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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  $\gamma$ -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  $\mu$ m), 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).  $\beta$ -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  $\beta$ -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 broadspectrum phenazine antibiotic inhibiting bacteria and fungi. Antibiotics of  $\beta$ -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, Lysobacterindicative 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

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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=BlastH ome). 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,  $10^0$ ,  $10^1$ ,  $10^2$  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  $10^5$  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'-TGTTGGGGGGGCAACTTGGCCCTCA;

L2: 3'-CCTTGGGTGGCGAGTGGCGGACGGGTGAGGAATACG; L3: 3'-TCGGAATCTGCCTATTTGTGGGGGGATAAC; 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
Lysobacter enzymogenes	C3
L. enzymogenes	C3R5
L. enzymogenes	OH11
L. enzymogenes	N4-7
L. enzymogenes	495 (type strain)
Lysobacter antibioticus	UASM 3C (type strain)
Stenotrophomonas maltophilia	34S1
Stenotrophomonas maltophilia	13270
Stenotrophomonas maltophilia	19867
Enterobacter cloacae	E1
Escherichia coli	XL1
Xanthomonas campestris pv. campestris	A249
Bacillus pumilus	INR7

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

ddH2O: $37\mu$ LLid temperature: $105^{\circ}$ C $10XPCR$ Buffer: $5\mu$ L1: T=94^{\circ}C 0:1:00dNTP: $1\mu$ L2: T=94^{\circ}C 0:00:15Taq DNA polymerase: $0.5\mu$ L3: T=52^{\circ}C 0:00:30MgCl2: $2.5\mu$ L4: T=72^{\circ}C 0:02:00Primer 27F: $1\mu$ L2-4 repeat 30 cyclesPrimer 1525R: $1\mu$ L5: T=72^{\circ}C 07:00
$10$ XPCR Buffer: $5\mu$ L1: T=94°C 0:1:00 $d$ NTP: $1\mu$ L2: T=94°C 0:00:15Taq DNA polymerase: $0.5\mu$ L3: T=52°C 0:00:30MgCl2: $2.5\mu$ L4: T=72°C 0:02:00Primer 27F: $1\mu$ L2-4 repeat 30 cyclesPrimer 1525R: $1\mu$ L5: T=72°C 07:00
dNTP: $1\mu L$ 2: T=94°C 0:00:15Taq DNA polymerase: $0.5\mu L$ 3: T=52°C 0:00:30MgCl2: $2.5\mu L$ 4: T=72°C 0:02:00Primer 27F: $1\mu L$ 2-4 repeat 30 cyclesPrimer 1525R: $1\mu L$ 5: T=72°C 07:00
Taq DNA polymerase: 0.5μL       3: T=52°C 0:00:30         MgCl2: 2.5μL       4: T=72°C 0:02:00         Primer 27F: 1μL       2-4 repeat 30 cycles         Primer 1525R: 1μL       5: T=72°C 07:00
MgCl2: 2.5μL       4: T=72°C 0:02:00         Primer 27F: 1μL       2-4 repeat 30 cycles         Primer 1525R: 1μL       5: T=72°C 07:00
Primer 27F: 1μL         2-4 repeat 30 cycles           Primer 1525R: 1μL         5: T=72°C 07:00
Primer 1525R: 1μL 5: T=72°C 07:00
Taq DNA polymerase: 0.5μL 6: Hold, 4°C
DNA Template: 2µL
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 <sup>TM</sup> Cyan/Orange Loading Buffer (Invitrogen Catalog #: 10482-028)
Universal bacterial primer 27F: AGAGTTTGATCCTGGCTCAG (20mM)
(Invitrogen)
Universal bacterial primer 1525R: AGGAGGTGATCCAGCC (20mM) (Invitrogen)

Sample	Location	County	Plant cover	Collection
number(s)		-		Date
146	Halaay National forest	Thomas	Crosses under conifer trace	10/2006
1,4,0	Halsey National forest	Inomas	Grasses under confier trees	10/2006
2,3,5	Halsey National forest	Thomas	Grasses	10/2006
_	<b>D</b>	XX /1 1		5/2005
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,	Scottsbluff	Scottsbluff	Sugarbeet	8/2007
31				
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,	Scottsbluff	Scottsbluff	Dry bean	8/2007
33, 35, 37				
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

