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Influence and characterization of microbial contaminants associated with the FDA BAM method used to detect Listeria monocytogenes from romaine lettuce

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INFLUENCE AND CHARACTERIZATION OF MICROBIAL CONTAMINANTS ASSOCIATED WITH THE FDA BAM METHOD USED TO DETECT *LISTERIA MONOCYTOGENES* FROM ROMAINE LETTUCE

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Christopher E. Bach

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of

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I would like to dedicate this thesis to my grandfather, Robert T. White.

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ABSTRACT

Bach, Christopher E., Purdue University, May 2015. Influence and characterization of microbial contaminants associated with the FDA BAM method used to detect *Listeria monocytogenes* from Romaine lettuce. Major Professor: Robert E. Pruitt.

Over the past few decades in the US, fresh produce commodities have become increasingly prevalent vehicles for the attribution of foodborne illness. Recent outbreaks of the bacterial foodborne pathogen *Listeria monocytogenes* linked to fresh produce highlight this immediate issue facing food safety. The most widely used method to screen *L. monocytogenes* from food matrices in the US is the Bacteriological Analytical Manual (BAM) developed by the Food and Drug Administration (FDA). Detection of this pathogen from all foods is primarily accomplished by using four FDA approved *Listeria* selective media: Oxford (OXA), modified Oxford (MOX), Lithium chloride-phenylethanol-moxalactam fortified with esculin and iron (LPM), or PALCAM. Currently, there is a scarcity of evaluations concerning methods for isolation of *L. monocytogenes* from produce items. Thus, the first objective of this thesis work was to assess traditional FDA media and the commercial medium RAPID'*L.mono* for their use in detecting *L. monocytogenes* from the popular fresh produce item Romaine lettuce. Our results revealed that all four FDA media readily select for bacteria that based on their growth on the selective

media appear to be *L. monocytogenes* but in fact belonged to other genera. The presence of these false positives ultimately limited the utility of each medium to detect Romaine lettuce samples that were found to be negative for *L. monocytogenes.* The commercial medium RAPID'*L.mono* was very accurate for detecting *L. monocytogenes*, as no false positives were characteristic of the pathogen on this medium. Testing false positives across media revealed that isolates recovered from MOX, OXA and PALCAM displayed broad positive behavior on other media. In contrast, the majority of isolates collected from LPM were found to have positive behavior restricted to that medium alone. The second objective of this thesis work was to perform whole genome sequencing of false positives taxonomically identified as *Cellulomonas* spp. to recover phylogenetic insights, determine how isolates survive selective plating and identify putative antibiotic target genes. Our phylogenetic analysis strongly supported that our isolates are species within the genus *Cellulomonas.* Resistance or susceptibility to antibiotics utilized in FDA media may be conferred by gene repertoires unique to certain isolates. We identified one potential antibiotic target gene present in *Cellulomonas* isolates that can be considered for future development of a selective medium to eliminate these false positives.

CHAPTER 1. GENERAL INTRODUCTION

1.1 General background on food safety

Food safety represents an essential component in the effort to sustain global food security, human health and consumer protection. Since the initial discovery that foods can vector human pathogens, the central goal has been to understand the microbiology, distribution and relationships that allow these organisms to persist in the food matrix. Insight into these elements has brought about advances in creating novel control strategies, devising detection methods, and building epidemiological models to understand disease. However, as food production and consumption change, it is necessary to determine how these changes influence the safety of the food we consume.

In the US, the Centers for Disease Control (CDC) estimates 48 million cases of foodborne illness, resulting in 3,000 deaths and 128,000 hospitalizations, occur annually (Scallan et al., 2011). The economic burden of foodborne illness can be enormous and healthcare costs associated with treating disease hover around $15 billion per year (USDA 2014a). Additionally, public fear over outbreaks associated with particular food items can resonate throughout the food industry negatively impacting retail sales. However, benefits arising from food safety measures can have

positive impacts both economically and for public health (Ivanek et al., 2005). Elucidating trends in foodborne illness is essential for legislators, public health officials and stakeholders to make data-driven decisions on allocating resources and shaping policies that affect food safety.

1.2 Trends in food safety of fresh produce

One current theme in food safety is that fresh fruits and vegetables have become more prevalent sources of foodborne illness. In the US, between 1970 and 1990 the number of outbreaks of foodborne illness associated with fresh produce increased from < 1% to 6% (Sivapalasingam et al., 2004). Since the early 1990s the frequency of outbreaks vectored by produce have varied over time. From 1995 to 2002 the CDC reported a sharp increase in the number of produce related outbreaks ultimately propelling this food group into the spotlight as a more prominent vehicle of foodborne illness (Olson et al., 2000; Painter et al., 2006). Subsequently this trend decreased but was then followed by several years in which produce commodities were reported as dominant causes implicated in foodborne illness outbreaks. To this day, the CDC has continued to highlight fruit or vegetable products as the most common vehicles for foodborne illness (CDC 2014a; CDC 2014b; CDC 2014c). Currently in the US it is estimated that produce is responsible for roughly 46% of all foodborne illness cases, while foods such as meat and poultry cause 22% (Painter et al., 2013).

When we shift to the perspective of total number of cases (i.e. total number of illnesses caused by an outbreak) resulting from outbreaks in a given year, fruits

and vegetables consistently rank high, or in some years, even highest among outbreaks with known vehicle of transmission (CDC 2014a;Olson et al., 2000; Painter et al., 2006). Hence years wherein produce-linked outbreaks might be moderately lower, the magnitude of these outbreaks often result in a substantial number of cases in proportion to the other food items. Within the produce group, outbreaks caused by leafy green items have generally been identified as the leading vehicle for the transmission of foodborne illness (Sapers et al., 2014). Furthermore, among the defined etiological agents responsible for causing produce-linked outbreaks, we find viruses and bacteria to be the most frequent. *Salmonella* spp. or *Escherichia coli* 0157:H7 are the most prevalent bacterial agents and norovirus typically dominates outbreaks of viral origin.

Several trends in food safety and consumption may offer insight into this epidemiological change in foodborne illness. Over the past few decades, produce commodities have been recognized as one of the most rapidly growing agricultural markets and in the US per-capita consumption of these foods has steadily increased since 1970 (Lynch et al., 2009; USDA 2014b). The high year-round demand for produce items has ultimately resulted in increasingly globalized trading of these commodities and as much as 60% of produce consumed in the USA is imported (CDC 2012a). Thus these food products may have to travel longer distances to reach the intended consumer presumably increasing the probability of contamination. Another proposed explanation to the increase in produce-associated outbreaks is more intensive and enhanced monitoring of foodborne illness (Berger et al., 2010).

It should be noted that since increasing surveillance of foodborne disease, epidemiological trends have also revealed decreases in disease incidence for certain etiological agents and outbreaks associated with particular food items (CDC 2014a). So the increase in produce-associated outbreaks might extend beyond the artifact of improved monitoring abilities. Lastly, produce commodities contaminated with foodborne pathogens might be at higher risk to cause infection since these foods are typically consumed raw.

1.3 Characterizing relationships between foodborne pathogens and plants

1.3.1 Mechanisms for contamination of fresh produce by human pathogens

In the effort to elucidate these trends, researchers have begun to define a working model for contamination of produce by human pathogens (Barak and Schroeder, 2012). The prevailing understanding for produce adulteration likely begins with animal or environmental reservoirs harboring human pathogens in close proximity to a produce-growing location (Oliveira et al., 2012). Additionally, contamination can occur as early as the seed stage (Landry et al., 2014). Human pathogens present in animal feces or soil then undergo a mobilization event by rain, flooding or surface water. Following dispersal, human pathogens come into contact with plants through soil, irrigation systems or other vectoring agents to ultimately colonize above or belowground parts of the plant (Barak and Schroeder, 2012). During contamination in the field, human pathogens may survive for weeks or several months on a plant surface and for extended periods of time in soil. Over the course of the growing season, populations of human pathogens readily decline and

therefore typically do not persist in high numbers. Pre-harvest contamination can then be sustained by introduction of the pathogen into the produce-processing environment where the pathogen can continually adulterate plant commodities destined for human consumption (Olaimat and Holley, 2012).

All of these steps in this model have been experimentally confirmed and for some outbreaks, trace back studies from epidemiological surveys have verified various steps as well (Sapers et al., 2014). Presently, the vast majority of the produce contamination research has focused on the enteric human pathogens, which includes *Salmonella* spp. and *E. coli* 0157. Many leafy green outbreaks caused by *E. coli* 0157 appear to have direct links to improperly composted manure and fecal contaminated irrigation water (Cooley et al., 2007). For instance, trace back of the 2006 *E. coli* 0157 spinach outbreak found identical strains in cattle feces and water sources adjacent to the produce-production area directly involved in the outbreak (CFERT 2007). Furthermore, there is mounting evidence that certain *Salmonella* spp. persist in the natural environment with sources such as soil, watersheds and wild animals serving as viable reservoirs (Winfield and Groisman, 2003; Strawn et al., 2012). The most significant contamination routes that contribute to pre-harvest produce contamination are through spray irrigation using pathogen tainted water sources and manure application. Among the foodborne pathogens frequently implicated in produce outbreaks, we also see trends emerging with respect to produce type. Foodborne illness caused by *Salmonella* spp. is often associated with tomato or sprout contamination and *E. coli* 0157 with lettuce or

spinach outbreaks (Berger et al., 2010). Ultimately these pathogen specificities towards produce type suggest unique plant-microbe interactions (Brandl, 2006).

1.3.2 Interactions between human pathogens and plants

Defining the biological interactions between foodborne pathogens and produce has focused on identifying traits associated with colonization and characterizing microbial behavior. Historically, enteric foodborne pathogens were known to have a strict association with animal hosts. However, we now understand that plants can serve as viable alternate hosts to vector these organisms. What defines the microbial community associated with a plant is determined by the ability of microorganisms to colonize specific niches on or within a plant (Lindow and Brandl, 2003). Microbes inhabiting the plant surface must be able to withstand a fairly inhospitable environment that includes fluctuations in temperature, relative humidity, free water, UV radiation and nutrient availability (Lindow and Leveau, 2002). Identifying such traits that enable foodborne pathogens to survive on produce items has been a central focus to disentangle their unique association with plants.

One of the most important microbial traits for colonization of the phyllosphere (i.e. leaf surface) is attachment or adhesion. Several investigations have demonstrated a significant role of aggregative fimbriae (i.e. attachment pili) and type-3-secretion system (T3SS) in mediating attachment of *E. coli* 0157 and *Salmonella* to plant surfaces allowing them to survive as epiphytes (Barak et al., 2005; Kyle et al., 2010; Saldaña et al., 2011). Additionally, flagellar components have

been identified as important factors for *L. monocytogenes* colonization of radish (Gorski et al., 2003). T3SSs give rise to many important functions in gram-negative bacteria, including the biosynthesis of flagella as well as aiding in interactions with eukaryotic organisms. Interestingly, it has become increasingly recognized that T3SSs can facilitate cross-domain relationships enabling gram-negative bacteria to colonize different hosts (Preston, 2007). For instance, molecular mechanisms involved in T3SS of *Pseudomonas aeruginosa* are important for pathogenic interactions in both plants and animals (Pallen et al., 2005).

Insight into the interface at which human pathogens and plants interact has also begun to unravel unique colonization behaviors, suggesting these organisms can respond to environmental cues, occupy specific niches and experience physiological changes required to survive. On Romaine lettuce, colonization by *E. coli* 0157 and *Salmonella enterica* was strongly associated with younger leaves (i.e. inner lettuce leaves) that provided favorable growth conditions presumably due to greater availability of nutrients and free water (Brandl and Amundson, 2008). Evidence of chemotropic behavior in *Salmonella* revealed the bacterium preferentially aggregated near stomata, leading to penetration of the stomata and occupation of the sub-stomatal space (Kroupitski et al., 2009). For *E. coli* 0157, we also see distinct localization near stomata, trichomes and plant veins (Brandl and Amundson, 2008). Such behaviors might reflect a microbial strategy to circumvent the harsh environment associated with the phyllosphere. Wounding of the leaf surface caused by mechanical damage or from soft-rot plant pathogens, like *Erwinia*

chrysanthemi, have also been reported to enhance attachment, survival and even growth of human pathogens (Brandl, 2008; Brandl et al., 2013). Colonization by human pathogens may also be largely determined by cultivar dependent interactions for a given produce item. *Escherichia coli* 0157 and *Salmonella* colonization efficiencies vary significantly among produce cultivars (Barak et al., 2011; Quilliam et al., 2012). There is evidence that plants can perceive conserved structures in human pathogens including flagella and lipopolysaccharide, resulting in induction of the innate immune response pathway in plants (Melotto et al., 2014). However, plant immune system responses, genetic mechanisms and physiological differences that influence cultivar interactions with human pathogens remain to be discovered. The rhizosphere also presents itself as a suitable environment for human pathogens and may play a role in aiding or suppressing subsequent colonization of the phyllosphere (Barak et al., 2008; Barak and Schroeder, 2012). Initial attraction to the rhizosphere by human pathogens is presumed to be dependent on root exudates (Klerks et al., 2007). Furthermore, interactions with the soil matrix have demonstrated improved survival of *E. coli* 0157 in soils with higher clay content (Gagliardi and Karns, 2002).

Colonization by human pathogens on roots, near stomata and other natural plant openings (e.g. wounds) can act as portals for active or passive entry, therefore enabling these organisms to internalize and persist as endophytes. For both *Salmonella* and *E. coli* 0157, there is strong evidence to support internalization in plant tissues and has been reported for a number of produce items such as lettuce,

tomato, spinach, mung bean, peanut and apple (Deering et al., 2012a). Internalization studies have also highlighted the ability of human pathogens to be transported to various tissues throughout the plant. Compared to developed plants, seeds may be particularly susceptible to contamination due to fewer natural protective barriers. Deering et al., (2011 & 2012b) demonstrated that following germination of seeds inoculated with *Salmonella enterica* and *E. coli* 0157, internalization was found for both pathogens in all major plant tissues. From a food safety perspective, internalization of human pathogens in plants is of great concern since this mechanism can offer physical protection from sanitizing treatments and also promote favorable conditions for survival. Collectively, interactions ranging from the molecular level to unique survival behaviors ultimately suggest an intimate relationship between the plant host and human pathogens.

1.4 Background on the bacterial foodborne pathogen *Listeria monocytogenes*

1.4.1 General history of *Listeria monocytogenes*

The first description and documented pathogenicity of *L. monocytogenes* in rabbits appeared in 1926 (Murray et al., 1926). For many years *L. monocytogenes* was believed to be a pathogen primarily restricted to animals as only a few isolated infections were ever identified in humans (Rocourt and Buchrieser, 2007). This previously held notion was challenged and subsequently changed in 1981 when the first ever-recorded human outbreak of *L. monocytogenes* was linked to the consumption of contaminated coleslaw (Schlech et al., 1983). Today, over 99% of all recorded sporadic disease cases and outbreaks caused by *L. monocytogenes* are of

food origin (Mead et al., 1999). Although disease incidence resulting from *L. monocytogenes* outbreaks have decreased by roughly 42%, the pathogen remains a significant public health concern averaging 1600 cases of infection per year (Scallan et al., 2011; CDC 2014d).

The genus *Listeria* is composed of 15 species and only two species, *L. ivanovii* and *L. monocytogenes* display pathogenicity in animals or humans. Infections caused by *L. ivanovii* are extremely rare in humans and therefore pathogenicity in this species is not considered a serious threat. *Listeria* is a member of the Firmicutes phylum, characterized as gram positive, rod forming, aerobic, facultatively anaerobic, low G + C content genome and non-spore forming (McLauchlin and Rees, 2009). Many species within the genus *Listeria*, including *L. monocytogenes* are widely distributed in nature (Chapin et al., 2014). The pathogen can live as a saprophyte in soil, inhabit aquatic areas, silage and sewage. The ability of *L. monocytogenes* to occupy many different niches is believed to contribute to its survival abilities as a foodborne pathogen.

Listeria monocytogenes has been isolated from nearly every food matrix including meat, dairy, seafood, fruits and vegetables (Farber and Peterkin, 1991). Historically outbreaks appear to be strongly linked to the consumption of ready-to-eat (rte) deli meats and certain styles of cheese. Control of this pathogen in food can be especially challenging because of its ability to multiply under refrigeration temperatures, low water activity, high salt concentration, wide pH range and ability to form biofilms (Vasseur et al., 1999; Valderrama and Cutter, 2013).

Any single processing activity from farm to fork has the potential to serve as a viable contamination point for *L. monocytogenes* and has been fairly well documented in disease epidemics and academic research. However, it appears that the food processing environment and retail establishments have emerged as the most significant contamination points along the food production continuum (Pan et al., 2006; Simmons et al., 2014). In many outbreak cases, contamination has been linked to poor environmental hygiene of the food processing facility. One factor that presumably led to the 2011 cantaloupe outbreak was attributed to inadequate sanitization of machinery used to clean cantaloupe (McCollum et al., 2013). The largest enigma surrounding food contamination today is how the pathogen initially becomes introduced into the food-processing environment.

