antagonism. Proteases, chitinases, glucanases, lipases and phospholipases produced by *Lysobacter* together can degrade the cell walls of all groups of plant pathogens. Proteases were the earliest enzyme group studied for biocontrol activity and were first thought to be involved in against nematodes (Katznelson *et al.* 1964). Later, proteases were also reported to be active against some gram-positive and gram-negative bacteria (Ensign and Wolfe 1965 and 1966). And it is still studied as a very important mechanism of biocontrol in recent years (Ahmed *et al.* 2003, Chohnan *et al.* 2004). Chitin is a very important component of fungal cell walls; hence most biocontrol agents for fungi possess chitinase activity. Chitinase activity of *L. enzymogenes* strain C3 has been reported to be involved in biological control activity for *Bipolaris sorokiniana* (Zhang and Yuen 2000). β -1,3-Glucans are critical components of cell wall structure in fungi and oomycetes, comprising over 80% of the cell wall polysaccharides (Blaschek *et al.* 1992). Palumbo *et al.* (2005) proved that *L.*

enzymogenes strains C3 and N47 have β -1, 3-glucanase producing systems, which enable C3 to have potential to decompose cell wall.

Besides enzyme activity, antibiotics produced by *Lysobacter* spp. are also contributed significantly to its biocontrol system. As early as 1966, Peterson *et al.* found myxin produced by *Lysobacter* strain, which was classified as *Sorangium*, as a broad-spectrum phenazine antibiotic inhibiting bacteria and fungi. Antibiotics of β -lactams containing substituted side chains, macrocyclic lactams, and macrocyclic peptides to control MRSA (caused by *Staphylococcus aureus*) were reported to be produced by *Lysobacter* spp. (Kato *et al.* 1997; Kato *et al.* 1998). Later, Christensen (2001) reported an antibiotic with a wide spectrum produced by *L. antibioticus* identified as 1-hydroxy-6-methoxyphenazine. A family of antibiotics consisting of dihydromaltophilin, called heat-stable antifungal factor (HSAF), is produced by *L. enzymogenes* strain C3 and was proved to be responsible for control of fungi and oomycetes by disruption of the fungal polarized growth (Yu *et al.* 2007, Li *et al.* 2006). A similar compound was also found that is produced by *L. enzymogenes* strain 3.1T8 showing inhibitory activity against oospores and cyst germination (Folman *et al.* 2004). At the same time, xanthobaccins A, produced by *Lysobacter* sp. SB-K88 was identified as a macrocyclic lactam and a structural analogue of dihydromaltophilin (Nakayama *et al.* 1999, Yu *et al.* 2007). Broad production of maltophilin-related antibiotics is a possible shared trait by *Lysobacter* spp., which

implies the ecological importance of *Lysobacter* biocontrol strains since these antibiotics are known to be effective on fungal inhibition.

Role of *Lysobacter* spp. in Nature

Christensen and Cook (1978) described *Lysobacter* spp. to be ubiquitous inhabitants of soil and water. All *L. brunescens* strains were isolated from water in Christensen & Cook's paper (1978). After that, several *Lysobacter* strains were found in diverse environments by ribosomal nucleic acid analysis or enriched isolation. 16S ribosomal RNA sequences found in hydrothermal vents and Mt. Pinatubo mud flows (Folman *et al.* 2003; Ogiwara unpublished) were later reported to correspond to those of *Lysobacter* spp. (Folman *et al.* 2003; Sullivan *et al.* 2003). Similarly, *Lysobacter*-indicative 16S rRNA gene sequences were reported from tar pits (Kim and Crowley 2007). The occurrence of living cells of *Lysobacter* in these extreme environments, however, was not confirmed. Strains of *Lysobacter* were isolated from diverse sources in different parts in the world, including Kentucky Bluegrass foliage in Nebraska (Giesler and Yuen 1998); root tips of hydroponic cucumber plants in the Netherlands (Folman *et al.* 2003); groundwater of a basement tile drain in Michigan, USA (Sullivan *et al.* 2003); upflow anaerobic blanket sludge reactors, Korea (Bae *et al.* 2005); plant rhizosphere soils, China (Jiang *et al.* 2005); Kartchner Caverns limestone cave, AZ, USA (Ikner *et al.* 2006); ginseng field near Daechung lake, Korea (Lee *et al.* 2006); greenhouse soils of Daejeon and Yangpyeong regions in Korea (Weon *et al.* 2006); deep-sea sponge in Philippine Sea (Romanenko *et al.* 2008); and field rhizosphere of rice, Korea (Aslam *et al.* 2009). These results

collective suggest that *Lysobacter* spp. are cosmopolitan, and that they inhabit not only ordinary aerobic environments, but also could occur in extreme and anaerobic environments. It has not been verified that under these conditions, there are actually live cells of *Lysobacter* or just residual DNA fragments.

Lysobacter spp. have been shown to be non-fastidious as to their nutrient requirements. Various media have been used routinely for culturing *Lysobacter*, such as Luria Bertani (LB) agar, nutrient broth (NB), sporulation agar (SA) (Sullivan *et al.* 2003), Difco R2A medium (Ikner *et al.* 2007) and 10% tryptic soy agar (TSA) (Giesler and Yuen 1998). Being highly chitinolytic and aggressive in lysing fungal hyphae, *Lysobacter* strains have been isolated from nature using chitin medium (Christensen and Cook 1978) and enrichment culture with fungal mycelia as a bait (Kobayashi and El-Barrad, 1996). However, none of these media is selective for *Lysobacter*. Selective medium are used for the growth of only select microorganisms, which is very important for bacterial population study. *Lysobacter* strains are slow growers and are under low population in nature, thus they are not very competitive than other natural microorganisms. Therefore, selective medium for *Lysobacter* can enable isolation of *Lysobacter* from nature by enriching their population and minimizing competition brought by other organisms.

Prospect

While anecdotal evidence show *Lysobacter spp.* occur in different parts and various environments in the world, but there has been no systematic study on the population distribution of the genus *Lysobacter* or *Lysobacter enzymogenes* within certain geographic location. Although Christensen and Cook (1978) described *Lysobacter* to be ubiquitous inhabitants of soil, they did not document the locations from which samples were collected nor did they provide direct quantitative data as proof of the frequency at which *Lysobacter spp.* could be isolated from soils. The information on population distribution is very important to prove the hypothesis that *Lysobacter* are ubiquitous in soils.