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

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	Lysobacter	Non-Lysobacter
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	+	-

			Yeast Broth (g/L)			Chit	in Broth	(g/L)			
C C	oncentration										
		0	0.1	0.25	0.5	1.0	0	0.1	0.25	0.5	1.0
Population	n in soil										
CFU/g	Days Strain	036	036	036	036	036	036	036	036	036	036
0	C3R5										
1	C3R5										
10	C3R5										
100	C3R5			+	• + +	• + +				• + +	• + +

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

Table 6. Effectiveness of PCR and enrichment culturing methods in detecting Lysobacter

spp. in 58 soil samples.

	Number of samples after	Total number of
Category	1 round (% of total)	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	19 (33)	40 (69)
categories 1 and 3)		
Total positive by culturing (=sum of	5 (9)	6 (10)
categories 2 and 3)		

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

Plant cover	Postive/negative for	Number of samples	Sample location
	Lysobacter		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,	Positive	5	22, 29, 41, 45, 56*
sorghum, wheat)			
Cereals (corn,	Negative	4	12, 43, 52, 54
sorghum, wheat)			
Other (common	Positive	12	18, 21, 25, 26, 27*,
bean, conifer, potato			30, 31, 32, 33, 35,
soybean, sugarbeet,			37, 50
sunflower)			
Other (common	Negative	6	17, 19, 20, 28, 36,
bean, conifer, potato			51#
soybean, sugarbeet,			
sunflower)			
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	caagtcgaac	ggcagcacag	aggagcttgc	tccttgggtg
121	gcgagtggcg	gacgggtgag	gaaaacg <mark>TCG</mark>	GAATCTGCCT	ATTTGTGGGG	<b>GATAAC</b> gtag
181	ggaaacttac	gctaataccg	catacgacct	acgggtgaaa	gtgggggacc	gcaagg <mark>CCTT</mark>
241	CACGCAGATA	GAT <mark>GAGCCGA</mark>	CGTCGGATTA	<b>GCTAGTT</b> ggc	ggggtaaagg	cccaccaagg
301	cgacgatccg	tagctggtct	gagaggatga	tcagccacac	tggaactgag	acacggtcca
361	gactcctacg	ggaggcagca	gtggggaata	ttggacaatg	ggcgcaagcc	tgatccagcc
421	atgccgcgtg	tgtgaagaag	gccttcgggt	tgtaaagcac	ttttgtccgg	aaagaaaagc
481	ttagggttaa	taaccctgag	tcatgacggt	accggaagaa	taagcaccgg	ctaacttcgt
541	gccagcagcc	gcggtaatac	gaagggtgca	agcgttactc	ggaattactg	ggcgtaaagc
601	gtgcgtaggt	ggtttgttaa	gtctgatgtg	aaagccctgg	gctcaacctg	ggaatggcat
661	tggaaactgg	ct <b>TACTAGAG</b>	TGCGGTAGAG	ggtagcggaa	ttcccggtgt	agcagtgaaa
721	tgcgtagata	tcggGAGGAA	CATCTGTGGC	GAAGGCGGCt	acctggacca	gcactgacac
781	tgaggcacga	aagcgtgggg	agcaaacagg	attagatacc	ctggtagtcc	acgccctaaa
841	cgatgcgaac	tgga <b>TGTTGG</b>	GGGCAACTTG	<b>GCCCTCA</b> gta	tcgaagctaa	cggcgttaag
901	ttegeeegee	tgggaagtac	ggtcgcaaga	ctgaaactca	aaggaattga	cgggggcccg
961	cacaagcggt	ggagtatgtg	gtttaattcg	atgcaacgcg	cagaacctta	cctggccttg
1021	acatgtcgag	aactttccag	agatggattg	gtgccttcgg	gaactcgaac	acaggtgctg
1081	catggctgtc	gtcagctcgt	gtcgtgagat	gttgggttaa	gtcccgcaac	gagcgcaacc
1141	cttgtcctta	gttgccagca	cgtaatggtg	ggaactctaa	ggagaccgcc	ggtgacaaac
1201	cggaggaagg	tggggatgac	gtcaagtcat	catggccctt	acggccaggg	ctacacacgt
1261	actacaatgg	tagggacaga	gggctgcaaa	cccgcgaggg	caagccaatc	ccagaaaccc
1321	tatctcagtc	cggattggag	tctgcaactc	gactccatga	agtcggaatc	gctagtaata
1381	gcagatcagc	attgctgcgg	tgaatacgtt	cccgggcctt	gtacacaccg	cccgtcacac
1441	catgggagtt	tgttgcacca	gaagcaggta	gcttaacctt	cgggagggcg	cttgccacgg
1501	tgtggccgat	gacggggtga	agtcgtaaca	aggtaccgta	tcggaaggtg	GGCTGGATC
1561	<b>ACCTCCTT</b> aa	tece				