1.4.2 *Listeria monocytogenes* virulence and disease

Listeria monocytogenes is the primary etiologic agent responsible for causing the disease listeriosis in humans (Briers et al., 2011). Pathogenicity in *L. monocytogenes* is unique compared to other foodborne pathogens in that *L. monocytogenes* is not typically associated with gastroenteritis, but can instead manifest into more serious infections such as meningitis, sepsis and encephalitis. Select individuals including elderly, immune compromised, pregnant women and neonates have emerged as the most vulnerable to listeriosis, accounting for approximately 90% of all reported infections (CDC, 2013). The exceptional virulence of *L. monocytogenes* to these groups makes this organism one of the most lethal foodborne pathogens with mortality rates averaging 20-30%.

The species *L. monocytogenes* can be further classified into 13 unique serotypes (i.e. characterization via surface antigens) that give rise to four independent evolutionary lineages (Haase et al., 2014). Interestingly, serotypes 1/2a, 1/2b and 4b are vastly overrepresented in disease cases and account for over 90% of documented outbreaks worldwide (Kathariou, 2002). Further unique patterns among the serotypes emerge with respect to food contamination and infection trends. Serogroup 1/2 appears to be more routinely isolated from foods relative to other subspecies while serotype 4b is generally more associated with clinical disease cases (Orsi et al., 2011). All serotypes have the ability to cause disease, however, the degree of pathogenicity can differ among strains. The reason for variability in pathogenic potential is not fully understood, especially since virulence genes are highly conserved among sub species. Comparative genomics suggests serotypes may differ in their respective abilities to cross certain cellular membranes (i.e. epithelial, blood brain barrier, etc), which is a necessary component for virulence during the infection cycle (Gilmour et al., 2010).

1.4.3 *Listeria monocytogenes* and fresh produce

The coleslaw outbreak of 1981 served as the impetus for academic researchers to document the isolation and study the behavior of this pathogen from fresh fruits and vegetables. There is a strong consensus among the current literature that *L. monocytogenes* has the ability to survive and even grow on a variety of produce items over a wide range of conditions. On broad-leaf endive, pathogen growth was observed to proceed with minimal interference at various temperatures

in the presence of phyllosphere microflora and the extent of spoilage was shown to positively correlate with *L. monocytogenes* growth (Carlin et al., 1995). Growth dynamics across different lettuce varieties has been shown to be significantly different and indeed growth rates can vary with respect to lettuce substrate (Carlin and Nguyen-the, 1994). Additionally, there also appears to be marked differences of growth behavior between *L. monocytogenes* strains in rte mixed salads (Skalina and Nikolajeva, 2010). Research concerning *L. monocytogenes* and its interaction with native microflora of fresh vegetables is very sparse, but there is some evidence that commensal bacteria can antagonize pathogen growth. Certain produce substrates also have the capacity to inhibit growth or inactivate the pathogen as was shown with chopped tomatoes and carrot (Beuchat and Brackett, 1990, 1991). In comparison to other food groups such as meat items, it is generally accepted that growth rates of *L. monocytogenes* on vegetables are not as substantial (Oliveira et al., 2010).

Historically, the number of *L. monocytogenes* outbreaks ultimately traced back to produce is very small relative to foods such as rte meats. Since 2007 in the US, six documented outbreaks of listeriosis have been linked to the consumption of fruits or vegetables (Cartwright et al., 2013; CDC 2014e; CDC 2014f). Nevertheless it is a common foodborne pathogen implicated in class one recalls of produce commodities (Dey et al., 2013). There are three types of recall events in the US, which are based on the potential threat of a product to cause harm in humans or animals. Class one recalls rank highest in terms of a products threat to public health

and may result in serious harm or death (FDA, 2009). Class two or three recalls are only issued when it is determined that a product is slightly harmful or defective (FDA, 2009).

Prevalence rates of *L. monocytogenes* among fresh vegetables are also quite low. A large meta-analysis drawing on 7 years of internationally published data found 3% of produce samples to be positive for *L. monocytogenes* (Crépet et al., 2007). Low prevalence on produce has also been reported for other foodborne pathogens such as *Salmonella* spp. (Gorski et al., 2011). Overall, prevalence studies have exemplified the heterogeneous distribution of *L. monocytogenes* produce contamination, which makes assessing prevalence and identifying at-risk commodities very challenging. A key attribute to assessing prevalence might lie in the uniformity of contamination on produce items. For instance, does *L. monocytogenes* colonize different parts of the plant equally well, or is colonization restricted to very specific sites? If the latter were true, sampling methods would have to account for this behavior to accurately capture prevalence. In our own next generation sequencing data we have found bacterial communities to be spatially distinct depending on where they are sampled from on a lettuce leaf (Bach and Pruitt unpublished). Further addressing colonization behavior of *L. monocytogenes* during pre-harvest and post-harvest may allow for improved estimates of prevalence from produce items.

In regards to post-harvest pathogen control strategies, produce items preserved in modified atmosphere packaging appear to have little to no utility in

inhibiting *L. monocytogenes* growth (Brackett 2007). In some instances, certain produce packaging atmospheres (e.g. low O_2 concentrations) have even been reported to enhance the growth of *L. monocytogenes* (O'Beirne et al., 2015). Sanitization methods (e.g. chlorine dioxide, peroxyacetic acid, ozone, etc.) used to control microbial populations on produce can have modest efficacies in inactivating *L. monocytogenes* but do not possess the antimicrobial power to completely eliminate the pathogen (Joshi et al., 2013). The utility of sanitizing treatments for produce is vastly reduced by biofilm formation of *L. monocytogenes*, which has been reported to occur within 48 h of inoculation on produce items such as lettuce (Ölmez and Temur, 2010). Although *L. monocytogenes* has the ability to grow at low temperatures, proper storage of produce at refrigeration temperature is essential to limiting and reducing growth of the pathogen. Persistence and survival in the produce-processing environment is poorly understood, but machinery used to mechanically process these items can conceivably serve as reservoirs for contamination (Kaminski et al., 2014). Given that the probability of contamination for this pathogen is likely highest during processing activities, it is essential that the processing environment maintain sanitary conditions. Contamination during pre-harvest activities remains largely unknown. However, in accordance with enteric pathogens, ensuring pathogen-free manure application and irrigation will hopefully reduce risk factors that contribute to contamination events (Park et al., 2012; Strawn et al., 2012).

The majority of *L. monocytogenes* produce research has typically used human

isolates implicated in dairy or meat outbreaks. These particular studies have served as invaluable resources in documenting and demonstrating the viability of certain fresh produce items to vector this pathogen. However, one of the limiting aspects to these early investigations is that they don't address pathogen behavior of isolates naturally present on fresh fruits and vegetables. Currently, researchers are focusing efforts on identifying serotypes, genetically characterizing and understanding the growth potential of *L. monocytogenes* strains isolated from naturally contaminated fresh vegetable items (Sant'Ana, Barbosa, et al., 2012; Sant'Ana, Igarashi, et al., 2012). It would be interesting to further characterize vegetable associated isolates through comparative genomics and utilize plant-microbe interaction approaches to identify factors that mediate colonization. On the applied side these isolates might prove to be useful models to study the efficacy of sanitization treatments and strategies for controlling contamination in the produce processing environment. Furthermore, on the basis of genetic characterization, we may find that the produce substrate generally does not support the persistence of highly virulent serotypes commonly associated with dairy items and rte meats. Such a finding might have broader implications on re-evaluating zero tolerance policies applied to produce items.

1.5 Methods for isolation and detection of *Listeria monocytogenes* from food

Culture-based methods prevail as the gold standard for isolating and subsequently detecting *L. monocytogenes* from all food matrices. In some respects these approaches remain necessary as confirmation of live *L. monocytogenes* cells

from foods is generally required to issue a class 1 recall. In the USA, two government-regulated protocols developed by the Food and Drug Administration (FDA) and United States Department of Agriculture (USDA) reign as the most widely used methods to detect and recover *L. monocytogenes* from all food commodities (Gasanov et al., 2005; US FDA BAM, 2011). Both methods utilize an enrichment step, in which a particular food is homogenized in a selective liquid medium. The purpose of the enrichment is to resuscitate physiologically stressed *L. monocytogenes* cells and increase concentrations of the pathogen to detectable levels when subsequently cultured on selective media. Proceeding with direct plating is not advised since at least 100-10,000 cells/g of food is generally required for recovery depending on food matrix and can be highly dependent on the physiological state of the pathogen (Golden et al., 1990). Once the enrichment stage is complete, samples are then cultured onto one of four selective solid media: Oxford (OXA), modified Oxford (MOX), Lithium chloride-phenylethanol-moxalactam (LPM), or PALCAM. All media also incorporate an indicator or detection component composed of esculin and iron. *Listeria* spp. present on the media hydrolyze esculin, which subsequently reacts with iron yielding a black precipitate that forms around the colony (Figure 1.5.1) (Rodriguez, 1984). This enables each medium to differentiate *Listeria* spp. from other non-esculin positive background microflora that might be present. One of the limiting aspects to traditional media, however, is that they cannot differentiate between *Listeria* spp. Following selective culture, colonies that are esculin positive and morphologically characteristic of *Listeria* spp. are selected and typically

identified using phenotypic markers for confirmation of the pathogen. The FDA also strongly recommends pairing commercial media with one of the afore mentioned media for the routine screening of *L. monocytogenes* from foods. Commercial media such as Rapid'L.Mono are supplemented with chromogenic reagents enabling them to rapidly differentiate *L. monocytogenes* from other *Listeria* spp. that might be present.

All FDA and USDA approved media were principally developed for the isolation of *L. monocytogenes* from dairy, meat or clinical specimens (Lee and McClain, 1986; Curtis et al., 1989; van Netten et al., 1989). The different environments intrinsic to a specific food matrix ultimately influence their respective microbial composition. For instance, meats are commonly associated with the gram-negative bacterial family Enterobacteriaceae, while gram-positive Lactobacilli often dominate cheeses (Doyle et al., 2007). Thus the option of four different media functionally expands detection strategies since each medium eliminates specific microbial contaminants depending on food sample, which in turn facilitates pathogen recovery. In regards to the food products intended to be used with these media, it is generally accepted that they perform well and false positive rates have been reported from 5-10% (Capita et al., 2001). False positives during this screen arise from non-*Listeria* background microflora that survive the selective agents present in the enrichment broth and media and are physiologically characteristic of *L. monocytogenes*. Ultimately it can be said that efficient isolation from food is both a function of the physiological state and concentration of *L. monocytogenes* combined

with the selective abilities of each medium to exclude food microbiota that obscure the detection of this pathogen.

One of the significant disadvantages to microbiological based methods is that they are relatively time consuming and from start to finish take approximately 7-10 days to complete. Isolation rates of the different enrichment protocols have also been shown to vary such that strain recovery can be dependent on enrichment medium. This was discovered early in the development of recovery methods when it was found that combining different enrichments consistently yielded higher rates of isolation relative to the use of a single enrichment broth (Warburton et al., 1991). Because growth dynamics of the pathogen coupled with co-enriching microflora can be difficult to quantify, the reason why recovery rates differ across enrichments is not entirely understood. However, some evidence has revealed that food microbiota along with certain *Listeria* spp. may compete with *L. monocytogenes* during enrichment and negatively affect recovery (Curiale and Lewus, 1994; Dailey et al., 2014).

Molecular methods have also served as powerful detection strategies from a wide number of food products. Polymerase chain reaction (PCR) can be used to target genus specific loci for the presence of *Listeria* spp. and virulence genes for confirmation of the species *L. monocytogenes*. Commercial real-time PCR test kits, such as the BAX system developed by Dupont, are available for screening for *Listeria* spp. and *L. monocytogenes* from food samples. This approach works by pairing traditional culture with molecular methods such that foods initially undergo

selective enrichment and then PCR is used as a prescreen to determine whether the enriched food sample is positive for *Listeria* spp. or *L. monocytogenes*. Following enrichment, BAX reports detection limits for as few as 10^4 cfu/ml of *Listeria* spp. and from start to finish results can be obtained in 2-3 days. Comparison between the BAX system and FDA protocol revealed no statistical difference in performance of either method for detecting *Listeria* spp. in rte meats (Wallace, 2013). Overall, one of the greatest advantages to the BAX system is the significant reduction in time and labor relative to traditional methods such as the FDA protocol. DNA sequencing methods are currently not implemented for the detection of *L. monocytogenes* during routine food screens. In chapter 2 of this thesis we demonstrate a Sanger sequencing approach of the 16S rRNA gene can serve as a reliable and accurate method to identify presumptive *Listeria* isolates from Romaine lettuce samples screened with the FDA protocol.

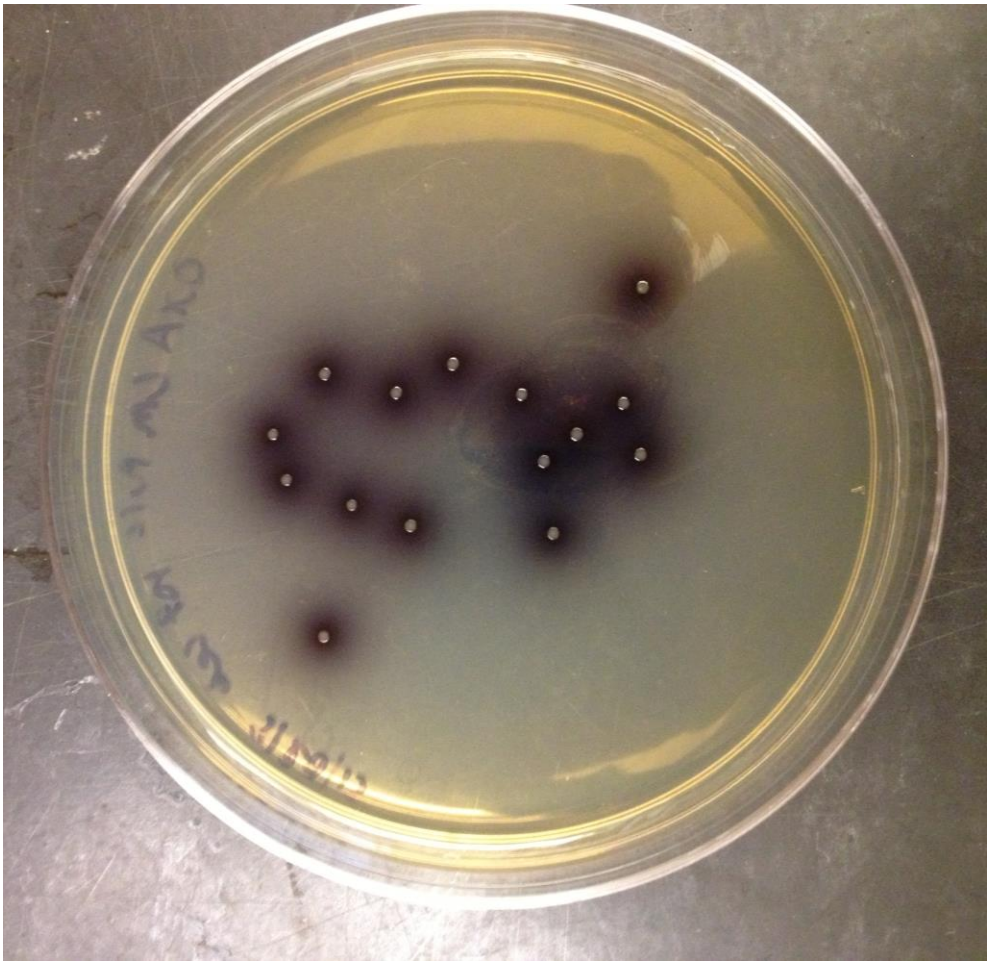

Figure 1.5.1. Pure culture of *L. monocytogenes* (strain 1035S) plated on OXA medium

1.6 References

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CHAPTER 2. EVALUATION OF THE FOOD AND DRUG ADMINISTRATION METHOD USED FOR THE DETECTION OF *LISTERIA MONOCYTOGENES* FROM ROMAINE LETTUCE

2.1 Introduction

In the United States we have observed a shift in the epidemiology of foodborne illness such that fresh produce commodities represent increasingly prevalent sources for the transmission of foodborne pathogens. Today, it is estimated that produce accounts for 46% of all foodborne illness in the US (Painter et al., 2013). Recent outbreaks of the bacterial foodborne pathogen *Listeria monocytogenes* associated with fresh produce emphasize this current issue facing food safety. One of the latest epidemiological surveys of *L. monocytogenes* noted several fresh produce items as novel food vehicles implicated in outbreaks (Cartwright et al., 2013). In 2011, cantaloupe contaminated with *L. monocytogenes* was responsible for one of the worst foodborne illness epidemics in US history that infected 147 people and resulted in 33 deaths (McCollum et al., 2013). Although historically *L. monocytogenes* has been responsible for a very small proportion of outbreaks linked to fresh produce, from 2003 to 2011 the pathogen accounted for 21% of class 1 recalls related to fresh fruits and vegetables (Dey et al., 2013). Emergence of novel food vehicles and persistent recall events associated with *L. monocytogenes* represent a significant threat to human health and security of the

fresh produce supply chain. Thus understanding the relationship between *L. monocytogenes* and fresh produce will hopefully improve food safety measures surrounding these foods.