Current studies on *Lysobacter* in the environment focus on two aspects: colonization and antagonism activity of biocontrol strains applied to soils and plant surfaces; and the presence of *Lysobacter spp.* as a component of the bacterial community in unique habitats. However, there is no research that connects the concepts of where *Lysobacter* can be found and the expression of biocontrol-related traits. *Lysobacter* is known to be potential biocontrol related group, so that it provides ideas further enhance study on ecology of *Lysobacter* in agricultural crop soil system by means of assessing *Lysobacter* population in agricultural field and understanding relationship with agricultural crops or field plants. Directly evaluating *Lysobacter* population in agricultural field system could be proposed as a new approach to better understand *Lysobacter's* role in nature.

Chapter 2 Introduction

When the bacterial genus *Lysobacter* was first described by Christensen and Cook (1978), it was known to inhabit soil and water and be antagonistic to a wide range of microorganisms. It was not until 20 years later when species and strains of *Lysobacter* were recognized to be of biological control importance (Sullivan *et al.* 2003). One explanation for the delay of recognition for *Lysobacter* is that it was difficult to distinguish from many other gram-negative genera on the basis of physiological tests. In fact, biological control strains of *Lysobacter* were classified in related genera *Stenotrophomonas* (Giesler and Yuen, 1998) and *Xanthomonas* (Sakka, *et al.* 1998) until they were reclassified using 16S rDNA sequence analysis (Sullivan *et al.* 2003). Another possible explanation is that *Lysobacter* species may be localized in distribution, and thus, biocontrol effective strains would be found only in certain locales. To date, however, there has been no systematic study to determine the distribution of a given *Lysobacter* sp. or the frequency of its occurrence within a particular geographic area. Christenson and Cook (1978) did not provide quantitative or geographic data to support their description of the genus being common in soils and water. A third explanation is that members of genus may commonly occur but strains with biological control ability are unique or uncommon in occurrence. Bacterial diversity studies using 16S rDNA have revealed the presence of *Lysobacter* spp. throughout the world and in very diverse environments (Lee *et al.* 2006, Schmalenberger and Tebbe 2003, Sigler and Turco 2002, Moyer, C., *et al.* 1995, Ikner *et al.* 2007, Ikner *et al.* 2007, Kim and Crowley 2006), suggesting that they are

indeed widely distributed. However, there has been no systematic comparison made across strains of any *Lysobacter* sp. for any given trait, with the exception of a study by Kilic-Ekici and Yuen (2003) that examined three strains of *L. enzymogenes* for the ability to activate induced resistance. The biggest hurdle to testing the validity of second and third hypotheses is the absence of reliable methods to detect and isolate *Lysobacter* strains from nature. PCR with taxon-specific primers offers an opportunity as a non-culture detection method (Scarpellini *et al.* 2004 and Sanguin *et al.* 2008) but *Lysobacter* specific primers have not yet been found. At the same time, conventional isolation methods used for genera such as *Pseudomonas* and *Bacillus* are thought to be ineffective for isolating *Lysobacter* from environmental samples such as soil because this group shows slower growth and exists in lower populations compared to other bacteria such as *Bacillus* and *Pseudomonas*. Christensen and Cook (1978) isolated *Lysobacter* strains using by first enriching soils with chitin, but their methodology was not clearly described nor validated by other researchers.

This study examines methods to detect and isolate *Lysobacter* spp. from the environment, which are the critical first steps toward answering the questions as to where *Lysobacter* species occur (distribution) and whether populations or strains vary in biocontrol related traits (diversity). Therefore, the objectives of this study were:

1. To create a non-culture based method for detecting *Lysobacter* in soils using *Lysobacter* specific PCR primers.

2. To develop a method to isolate *L. enzymogenes*, the most commonly reported species from soil based enrichment with chitin.
3. To compare the sensitivity of the two methods for detecting *Lysobacter* in soil.
4. To use the two methods to assess the distribution of *Lysobacter* spp. and *L. enzymogenes* in soil within Nebraska.

Chapter 3 Materials and Methods

Bacterial strains and culturing conditions

All bacterial strains (Table 1) were obtained from Gary Yuen's collection. They were stored at -75°C and cultured on tenth-strength tryptic soy agar (10% TSA) at 28°C for 2 days before use.

Development of a PCR based detection method for *Lysobacter*

Lysobacter specific primers were designed by first aligning 16S ribosomal DNA sequences from 250 *Lysobacter* strains listed in GeneBank using ClustalW multiple alignment program (<http://workbench.sdsc.edu/>). Conserved sequences were screened according to the number of nucleotides (15-50 bp total length, 3-10 binding length was desired), predicted size of the PCR product when the conserved sequences are paired with a universal reverse primer (350-1400 bp was desired), and location of conserved sequences (sequences at either 5' or 3' end of the 16S rDNA sequence map were excluded). Then, candidate sequences were searched for potential *Lysobacter* specificity (only *Lysobacter* shows in >85% identity) by nucleotide BLAST tool towards nr/nt nucleotide sequence database in NCBI website.

(http://www.ncbi.nlm.nih.gov/blast/Blast.cgi?CMD=Web&PAGE_TYPE=BlastHome). Finally, sequences with potential specificity and appropriate size were analyzed by MacVector primer design software (New Haven, CT) for primer quality. Putative primers were synthesized from the candidate sequences by

Invitrogen (CA, USA). They were evaluated for specificity by using them as the forward primers, along with universal bacterial 1525R as the backward primer, in comparative PCR amplification of DNA from strains of *Lysobacter* spp, (*L. enzymogenes* C3, N4-7 and 495, *L. antibioticus* type strain UASM 3C) and other bacterial species (*Stenotrophomonas maltophilia* 34S1, 13270. 19867, *Xanthomonas campestris* pv. *Campestris* 2A49, *Enterobacter cloacae* E1, *Escherichia coli* XL1, and *Bacillus pumilus* INR7). The PCR reagents and conditions in these tests are reported in Table 2. *Lysobacter* specific primer pairs will be discovered by only showing expected sized band in *Lysobacter* DNA amplification, but not in reactions with other bacterial species.