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. Electrophesis 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	TTCACGCAGATAGATGAGCCGACGTCGGATTAGCTAGTTGGCGGGGTAAAGGCCCACCAA
LeC3	T-CACGCAGATAGATGAGCCGACGTCGGATTAGCTAGTTGGCGGGGTAAAGGCCCACCAA
L.e.OH11	T-CACGCAGATAGATGAGCCGACGTCGGATTAGCTAGTTGGCGGGGTAAAGGCCCACCAA
L.antibiot	T-CGCGCAGATAGATGAGCCGACGTCGGATTAGCTAGTTGGCGGGGTAAAGGCCCACCAA
L.gummosus	T-CGCGCAGATAGATGAGCCGACGTCGGATTAGCTAGTTGGCGGGGTAAAGGCCCACCAA
L.koreensi	T-CGCGCAGATAGATGAGCCGACGTCGGATTAGCTAGTTGGCGGGGTAAAGGCCCACCAA
L.brunesce	T-CGCGCGATTAGATGAGCCGATGTCCGATTAGCTAGTTGGCGGGGTAATGGCCCACCAA
L.c.Ko07	T-CGCGCAGAAAGATGAGCCGACGTCGGATTAGCTAGTTGGAGGGGTAATGGCCCACCAA
L.daejeone	T-CACGCAGAAAGATGAGCCGACGCCGGATTAGCTAGTTGGCGGGGTAAAGGCCCACCAA
S. maltoph	T-TGCGCGATTGAATGAGCGGATGTCAGATTATCTATTTGGCGGGGGTAAAGGCCCACCAA

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 8. Soil sampling locations; number of soil samples with *Lysobacter* detected by PCR (red), and enrichment culture (blue) and total number of samples collected (black).



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 are 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 systematicallycollected 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 relatively wide-spread throughout the state. By mapping the geographic distribution of Lysobacter within Nebraska we conclude that 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.