Research concerning the microbiology of *L. monocytogenes* on fresh produce indicates this pathogen has the ability to persist and even multiply on a variety of leafy greens including lettuce, spinach, cabbage and bean sprouts over a wide range of conditions (e.g. pH, salt concentration, temperature, modified atmosphere packaging) (Carlin et al., 1995; Beuchat, 1996). The distribution of *L. monocytogenes* across vegetable products is assumed to be highly heterogeneous and if present, the pathogen is likely to exist in low concentrations (i.e. low CFU/g) (Crépet et al., 2007). Furthermore the ability of this pathogen to occupy a diverse range of natural habitats and linger in the food-processing environment makes these reservoirs relevant contamination concerns (Valderrama and Cutter, 2013; Chapin et al., 2014).

Regulation and compliance surrounding fresh produce in the US mandates a zero tolerance policy, meaning that concentrations of *L. monocytogenes* ≥ 1 CFU/g cannot be present since these foods are typically consumed raw. Because the infectious dose of *L. monocytogenes* is not well established in humans, many critics have questioned the pragmatism of this regulatory stance. For humans with intact immune systems, the median concentration of *L. monocytogenes* required to cause infection is estimated at 10^5 CFU/g (Doyle, 2007). Such concentrations of *L. monocytogenes* would be considered very high and uncommon for produce items.

Furthermore, certain strains of *L. monocytogenes* exhibit attenuated virulence. Recently, developed nations such as Canada and Australia have adopted new regulation for produce items and allow concentrations of *L. monocytogenes* below 100 CFU/g. Thus our abilities to accurately detect *L. monocytogenes* from fresh produce items are essential to preventing outbreaks and the accidental recall of non-contaminated foods.

One of the most widely used methods for the detection and enumeration of *L. monocytogenes* from food matrices is the Bacteriological Analytical Manual (BAM) developed by the US Food and Drug Administration (FDA) (Gasanov et al., 2005). The methods first prescribe homogenization of a food sample in a selective liquid medium called buffered listeria enrichment broth (BLEB) that is utilized during the selective enrichment stage to promote *L. monocytogenes* growth and eliminate background flora commonly found on foods. The enriched food sample is subsequently cultured onto one of four traditional solid FDA approved media: Oxford (OXA), modified Oxford (MOX), Lithium chloride-phenylethanol-moxalactam (LPM) fortified with esculin and iron, or PALCAM. Following culture on selective media, presumptive *Listeria* colonies are collected and typically identified using additional traditional microbiological techniques.

Methodology surrounding *L. monocytogenes* recovery was originally developed to address the need for a standard protocol to detect this pathogen from a particular food matrix. For instance, the FDA enrichment method was designed for the isolation of *L. monocytogenes* from dairy products and selective agar specifically

targeted meat or dairy items (Lee and McClain, 1986; Curtis et al., 1989; van Netten et al., 1989; Curtis and Lee, 1995). The enrichment broth and traditional media select for *Listeria* spp., and eliminate gram-negative bacteria, yeasts, molds and certain gram-positive bacteria (Table 2.1.1, Table 2.1.2). The option of four different media expands detection strategies to eliminate specific microbial contaminants (i.e. false positives, background microflora) associated with certain food matrices to improve the accuracy of detecting samples positive for *L. monocytogenes*. In addition to traditional isolation media, the FDA also recommends supplementing routine food screens with a commercial medium. One of the strong advantages of commercial media is that they have the ability to differentiate between *Listeria* spp., which is impossible on traditional media. Some researchers have also reported improved sensitivity (i.e. ability to detect a true positive sample) and selectivity (i.e. ability to detect a true negative sample) of commercial media for the detection of *L. monocytogenes* (Hegde et al., 2007; Park et al., 2012).

Although a respectable body of research exists in regards to studying the behavior of *L. monocytogenes* on produce, microbiological methods concerning its isolation from these particular food items have largely been ignored. A few research articles have attempted to address methods for the recovery of *L. monocytogenes* from cabbage (Hao et al., 1987). Early observations noted that high microbial loads precluded the utility of some media for recovering *L. monocytogenes* from raw cabbage (Cassiday et al., 1989; Golden et al., 1990). However, it is presently difficult to extrapolate the results by these former investigations since some media

formulations and isolation methods have become obsolescent or undergone significant modification. The consensus from early and recent evaluations of various selective enrichment media (i.e. Fraser broth, BLEB, USDA) and traditional media suggest these protocols are sufficient for the recovery of *L. monocytogenes* from produce items (Hayes et al., 1991; Warburton et al., 1991; Denver et al., 1993; Jamali et al., 2013). However, no contemporary evaluation exists for the FDA method assessing the impact of produce-associated microbiota during routine screening of *L. monocytogenes* from produce commodities. Given that the microbial composition on produce items such as Romaine lettuce is intrinsically different from foods such as Latin style cheeses, it raises an interesting question in terms of whether media possess comparable selective abilities for recovery and detection relative to meats or dairy items (Lusk et al., 2012; Jackson et al., 2013). Furthermore, it is essential to understand how endogenous microflora that survive selective enrichment and plating might influence the detection of *L. monocytogenes* from a particular food matrix (Brackett and Beuchat, 1989).

The central aim for Chapter 2 of this thesis work was to assess traditional FDA media and one commercial *Listeria* selective medium for their use in detecting *L. monocytogenes* from the popular fresh produce item Romaine lettuce. The first objective of this research was to follow the FDA BAM protocol, identify presumptive *Listeria* isolates and evaluate OXA, MOX, PALCAM and LPM media for their use in the detection of *L. monocytogenes* from BLEB enriched Romaine lettuce samples. We also chose to evaluate the basal formulation of OXA without antibiotics and compare

our results to OXA supplemented with antibiotics. Second, we wanted to classify false positive isolates associated with each medium to understand how these microbial contaminants influence the detection of *L. monocytogenes*. Third, we investigated whether the 24h or 48h enrichment incubation times influenced detection of false positive isolates. Fourth, once presumptive isolates were collected, we tested those identified as false positives against all media to understand and characterize their respective positive behaviors. Fifth, we were also curious to evaluate the commercial medium RAPID'*L.mono* and determine whether false positive isolates revealed similar chromogenic phenotypes to *Listeria* spp.

Table 2.1.1. Antimicrobial agents used in traditional FDA media and BLEB enrichment and their antimicrobial activity

Antimicrobial agent	Antimicrobial activity
Lithium chloride	G- bacteria
Acriflavine	G+ cocci
Nalidixic acid	G- bacteria
Moxalactam	Broad spectrum for G+ and G- bacteria
Ceftazidime	Broad spectrum for G+ bacteria, G- bacteria, molds, yeasts
Cyclohemixide	Yeasts, molds
Colistin sulfate	G- bacilli
Cefotetan	G- bacteria, G+ cocci
Fosfomycin	Broad spectrum for G+ cocci and G- rods
Polymyxin B Sulfate	G+ cocci, G- rods

Table 2.1.2. Usage of antimicrobial agents in traditional FDA media and BLEB enrichment. Presence of antimicrobial indicated by + and absence is intentionally left blank.

Antimicrobial compound	Antimicrobial activity					
	MOX	LPM	PALCAM	OXA	B-OXA	BLEB
Lithium chloride	+	+	+	+	+	+
Acriflavine			+	+		+
Nalidixic acid						+
Moxalactam	+	+				
Ceftazidime			+			
Cyclohemixide				+		+
Colistin sulfate	+			+		
Cefotetan				+		
Fosfomycin				+		
Polymyxin B Sulfate			+			

2.2 Materials and Methods

2.2.1 Detection of *Listeria monocytogenes* from Romaine lettuce using the FDA method

Detection and isolation of *L. monocytogenes* was accomplished by following a modified procedure outlined in the FDA BAM (US FDA BAM, 2011). A non-composited sample approach was utilized and *L. monocytogenes* confirmed samples were not preserved nor enumerated. 42 whole head commodity Romaine lettuce samples were purchased at local grocery stores in West Lafayette, Indiana over the course of 1 year. For each head of Romaine lettuce, 25 g samples of lettuce leaf tissue was pre-enriched by blending (Oster, Boca Raton FL, US) samples with 225 ml buffered listeria enrichment broth (BLEB) (Becton, Dickinson, Franklin Lakes NJ, US) and incubated at 30°C with shaking at 300 rpm. After four hours of initial incubation, selective antibiotics acriflavine hydrochloride (Spectrum, New Brunswick NJ, US) (10 mg/L), nalidixic acid (Alfa Aesar, Ward Hill MA, US) (40 mg/L) and cycloheximide (Sigma-Aldrich, St. Louis MO, US) (50mg/L) were added to enriched lettuce samples and incubated for a total of 48 h. Lettuce enrichments were sampled for *L. monocytogenes* at 24h and 48h enrichment incubation times as specified in the BAM. At 24 h and 48 h incubation times, enriched lettuce samples were serially diluted from 10^{-1} to 10^{-6} fold in sterile 0.1 M phosphate buffer pH 7.0 and cultured onto OXA, Basal OXA (B-OXA), MOX, LPM and PALCAM media (Becton, Dickinson, Franklin Lakes NJ, US). Selective media MOX, OXA, B-OXA and PALCAM were incubated at 35 °C and LPM at 30°C. Plates were monitored at 24 h and 48 h

selective culture incubation times for presumptive isolates. If present, up to 5 presumptive *Listeria* colonies were selected and streaked onto trypticase soy agar with 0.6% yeast extract (TSAye) (Becton, Dickinson, Franklin Lakes NJ, US) for purification. All isolates purified on TSAye were sub-cultured in brain heart infusion (BHI) (Becton, Dickinson, Franklin Lakes NJ, US) liquid medium for 24 h at 30°C and preserved in 7% dimethyl sulfoxide (Sigma-Aldrich, St. Louis MO, US)+ BHI at -80°C. It should be noted that 22 lettuce samples were analyzed together using PALCAM, LPM, MOX and B-OXA. Three separate lettuce samples were independently analyzed with PALCAM, bringing the sample total to 25 for that medium. Lastly, 17 additional lettuce samples screened using OXA were carried out separately from MOX, B-OXA, LPM and PALCAM. Our initial sampling goal was to obtain and identify 100 presumptive isolates for each medium evaluated in this study.

2.2.2 Identification of presumptive isolates

Colony polymerase chain reaction (PCR) was used to amplify the v3 to v6 region of the 16S rRNA gene (approx. 650 residues) for all presumptive isolates collected from enriched lettuce samples cultured on each medium. Oligonucleotide primers were purchased from Integrated DNA Technologies. Primers used were designed by Huse et al. (2008) (Table 2.2.1). Taq DNA polymerase was "homemade" and treated with ethidium monoazide to inhibit amplification of exogenous DNA. Final concentrations of PCR buffer consisted of 500 mM KCl, 100 mM Tris pH 9.0, 1% Triton X-100 and 20 mM $MgCl_2$. Each 20 µl PCR reaction consisted of 1 µl bacterial culture (i.e. template), 200 µm of dNTPs, 2 µl buffer, 0.3 µl 1:100 Taq DNA

polymerase, and 5 pmol of each forward and reverse primer. PCR was carried out on a thermal cycler (BioRad) as follows: 1 cycle of 3 min at 95 °C, 30 sec at 55 °C, 2 min at 72 °C followed by 39 cycles of 25 sec at 95 °C, 30 sec at 55 °C, 2 min at 72 °C. PCR samples were then purified using Qiagen PCR cleanup kit per manufacturers instructions. Using purified template from the PCR reaction we performed DNA Sanger sequencing with Big Dye reagent v3.1 (Thermo Fisher Scientific, Waltham MA, US). The v3-v6 regions of the 16S rRNA gene were sequenced bi-directionally for each isolate. Each 10 µl sequencing reaction consisted of 4 µl Big Dye reagent, 5 pmol forward or reverse primer and 4 ul of purified PCR product. DNA Sanger sequencing took place under the under the following conditions on a thermal cycler: 1 cycle of 2 min 25 sec at 95 °C, 20 sec at 50 °C, 4 min at 60 °C followed by 30 cycles of 25 sec at 95 °C, 20 sec at 50 °C, 4 min at 60 °C. Post sequencing purification was completed using big dye clean up columns (Edge BioSystems, Gaithersburg MD, US) and samples were submitted to the Purdue Genomics Center for analysis on an ABI sequencer (Thermo Fisher Scientific, Waltham MA, US). Once DNA sequences of presumptive isolates were obtained, the v3F and v6R sequence for each respective isolate was assembled using CAP3 (Huang and Madan, 1999). Sequences were then trimmed at the v3F and v6R primer positions to isolate the v3-v6 region of the 16S rRNA gene. Taxonomy for v3-v6 16S rRNA sequences from each respective isolate was assigned using software implemented in Global Alignment for Sequence Taxonomy (GAST) (Huse et al., 2008).

2.2.3 PCR confirmation of *Listeria monocytogenes* isolates

Species-specific primers were used for *L. monocytogenes* confirmation of presumptive isolates identified to the genus *Listeria.* PCR reaction mixtures were the same as described in identification of presumptive isolates methods section. Reaction conditions and primers used followed Hudson et al. (2001) (Table 2.2.2).

2.2.4 Media cross-comparison

We evaluated a total of 373 isolates confirmed to be false positive collected from MOX, OXA, PALCAM and LPM. Isolates collected from B-OXA were not included in the media-cross comparison study, as we were only interested to characterize false positives that were recovered from OXA with antibiotics. Stock BHI cultures of false positives were first streaked against the medium they were originally isolated from to confirm positive behavior and then streaked against each individual medium. Isolate behavior across each medium was recorded as positive or negative. Relationships between media and false positives were explored by displaying the results with a Venn diagram using Venny software (Oliveros 2007). Media were incubated for the same time and temperature as mentioned in Romaine Lettuce enrichment, detection and isolation of presumptive isolates methods section.

2.2.5 Characterizing isolate phenotypes on RAPID'*L.mono*

All 514 isolates collected from MOX, OXA, B-OXA, LPM and PALCAM were streaked against RAPID'*L.mono* to observe and record phenotypes associated with each isolate (See Appendix A for complete list of isolates). RAPID'*L.mono* has the ability to differentiate the following *Listeria* spp. based on unique chromogenic

phenotypes: *L. monocytogenes*, *L. innocua*, *L. welshimeri* and *L. ivanovii* (Table 2.2.3). RAPID'*L.mono* was incubated at 37 °C and isolate behavior was recorded at 24 h or 48 h time periods.

Table 2.2.1. PCR primers used to amplify the v3-v6 region 16S rRNA gene. Primers designed by Huse et al. (2008).

Primer	Sequence (5'-3')	16S rRNA coordinates	
		5' end	**3' end**
v3F	ACTCCTACGGGAGGCAGCAG	338	358
v6R	CgACARCCATgCASCACCT	1064	1044

Table 2.2.2. *Listeria* and *L. monocytogenes* PCR primers. Primers designed by Hudson et al. (2001).

Primer	Sequence (5'-3')	Specificity
L318F	GGGGAACCCACTATCTTTAGTC	*Listeria*
L559R	GGGCCTTTCCAGACCGCTTCA	*Listeria*
310F	GCCTGCAAGTCCTAAGACGCCAATC	*L. monocytogenes*
1016R	CTTGCAACTGCTCTTTAGTAACAGC	*L. monocytogenes*

Table 2.2.3. Chromogenic differentiation of *Listeria* spp. by RAPID'*L.mono*

***Listeria* spp.**	**Phenotype**	**Chromogenic activity**
L. monocytogenes	PIPLC+, xylose-	Purple colony, no halo
L. welshimeri	PIPLC-, xylose+	White colony, yellow halo
L. innocua	PIPLC-, xylose-	White colony, no halo
L. ivanovii	PIPLC+, xylose+	Green colony, yellow halo

2.3 Results

2.3.1 Evaluation of traditional FDA media

To assess whether traditional FDA media could efficiently select for *Listeria* spp. and inhibit lettuce-associated microflora we identified all presumptive isolates by sequencing the 16S rRNA gene. Across B-OXA, OXA, PALCAM, LPM and MOX a total of 514 presumptive isolates were collected from 42 enriched lettuce samples and identified to genus (Table 2.3.1; See Appendix A for complete list of isolates). Out of the total number of presumptive isolates, 34 were assigned to the genus *Listeria*. Using PCR we confirmed 19 of the 34 *Listeria* isolates to be *L. monocytogenes* (Table 2.3.1). Overall, four lettuce samples yielded *Listeria* spp., with two of these samples positive for *L. monocytogenes* (Table 2.3.2). The remaining 480 isolates were assigned to a genus other than *Listeria* and revealed a diverse composition of 16 genera (Table 2.3.1 and Figure 2.3.2). Each medium strongly selected for bacteria that were physiologically characteristic of *L. monocytogenes,* but according to their respective 16S rRNA sequence taxonomy, were confirmed to be from other genera (Figure 2.3.1 and Figure 2.3.2). The consistent recovery of false positive isolates resulted in high confirmation rates of non-*Listeria* spp. ranging from 87%-96% across media (Table 2.3.1). Each medium performed exceptionally poorly with respect to their ability in detecting Romaine lettuce samples where *Listeria* spp. or *L. monocytogenes* was found to not be present. This ultimately led to 64%-82% of samples that yielded presumptive isolates in which none were identified as *Listeria* spp. (Table 2.3.2).