One primer (L4) from above procedure identified to be specific to *Lysobacter* was used in PCR-based detection of *Lysobacter* in soil. DNA was extracted from 0.5 g amounts of soil using Mo Bio UltraClean Soil DNA Isolation Kit (Carlsbad, CA USA). Extracted DNA then was subjected to PCR amplification using L4 and a universal primer 1525R as the forward and backward primers, respectively. The existence of *Lysobacter*-indicative amplification products was confirmed by electrophoresis.

Development of enrichment culturing method for isolating *L. enzymogenes* from soil

Common antibiotics were screened for effects on strains of *L. enzymogenes* and other bacterial species to identify those that could be used as selective agents in media. Filter discs (10 mm diameter) were saturated with aqueous solution of ampicillin, penicillin, tetracycline, kanamycin, rifampicin or streptomycin, each at 50µg/ml or 200µg/ml. The disks were placed on the surface of agar plates on which strains of *S. maltophilia* or *Lysobacter* spp. (*L. antibioticus*, *L. enzymogenes* C3, N4-7, and 495) had been freshly transferred were cultured on media plates, on surface of which antibiotic filter discs were applied. The size of the growth inhibition zone developed around each disk was measured after 3 and 6 days of incubation.

Two substrates, chitin and yeast cells, were compared as carbon sources for growth of *L. enzymogenes*. Broth media containing ground chitin (Sigma, practical grade) or yeast cells (Red Star Active Dry Yeast, Milwaukee, WI) were prepared with 0.1, 0.25, 0.5, or 1.0 g of the carbon source in 1L double-distilled water and amended with the antibacterial drugs penicillin and kanamycin at 50mg/L and the fungicidal drug cycloheximide at 100mg/L. To evaluate the media for enrichment of *L. enzymogenes*, strain C3R5 (a spontaneous rifampicin-resistant mutant of C3) was added to an autoclaved soil to 0, 10⁰, 10¹, 10² CFU/g, and then 50 ml volumes of each broth medium was seeded with 1 g of a soil sample. After 0, 3, and 6 days incubation (shaking under 28 °C), 200 µL of each

culture were spread on plates of 10%TSA amended with rifampicin and cycloheximide (TSARC) to confirm the presence of C3R5 in the broth cultures.

Sensitivity comparison between detection methods

Preliminary forms of the PCR based detection and enrichment isolation methods were compared for sensitivity in detecting *L. enzymogenes* in soil. Samples of raw and autoclaved soil were amended with strain C3R5 at cell concentrations varying in ten-fold increments from 1 to 10⁵ CFU/g. The control was soil to which sterile water was added. For the PCR based method, DNA was extracted from 0.5 g of soil, and the extract was PCR-amplified as described above using sequence L4 as the forward primer. Positive detection of *Lysobacter* was based on the presence a 1,100 bp length PCR product in the electrophoresis. For the enrichment culture method, 1 g of soil sample was incubated in chitin broth, containing 0.5 g/L chitin for 3 days, and then spread or streaked onto TSARC.

Comparison of PCR and enrichment culturing methods on field soil samples

Soil samples were collected from 38 Nebraska sites within 21 counties with different plant cover, including turfgrass, agricultural crops (wheat, dry bean, corn, sorghum, sunflower and etc.) and forest (Table 3). One to 3 samples were collected from each site, with a total of 58 samples being collected. Each sample was taken from plant root area, containing two scoops (200 gram) of soil, with little to no root material, by a hand trowel and pooled together. Each soil sample were mixed, sealed and refrigerated before processing. Each sample was analyzed

once by the two detection methods. If a method failed to detect *Lysobacter* in a soil sample, then the process was repeated using another subsample of the same soil. The PCR based detection method was applied as described above. The enrichment culturing method involved drug-amended chitin broth, with 0.5 g/L ground chitin, as the enrichment medium and yeast-cell agar (YCA; 18 g Sigma agar, 5 g active dry yeast in 1 L ddH₂O, autoclave for 40 min add 50 mg/l penicillin, 50 mg/l kanamycin, and 100 mg/l cycloheximide) as the isolation medium. After 1 g of a soil sample was cultured in 50 ml chitin broth with shaking for 3 days, a loopful of the broth was streaked on a YCA plate. After 3 days, bacterial colonies surrounded by clear zones in which yeast cells were digested were purified by streaking on new YCA plates three times to get single colonies. Then non-*Lysobacter* isolates were eliminated using by a series of physiological tests (Table 4) as described in Schaad *et al.*, 2001.

Putative *Lysobacter* isolates were cultured in Luria-Bertani broth and total DNA was extracted using UltraClean Microbial DNA Isolation Kit (Mo Bio Laboratories, Inc.). Universal bacterial primers 27F and 1525R were used for PCR amplification of 16S rDNA. Universal bacterial primers 27F and 530F were used for sequencing. Samples of amplified DNA were sequenced by Center for Biotechnology, UNL, and the results were subjected to BLAST search to determine the closest identity.

Chapter 4 Results

PCR based detection method for *Lysobacter*

When 16S ribosomal DNA sequences from 250 *Lysobacter* strains were aligned and examined, six potential primer sequences are found to be conserved with appropriate size and location. These sequences are list below (location in C3 16S rDNA sequence map shown in Fig. 1).

L1: 3'-TGTTGGGGGCAACTTGGCCCTCA;

L2: 3'-CCTTGGGTGGCGAGTGGCGGACGGGTGAGGAATACG;

L3: 3'-TCGGAATCTGCCTATTTGTGGGGGATAAC;

L4: 3'-GAGCCGACGTCGGATTAGCTAGTT;

L5: 3'-GAGGAACATCTGTGGCGAAGGCGAC;

L6: 3'-TACTAGAGTGCGGTAGAG.

As shown in Fig. 2, all 6 potential conserved regions are in bacterial hypervariable region site (Neefs, *et al.* 1990), which suggests big chance to find *Lysobacter* specific sequence among those six candidates. Using each of these sequences separately as a forward primer, with 1525 universal bacterial primer as the reverse primer, in PCR amplification of 16S rDNA from *Lysobacter* spp. and other bacterial genera, sequence L4 was found to exclusively amplify DNA from

Lysobacter strains, producing a 1,100 bp product as revealed in electrophoresis (Fig. 3 and 4). Amplification using the other 5 sequences as primers produced non-specific PCR product from DNA of other bacteria strains as well as *Lysobacter* spp. Based on alignment between *Lysobacter* strains and closely related species *Stenotrophomonas maltophilia* around L4 area in 16S rDNA sequence, L4 shows high specific conservation in *Lysobacter* strains, but not in *S. maltophilia*, which has multiple nucleotide variations (Fig. 5).