# Chapter 6 Reference

- Ahmed, K., Chohnan, S., Ohashi, H., Hirata, T, Masaki, T., Sakiyama, F. 2003. Purification, bacteriolytic activity, and specificity of β-lytic protease from *Lysobacter* sp. IB-9374. Journal of Bioscience and Bioengineering 95:27-34.
- 2. Aslam, Z., Yasir, M., Jeon, C., Chung, Y. 2009. *Lysobacter oryzae* sp. nov., isolated from the rhizosphere of rice (*Oryza sativa* L.). Journal of Systematic and Evolutionary Microbiology 59: 675-680.
- 3. Bae, H. *et al.* 2005. *Lysobacter concretionis* sp. nov., isolated from anaerobic granules in an upflow anaerobic sludge blanket reactor. International Journal of Systematic and Evolutionary Microbiology 55: 1155–1161.
- 4. Banyko, J., Vyletelova, M. 2009. Determining the source of *Bacillus cereus* and *Bacillus licheniformis* isolated from raw milk, pasteurized milk and yoghurt. Letters in Applied Microbiology 48: 318–323.
- 5. Begunova, E., Stepnaya, O., Tsfasman, I., Kulaev, I. 2004. The effect of the extracellular bacteriolytic enzymes of *Lysobacter* sp. on gram-negative bacteria. Microbiology;73:267–70.
- 6. Binnerup, S., *et al.* 1993. Detection of viable, but non-culturable *Pseudomonas fluorescens* DF57 in soil using a microcolony epifluorescence technique. FEMS Microbiology Ecology 12 (2): 97-105.
- Blackmoore, M., Patel, N., Hillman, B., Kobayashi, D. 2009. Involvement of type IV secretion in *Lysobacter enzymogenes* pathogenesis of fungal and algal hosts. 2009 APS Annual Meeting.
- Blaschek, W., Kaesbauer, J., Kraus, J., and Franz, G. 1992. *Pythium aphanidermatum* culture cell-wall composition and isolation and structure of antitumour storage and solubilised cell-wall 1-3-β-D-glucans. Carbohydrate Research 231:293-307.
- Chen, J., Moore, W., Yuen, G., Kobayashi, D., Caswell-Chen, E. 2006. Influence of *Lysobacter enzymogenes* strain C3 on nematodes. Journal of Nematology 38: 233-239.
- Christensen, P. & Cook, F. D. (1978). *Lysobacter*, a new genus of nonfruiting, gliding bacteria with a high base ratio. International Journal of Systematic Bacteriology 28: 367–393
- 11. Christensen, P. 2001. Genus IV Lysobacter Christensen and Cook 1978. In

Bergey's Manual of Systematic Bacteriology ed. Brenner, D., Krieg, N., Staley, J. pp. 95–101. New York: Springer.

- Chohnan, S., Nonaka, J., Teramoto, K., Taniguchi, K., Kameda, Y., Tamura, H., et al. 2004. A second lysine-specific serine protease from *Lysobacter* sp. strain IB-9374. Journal of Bacteriology 186:5093-100.
- 13. Cikwell, R. 2000. Viable but nonculturable bacteria: a survival strategy. Journal of Infection and Chemotherapy Vol. 6 (2): 121-125.
- Costa, R., *et al.* 2006. Diversity and antagonistic potential of *Pseudomonas* spp. associated to the rhizosphere of maize grown in a subtropical organic farm. Soil Biology & Biochemistry 38: 2434–2447.
- 15. Colwell, R. 2000. Viable but nonculturable bacteria: a survival strategy. Journal of Infection and Chemotherapy 6: 121-125.
- Dereeper, A., *et al.* 2008. Phylogeny.fr: robust phylogenetic analysis for the nonspecialist. Nucleic Acids Research 36 (Web Server Issue): W465-9. Epub 2008 Apr 19.
- 17. Ensign, J., Wolfe, R. 1965. Lysis of bacterial cell walls by anenzyme isolated from a Myxobacter. Journal of Bacteriology 90: 395-402.
- 18. Ensign, J., Wolfe, R. 1966. Characterization of a small proteolytic enzyme which lyses bacterial cell walls. Journal of Bacteriology 91: 524-34.
- Folman, L., Postma, J., van Veen, J. 2003. Characterisation of *Lysobacter* enzymogenes (Christensen and Cook 1978) strain 3.1T8, a powerful antagonist of fungal diseases of cucumber. Microbiological Research 158: 107-115.
- Folman, L., De Klein, M., Postma, J., Van Veen, J. 2004. Production of antifungal compounds by *Lysobacter enzymogenes* isolate 3.1T8 under different conditions in relation to its efficacy as a biocontrol agent of *Pythium aphanidermatum* in cucumber. Biological Control 31: 145–154.
- 21. Giesler, L., and Yuen, G. 1998. Evaluation of *Stenotrophomonas maltophilia* strain C3 for biocontrol of brown patch disease. Crop Protection 17:509-513.
- 22. Hayward, A., Fegan, N., Fegan, M., Stirling, G. 2009. *Stenotrophomonas* and *Lysobacter*: ubiquitous plant-associated *gamma*-proteobacteria of developing significance in applied microbiology. Journal of Applied Microbiology, Online Published: 13 Jul 2009.
- 23. Ikner, L., *et al.* 2007. Culturable Microbial Diversity and the Impact of Tourism in Kartchner Caverns, Arizona. Microbial Ecology 53: 30–42.