The relative proportion of false positive genera was not consistent across media and each medium displayed a unique distribution of isolates (Figure 2.3.2). PALCAM frequently selected for the genera *Cellulomonas, Microbacterium* and *Rothia.* By comparison we found LPM strongly inhibited these same genera as they were recovered in very low frequency or completely absent. Both MOX and LPM revealed strong selection for the genus *Curtobacterium*. In contrast, *Curtobacterium* spp. were recovered at a much lower frequency on PALCAM, OXA and B-OXA. PALCAM, OXA and MOX appeared to share the most genera between media, albeit the frequencies of these genera varied depending on the medium. In some instances genera were unique to a particular medium, such as *Leuconostoc, Weissella* and *Vagococcus* that were only identified from LPM. Despite this variation between media, several genera including *Microbacterium*, *Cellulomonas* and *Curtobacterium* emerged as the most frequently recovered false positives across media (Figure 2.3.2). Collectively, these 3 genera accounted for 60% of all presumptive isolates identified in this study.

Further investigation of false positive genera revealed distinct associations with phylum and cellular morphology (Table 2.3.3). The taxonomy for false positive isolates recovered from OXA, PALCAM, LPM and MOX indicates they are all gram positive and members of the phyla Actinobacteria or Firmicutes. Thus traditional FDA media exhibited complete inhibition of false positives representing gram-negative bacteria. Gram-positive phyla were also vastly overrepresented on B-OXA with the exception of one gram-negative genus, *Serratia* that is a member of the

phylum Proteobacteria. Actinobacteria dominated false positives and accounted for 72% of isolates while Firmicutes were responsible for 21%. In addition to phylum level selection, there also appeared to be genus level cellular morphological features that were associated with recovery rates of false positive isolates. We found false positive genera displaying rod-forming cellular morphology accounted for 81% of isolates collected from B-OXA, OXA, MOX, LPM and PALCAM (Table 2.3.3). Additionally, the rod-forming group encompassed highly prevalent false positive genera such as *Cellulomonas, Microbacterium, Curtobacterium and Sanguibacter* (Figure 2.3.2). Comparatively, genera with coccid morphology were strongly inhibited across media, representing 13% of false positives recovered in this study.

Comparison of B-OXA to OXA revealed that selective supplements had minimal utility in inhibiting certain false positive genera. OXA appeared to only eliminate 4 of the 9 genera found on B-OXA, which included *Sanguibacter, Rothia, Marinilactibacillus* and *Serratia* (Figure 2.3.2). Inhibition of these genera by OXA did not improve the performance of this medium as it readily selected for other false positive isolates such as *Microbacterium* and *Cellulomonas*. PALCAM, LPM and MOX all strongly inhibited *Bacillus* spp. The genus *Staphylococcus* was also absent from all media except B-OXA. Additionally, LPM appeared to be the only medium that consistently selected for the genera *Enterococcus*, *Leuconostoc* and *Weissella*.

The 48 h enrichment incubation time yielded more false positive isolates compared to the 24 h (Figure 2.3.3). It was found that 83% of lettuce samples enriched for 48 h yielded false positive isolates while the 24 h enrichment produced

false positive isolates from 52% of samples (Figure 2.3.3). This trend was also similar for detection of *L. monocytogenes* and other *Listeria* spp. in which isolates were detected from three lettuce samples at the 48 h enrichment and only one at 24 h (Table 2.3.2). Detection of *L. monocytogenes* was only observed during the 48 h enrichment. Furthermore, for 2 enriched lettuce samples cultured on LPM, MOX and B-OXA at 24 h, false positive isolates preceded detection of *L. monocytogenes* or *Listeria* spp. that were subsequently recovered at 48 h.

Isolation of *Listeria* spp. was not always consistent across LPM, PALCAM, MOX and B-OXA. For one lettuce enrichment, *L. monocytogenes* was recovered from PALCAM, MOX and B-OXA but was not detected on LPM (Table 2.3.2). For that particular sample, false positive isolates were recovered from LPM at the 24 h enrichment time with no collection of presumptive isolates following at 48 h. For another lettuce sample, *Listeria* spp. were isolated from MOX and LPM but not from B-OXA and PALCAM. OXA revealed 2 lettuce samples contaminated with *Listeria* spp. with one sample confirmed positive for *L. monocytogenes* (Table 2.3.2). Comparing the recovery of *Listeria* spp. across all media tested in this study is impossible since samples screened with OXA were carried out separately from the PALCAM, LPM, MOX and B-OXA. Additionally, we fell slightly short of our initial sampling goal of 100 isolates from PALCAM and MOX. This was because some isolates were difficult to sequence or upon re-streaking onto the medium they were originally isolated from were confirmed as negative.

2.3.2 Media cross-comparison

A total of 373 false positive isolates were cross-compared against OXA, MOX, PALCAM and LPM to further characterize their respective positive behaviors (Figure 2.3.4). Overall, 74 isolates were found to be positive on all media. Within the 74 isolates positive on all media, 56 (76%) were originally isolated from OXA and PALCAM. MOX and LPM appeared to share the greatest number of isolates between any two media. This was likely due to the strong selection of *Curtobacterium* by both media (Figure 2.3.2). The vast majority of isolates originally collected from MOX, OXA and PALCAM were positive on the other media and found to be from the genera *Curtobacterium*, *Microbacterium* or *Cellulomonas*. Conversely, 59 (59%) isolates collected from LPM were shown to have positive behavior restricted to that medium alone. Most of the genera found to display positive behavior on LPM were strictly associated with that medium and never isolated from the other media. All media combinations had some degree of sharing of isolates except for LPM and PALCAM, which revealed no shared false positives (Figure 2.3.4). Within a particular genus such as *Cellulomonas*, *Microbacterium* or *Curtobacterium*, positive behavior was not always conserved across each medium and isolates displayed unique media sensitivities.

2.3.3 Phenotypes of presumptive isolates streaked onto RAPID'*L.mono*

All isolates confirmed as *L. monocytogenes* from lettuce enrichments displayed PIPLC +/xylose – activity yielding the characteristic blue/purple color colony. No false positive genera revealed PIPLC +/xylose – behavior. Streaking

Listeria spp. onto RAPID'*L.mono* revealed white colonies with yellow halos, possibly identifying these isolates as *L. welshimeri*. Some false positive genera frequently yielded chromogenic phenotypes similar to other *Listeria* spp. For instance, genera such as *Microbacterium* and *Curtobacterium* displayed PIPLC –/xylose – behavior indicative of *L. innocua*. Genera including *Curtobacterium* and *Cellulomonas* were also able to confer PIPLC –/xylose + phenotype resembling *L. welshimeri*. No chromogenic phenotypes similar to *L. ivanovii* were observed for any isolates collected in this study. The vast majority of genera that were completely inhibited were originally isolated from LPM and included *Enterococcus*, *Weissella* and *Leuconostoc*. Results of RAPID'*L.mono* should be carefully interpreted as we noticed white/yellow halo colonies in close proximity to blue/purple *L. monocytogenes* colonies could appear as greenish indicating the presence of *L. ivanovii*.

Table 2.3.1. Summary of presumptive isolates collected from PALCAM, LPM, MOX, OXA and B-OXA.

Medium	Presumptive isolates collected	*Listeria* spp.	*L. monocytogenes*	Other genera	Non-*listeria* spp. confirmation rate
PALCAM	94	5	5	89	89/94 (95%)
LPM	100	4	0	96	96/100 (96%)
MOX	98	7	5	91	91/98 (93%)
OXA	111	14	5	97	97/111 (87%)
B-OXA	111	4	4	107	107/111 (96%)
Total	514	34	19	480	480/514 (93%)

Table 2.3.2. Recovery rates of *Listeria spp., L. monocytogenes* and false positive samples. False positive samples represent lettuce enrichments that yielded presumptive isolates in which none were identified as *Listeria* spp.

Medium	Number of Romaine Lettuce samples	Positive for *Listeria* spp.		Positive for *L. monocytogenes*		False positive samples
		24h	48h	24h	48h	
PALCAM	25	0 (0%)	1 (4%)	0 (0%)	1 (4%)	18 (72%)
LPM	22	0 (0%)	1 (5%)	0 (0%)	0 (0%)	18 (82%)
MOX	22	0 (0%)	2 (9%)	0 (0%)	1 (5%)	14 (64%)
OXA	17	1 (6%)	2 (12%)	0 (0%)	1 (6%)	14 (82%)
B-OXA	22	0 (0%)	1 (5%)	0 (0%)	1 (5%)	18 (82%)
Total	42	1 (2%)	3 (7%)	0 (0%)	2 (5%)	–

Table 2.3.3. Frequency of genera observed across all media, associated phyla and shape

Genus	Frequency (%)	Phylum	Shape
Sanguibacter	6.6	Actinobacteria	Rod
Staphylococcus	3.1	Firmicutes	Cocci
Curtobacterium	20.2	Actinobacteria	Rod
Weissella	3.3	Firmicutes	Cocci, Rod
Leuconostoc	3.1	Firmicutes	Cocci, Rod
Cellulomonas	14.2	Actinobacteria	Rod
Arthrobacter	0.2	Actinobacteria	Cocci, Rod
Marinilactibacillus	1.2	Firmicutes	Rod
Enterococcus	4.7	Firmicutes	Cocci
Isoptericola	0.2	Actinobacteria	Cocci, Rod
Vagococcus	0.6	Firmicutes	Cocci
Psuedoclavibacter	0.4	Actinobacteria	Rod
Serratia	0.6	Proteobacteria	Rod
Bacillus	5.1	Firmicutes	Rod
Microbacterium	25.7	Actinobacteria	Rod
Rothia	4.3	Actinobacteria	Cocci
Listeria	6.6	Firmicutes	Rod

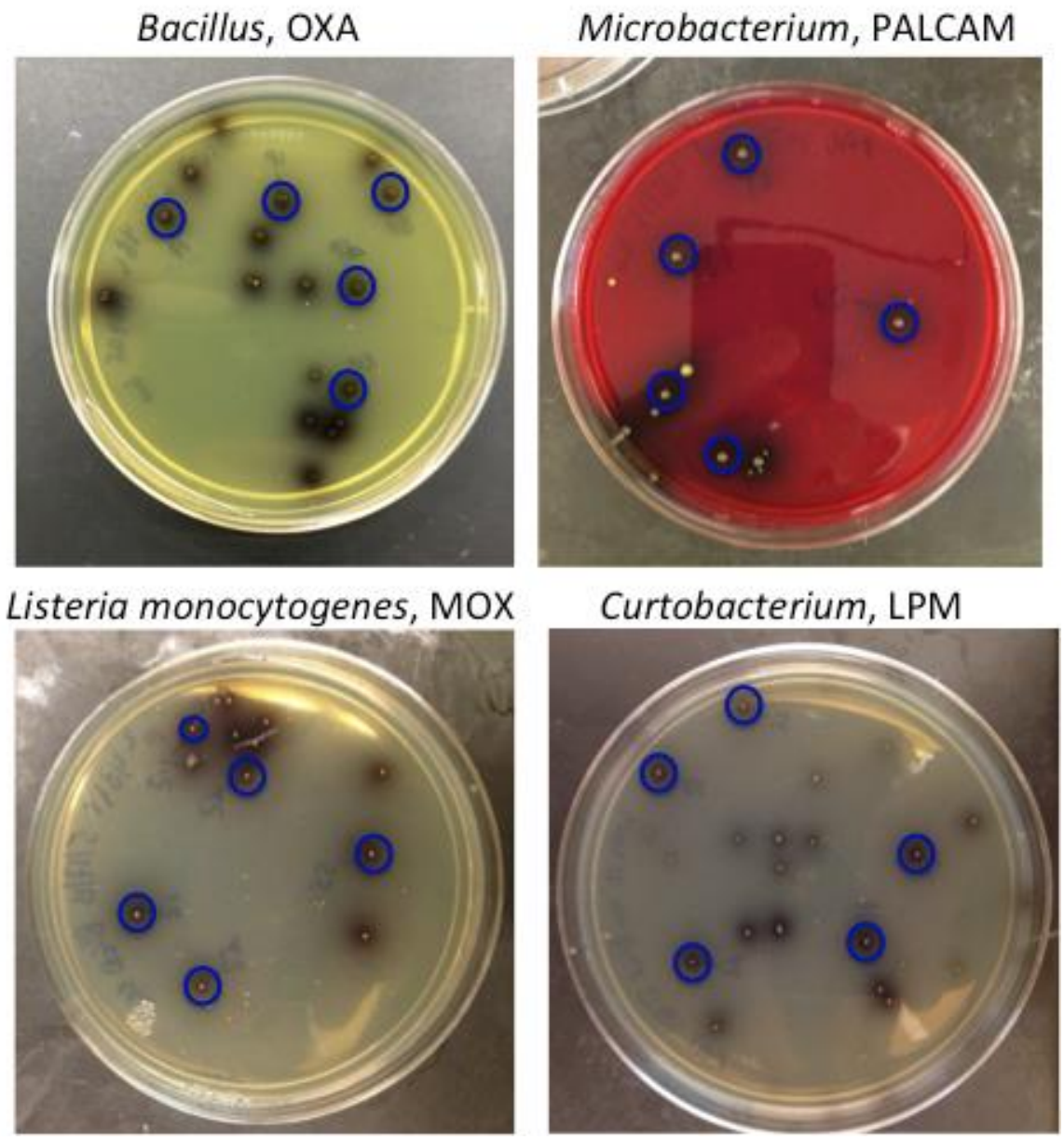

Figure 2.3.1. Picture displaying false positive genera recovered on LPM, OXA and PALCAM from Romaine lettuce enrichments. One of the lettuce samples positive for *L. monocytogenes* detected on MOX is shown for comparison. Colonies circled in blue were picked and had genus identification as labeled next to each medium.

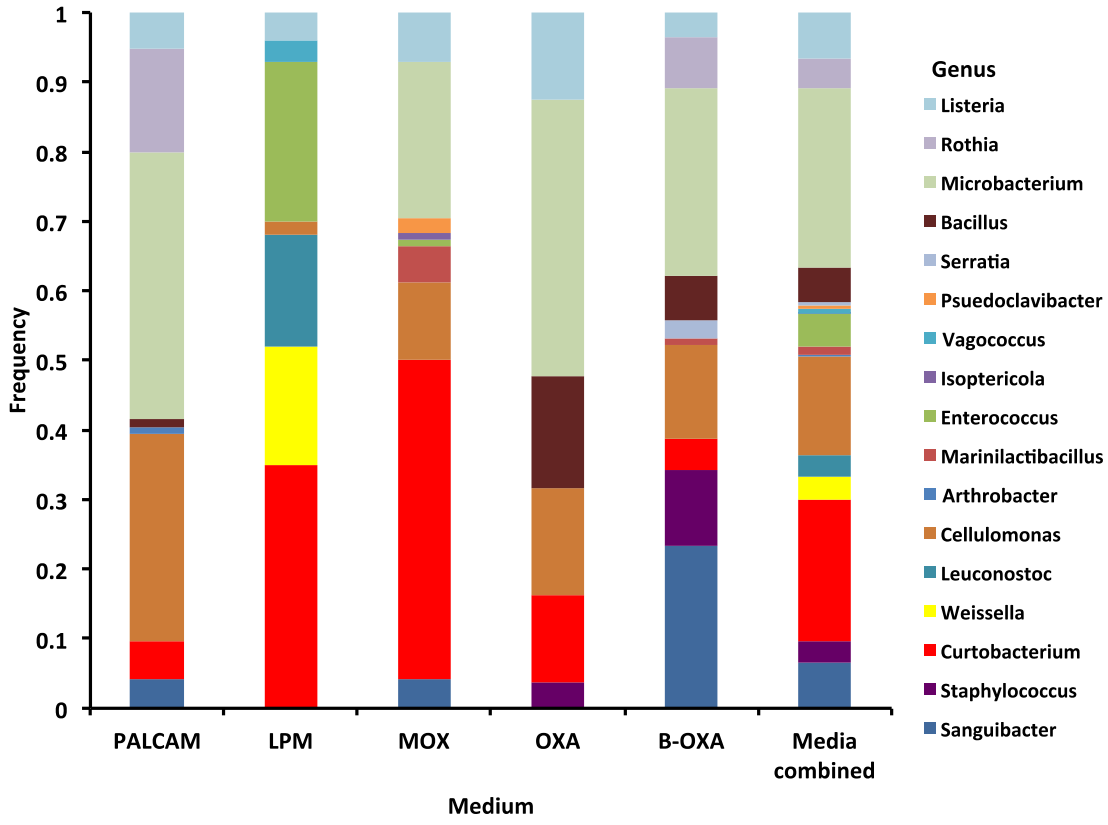

Figure 2.3.2. Relative frequency of presumptive isolates assigned to genus from PALCAM, LPM, MOX, OXA, B-OXA. Media combined represents frequency of all genera collected across all media.