Based on the initial results with L4, an experiment was conducted in which the primer was used to amplify DNA extracted from six field soil samples in which the existence of *Lysobacter* spp. was suspected. For nearly all of the soil samples tested, either a 1,100 bp band or no PCR product was found in the electrophoresis. The exception was a 1,500 bp band amplified from one soil (Fig. 6). Upon sequencing of the DNA in these bands, the 1,100 bp bands were found to be *Lysobacter*. Using 1525R as primer, the 1,500 bp band corresponded to *Stenotrophomonas maltophilia* (92% identity) by sequencing and BLAST search.

Enrichment culturing for *L. enzymogenes*

In the screening of antibacterial drugs for activity against *L. enzymogenes* and other bacterial species, all strains of *L. enzymogenes*, *L. antibioticus*, and *Stenotrophomonas maltophilia* were insensitive to penicillin and kanamycin at

200 mg/L. Therefore, these antibiotics, along with the fungicide cycloheximide, were added to all subsequent media used in culturing *L. enzymogenes* from soil.

In comparing chitin and yeast cells as carbon substrates for enrichment culturing of *L. enzymogenes* C3R5 from soil, the two substrates were similarly effective in enriching populations of C3R5 so that it could be detected by growth on TSARC. For either substrate, 0.5 g/L as sufficient for use in a broth form. Both required that the *L. enzymogenes* population in the soil be at least 100 CFU/g. (Table 5)

Because chitinolysis is one distinguishing feature of *L. enzymogenes* (Christensen & Cook 1978) and yeast cells, which contain high concentrations of proteins and other carbohydrates, is presumably a much less exclusive substrate, chitin was chosen as the carbon source for the enrichment broth, while yeast cells was used as the carbon source in the agar medium (YCA) for isolating chitinolytic bacteria growing in the enrichment broth.

Comparative sensitivity of enrichment culturing and PCR in detecting *L. enzymogenes* in soil

When the two methods were used to assay the same soils containing various cell concentrations of *L. enzymogenes* C3R5, PCR amplification using sequence L4 as a primer was much more sensitive in detecting C3R5. As in the previous

experiment, populations of C3R5 higher than 10^2 CFU/g could be cultured and isolated from a sterilized soil, but effective culturing of C3R5 added to raw soil required that its population be at least 10^4 CFU/g (data not shown). The PCR method on the other hand, could detect C3R5 in sterilized or raw soil at populations as low as 10^2 CFU/g (Fig. 7).

Validation of enrichment culturing and PCR methods using Nebraska field soils

PCR was more effective of the two methods in detecting *Lysobacter* spp. in field soil samples (Table 6). Two rounds of DNA extraction and PCR amplification were required for the PCR method. In the first round, *Lysobacter* was detected in 19 (33%) of the 58 samples by PCR. A second round of PCR amplification on soil samples that were negative in the first round more than doubled the total number of PCR-positive samples to 40 (69% of all samples). In contrast, only 6 (10%) of the 58 soil samples proved positive for *Lysobacter* by enrichment culturing, five of these were in the first round and in samples that were also positive by PCR. One soil sample (number 51) was positive by the enrichment culturing method but not by the PCR method. Out of 15 strains of *Lysobacter* isolated from the six soil samples, all were classified as *L. enzymogenes* related based on >95% identity in 16S rDNA sequence with known strains except for one strain that was more closely related to *L. antibioticus* and *L. gummosus* (97% and 96 % identity, respectively).

The distribution of *Lysobacter* and *L. enzymogenes* in soil within Nebraska

Lysobacter spp. and *L. enzymogenes* appeared to be generally distributed throughout Nebraska in that positive soil samples were not restricted to any particular region in the state. However, detection number for grass field sample is 24 out of 31, whereas 17 out of 27 for non-grass samples (Fig. 8). Based on chi-square test, probability is only 0.1891, which suggests that detection numbers for those two sample types are significantly different, and detection in grass samples is more frequent. The genus and species, however, were not found in all areas of the state, nor were they found in all of the multiple samples collected from any given area.

Sample 27 yielded a strain of *L. enzymogenes* and also strain that was equally identical to *L. gummosus* and *L. antibioticus* by way of its 16S rDNA sequence. The remaining 13 strains of *Lysobacter* isolated from Nebraska soil corresponded to *L. enzymogenes*. Since enrichment culturing method is biased towards *L. enzymogenes*, it is not surprising that most isolates are related with *L. enzymogenes*. Relationships between 16S rDNA sequences of isolates and known *Lysobacter* species was studied by phylogeny analysis (Dereeper *et al.* 2008) using HKY85 model (Fig. 9). From phylogeny analysis result, there is no clear correlation between subgroups and different plant cover types, or geographic locations.

Table 1. Bacterial strains used in this study.

Bacterial Species	Strain
<i>Lysobacter enzymogenes</i>	C3
<i>L. enzymogenes</i>	C3R5
<i>L. enzymogenes</i>	OH11
<i>L. enzymogenes</i>	N4-7
<i>L. enzymogenes</i>	495 (type strain)
<i>Lysobacter antibioticus</i>	UASM 3C (type strain)
<i>Stenotrophomonas maltophilia</i>	34S1
<i>Stenotrophomonas maltophilia</i>	13270
<i>Stenotrophomonas maltophilia</i>	19867
<i>Enterobacter cloacae</i>	E1
<i>Escherichia coli</i>	XL1
<i>Xanthomonas campestris</i> pv. <i>campestris</i>	A249
<i>Bacillus pumilus</i>	INR7

Table 2. PCR reagents and conditions in these tests are reported in

PCR reagents	PCR cycles
ddH ₂ O: 37μL 10XPCR Buffer: 5μL dNTP: 1μL Taq DNA polymerase: 0.5μL MgCl ₂ : 2.5μL Primer 27F: 1μL Primer 1525R: 1μL Taq DNA polymerase: 0.5μL DNA Template: 2μL	Lid temperature: 105°C 1: T=94°C 0:1:00 2: T=94°C 0:00:15 3: T=52°C 0:00:30 4: T=72°C 0:02:00 2-4 repeat 30 cycles 5: T=72°C 07:00 6: Hold, 4°C
PCR reagents sources	
Taq DNA polymerase, Recombinant (Invitrogen Catalog #: 10342-020) 100 mM dNTP (Invitrogen Catalog #: Set 10297-018) 1 Kb Plus DNA Ladder™ (Invitrogen Catalog #: 10787-018) TrackIt™ Cyan/Orange Loading Buffer (Invitrogen Catalog #: 10482-028) Universal bacterial primer 27F: AGAGTTTGATCCTGGCTCAG (20mM) (Invitrogen) Universal bacterial primer 1525R: AGGAGGTGATCCAGCC (20mM) (Invitrogen)	

Table 3. Soil samples collected from Nebraska locations and tested for presence of *Lysobacter* spp.