- 24. Ji, S., *et al.* 2007. Metagenomic analysis of BTEX-contaminated forest soil microcosm. Journal of Microbiology and Biotechnology. Vol. 17 (4): 668-672.
- Jiang, Y., Hu, B., Liu, F. 2005. Selection and Identification of Antagonistic Bacteria against Soil borne Plant Pathogens. Chinese Journal of Biological Control 21 (4): 260-264.
- Jung, H., Ten, L., Im, W., Yoo, S., Lee, S. 2008. *Lysobacter ginsengisoli* sp. nov., a novel species isolated from soil in Pocheon Provincye, South Korea. Journal of Microbiology and Biotechnology 18 (9): 1496-1499.
- 27. Khan, I., and Yadav, J. 2004. Real-time PCR assays for genus-specific detection and quantification of culturable and non-culturable mycobacteria and pseudomonads in metalworking fluids. Molecular and Cellular Probes 18: 67–73.
- Kato, A., Nakaya, S., Ohashi, Y., Hirata, H. 1997. WAP-8294A2, a novel anti-MRSA antibiotic produced by *Lysobacter* sp. Journal of the American Chemical Society 119: 6680-6681.
- 29. Kato, A., Nakaya, S., Kokubo, N., Aiba, Y. 1998. A New Anti-MRSA Antibiotic Complex, WAP-8294A. The Journal of Antibiotics 51(10): 929-935.
- Katznelson, H., Gillespie, D., Cook, F. 1964. Studies on the relationships between nematodes and other soil microorganisms. Canadian Journal of Microbiology 10: 699-704.
- Kilic-Ekici, O. and Yuen, G. 2003. Induced resistance as a mechanism of biological control by *Lysobacter enzymogenes* strain C3.Phytopathology 93: 1103-10.
- 32. Kilic-Ekici, O., and Yuen, G. 2004. Comparison of strains of *Lysobacter enzymogenes* and PGPR for induction of resistance against *Bipolaris sorokiniana* in tall fescue. Biological Control 30: 446-455.
- Kim, J., and Crowley, D. 2007. Microbial Diversity in Natural Asphalts of the Rancho La Brea Tar Pits. Applied and Environmental Microbiology July: 4579– 4591.
- Kobayashi, D., and El-Barrad, N. 1996. Selection of bacterial antagonists using enrichment cultures for the control of summer patch disease in Kentucky bluegrass. Current Microbiology 32, 106–110.
- 35. Kobayashi, D., *et al.* 2005. A *clp* gene homologue belonging to the Crp gene family globally regulates lytic enzyme production, antimicrobial activity, and biological control activity expressed by *Lysobacter enzymogenes* strain C3.

Applied and Environmental Microbiology 71: 261–269.