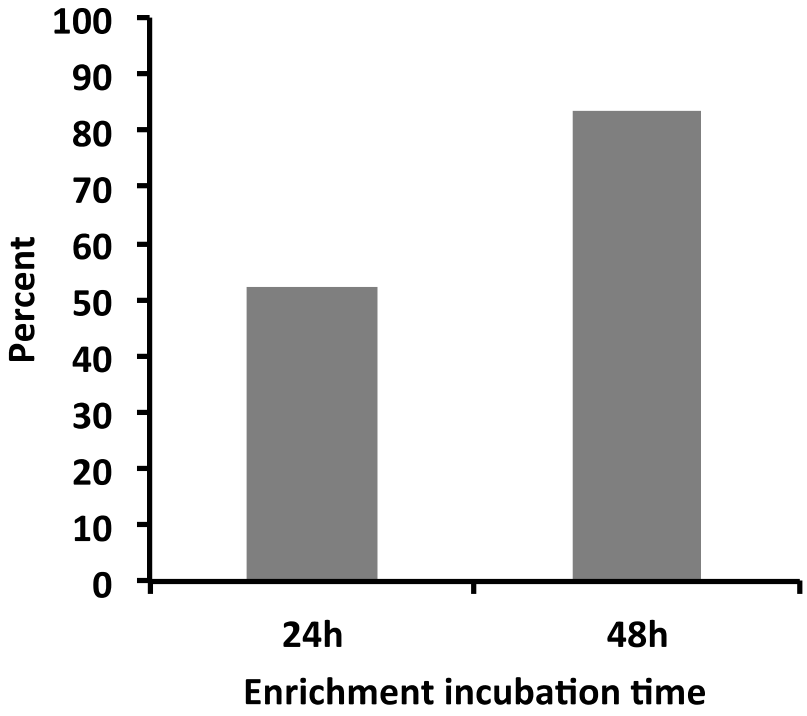

Figure 2.3.3. Percent of Romaine lettuce samples yielding false positive isolates by enrichment incubation time (n=42)

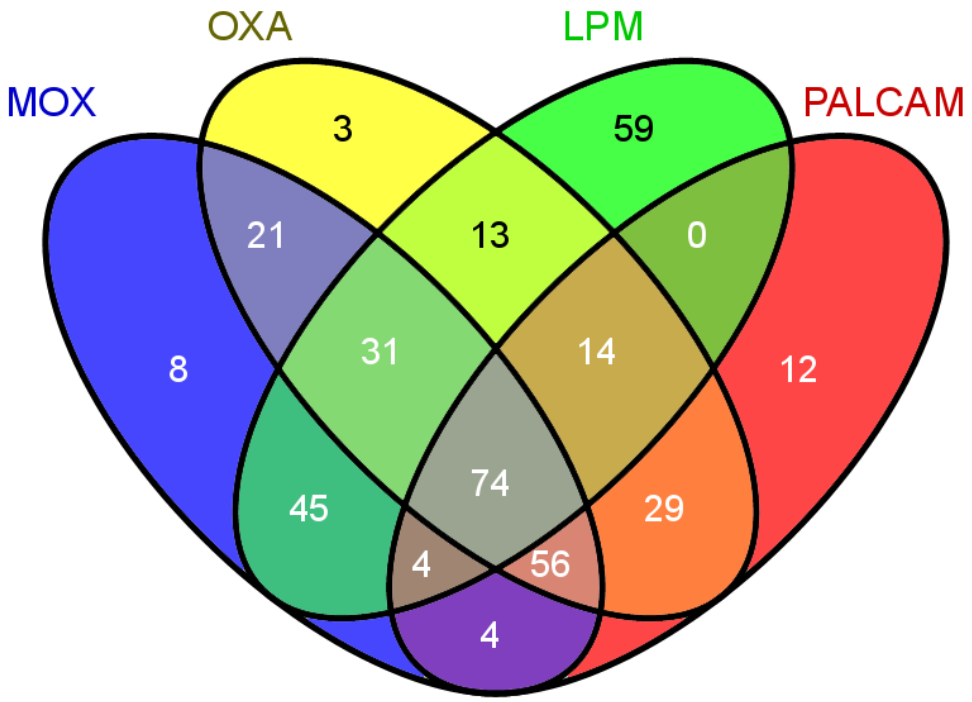

Figure 2.3.4. Media cross-comparison displaying relationships of false positives across each medium. The numbers at each intersection represent isolates that were found to be positive.

2.4 Discussion

2.4.1 Evaluation of traditional FDA media

Romaine lettuce enrichments cultured on all four FDA media efficiently selected for bacteria that were physiologically characteristic of *L. monocytogenes,* but were determined to be from other genera (Table 2.3.1; Figure 2.3.1 and Figure 2.3.2). This led to 64%-82% of BLEB enriched lettuce samples initially appearing positive across MOX, LPM, OXA, B-OXA and PALCAM, but did not yield presumptive isolates that were identified as *Listeria* spp. (Table 2.3.2). Given that up to five presumptive isolates were picked from lettuce enrichments cultured on FDA media, it is possible for *Listeria* spp. and *L. monocytogenes* to have escaped detection. Thus some samples may have been unofficially determined as negative because sampling depth was too low to recover *L. monocytogenes*. This may have especially been true for enrichments where false positives were picked at the 24 h enrichment and detection of *L. monocytogenes* emerged at 48 h.

The rate at which false positives were recovered from lettuce enrichments relative to *Listeria* spp. was exorbitantly high and fairly consistent between media (Table 2.3.1). Additionally, our analysis of B-OXA and OXA revealed antimicrobial supplements did not improve the performance of OXA as it regularly selected for other false positive genera (Table 2.3.1; Figure 2.3.2). These observations provided strong evidence that selective agents present in the enrichment stage and in each medium are inadequate for inhibiting lettuce-associated microbial contaminants. Our findings of high false positive rates across traditional FDA media greatly

contrast with what researchers have reported for foods such as poultry (Capita et al., 2001). The poor performance of the FDA method might be attributed to its original development for isolating *L. monocytogenes* from dairy, meat and poultry products. Overall, the utility of traditional FDA media as a routine screen to determine presumptive *L. monocytogenes* lettuce samples is greatly reduced by persistent false positive isolates that complicate the detection of this pathogen and greatly influence the interpretation of this assay.

The strong selection of false positives displaying rod-shaped morphology likely represents a key characteristic for their ability to survive selective enrichment (Table 2.3.3). The antimicrobials present during the enrichment specifically target yeasts, molds, gram-negative bacteria and gram-positive cocci (Table 2.1.1 and Table 2.1.2). Thus the spectrum of antibiotics present during the enrichment lacks coverage of gram-positive rods, presumably enabling these microbial contaminants to efficiently co-enrich. Eliminating these false positives during the enrichment might represent a significant challenge as *L. monocytogenes* has rod-shaped cellular morphology. By specifically inhibiting rod-shaped false positives during enrichment one would have to ensure that antimicrobial agents do not negatively influence *L. monocytogenes* growth. However, this limitation could potentially be overcome by designing a medium to select against gram-positive rod-shape microbial contaminants.

The variation in relative frequency of false positive genera across each medium demonstrates that media appear to possess inhibitory action against some

isolates more effectively than others (Figure 2.3.2). From these observations we can confidently conclude that the most problematic false positive genera include *Microbacterium*, *Curtobacterium, Cellulomonas and Enterococcus,* as these genera were the most frequently recovered from traditional FDA media. The selective supplements in PALCAM and OXA appeared to be highly effective in eliminating or reducing the genera *Enterococcus* and *Curtobacterium.* Fosfomycin present in OXA is well known for its bactericidal effects against *Enterococcus* spp. (Michalopoulos et al., 2011). The sole antimicrobial compound in OXA or PALCAM responsible for the low recovery of *Curtobacteirum* can't be determined, although ceftazidime, a broad-spectrum cephalosporin present in PALCAM, may have yielded antimicrobial effects against this genus. LPM was highly effective in inhibiting *Cellulomonas* spp. *and Microbacterium* spp. LPM is unique compared to the other media in that it incorporates glycine anhydride into its base formula. D-amino acids such as glycine have reported concentration dependent antimicrobial effects in certain bacteria (Hishinuma et al., 1969). Thus the addition of glycine anhydride may have a role in selecting against the genera *Cellulomonas* and *Microbacterium* on LPM. Therefore combining the base formula of LPM with the selective supplements present in OXA or PALCAM may provide a highly selective medium to eliminate abundant false positive genera from BLEB enriched Romaine lettuce samples.

We did observe 2 lettuce enrichments where *L. monocytogenes* or *Listeria* spp. were inconsistently detected across MOX, B-OXA LPM and PALCAM. This trend is probably not unique to BLEB lettuce enrichments, as inconsistencies in recovery

across traditional media have been commonly reported in other food matrices (Denver et al., 1993). However, one the most concerning findings was *Listeria* spp. that escaped detection within the 24 h enrichment, where false positives were initially isolated and detection of *Listeria* emerged at 48 h. Furthermore, *L. monocytogenes* was only recovered from the 48 h enrichment. This observation strongly contrasts with published early enrichment evaluations where 89% of food samples were reported to recover *L. monocytogenes* within 24 h of enrichment (Warburton et al., 1991). Our results might be explained by false positive isolates that masked detection within the initial 24 h enrichment. Alternatively, the isolation of *Listeria* spp. during the 48 h enrichment suggests that lettuce-associated microflora present during Romaine lettuce enrichments could affect growth dynamics thereby limiting the detection threshold to 48 h.

Currently, efforts have focused on identifying foodborne microbiota that survive selective enrichment and actively compete with *L. monocytogenes*. Competition between *L. moncytogenes* and *Entercoccus* spp. was recently documented using BLEB enriched processed milk samples (Dailey et al., 2014). The authors reported various sensitivities of *L. monocytogenes* growth in response to co-enrichment with *Enterococcus* spp., such that these competing organisms negatively influenced pathogen growth. In our study, *Enterococcus* spp. were isolated from several BLEB lettuce enrichments cultured on LPM but were virtually absent on the other media. *Enterococcus* belongs to order Lactobacillales that consists of lactic acid bacteria (LAB), which are fairly well known for displaying inhibitory action

against *L. monocytogenes* growth (Al-Zeyara et al., 2011). Foodborne LAB are known producers of bacteriocins and because of their unique fermenting abilities can influence the pH of their surrounding environment (Coelho et al., 2014). We also observed several other genera belonging to the LAB clade such as *Weisellia* and *Leuconostoc* that were recovered from LPM. Whether the LAB genera recovered in our study have antagonistic effects on *L. monocytogenes* growth can't be determined from the present study. However, it is important to recognize these microflora survive and actively multiply along with *L. monocytogenes* during lettuce BLEB enrichments.

The number of lettuce samples yielding false positive isolates appeared to be more strongly associated with the 48 h enrichment (Figure 2.3.3). Interestingly, this trend also seemed to be consistent with recovery of *Listeria* spp. across all media. For the 4 lettuce samples that yielded *Listeria* spp., 3 of these recovery events occurred during the 48 h enrichment (Table 2.3.2). The issue of false positives emerging during the 48 h enrichment could be marginally remedied if we had strong assurance of consistently detecting *Listeria* within 24 of enrichment. It might have been useful to monitor total aerobic background microflora during 24 h and 48 h BLEB lettuce enrichments to see whether population differentials contribute to sensitivity of recovering *L. monocytogenes*. Currently, competition between foodborne microflora and *L. monocytogenes* during BLEB enrichments of produce items remains unknown.

We found our DNA sequencing approach using the 16S rRNA gene to be a powerful method to rapidly identify presumptive *Listeria* isolates to genus. Use of DNA sequencing to identify presumptive isolates would be considered a novel approach within food safety testing, as it is not a standard technique. Recently, Hellberg et al., 2013 demonstrated that partial sequencing of the polymorphic v2 region from the 16S rRNA gene can allow differentiation of *Listeria* spp., including *L. monocytogenes*. The researchers were able to successfully apply their method by identifying *L. monocytogenes* from spiked food samples that were screened using the FDA BAM protocol. Conceivably, one could enhance this approach by building a 16S rRNA database composed of all known microbial contaminants and *Listeria* spp. to fully integrate a sequencing approach for rapid identification of presumptive isolates during food screens. As demonstrated in our work, DNA sequencing of the 16S rRNA gene can offer many advantages to identification of putative positive isolates, especially over traditional and subjective phenotyping methods.

Our finding of high recovery rates of false positives across traditional media has never been reported, even in recent media evaluations that included other fresh produce commodities such as mixed salad, coleslaw, tomato and lettuce (Jamali et al., 2013). However, evaluations of enrichment and selective plating media strictly focus on the recovery aspect of *Listeria* spp. or *L. monocytogenes* from food matrices. That is, evaluations typically report presence or absence of *L. monocytogenes* to determine whether detection rates are similar across media. Yet, a consistent theme in many of these evaluations is omission of investigating food-associated microbiota

that may influence recovery and detection of *L. monocytogenes*. The issue with presence or absence and PCR based approaches to media evaluations is that false positives are likely to go unreported and remain unclassified. Our characterization of false positive genera from Romaine lettuce samples revealed that microbial contaminants confounded the detection of *L. monocytogenes* and readily influenced the interpretation of this screen such that many lettuce samples initially appeared positive but ultimately did not yield *Listeria* spp.

2.4.2 Media cross-comparison

The media cross-comparison study allowed us to investigate inter-media relationships between false positive isolates and further characterize their respective behaviors (Figure 2.3.4). False positives originally recovered from OXA, MOX and PALCAM displayed broad positive behavior across the other media. Typically isolates displaying this activity were from the same genera representing *Microbacterium*, *Curtobacterium* or *Cellulomonas*. LPM was the most unique medium that revealed the largest proportion of positive isolates restricted to any single medium. Positive behavior was not always conserved within a genus as well, as many isolates displayed unique activity across media ultimately suggesting diversity at the species level or even the strain level. Further addressing this variable positive behavior will allow us to understand which antimicrobial supplements present in traditional media are most affective against false positive genera collected in this study.

2.4.3 Phenotypes of presumptive isolates streaked onto RAPID'*L.mono*

Our investigation of false positive behavior on RAPID'*L.mono* revealed no isolates displayed chromogenic activity similar *L. monocytogenes*. Thus is can be said false positives collected from lettuce enrichments on traditional FDA media are not likely to influence detection of *L. monocytogenes* on RAPID'*L.mono*. Overall this commercial medium would be beneficial for screening presumptive isolates for the presence of *L. monocytogenes*. Because we didn't directly evaluate lettuce enrichments cultured on this commercial medium, it's unknown whether other foodborne microflora present during BLEB enrichments can display similar characteristics to *L. monocytogenes* or whether recovery of this pathogen is comparable to traditional FDA media.

We found that several isolates from the genus *Microbacterium*, *Curtobacterium* or *Cellulomonas* were able to confer phenotypes indicative of *L. innocua* and *L. welshimeri*. This finding ultimately limited the utility of this medium to accurately screen presumptive isolates for *L. innocua* and *L. welshimeri*. The poor discriminatory power between background microflora and these *Listeria* spp. on RAPID'*L.mono* has been previously reported for other foods (Greenwood et al., 2005). From a food safety standpoint, because both of these species are not considered pathogenic to humans, such a finding might be less of a concern if investigators are not interested in assessing *Listeria* spp. on Romaine lettuce. However, for researchers using RAPID'*L.mono* to assess prevalence of *L. ivanovii* and

L. innocua on lettuce, results must be carefully interpreted and confirmation of these species by PCR, DNA sequencing or biochemical characterization should follow.

The usefulness in pairing commercial media with traditional *Listeria* selective media for detection of *L. monocytogenes* from food is strongly supported in the literature (Hegde et al., 2007; Aragon-Alegro et al., 2008; Park et al., 2012). We can conclude that our results are generally in agreement with these former studies. Due to our finding of high false positive rates across traditional FDA media, pairing with RAPID'*L.mono* during routine lettuce screens would appear to be highly beneficial for detecting *L. monocytogenes*.

2.4.4 Conclusions

Overall, the entire FDA BAM protocol from the enrichment to traditional media appears to be unsuitable for the routine screening of *L. monocytogenes* from Romaine lettuce. OXA, PALCAM, LPM and MOX all efficiently select for false positive genera that significantly influence the interpretation of this screen. The option of four different media makes choosing a particular medium for routine screening and basic research investigations difficult. Researchers might be able to simplify this choice by empirically evaluating media for other fresh produce items to determine an optimal isolation medium. Going forward, evaluating improved enrichment and selective media should be highly considered. In the meantime, traditional FDA media should be paired with a commercial medium such as RAPID'*L.mono* when using the FDA protocol to screen Romaine lettuce samples for *L. monocytogenes*. Our media-cross comparison yielded valuable insight into false positive behavior across

media and may provide insight into specific antimicrobial supplements that actively select against certain genera. Furthermore, analyzing whole genome sequences of false positives would be useful for mining genes to help identify antimicrobial compounds effective against these genera.

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CHAPTER 3. CHARACTERIZATION OF FALSE POSITIVE *CELLULOMONAS* ISOLATES: GENOME ANALYSIS, ANTIBIOTIC RESISTANCE GENES, ANTIBIOTIC TARGET GENES AND PHYLOGENETIC ANALYSIS

3.1 Introduction

The emergence of next generation sequencing (NGS) technology has vastly accelerated and contributed to the endeavor to understand the microbial world. Recent advances ranging from sequencing genomes to characterizing the human microbiome truly underscore the capabilities of this technology (Turnbaugh et al., 2007). One of the most remarkable insights into these explorations is the enormity of prokaryotic diversity. Using NGS approaches the number of species of bacteria in soil have been estimated at 52,000 while computational methods suggest these numbers are as high as 10^7 species (Gans, 2005; Roesch et al., 2007).