Sample number(s)	Location	County	Plant cover	Collection Date
1,4,6	Halsey National forest	Thomas	Grasses under conifer trees	10/2006
2,3,5	Halsey National forest	Thomas	Grasses	10/2006
7	Barlette	Wheeler	Grasses	5/2007
8	Chambers	Holt	Grass with trees	5/2007
9	Spalding	Greeley	Grass with trees	5/2007
10	St. Edward	Boone	Grasses	5/2007
11	Silver creek	Merrick	Grasses	5/2007
12	Shelby	Polk	Corn, previously soybean	5/2007
13	Shelby	Polk	Grasses	5/2007
14-16	Sand Hills area	Valley	Grasses	6/2007
17, 18	Scottsbluff	Scottsbluff	Potato	8/2007
19- 21, 28, 31	Scottsbluff	Scottsbluff	Sugarbeet	8/2007
22, 29	Scottsbluff	Scottsbluff	Corn (100 year continuous culture)	8/2007
23, 24	Halsey National forest	Thomas	Grassland under trees	8/2007
25-27, 30, 33, 35, 37	Scottsbluff	Scottsbluff	Dry bean	8/2007
32	Scottsbluff	Scottsbluff	Conifer trees	8/2007
34	Scottsbluff	Scottsbluff	Grasses near corn field	8/2007
36	Scottsbluff	Scottsbluff	Sunflower	8/2007
38-40	Mead	Saunders	Kentucky bluegrass lawn	6/2008
41	Cherry	Cherry	Corn field w/ nematode, previously in grasses	6/2008
42	Box Butte	Box Butte	Grasses	6/2008

43	Box Butte	Box Butte	Wheat w/ bacterial spot	6/2008
44	Cherry	Cherry	Sand hills grasses near corn field	6/2008
45	Cherry	Cherry	Corn	6/2008
46	Box Butte	Box Butte	Grasses	6/2008
47,48	Ashfall State Park	Antelope	Grasses	6/2008
49	Beatrice	Gage	Grasses near wheat field	10/2008
50	Fairbury	Jefferson	Soybean; previously in corn	10/2008
51	Hebron	Thayer	Soybean (outside of grassland)	10/2008
52	Red Cloud	Webster	Fallow; previously wheat	10/2008
53	Naponee	Franklin	Grasses	10/2008
54	Alma	Harlan	Sorghum	10/2008
55	Alma	Harlan	Grasses near sorghum field	10/2008
56	McCook	Red Willow	Sorghum	10/2008
57	McCook	Red Willow	Grasses near sorghum field	10/2008
58	Nelson	Nuckolls	Grasses	10/2008

Table 4. Bioassay tests and expected results

Tests	<i>Lysobacter</i>	Non- <i>Lysobacter</i>
Growth @ 28°C and 37°C	28°C (No or min. growth @37°C)	37°C (No or min. growth @28°C)
KOH test (3% KOH solution)	+ (Gram -)	- (Gram -)
Oxidase activity test	+	-
Flagella motility test	-	+
Gliding motility	+	-

Table 5. Result for detecting C3R5 from soil using Yeast and Chitin Broth.

		Yeast Broth (g/L)					Chitin Broth (g/L)				
Concentration		0	0.1	0.25	0.5	1.0	0	0.1	0.25	0.5	1.0
CFU/g	Population in soil										
	Days	0 3 6	0 3 6	0 3 6	0 3 6	0 3 6	0 3 6	0 3 6	0 3 6	0 3 6	0 3 6
	Strain										
0	C3R5	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -
1	C3R5	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -
10	C3R5	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -
100	C3R5	- - -	- - -	- - +	- + +	- + +	- - -	- - -	- - -	- + +	- + +

Table 6. Effectiveness of PCR and enrichment culturing methods in detecting *Lysobacter* spp. in 58 soil samples.

Category	Number of samples after 1 round (% of total)	Total number of samples after 2 rounds (% of total)
1. Positive by PCR/ negative by culturing	14 (24)	35 (60)
2. Positive by culturing/ negative by PCR	0 (0)	1 (2)
3. Positive by PCR and culturing	5 (9)	5 (9)
4. Negative by PCR and culturing	39 (67)	12 (21)
Total positive by PCR (=sum of categories 1 and 3)	19 (33)	40 (69)
Total positive by culturing (=sum of categories 2 and 3)	5 (9)	6 (10)

Table 7. Occurrence of *Lysobacter* spp. based on plant cover.

Plant cover	Postive/negative for <i>Lysobacter</i>	Number of samples	Sample location number (identified in Table 3)
Grasses	Positive	24	1, 2, 3, 4, 6, 9, 14-16, 23, 24, 38*, 39, 40*, 42, 44, 46-49, 53*, 55, 57, 58
Grasses	Negative	7	5, 7, 8, 10, 11, 13, 34
Cereals (corn, sorghum, wheat)	Positive	5	22, 29, 41, 45, 56*
Cereals (corn, sorghum, wheat)	Negative	4	12, 43, 52, 54
Other (common bean, conifer, potato soybean, sugarbeet, sunflower)	Positive	12	18, 21, 25, 26, 27*, 30, 31, 32, 33, 35, 37, 50
Other (common bean, conifer, potato soybean, sugarbeet, sunflower)	Negative	6	17, 19, 20, 28, 36, 51#
Total non-grasses	Positive	17	
Total non-grasses	Negative	10	