- 36. Lee, J, *et al.* 2006. *Lysobacter koreensis* sp. nov., isolated from a ginseng field. International Journal of Systematic and Evolutionary Microbiology 56: 231–235.
- 37. Lee, M., *et al.* 2006. Dominance of *Lysobacter* sp. in the rhizosphere of two coastal sand dune plant species, *Calystegia soldanella* and *Elymus mollis*. Antonie van Leeuwenhoek 90:19-27.
- Li, S., Du, L., Yuen, G., and Harris, S. 2006. Distinct ceramide synthases regulate polarized growth in the filamentous fungus *Aspergillus nidulans*. Molecular Biology of the Cell 17: 1218–1227.
- 39. Moyer, C., *et al.* 1995. Phylogenetic Diversity of the Bacterial Community from a Microbial Mat at an Active, Hydrothermal Vent System, Loihi Seamount, Hawaii. Applied and Environmental Microbiology Apr.: 1555–1562.
- 40. Nakayama, T., Homma, Y., Hashidoko, Y., Mizutani, J., Tahara, S. 1999. Possible role of xanthobaccins produced by *Stenotrophomonas* sp. strain SB-K88 in suppression of sugar beet damping-off disease. Applied Environmental Microbiology 65: 4334-4339.
- Neefs, J., Van De Peer, Y., Hendriks, L. and De Wachter, R. (1990) Compilation of small ribosomal subunit RNA sequences. Nucleic Acids Resarch, 18: 2237-2317.
- 42. Oliver, J. 2005. The viable but nonculturable state in bacteria. The Journal of Microbiology vol. 43, special issue: 93-100.
- 43. Palumbo J., *et al.* 2005. Mutagenesis of b-1,3-glucanase genes in *Lysobacter enzymogenes* strain C3 results in reduced biological control activity towards Bipolaris leaf spot of tall fescue and Pythium damping off of sugarbeet. Phytopathology 95:701-707.
- 44. Palumbo, J., Sullivan, R., Kobayashi, D. 2003. Molecular characterization and expression in *Escherichia coli* of three b-1,3-glucanase genes from *Lysobacter enzymogenes* strain N4-7. Journal of Bacteriology 185: 4362-70.
- 45. Park, J., Kim, R., Aslam, Z., Jeon, C., Chung, Y. 2008. *Lysobacter* capsici sp. nov., with antimicrobial activity, isolated from the rhizosphere of pepper, and emended description of the genus *Lysobacter*. International Journal of Systematic and Evolutionary Microbiology 58, 387-392.
- 46. Patel, N., Blackmoore, M., Hillman, B., Kobayashi, D. 2009. Evidence for the role of Type VI secretion during *Lysobacter enzymogenes* pathogenesis of fungal hosts. 2009 APS Annual Meeting.

- 47. Pellett, S. Bigley, D., Grimesi, D. 1983. Distribution of *Pseudomonas aeruginosa* in a Riverine. Applied and Environment Microbiology Vol 50 No 1: 328-332.
- 48. Peterson, E., Gillespie, D., Cook, F. 1966. A wide spectrum antibiotic produced by a species of *Sorangium*. Canadian Journal of Microbiology 12: 221-30.
- 49. Picard, C., Bosco, M. 2008. Genotypic and phenotypic diversity in populations of plant-probiotic *Pseudomonas* spp. colonizing roots. Naturwissenschaften 95:1–16.
- Postma, J., Stevens, L., Wiegers, G., Davelaar, E., Nijhuis, E. 2009. Biological control of *Pythium aphanidermatum* in cucumber with a combined application of *Lysobacter enzymogenes* strain 3.1T8 and chitosan. Biological Control 48 (3): 301-309.
- 51. Reedy, R., kobayashi, D. 2003. Induction of the type III secretory pathway in the biocontrol bacterium *Lysobacter enzymogenes* strain C3 in the presence of fungal cell wall constituents. 2003 APS Annual Meeting.
- Romanenko, L., *et al.* 2008. *Lysobacter spongiicola* sp. nov., isolated from a deep-sea sponge. International Journal of Systematic and Evolutionary Microbiology 58, 370-374.
- 53. Sanguin, H., *et al.* 2008. Development of a 16S rRNA microarray approach for the monitoring of rhizosphere *Pseudomonas* populations associated with the decline of take-all disease of wheat. Soil Biology & Biochemistry 40: 1028–1039.
- Scarpellini, M., Franzetti, L., and Galli, A. 2004. Development of PCR assay to identify *Pseudomonas fluorescens* and its biotype. FEMS Microbiology Letters 236: 257–260.
- 55. Schaad, N., Jones, J., and Chun, W. (2001). Laboratory guide for identification of plant pathogenic bacteria. Third Edition.
- 56. Schmalenberger, A. and Tebbe, C. 2003. Bacterial diversity in maize rhizospheres: conclusions on the use of genetic profiles based on PCR-amplified partial small subunit rRNA genes in ecological studies. Molecular Ecology 12: 251-262.
- 57. Sessitsch, A., Kan, F., and Pfeifer, U. 2003. Diversity and community structure of culturable *Bacillus* spp. populations in the rhizospheres of transgenic potatoes expressing the lytic peptide cecropin B. Applied Soil Ecology 22: 149–158.
- Sigler, W., and Turco, R. 2002. The impact of chlorothalonil application on soil bacterial and fungal populations as assessed by denaturing gradient gel electrophoresis. Applied Soil Ecology 21: 107–118.