Our ability to accurately capture this microbial diversity and describe novel species is dependent on the use of molecular markers. For bacteria, the 16S small subunit ribosomal RNA (rRNA) gene has been the most widely utilized marker. The 16S rRNA gene yields a strong taxonomic signal due to its mosaic of highly conserved regions combined with regions that are more variable in nucleotide composition (Olsen and Woese, 1993). These molecular features of the 16S rRNA gene allow it to be used to distinguish between distantly and closely related bacteria, therefore owing to its utility for accurately classifying bacteria. The utility

of this particular gene was discovered by Carl Woese and served as an invaluable molecular tool to phylogenetically resolve the major bacterial phyla (i.e. gram positive bacteria, cyanobacteria, purple bacteria, etc.,) (Woese, 1987, 1990). However, as we further progress into the genomics era, the increased availability of whole genome data should enable us to draw more robust phylogenetic inferences between novel and previously characterized prokaryotes.

Previously, in Chapter 2 of this thesis we utilized a portion of the 16S rRNA gene to identify presumptive isolates to genus. This approach allowed us to identify a number of false positive genera that complicated detection of *Listeria monocytogenes* from traditional US Food and Drug Administration (FDA) media. Of note, we found isolates identified as *Cellulomonas* spp. to be one of the more problematic false positives on MOX, OXA, B-OXA and PALCAM. One of the peculiarities of this finding is that *Cellulomonas* spp. are not typically associated with plants (Stackebrandt and Schumann, 2014). Rather, these bacteria most commonly inhabit the soil environment and are best known for their cellulolytic activity (Stackebrandt and Schumann, 2014). This finding was what spurred further inquiry into the relationship of our presumed *Cellulomonas* isolates to other species within this genus.

Phenotypes of *Cellulomonas* isolates recovered from lettuce enrichments were not conserved across media, and in fact we observed several unique susceptible and resistant phenotypes. This ultimately suggested diversity at the species or even strain level for these isolates. Given that we observed several

distinct media phenotypes for these isolates and recovered these *Cellumonomas* spp. from a habitat that would be considered fairly novel, we were left with a couple of intriguing questions. First, what factors determine susceptibility and resistance to media antibiotics within this particular set of false positive isolates? Second, are these false positive isolates members of the genus *Cellulomonas* and if so are they novel species, or do they represent a novel group of bacteria that are closely related to this genus?

In Chapter 3 of this thesis we crafted our objectives to further explore these questions by performing whole genome sequencing of 13 *Cellulomonas* false positive isolates with unique media-phenotype behaviors. First, we were interested to draw inferences from isolate genome annotations to gain insight into factors that mediate the various phenotypes displayed towards media antibiotics. Second, we wanted to resolve the phylogenetic relationship of the 13 *Cellulomonas* isolates to known *Cellulomonas* spp. and other closely related bacteria. Our investigation of phylogenetic relationships employed two approaches: genome data was used to determine genus-level relationships, then the 16S rRNA gene was used to determine species-level relationships. Third, using our genome data from the 13 isolates, we wanted to identify conserved antibiotic target genes so that going forward one might be able to test antibiotics to eliminate these false positives.

3.2 Methods

3.2.1 Selection of *Cellulomonas* isolates, genome sequencing and assembly

We were interested in performing whole genome sequencing of *Cellulomonas* isolates that displayed unique phenotypes on MOX, B-OXA, PALCAM and LPM. These different phenotypes appeared over the course of nine enriched lettuce samples. From each of these lettuce samples we selected as many isolates as there were unique phenotypes displayed across MOX, B-OXA, PALCAM and LPM (Table 3.2.1). From the nine lettuce enrichments we selected a total of 13 *Cellulomonas* isolates. Once the 13 isolates were chosen, they were cultured overnight in brain heart infusion broth at 30 °C. Genomes were extracted using the GenElute Bacterial Genomic DNA kit (Sigma-Aldrich, St. Louis MO, US). Once purified genomic DNA was obtained, samples were submitted to the Purdue University Genomics Center for sequencing on the MiSeq platform (Illumina, San Diego CA, US). Once sequencing was complete, genomes were assembled *de novo* using AbySS software (Simpson et al., 2009). It should be noted that false positive *Cellulomonas* isolates collected from OXA were not used in this study, as genome sequencing was performed before we began identifying isolates collected from this medium.

3.2.2 Genome annotation

An in-house pipeline was used to annotate assembled genomes of all 13 isolates. To explain briefly, gene models were predicted using Prodigal (v2.60) (Hyatt et al., 2010); annotation of gene models was accomplished through software implemented in Prokka (v1.90) (Seemann, 2014); tRNAscan (v1.21) was used to

predict transfer RNA genes (Lowe and Eddy, 1997); RNAmmer (v1.20) was employed to predict ribosomal RNA genes (Lagesen et al., 2007). Proteomes from all isolate genomes were included in orthology analysis using the software package Proteinortho5 (v5.10) (Lechner et al., 2011). Orthologous relationships between isolates were visualized using FriPan (https://github.com/Victorian-Bioinformatics-Consortium/FriPan/). Genome-relatedness between isolates was explored through pairwise comparisons of whole genome sequences to calculate average nucleotide identity (ANI) (Goris et al., 2007). It should be noted that ANI between genomes is not influenced by genome size, as this program only considers alignable regions when calculating percent identity between the query and reference genome.

3.2.3 Multilocus phylogenetic analysis using closely related proteomes

In order to determine the genus of our isolates, we employed a multilocus phylogenetics approach. Using the RAST SEED server we identified nine genomes closely related to the genomes of our 13 isolates (Overbeek et al., 2005). These nine closely related genomes included: *Cellulomonas fimi* ATCC 484, *Cellulomonas flavigena* DSM 20109, *Cellulomonas gilvis* ATCC 13127, *Bacillus subtilis* strain 168, *Clavibacter michiganensis subsp sepedonicus*, *Jonesia denitrificans* DSM 20603, *Micrococcus luteus* NCTC 2665, *Sanguibacter kideii* DSM 10542 and *Xylanimonas cellulosilytica* DSM 15894 (See Appendix C for genome accessions). Proteomes of these nine genomes were retrieved from the National Center for Biotechnology genome database (http://www.ncbi.nlm.nih.gov). It should be noted that our phylogenetic analysis included the type species for the genus *Cellulomonas*, which is

Cellulomonas flavigena. The program AMPHORA (v2.0) was used to identify highly conserved protein-coding genes across all of the genomes (Wu and Eisen, 2008). Once conserved sequences were identified from each genome, they were aligned and trimmed by AMPHORA. 30 protein sequences were subsequently concatenated using FasconCAT (v1.0) (Kück and Meusemann, 2010) to build a super matrix consisting of 6,351 amino acid residues. To estimate genus-level phylogenetic relationships between the 22 isolates we used RAxML (v8.0.19) (Stamatakis et al., 2008). RAxML analysis employed a gamma model of rate heterogeneity, JTT amino acid substitution matrix, 100 bootstrap inferences and maximum likelihood (ML) search. Trees were rooted using *Bacillus subtilis* strain 168 as the out group taxon. *B. subtilis* was chosen as the out-group based on a recent paper by Christopherson et al. (2013) that investigated multilocus phylogenetic relationships of *Cellulomonas* spp. to other closely related bacteria. The best-scoring maximum likelihood tree was viewed and edited in FigTree (v1.4.2) (http://tree.bio.ed.ac.uk/software/figtree/).

3.2.4 Phylogenetic analysis using the 16S rRNA gene

To determine whether our isolates shared species-level relationships to members within the genus *Cellulomonas* we constructed a phylogeny using the 16S rRNA gene. Our methods followed a recently published phylogeny of the genus *Cellulomonas* described in Ahmed et al. (2014). The 16S rRNA gene from all 23 type species of the genus *Cellulomonas* and *Cellulosimicrobium cellulans* were retrieved from EzTaxon, a prokaryotic sequence database (Kim et al., 2012) (See Appendix B for list of type strains). Sequences were then aligned using ClustalX (v2.1) (Larkin et

al., 2007) and trimmed with Gblocks (v0.91b) (Castresana, 2000). A phylogenetic tree was made in MEGA (v6.06) using the neighbor joining method, Kimura two-parameter model and 1000 bootstrap inferences (Tamura et al., 2013). The tree was rooted with *Cellulosimicrobium cellulans* as the out-group taxon and visualized in MEGA.

3.2.5 Identification of antibiotic resistance genes and antibiotic target genes

Whole genome sequences of all 13 isolates were queried against the Comprehensive Antibiotic Resistance Database (CARD) to predict antibiotic resistance gene clusters (McArthur et al., 2013). CARD is a genomics pipeline that is available to researchers interested in studying antibiotic resistance and antibiotic target genes from a wide variety of bacteria. To identify putative antibiotic target genes for isolates, we first downloaded a sequence database of known antibiotic target genes from the CARD website (http://arpcard.mcmaster.ca). Then we used Proteinortho5 to search for orthology between the proteomes of our 13 isolates and proteins from the antibiotic target gene database. Candidate antibiotic target genes were reported if and only if we observed orthology between protein-coding genes present in all 13 isolates to a specific gene in the database.

Table 3.2.1. *Cellumonas* isolates and their respective phenotype corresponding to each medium. Lettuce enrichment refers to lettuce sample that was enriched for screening of *Listeria monocytogenes* from Chapter 2 of this thesis.

		Phenotype by medium			
Isolate	Lettuce enrichment	MOX	B-OXA	LPM	PALCAM
MOX13	3	+	+	+	+
MOX21	5	+	+	+	+
MOX31	8	+	+	+	+
MOX36	10	+	+	-	+
B-OXA1	1	+	+	+	+
B-OXA19	4	-	+	-	+
B-OXA26	6	-	+	-	+
B-OXAl38	8	-	+	-	+
B-OXA42	9	-	+	-	+
PALCAM14	3	-	+	-	+
PALCAM26	6	+	+	+	+
PALCAM35	8	+	+	-	+
PALCAM41	12	+	+	-	+

3.3 Results

3.3.1 Genome description and comparison

Genome size ranged from 3.84 Mb to 4.92 Mb across the 13 isolates. GC content of all isolates averaged 75% (Table 3.3.1). Percent genome coding was highly consistent across isolates, but the total number of putative gene models varied considerably with genome size. tRNA prediction found genomes ranged from 47 to 52 tRNA genes with the exception of B-OXA38 that recovered 94 (Table 3.3.1). Genomes displayed a high degree of orthology and shared 1,985 single copy orthologs, indicating that this set of genes likely makes up the core genome of all 13 isolates. Although isolates appeared to share some functional conservation, many genes were presumably novel or unique to a particular isolate or 'species' as visualized using FriPan (Figure 3.3.1). ANI comparisons recovered several isolate genomes that were highly related and shared over 95% identity (Table 3.3.2). For instance, isolates B-OXA1, MOX31 and MOX36 shared an ANI of > 97% (Table 3.3.2). Even when isolate genomes shared ANI values of > 97% we still observed differences in media phenotype behavior, as was the case with MOX31 and MOX36. ANI also revealed some modest sequence diversity between isolates with the majority sharing anywhere from 85% to 90% of their genomes (Table 3.3.2). From this information we were not able to deduce any immediate specific genome features or genome-relatedness that corresponded to the various isolate phenotypes on media.

3.3.2 Multilocus phylogenetic analysis

In order to better understand the relationship of our isolates to the genus *Cellulomonas*, we employed a multilocus phylogenetic approach using highly conserved protein-coding sequences. From the 22 genomes, we identified 30 highly conserved protein-coding genes that were included in our analysis: *frr*, *nusA, pgk, pyrG, rplA, rplB, rplC, rplD, rplE, rplF, rplK, rplL, rplM, rplN, rplP, rplS, rplT, rpmA, rpoB, rpsB, rpsC, rpsE, rpsL, rpsJ, rpsK, rpsM, rpsS, smpB, tsf and dnaG.*

Our phylogenetic analysis found our 13 isolates formed a strongly supported (100 Bootstrap Probability) monophyletic clade composed of just our false positive isolates from Romaine lettuce (Figure 3.3.2). The clade with our false positive isolates is sister to the clade composed of known *Cellulomonas* spp. This analysis suggests our isolates are either in the genus *Cellulomonas* or comprise a sister genus to *Cellulomonas* (Figure 3.3.2). Due to the fact that only three *Cellulomonas* spp. have available genome data, species-level relationships could not be resolved.

3.3.3 Phylogenetic analysis of the 16S rRNA gene

In order to determine species-level relationships, we constructed a phylogenetic tree using the 16S rRNA gene from all known *Cellulomonas* spp. Our analysis strongly supports the inclusion of our 13 isolates within the genus *Cellulomonas* (Figure 3.3.3). Our analysis derived three well-supported clades within *Cellulomonas*, with our 13 isolates only present in clade III (Figure 3.3.3). Three currently described *Cellulomonas* spp. were present in clade III: *C. pakistanensis*, *C. denverensis* and *C. hominis*. PALCAM14, PALCAM26, B-OXA38 and

B-OXA26 form a monophyletic group that is sister to *C. pakistanensis* (Figure 3.3.3). Another set of isolates, PALCAM35, MOX21, B-OXA19, MOX13 and PALCAM41 is sister to the *C. pakistanensis* group. B-OXA42 is sister to *C. denvernesis* and could represent a novel species (Figure 3.3.3). MOX31, MOX36 and B-OXA1 form a monophyletic clade that is sister to the rest of the isolates in clade III and could represent a second novel species. Due to the limited number of 16S rRNA sequences available for strains of *C. pakistansensis,* we could not further identify novel species for isolates that had a sister relationship to *C. pakistansensis.*

3.3.4 Antibiotic resistance and antibiotic gene targets

Using the CARD database we identified a number of putative antibiotic gene clusters. Overall, within this set of potential resistance genes, only two genes identified as qacA and qacB might have a role in aiding resistance of certain isolates to acriflavine, which is an intercalating dye used in OXA, PALCAM and buffered *Listeria* enrichment broth (BLEB) (Table 3.3.3 and Table 3.3.4). Other than qacA and qacB, no other putative resistance genes recovered by CARD would appear to play a role in allowing isolates to survive selective enrichment and plating. Genes involved in resistance novobiocins and lincosamides were highly conserved among isolates (Table 3.3.3 and Table 3.3.4). We also found antibiotic resistance clusters that were unique to a particular isolate. For instance, B-OXA1 was the only isolate with genes predicted to be involved in resistance to glycopeptides.

All traditional FDA media incorporate broad-spectrum antibiotics known as cephalosporins (US FDA BAM, 2011). Bacteria are able to gain resistance against

certain classes of cephalosporins by producing special enzymes such as beta-lactamases (Tenover, 2006). Our results from CARD failed to identify any matches from their database to beta-lacatamase genes present in our isolates. However, after we performed a search of our annotated genes from all isolates we recovered a number of gene models identified as beta-lactamase. In addition, we also found several genes annotated as multi-drug resistance proteins and multi-drug resistance abc transporters that were not reported by CARD.

Orthology analysis between the proteomes of our 13 isolates and protein sequences from the antibiotic target database yielded one potential antibiotic target gene present in all of our 13 isolates (Table 3.3.5). The one antibiotic target gene found in the 13 isolates was found to be translation elongation factor G. We also identified a potential antibiotic molecule affective this target gene as fusidic acid.

Table 3.3.1. Breakdown of genome attribute for each *Cellulomonas* isolate

	Isolate												
Attribute	**MOX 13**	**MOX21**	**MOX31**	**MOX 36**	**B-OXA 1**	**B-OXA 19**	**B-OXA 26**	**B-OXA 38**	**B-OXA 42**	**PALCAM 14**	**PALCAM26**	**PALCAM35**	**PALCAM 41**
Genome size	4345526	4710737	4083209	3843571	3979894	4724853	4339056	4927613	4019332	4439627	4268011	4359264	4332446
Coverage	153.4	187.1	226.3	144.9	119.4	204.9	232.8	192.2	241.0	156.6	203.5	219.5	233.9
Scaffolds	14	27	31	15	21	24	33	32	15	49	14	34	34
Percent GC	75.4	75.4	75.5	75.6	75.5	75.2	75.8	74.9	74.6	75.4	75.4	75.4	75.4
tRNA	47	47	48	47	47	48	47	94	48	51	47	52	47
16S rRNA genes	6	6	6	6	6	5	6	6	5	3	4	6	6
Predicted coding sequences	3832	4181	3683	3474	3639	4187	3905	4625	3602	4009	3842	3880	3856
% Genome coding	89.9	90.3	90.4	90.5	90.7	90.3	89.8	90.0	90.7	90.4	90.7	89.5	90.0

Table 3.3.2. ANI values for all 13 *Cellulomonas* isolates. Values in bold represent highly related genome sequences between isolates.