*, # indicate positive detection of *Lysobacter* spp. by PCR and enrichment culture and by enrichment culturing only. All other *Lysobacter*-positive were positive by way of PCR only.

```

1  tcgacctgca ggcggccgca ctagtgatta gagtttgatc ctggctcaga gtgaacgctg
61  ggcggcaggc ctaacacatg caagtogaac ggcagcacag aggagcttgc tccttgggtg
121 gcgagtggcg gacgggtgag gaaaacgTCG GAATCTGCCT ATTTGTGGGG GATAACgtag
181 ggaaacttac gctaataccg catacagacct acgggtgaaa gtgggggacc gcaaggCCTT
241 CACGCAGATA GATGAGCCGA CGTCGGATTA GCTAGTTggc ggggtaaagg cccaccaagg
301 cgacgatccg tagctggtct gagaggatga tcagccacac tggaaactgag acacggtcca
361 gactcctacg ggaggcagca gtggggaata ttggacaatg ggcgcaagcc tgatccagcc
421 atgcccgctg tgtgaagaag gccttcgggt tgtaaagcac ttttgtccgg aaagaaaagc
481 ttagggttaa taaccctgag tcatgacggg accggaagaa taagcaccgg ctaacttcgt
541 gccagcagcc gcggtaatac gaagggtgca agcgttactc ggaattactg ggcgtaaagc
601 gtgcgtaggt gtttggttaa gtctgatgtg aaagccctgg gctcaacctg ggaatggcat
661 tggaaactgg ctTACTAGAG TCGGTTAGAG ggtagcggaa ttcccggtgt agcagtgaaa
721 tgcgtagata tcggGAGGAA CATCTGTGGC GAAGGGCGCt acctggacca gcactgacac
781 tgaggcacga aagcgtgggg agcaaacagg attagatacc ctggtagtcc acgcctaaa
841 cgatgcgaac tggTGTTGG GGGCAACTTG GCCCTCAgta tcgaagctaa cggcgttaag
901 ttgcccgcc tgggaagtac ggtcgcaaga ctgaaactca aaggaattga cgggggcccg
961 cacaagcggg ggagtatgtg gtttaattcg atgcaacgcg cagaacctta cctggccttg
1021 acatgtcgag aactttccag agatggattg gtgccttcgg gaactcgaac acaggtgctg
1081 catggctgtc gtcagctcgt gtcgtgagat gttgggttaa gtcccgaac gagegcaacc
1141 cttgtcctta gttgccagca cgtaatggtg ggaactctaa ggagaccgcc ggtgacaaac
1201 cggaggaagg tgggatgac gtcaagtcac catggccctt acggccaggg ctacacacgt
1261 actacaatgg tagggacaga gggctgcaaa cccgagaggg caagccaatc ccagaaacc
1321 tatctcagtc cggattggag tctgcaactc gactccatga agtcggaatc gctagtataa
1381 gcagatcagc attgctgceg tgaatacgtt cccgggcctt gtacacaccg cccgtcacac
1441 catgggagtt tgttcacca gaagcaggta gcttaacctt cgggagggcg cttgccacgg
1501 tgtggccgat gacgggggtga agtcgtaaca aggtaccgta tcggaaggtg cGGCTGATC
1561 ACCTCCTTaa tccc

```

Figure 1. Six potential *Lysobacter* conserved sequences in *L. enzymogenes* N4-7 16S rDNA map (L1: green, 855-877; L2: light blue, shared partially with L4, 237-262; L3: red, 148-176; L4: dark blue, shared partially with L2, 254-277; L5: purple, 735-759; L6: orange, 673-690), and 1525R universal bacterial primer (in yellow and red).

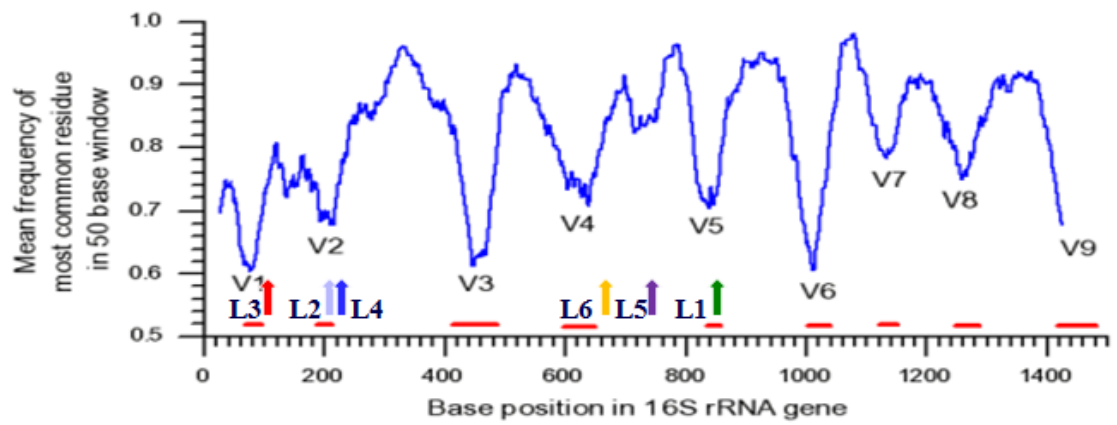


Figure 2. Six potential *Lysobacter* conserved sequences in bacterial hypervariable regions map within the 16S rRNA gene (Neefs *et al.* 1990).

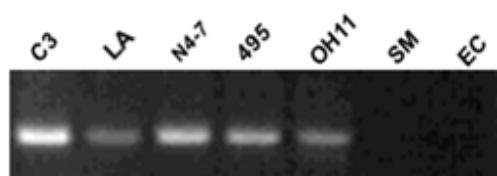


Figure 3. Electrophoresis gel with products from amplification by L4 and 1525R universal primer of 16S rDNA from *Lysobacter enzymogenes* strain C3, N4-7, OH11, and 495, *Lysobacter antibioticus* (LA), *Stenotrophomonas maltophilia* strain 34S1(SM) and *Escherichia coli* (EC).