- Sullivan, R., *et al.* 2003. Taxonomic positioning of two biological control agents for plant diseases as *Lysobacter enzymogenes* based on phylogenetic analysis of 16S rDNA, fatty acid composition and phenotypic characteristics. Journal of Applied Microbiology 94:1079-1086.
- 60. Tarnawski, S. *et al.* 2003. Examination of Gould's modified S1 (mS1) selective medium and Angle's non-selective medium for collecting diversity of *Pseudomonas* spp. in soil and root environments. FEMS Microbiology Ecology 45, 97–104.
- 61. Tarnawski, S. *et al.* 2006.Phenotypic structure of *Pseudomonas* populations is altered under elevated pCO2 in the rhizosphere of perennial grasses. Soil Biology & Biochemistry 38: 1193–1201.
- 62. Ten, L., Jung, H., Im, W., Yoo, S., Lee, S. 2008. *Lysobacter daecheongensis* sp. nov., isolated from sediment of stream near the Daechung dam in South Korea. Journal of Microbiology 46: 519–524.
- 63. Tran, H., Kruijt, M., Raaijmakers, J. 2008. Diversity and activity of biosurfactantproducing *Pseudomonas* in the rhizosphere of black pepper in Vietnam. Journal of Applied Microbiology 104: 839–851.
- 64. van Elsas, J., Duarte, G., Rosado A., Smalla K. 1998. Microbiological and molecular methods for monitoring microbial inoculants and their effects in the environment. J Microbiol Methods 32:133–154.
- 65. Wakisaka, Y., Koizumi, K. 1982. An Enrichment Isolation Procedure for Minor *Bacillus* Populations. Journal of Antibiotics (Tokyo) Vol. 35 (4): 450-457.
- 66. Weon, H., *et al.* 2006. Two novel species, *Lysobacter daejeonensis* sp. nov. and *Lysobacter yangpyeongensis* sp. nov., isolated from Korean greenhouse soils. International Journal of Systematic and Evolutionary Microbiology 56:947-51.
- 67. Weon, H., *et al.* 2007. *Lysobacter niabensis* sp nov and *Lysobacter niastensis* sp nov., isolated from greenhouse soils in Korea. International Journal of Systematic and Evolutionary Microbiology Vol. 57 (Part 3): 548-551.
- 68. Yadav, J., Selvaraju, S. and Khan, I. 2006. Enhanced recovery and real-time PCRbased quantification of mycobacteria from metalworking fluids. Journal of ASTM International 3: 1–18.
- Yassin, A., *et al.* 2007. *Lysobacter defluvii* sp. nov., isolated from municipal solid waste. International Journal of Systematic and Evolutionary Microbiology 57:1131-1136.
- 70. Yu, F., Zaleta-Rivera, K., Zhu, X., Huffman, J., Millet, J., Harris, S., et al. 2007.

Structure and biosynthesis of HSAF, a broad spectrum antimycotic with a novel mode of action. Antimicrobial Agents and Chemotherapy 51: 64-72.

- 71. Yuen, G., *et al.* 2001. Bean rust biological control using bacterial agents. Crop Protection 20: 395–402.
- 72. Yuen, G., Li, S., Harris, S., Yu, F., Du, L. 2005. An antibiotic produced by the biocontrol agent *Lysobacter enzymogenes* C3 inhibits fungal growth by blocking ceramide synthesis. Phytopathology 95: S116.
- 73. Yuen, G., et al. 2003. Biocontrol of fusarium head blight in wheat by *Lysobacter enzymogenes* C3. Phytopathology 93: S93.
- 74. Zhang, Z., and Yuen, G. 1999. Biological control of *Bipolaris sorokiniana* on tall fescue by *Stenotrophomonas maltophilia* C3. Phytopathology 89: 817–822.
- 75. Zhang, Z., and Yuen, G. 2000. The role of chitinase production by *Stenotrophomonas maltophilia* strain C3 in biological control of *Bipolaris sorokiniana*. Phytopathology 90: 384-389.