	B-OXA1	MOX13	MOX21	MOX31	MOX36	B-OXA19	B-OXA26	B-OXA38	B-OXA42	PALCAM14	PALCAM26	PALCAM35	PALCAM41
B-OXA1		85.04	84.97	**97.94**	**98.84**	84.92	85.14	84.88	84.86	84.92	84.92	85.21	84.99
MOX13			92.67	84.97	84.99	92.48	89.67	89.87	84.28	89.82	89.82	88.05	**97.81**
MOX21				84.88	84.99	**94.62**	90	90.87	84.33	90.86	90.83	88.13	92.6
MOX31					**97.98**	85.01	85.15	84.95	84.92	84.9	84.95	85.28	84.92
MOX36						84.99	85.17	84.97	84.93	84.96	84.95	85.19	84.87
B-OXA19							90.03	90.69	84.52	90.68	90.69	88.17	92.55
B-OXA26								91.31	84.5	91.23	91.31	88.68	89.63
B-OXA38									84.41	**98.75**	**97.41**	88.44	89.78
B-OXA42										84.3	84.34	84.23	84.38
PALCAM14											**97.46**	88.41	89.71
PALCAM26												88.39	89.84
PALCAM35													88
PALCAM41													

Table 3.3.3. Putative antibiotic resistance gene clusters identified by CARD for each *Cellulomonas* isolate. ND = No antibiotic genes detected

Isolate	Antibiotic resistance gene clusters identified by CARD
B-OXA1	VanRO, Erm(30), novA, tet43, ImrB
MOX13	ImrB, Erm(30), novA, tet43, mepA
MOX21	mepA, ImrB, novA
MOX31	ImrB, novA
MOX36	ImrB, novA
B-OXA19	mepA, ImrB, novA
B-OXA26	ImrB, novA
B-OXA38	qacA, qacB, novA, ImrB
B-OXA42	ImrB, novA
PALCAM14	qacA, qacB, novA, ImrB
PALCAM26	ND
PALCAM35	ImrB, novA
PALCAM41	novA, Erm(30), tet43, mepA, ImrB

Table 3.3.4. Antibiotic resistance genes and their associated resistance to antibiotic agents

Antibiotic resistance gene	Associated antibiotic resistance
novA	Novobiocin
Erm(30)	pikromycin, narbomycin, methymycin, neomethymycin
tet43	tetracycline
mepA	bis-indoles, multidrug resistance
ImrB	lincosamides
VanRO	glycopeptides (i.e. vancomycin, teicoplanin)
qacA	intercalating dyes, quaternary ammonium compounds, diamidines, biguanidines
qacB	intercalating dyes, quaternary ammonium compounds, diamidines, biguanidines

Table 3.3.5. Antibiotic target gene found in all 13 *Cellulomonas* isolates and potential antibiotic agent associated with target gene

Antibiotic target gene detected in all 13 isolates	Antibiotic associated with target gene
translation elongation factor G	Fusidic acid

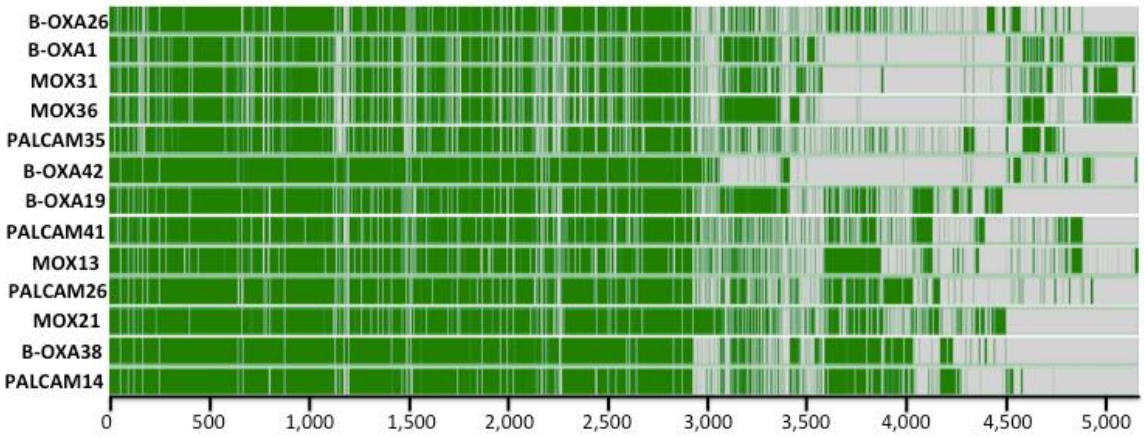

Figure 3.3.1. FriPan visualization map displaying gene orthology across all 13 *Cellulomonas* isolates. Green indicates gene orthology between isolates and grey represents absence of orthology.

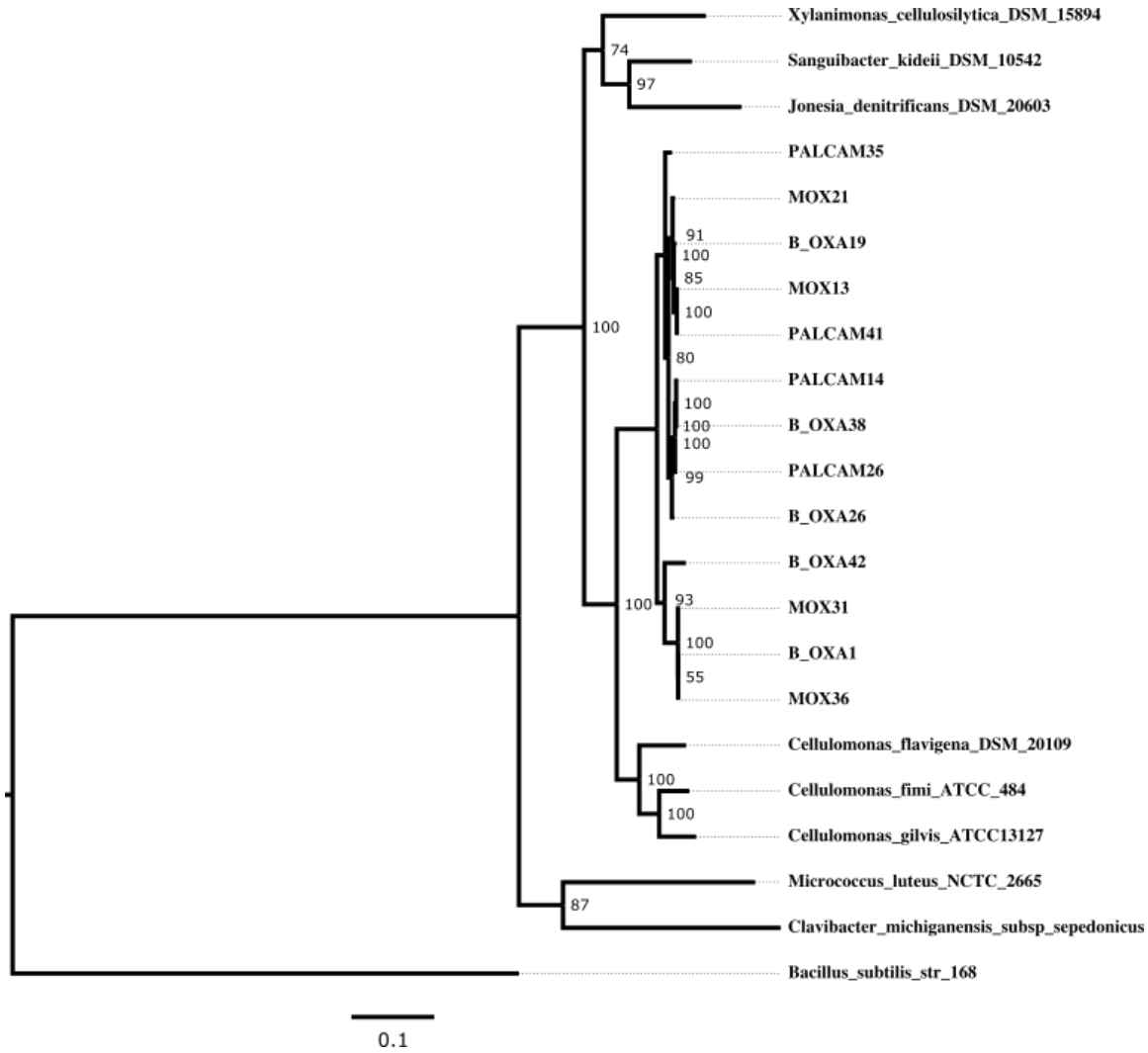

Figure 3.3.2. Multilicous phylogenetic tree. Best scoring maximum likelihood tree constructed from 30 highly conserved protein-coding genes of the 13 false positive *Cellulomonas* isolates, 3 type species of the genus *Cellulomonas,* including the type species *C. flavigena* and six closely related bacteria. *Bacillus subtilis* served as the out-group. The tree was made using RAxML and employed a gamma model of rate heterogeneity, JTT amino acid substitution matrix, 100 bootstrap inferences, maximum likelihood search.

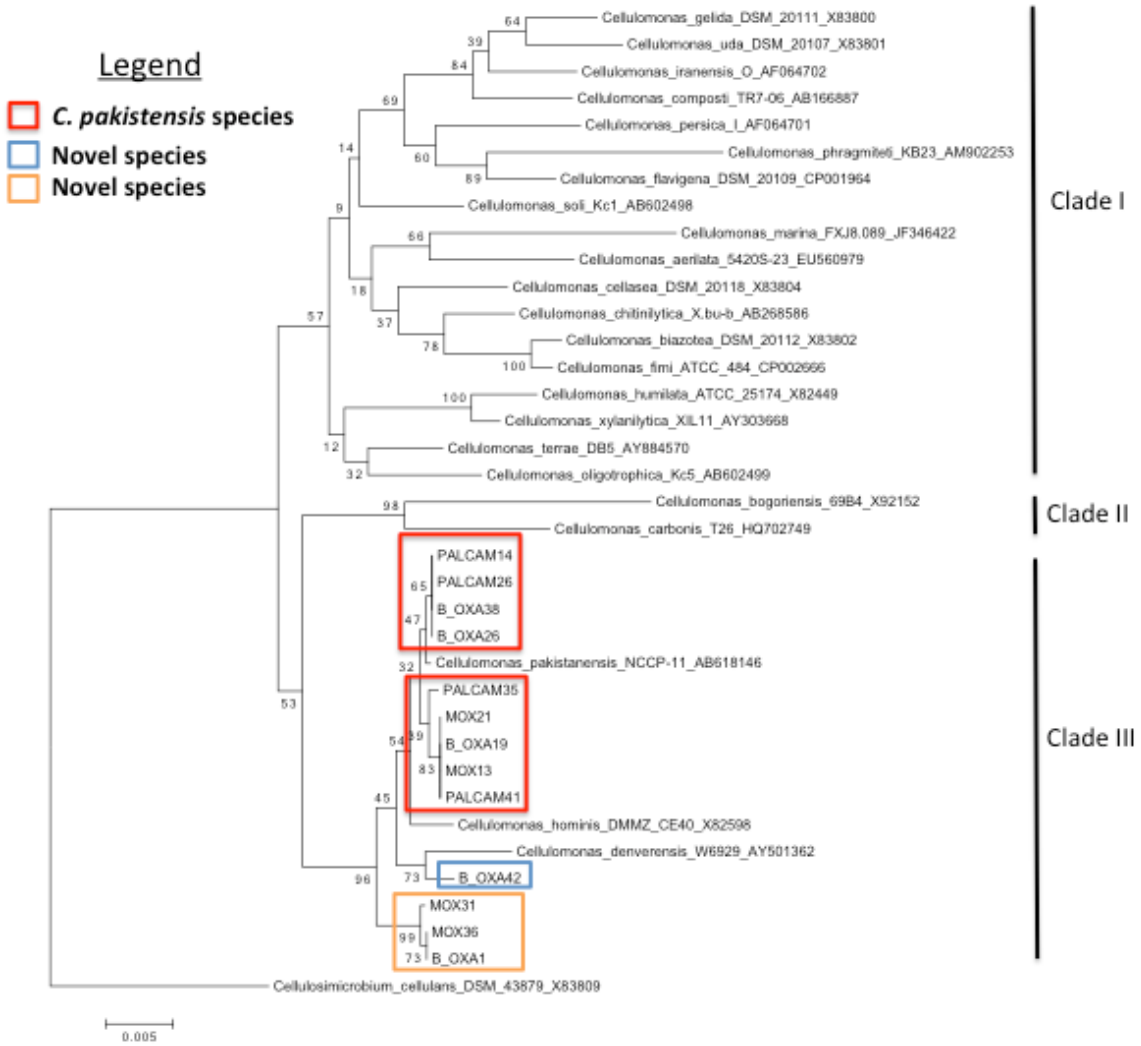

Figure 3.3.3. 16S rRNA Phylogenetic tree. Tree was constructed using the 16S rRNA gene from the 13 false positive *Cellulomonas* isolates and all 23 known species of the genus *Cellulomonas*. *Cellulosimicrobium cellulans* served as the out-group. Phylogenetic relationships were estimated in MEGA using the neighbor joining method, Kimura two-parameter model and 1000 bootstrap inferences. *Cellulomonas* isolates found to be *C. pakistanensis* are highlighted in the red box, and novel species are indicated in the blue and yellow boxes.

3.4 Discussion

3.4.1 Genome analysis

Across isolates we observed a broad variation in genome size that correlated with the number of putative gene models (Table 3.3.1). Some of this variation may be due to the fact that all isolate genomes are presently in a draft state, meaning that some regions of the genome could be missing. Nevertheless, our orthology analysis indicated that many isolates possess a repertoire of novel genes only found in certain isolates (Figure 3.3.1). For free-living bacteria, differences in genome size have been reported to correlate with genes involved in energy, metabolism and regulation (Konstantinidis and Tiedje, 2004). Therefore it is possible unique genes among isolates might serve as nutrient acquisition and survival strategies for these *Cellulomonas* spp. inhabiting the lettuce phyllosphere.

3.4.2 Phylogenetic analysis

Based on the phylogenies constructed from the 16S rRNA gene and conserved loci from whole genome data we can confidently conclude that our isolates are species within the genus *Cellulomonas* (Figure 3.3.2 and Figure 3.3.3). Phylogenetic analysis using a multilocus approach allowed us to determine that our 13 isolates were either in the genus *Cellulomonas* or comprise a sister genus. Nevertheless, even though they inhabit a novel niche, our results confirm these isolates are closely related to *Cellulomonas* (Figure 3.3.2). From the multilocus analysis, species-level relationships remained ambiguous because of the lack of available sequenced genomes. We were able to overcome this limitation by building

a phylogeny using the 16S rRNA gene from all members of the genus *Cellulomonas* (Figure 3.3.3). This approach enabled us to identify closely related species and novel species within our collection of isolates (Figure 3.3.3). At the very least, it appears that some of our isolates represent two novel species. The remaining isolates are presumably *C. pakistanensis.* Further phenotyping and genotyping of isolates closely related to *C. pakistanensis* may in fact lead to identification of more novel species.

All of our isolates formed a distinct clade (i.e. clade III) with known species *C. pakistanensis*, *C. denverensis* and *C. hominis* (Figure 3.3.3). *Cellulomonas pakistanensis* is the most recently of these and was originally isolated from paddy rice (Ahmed et al., 2014). Thus the plant association of *C. pakistanensis* would appear to be consistent with the habitat from which our *Cellulomonas* isolates were recovered. One of the more curious relationships, however, was the close association of our isolates to *C. denverensis* and *C. hominis*, both of which are opportunistic pathogens that were isolated from humans (Brown et al., 2005; Ohtaki et al., 2009). Looking over the literature, it's not entirely clear how these opportunistic pathogens were acquired. Given that the rest of the *Cellulomonas* spp. in clade III were isolated from plants, it is conceivable that infection caused by *C. denverensis* and *C. hominis* could have been acquired through contact with plants or produce.

From the 16S phylogeny it would seem that clade III is more adapted to a plant lifestyle relative to members of clade I, which are most commonly associated with the soil environment (Figure 3.3.3). Future research can focus on genomic

differences between isolates in each of the different clades, which may contribute to the observed ecological differences among species. Additionally, evaluating gene orthology between our isolates and other members of this group may provide further insight as to what makes these organisms successfully adapted to plants. We might also find key components that enable certain species to live as opportunistic pathogens.

3.4.3 Media-phenotype relationships and potential antibiotic target genes

From our basic genome analysis and query of antibiotic resistance databases it's presently difficult to assess specific genome-level features among the bacterial isolates to fully explain the observed media-phenotype relationship (Table 3.3.1, Table 3.3.3 and Table 3.3.4). Had we found a group of false positive isolates that were highly clonal, we might be able to predict that resistance and susceptibility to media antibiotics is conferred through mutations. For instance, mutations affecting the binding site of a protein target may limit the ability of an antibiotic agent to act on that particular protein (Tenover, 2006). Although this sort of resistance mechanism could still be true for our isolates, because we observed variation in gene content, presence or absence of unique genes may determine whether isolates are susceptible or resistant to antibiotics present in traditional FDA media. *Cellulomonas* spp. are defined as gram positive and have rod-shaped cellular morphology (Stackebrandt and Schumann, 2014). For certain gram-positive rod-shaped bacteria such as *L. monocytogenes* and *Corneybacterium* spp., multidrug resistance is intrinsic to these organisms (Baquero, 1997; Otsuka et al., 2006). The

annotated gene models of the false positive *Cellulomonas* isolates recovered several multidrug resistance genes. Therefore it is possible that multidrug resistance may also play a role in mediating survival of *Cellulomonas* isolates during selective enrichment and plating. However, even in light of possessing these genes, some isolates still displayed susceptibility towards certain media.

Using an orthology-based approach we identified one antimicrobial target gene, translation elongation factor G, which was present in all 13 isolates (Table 3.3.5). One notable antibiotic agent that has activity towards this target gene in gram-positive bacteria is fusidic acid. A recent investigation testing the antimicrobial activity of fusidic acid on *L. monocytogenes* found the majority of strains were resistant to this drug (Conter et al., 2009). Going forward we can now test fusidic acid to determine whether this antibiotic is effective against this collection of *Cellulomonas* isolates. If this approach is successful, we could sequence the genomes of all false positive isolates recovered in Chapter 2 of this thesis to identify a set of conserved antibiotic target genes and develop a highly selective medium to eliminate these microbial contaminants from BLEB enriched Romaine lettuce samples.