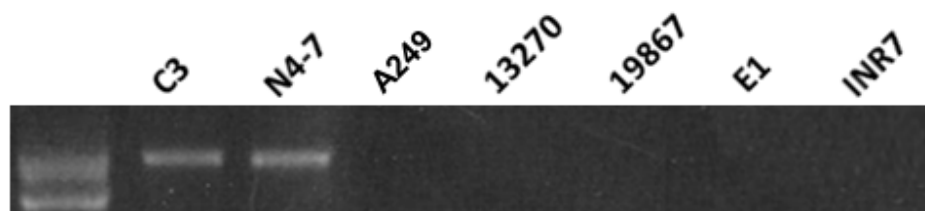


Figure 4. Amplification by L4 and 1525R from 16S rDNA of *Lysobacter enzymogenes* strain C3 and N4-7 495, *Xanthomonas campestris pv. campestris* strain A249, *Stenotrophomonas maltophilia* strain 13270 and 19867, *Enterobacter cloacae* strain E1 and *Bacillus pumilus* strain INR7.

```

L.e 495      TTCACGCAGATAGATGAGCCGACGTCGGATTAGCTAGTTGGCGGGGTAAAGGCCACCAA
LeC3        T-CACGCAGATAGATGAGCCGACGTCGGATTAGCTAGTTGGCGGGGTAAAGGCCACCAA
L.e.OH11    T-CACGCAGATAGATGAGCCGACGTCGGATTAGCTAGTTGGCGGGGTAAAGGCCACCAA
L.antibiot  T-CGCGCAGATAGATGAGCCGACGTCGGATTAGCTAGTTGGCGGGGTAAAGGCCACCAA
L.gummosus  T-CGCGCAGATAGATGAGCCGACGTCGGATTAGCTAGTTGGCGGGGTAAAGGCCACCAA
L.koreensi  T-CGCGCAGATAGATGAGCCGACGTCGGATTAGCTAGTTGGCGGGGTAAAGGCCACCAA
L.brunesce  T-CGCGCGATTAGATGAGCCGATGTCAGATTAGCTAGTTGGCGGGGTAAAGGCCACCAA
L.c.Ko07    T-CGCGCAGAAAGATGAGCCGACGTCGGATTAGCTAGTTGGAGGGGTAAAGGCCACCAA
L.daejeone  T-CACGCAGAAAGATGAGCCGACGTCGGATTAGCTAGTTGGCGGGGTAAAGGCCACCAA
S. maltoph  T-TGCGCGATTGAATGAGCGGATGTCAGATTATCTATTTGGCGGGGTAAAGGCCACCAA

```

Figure 5. Alignment in L4 sequence area between *Lysobacter* strains (L.e 495: *L. enzymogenes* 495; LeC3: *L. enzymogenes* C3; L.e.OH11: *L. enzymogenes* OH11; L.antibiot: *L. antibioticus*; L.gummosus: *L. gummosus*; L.koreensi: *L. koreensis*; L.brunesce: *L. brunescens*; L.c.ko07: *L. concretionis* Ko07; L.daejeone: *L. daejeonensis*) and closely related species *Stenotrophomonas maltophilia* (S.maltoph). Red indicates mostly conserved nucleotide, and blue shows variable nucleotide.

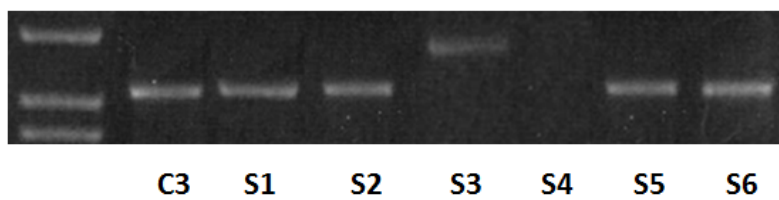


Figure 6. L4 with 1525R used to amplify DNA extracted from six field soil samples (S1-S6) with C3 as control.

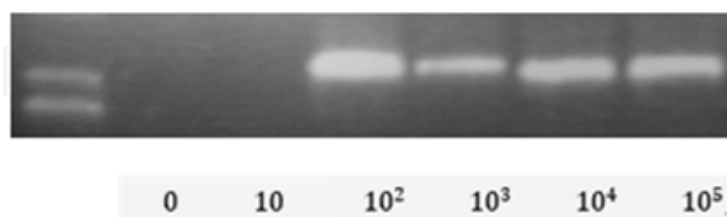


Figure 7. PCR Detection of different concentration of C3R5 in sterile soil by using L4 and 1525R.

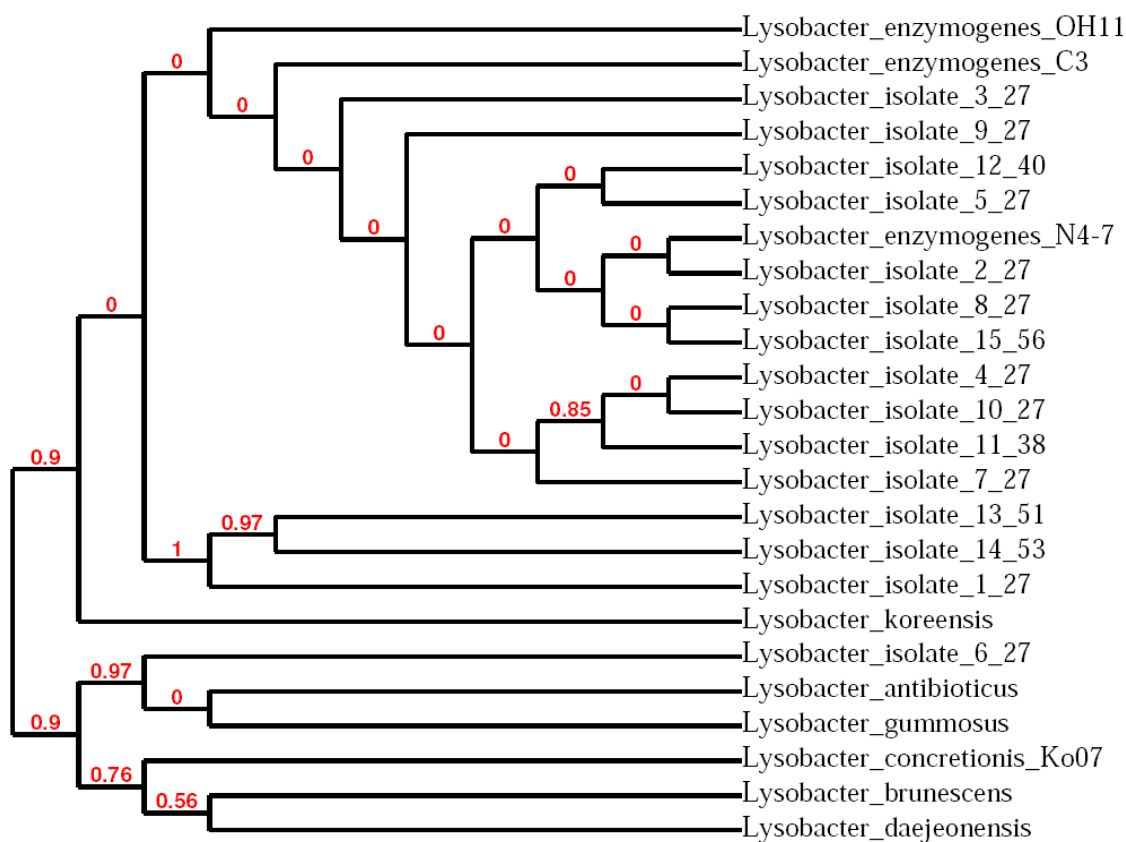


Figure 9. Phylogenetic analysis (model: HKY85) of 15 strains of *Lysobacter* species isolated from Nebraska soils (*Lysobacter* isolate 1-15, with source sample number 27, 38, 40, 51, 53, 56) and nine reported strains from seven species (values in red indicate branch support values).

Chapter 5 Discussion

Since the original description of *Lysobacter* by Christiansen and Cook (1978), there have been no reported population studies that focused on the genus *Lysobacter* or any species within this genus. The occurrence of *Lysobacter* spp. was revealed in some studies on soil bacterial community structure or diversity studies (Lee *et al.* 2006, Schmalenberger and Tebbe 2003, Sigler and Turco 2002, Moyer *et al.* 1995, Ikner *et al.* 2007), but conclusions cannot be drawn from diversity studies that did not mention *Lysobacter* spp. because such studies tend to be biased to the most numerous organisms and involve small sample numbers. This study is the first to focus on *Lysobacter* spp. population and geographic distribution.

This study is the first to employ a DNA-based detection method developed specifically for *Lysobacter* spp. This DNA based detection method proved to be very sensitive in detecting *Lysobacter* from soil. Similar methods have been used in investigating other common bacteria in soil. For example, Scarpellini *et al.* (2004) and Sanguin *et al.* (2008) designed *Pseudomonas* specific primers and amplified 16S rDNA of extracted DNA from bacterial suspensions or rhizosphere soil to assess population structure of *Pseudomonas*. In another study, populations of *Bacillus* spp. in forest soils were analyzed by amplifying 23S rDNA and 16S rDNA from soil samples using Bacilli-specific primer sets (Ji *et al.* 2007). Even

though it is proved that PCR detection method is much more sensitive than enrichment culturing method, there is one soil sample detected only by enrichment culturing method. However, by PCR using L4, the isolate from this soil sample can be verified. The failure of PCR to detect *Lysobacter* in this particular soil sample may be caused due to insufficient subsampling. Another interesting fact is that enrichment culturing method is more sensitive in detecting C3R5 in sterile soil than in raw soil. For culturing method, there was a 3 day culturing period, during which different rates of population change in the soil could account for the different level of sensitivity. A possible reason is that in raw soil, existed microorganisms are still present during this period, so there is more severe competition for *Lysobacter* to survive in it. Higher initial population may help *Lysobacter*'s survival, which also results in detection.

The PCR detection method used in this study has some limitations. First, the primer used in DNA based method is genus specific; DNA sequences conserved only in *L. enzymogenes* could not be found. Second, because of the non-uniform distribution of bacteria in soil and the small amount of soil that can be extracted for DNA, multiple subsamples of a soil sample must be extracted. We have not examined the benefits of extracting more than two subsamples from each soil sample; it is conceivable that more subsamples would improve detection accuracy but would also greatly increase the time and cost per sample. Third, the method does not provide a living culture for further study.

Because many bacterial genera in soil have similar nutritional and drug-resistance characteristics as *Lysobacter*, it was not possible to create a medium that is highly specific for the genus. Therefore, we had to first culture a spectrum of organisms from field samples and then identify the cultured organisms through physiological and genetic characterization, a strategy commonly used in studying populations and diversity of other soilborne bacteria (van Elsas *et al.* 1998). Our isolation method was made more specific for *L. enzymogenes* through the use of antibiotics. This was reflected in the fact that we isolated only one strain of *Lysobacter* that was not *L. enzymogenes*. The bias of the isolation method for *L. enzymogenes* may be one reason why we were not able to isolate members of the genus from most of the soil samples that were *Lysobacter* positive in the PCR assay. Alternatively, the culturing method has much lower sensitivity than the PCR method and populations of *Lysobacter* in the soils may have been too low to be isolated. Yet another possible explanation is that the populations were in a viable but non-culturable state. This physiological state may occur when bacterial cells are subjected to changes in temperature, nutrients, pH, or other conditions (Colwell 2000, Oliver 2005). The dissimilarity in our results using the two methods are in line with those from microbial diversity studies (Ikner *et al* 2007, Lee *et al.* 2006) that indicated the presence of *Lysobacter* spp. in soils using DNA based techniques but were not successful in culturing the organism from the same soils.

Despite the limitations inherent in each of the two methods used in this study, each method is specific to a taxonomic group and, when used together, are complementary. By using these two methods and assaying systematically-collected samples, we can conclude with greater confidence where *Lysobacter* and *L. enzymogenes* do or do not occur. Although not all areas in Nebraska were sampled in this study and sample numbers were low in some locations, we can conclude from the detection of *Lysobacter* in 71% of samples that the genus is relatively wide-spread throughout the state. By mapping the geographic distribution of *Lysobacter* within Nebraska we conclude that it is not restricted by soil type. Plant cover appears to have an influence as *Lysobacter* was detected at a higher frequency in samples from areas with perennial grass cover than non-grass areas. It may be due to the continuous presence of live roots in perennial grasses providing a stable environment for microorganisms. In contrast, the plant root system in agricultural soils is disturbed each year so that the rhizosphere environment is dramatically changed frequently, which may be not conducive for *Lysobacter* population growth.

It remains to be determined whether biotic and abiotic factors affect populations of *Lysobacter* in a quantitative manner. Because *L. enzymogenes* was confirmed in so few locations, further work is necessary before conclusions about the species

can be drawn, but our preliminary evidence suggests that there are subpopulations of the species within the state.

By assessing population structure and distribution of *Lysobacter* in nature, the role of *Lysobacter* in the microbial community, as well as its interactions with other organisms including plants, can be better understood. Ultimately, this may lead to strategies to conserve and enhance its populations in the field and thereby help improve the utilization of *Lysobacter* for biocontrol.

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