3.5 References

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APPENDICES

Appendix A Isolates used in this thesis collected from BLEB enriched lettuce samples on MOX, B-OXA, OXA, LPM and PALCAM

Table A 1

Isolate	v3-v6 GAST taxonomy (genus)
MOX1E1T48.130322	*Isoptericola*
MOX2E1T48.130322	*Curtobacterium*
MOX3E1T48.130322	*Curtobacterium*
MOX4E1T48.130322	*Microbacterium*
MOX5E1T48.130322	*Curtobacterium*
MOX6E2T48.130329	*Microbacterium*
MOX7E2T48.130329	*Microbacterium*
MOX8E2T48.130329	*Curtobacterium*
MOX9E2T48.130329	*Microbacterium*
MOX10E2T48.130329	*Microbacterium*
MOX11E3T48.130403	*Curtobacterium*
MOX12E3T48.130403	*Curtobacterium*
MOX13E3T48.130403	*Cellulomonas*
MOX14E3T48.130403	*Cellulomonas*
MOX15E3T48.130403	*Cellulomonas*
MOX16E4T48.130405	*Curtobacterium*
MOX17E4T48.130405	*Curtobacterium*
MOX18E4T48.130405	*Curtobacterium*
MOX19E4T48.130405	*Microbacterium*
MOX20E4T48.130405	*Microbacterium*
MOX21E5T48.130410	*Cellulomonas*
MOX22E5T48.130410	*Microbacterium*
MOX23E5T48.130410	*Cellulomonas*
MOX24E5T48.130410	*Curtobacterium*
MOX25E5T48.130410	*Microbacterium*
MOX26E6T48.130417	*Curtobacterium*
MOX27E6T48.130417	*Curtobacterium*
MOX28E6T48.130417	*Curtobacterium*
MOX29E6T48.130417	*Curtobacterium*
MOX30E6T48.130417	*Curtobacterium*
MOX31E8T48.130426	*Cellulomonas*
MOX32E8T48.130426	*Cellulomonas*
MOX34E8T48.130426	*Cellulomonas*
MOX35E8T48.130426	*Cellulomonas*
MOX36E10T48.130506	*Cellulomonas*

Table A 1 Continued

Isolate	v3-v6 GAST taxonomy (genus)
MOX37E10T48.130506	*Sanguibacter*
MOX38E10T48.130506	*Sanguibacter*
MOX40E10T48.130506	*Sanguibacter*
MOX46E12T24.130510	*Microbacterium*
MOX47E12T24.130510	*Curtobacterium*
MOX48E12T24.130510	*Microbacterium*
MOX49E12T24.130510	*Curtobacterium*
MOX50E12T24.130510	*Curtobacterium*
MOX51E13T48.130515	*Listeria*
MOX52E13T48.130515	*Listeria*
MOX53E13T48.130515	*Listeria*
MOX54E13T48.130515	*Listeria*
MOX55E13T48.130515	*Listeria*
MOX56E17T48.130610	*Pseudoclavibacter*
MOX57E17T48.130610	*Microbacterium*
MOX58E17T48.130610	*Psuedoclavibacteri*
MOX59E17T48.130610	*Curtobacterium*
MOX60E17T48.130610	*Sanguibacter*
MOX61E18T48.130612	*Marinilactibacillus*
MOX62E18T48.130612	*Marinilactibacillus*
MOX63E18T48.130612	*Marinilactibacillus*
MOX64E18T48.130612	*Marinilactibacillus*
MOX65E18T48.130612	*Marinilactibacillus*
MOX66E19T24.130617	*Curtobacterium*
MOX68E19T24.130617	*Curtobacterium*
MOX69E19T24.130617	*Curtobacterium*
MOX70E19T24.130617	*Curtobacterium*
MOX71E19T48.130617	*Curtobacterium*
MOX72E19T48.130617	*Microbacterium*
MOX73E19T48.130617	*Microbacterium*
MOX74E19T48.130617	*Microbacterium*
MOX75E19T48.130617	*Microbacterium*
MOX76E20T24.130716	*Enterococcus*
MOX77E20T24.130716	*Curtobacterium*
MOX78E20T24.130716	*Curtobacterium*
MOX80E20T24.130716	*Curtobacterium*
MOX81E20T48.130716	*Curtobacterium*
MOX82E20T48.130716	*Microbacterium*
MOX83E20T48.130716	*Curtobacterium*

Table A 1 Continued

Isolate	v3-v6 GAST taxonomy (genus)
MOX84E20T48.130716	*Microbacterium*
MOX85E20T48.130716	*Curtobacterium*
MOX86E21T24.130806	*Curtobacterium*
MOX88E21T24.130806	*Curtobacterium*
MOX89E21T24.130806	*Curtobacterium*
MOX90E21T24.130806	*Curtobacterium*
MOX91E21T48.130806	*Curtobacterium*
MOX92E21T48.130806	*Curtobacterium*
MOX93E21T48.130806	*Microbacterium*
MOX94E21T48.130806	*Curtobacterium*
MOX95E21T48.130806	*Curtobacterium*
MOX96E22T24.130816	*Curtobacterium*
MOX97E22T24.130816	*Microbacterium*
MOX98E22T24.130816	*Curtobacterium*
MOX99E22T24.130816	*Microbacterium*
MOX101E22T48.130816	*Listeria*
MOX102E22T48.130816	*Curtobacterium*
MOX103E22T48.130816	*Microbacterium*
MOX104E22T48.130816	*Curtobacterium*
MOX105E22T48.130816	*Listeria*
B-OXA1E1T48.130322	*Cellulomonas*
B-OXA3E1T48.130322	*Microbacterium*
B-OXA4E1T48.130322	*Microbacterium*
B-OXA5E1T48.130322	*Microbacterium*
B-OXA6E2T48.130329	*Microbacterium*
B-OXA7E2T48.130329	*Microbacterium*
B-OXA8E2T48.130329	*Microbacterium*
B-OXA9E2T48.130329	*Microbacterium*
B-OXA10E2T48.130329	*Microbacterium*
B-OXA11E3T48.130403	*Cellulomonas*
B-OXA12E3T48.130403	*Cellulomonas*
B-OXA13E3T48.130403	*Cellulomonas*
B-OXA15E3T48.130403	*Cellulomonas*
B-OXA16E4T48.130405	*Microbacterium*
B-OXA17E4T48.130405	*Bacillus*
B-OXA19E4T48.130405	*Cellulomonas*
B-OXA20E4T48.130405	*Sanguibacter*
B-OXA21E5T48.130410	*Bacillus*
B-OXA22E5T48.130410	*Sanguibacter*

Table A 1 Continued

Isolate	v3-v6 GAST taxonomy (genus)
B-OXA23E5T48.130410	*Bacillus*
B-OXA25E5T48.130410	*Sanguibacter*
B-OXA26E6T48.130417	*Cellulomonas*
B-OXA27E6T48.130417	*Cellulomonas*
B-OXA29E6T48.130417	*Cellulomonas*
B-OXA30E6T48.130417	*Marinilactibacillus*
B-OXA31E7T48.130419	*Sanguibacter*
B-OXA32E7T48.130419	*Sanguibacter*
B-OXA33E7T48.130419	*Sanguibacter*
B-OXA34E7T48.130419	*Sanguibacter*
B-OXA35E7T48.130419	*Sanguibacter*
B-OXA36E8T48.130426	*Cellulomonas*
B-OXA37E8T48.130426	*Cellulomonas*
B-OXA38E8T48.130426	*Cellulomonas*
B-OXA39E8T48.130426	*Cellulomonas*
B-OXA40E8T48.130426	*Sanguibacter*
B-OXA41E9T48.130501	*Sanguibacter*
B-OXA42E9T48.130501	*Cellulomonas*
B-OXA43E9T48.130501	*Sanguibacter*
B-OXA44E9T48.130501	*Sanguibacter*
B-OXA45E9T48.130501	*Microbacterium*
B-OXA46E10T48.130506	*Sanguibacter*
B-OXA47E10T48.130506	*Sanguibacter*
B-OXA48E10T48.130506	*Sanguibacter*
B-OXA49E10T48.130506	*Sanguibacter*
B-OXA50E10T48.130506	*Sanguibacter*
B-OXA51E11T48.130508	*Bacillus*
B-OXA52E11T48.130508	*Serratia*
B-OXA53E11T48.130508	*Serratia*
B-OXA54E11T48.130508	*Bacillus*
B-OXA55E11T48.130508	*Serratia*
B-OXA56E12T24.130510	*Sanguibacter*
B-OXA57E12T24.130510	*Sanguibacter*
B-OXA58E12T24.130510	*Sanguibacter*
B-OXA60E12T24.130510	*Bacillus*
B-OXA63E13T24.130515	*Sanguibacter*
B-OXA64E13T24.130515	*Sanguibacter*
B-OXA66E13T48.130515	*Listeria*
B-OXA67E13T48.130515	*Listeria*

Table A 1 Continued

Isolate	v3-v6 GAST taxonomy (genus)
B-OXA68E13T48.130515	*Sanguibacter*
B-OXA69E13T48.130515	*Listeria*
B-OXA70E13T48.130515	*Listeria*
B-OXA71E14T24.130517	*Rothia*
B-OXA72E14T24.130517	*Sanguibacter*
B-OXA74E14T24.130517	*Rothia*
B-OXA75E14T24.130517	*Rothia*
B-OXA77E14T48.130517	*Rothia*
B-OXA78E14T48.130517	*Rothia*
B-OXA79E14T48.130517	*Rothia*
B-OXA80E14T48.130517	*Rothia*
B-OXA81E15T24.130603	*Microbacterium*
B-OXA82E15T24.130603	*Staphylococcus*
B-OXA83E15T24.130603	*Microbacterium*
B-OXA84E15T24.130603	*Microbacterium*
B-OXA85E15T24.130603	*Microbacterium*
B-OXA86E15T48.130603	*Staphylococcus*
B-OXA87E15T48.130603	*Staphylococcus*
B-OXA88E15T48.130603	*Staphylococcus*
B-OXA89E15T48.130603	*Staphylococcus*
B-OXA90E15T48.130603	*Microbacterium*
B-OXA91E16T48.130605	*Staphylococcus*
B-OXA92E16T48.130605	*Staphylococcus*
B-OXA93E16T48.130605	*Staphylococcus*
B-OXA94E16T48.130605	*Staphylococcus*
B-OXA95E16T48.130605	*Staphylococcus*
B-OXA96E20T24.130716	*Bacillus*
B-OXA97E20T24.130716	*Microbacterium*
B-OXA98E20T24.130716	*Microbacterium*
B-OXA99E20T24.130716	*Microbacterium*
B-OXA100E20T24.130716	*Curtobacterium*
B-OXA101E20T48.130716	*Microbacterium*
B-OXA102E20T48.130716	*Microbacterium*
B-OXA103E20T48.130716	*Sanguibacter*
B-OXA104E20T48.130716	*Microbacterium*
B-OXA106E21T24.130806	*Curtobacterium*
B-OXA107E21T24.130806	*Microbacterium*
B-OXA108E21T24.130806	*Curtobacterium*
B-OXA109E21T24.130806	*Microbacterium*

Table A 1 Continued

Isolate	v3-v6 GAST taxonomy (genus)
B-OXA110E21T24.130806	*Curtobacterium*
B-OXA112E21T48.130806	*Curtobacterium*
B-OXA113E21T48.130806	*Microbacterium*
B-OXA114E21T48.130806	*Staphylococcus*
B-OXA115E21T48.130806	*Staphylococcus*
B-OXA116E22T24.130816	*Microbacterium*
B-OXA119E22T24.130816	*Microbacterium*
B-OXA120E22T24.130816	*Microbacterium*
PALCAM1E1T48.130322	*Microbacterium*
PALCAM2E1T48.130322	*Microbacterium*
PALCAM3E1T48.130322	*Microbacterium*
PALCAM5E1T48.130322	*Microbacterium*
PALCAM6E2T48.130329	*Bacillus*
PALCAM7E2T48.130329	*Microbacterium*
PALCAM8E2T48.130329	*Microbacterium*
PALCAM9E2T48.130329	*Microbacterium*
PALCAM10E2T48.130329	*Microbacterium*
PALCAM11E3T48.130403	*Microbacterium*
PALCAM12E3T48.130403	*Microbacterium*
PALCAM13E3T48.130403	*Cellulomonas*
PALCAM14E3T48.130403	*Cellulomonas*
PALCAM15E3T48.130403	*Cellulomonas*
PALCAM16E4T48.130405	*Microbacterium*
PALCAM17E4T48.130405	*Microbacterium*
PALCAM18E4T48.130405	*Cellulomonas*
PALCAM19E4T48.130405	*Microbacterium*
PALCAM20E4T48.130405	*Cellulomonas*
PALCAM21E5T48.130410	*Cellulomonas*
PALCAM22E5T48.130410	*Microbacterium*
PALCAM23E5T48.130410	*Microbacterium*
PALCAM24E5T48.130410	*Cellulomonas*
PALCAM25E5T48.130410	*Microbacterium*
PALCAM26E6T48.130417	*Cellulomonas*
PALCAM27E6T48.130417	*Cellulomonas*
PALCAM28E6T48.130417	*Cellulomonas*
PALCAM30E6T48.130417	*Cellulomonas*
PALCAM31E8T48.130426	*Cellulomonas*
PALCAM32E8T48.130426	*Cellulomonas*
PALCAM33E8T48.130426	*Cellulomonas*

Table A 1 Continued

Isolate	v3-v6 GAST taxonomy (genus)
PALCAM34E8T48.130426	*Cellulomonas*
PALCAM35E8T48.130426	*Cellulomonas*
PALCAM36E10T48.130506	*Sanguibacter*
PALCAM37E10T48.130506	*Cellulomonas*
PALCAM38E10T48.130506	*Sanguibacter*
PALCAM39E10T48.130506	*Sanguibacter*
PALCAM40E10T48.130506	*Sanguibacter*
PALCAM42E12T48.130510	*Cellulomonas*
PALCAM43E12T48.130510	*Cellulomonas*
PALCAM44E12T48.130510	*Cellulomonas*
PALCAM45E12T48.130510	*Cellulomonas*
PALCAM46E13T48.130515	*Listeria*
PALCAM47E13T48.130515	*Listeria*
PALCAM48E13T48.130515	*Listeria*
PALCAM49E13T48.130515	*Listeria*
PALCAM50E13T48.130515	*Listeria*
PALCAM51E14T24.130517	*Rothia*
PALCAM52E14T24.130517	*Rothia*
PALCAM53E14T24.130517	*Rothia*
PALCAM54E14T24.130517	*Rothia*
PALCAM55E14T24.130517	*Rothia*
PALCAM56E14T48.130517	*Rothia*
PALCAM57E14T48.130517	*Rothia*
PALCAM58E14T48.130517	*Rothia*
PALCAM59E14T48.130517	*Rothia*
PALCAM60E14T48.130517	*Rothia*
PALCAM66E15T48.130603	*Microbacterium*
PALCAM67E15T48.130603	*Microbacterium*
PALCAM68E15T48.130603	*Microbacterium*
PALCAM69E15T48.130603	*Microbacterium*
PALCAM70E15T48.130603	*Microbacterium*
PALCAM72E16T48.130605	*Rothia*
PALCAM73E16T48.130605	*Rothia*
PALCAM74E16T48.130605	*Microbacterium*
PALCAM75E16T48.130605	*Rothia*
PALCAM78E17T24.130610	*Arthrobacter*
PALCAM79E17T24.130610	*Microbacterium*
PALCAM80E17T24.130610	*Microbacterium*
PALCAM86E18T24.130612	*Microbacterium*

Table A 1 Continued

Isolate	v3-v6 GAST taxonomy (genus)
PALCAM88E18T24.130612	*Microbacterium*
PALCAM89E18T24.130612	*Rothia*
PALCAM90E18T24.130612	*Microbacterium*
PALCAM91E20T24.130716	*Microbacterium*
PALCAM92E20T24.130716	*Microbacterium*
PALCAM93E20T24.130716	*Microbacterium*
PALCAM94E20T24.130716	*Microbacterium*
PALCAM95E20T24.130716	*Microbacterium*
PALCAM97E22T48.130806	*Microbacterium*
PALCAM98E22T48.130806	*Microbacterium*
PALCAM101E44T24.141016	*Curtobacterium*
PALCAM102E44T24.141016	*Curtobacterium*
PALCAM103E44T24.141016	*Curtobacterium*
PALCAM104E44T24.141016	*Curtobacterium*
PALCAM105E44T24.141016	*Curtobacterium*
PALCAM106E42T48.141016	*Cellulomonas*
PALCAM107E42T48.141016	*Cellulomonas*
PALCAM108E42T48.141016	*Cellulomonas*
PALCAM109E42T48.141016	*Cellulomonas*
PALCAM110E42T48.141016	*Cellulomonas*
LPM1E1T48.130322	*Curtobacterium*
LPM2E1T48.130322	*Curtobacterium*
LPM3E1T48.130322	*Curtobacterium*
LPM4E1T48.130322	*Curtobacterium*
LPM5E1T48.130322	*Curtobacterium*
LPM6E2T48.130329	*Enterococcus*
LPM7E2T48.130329	*Enterococcus*
LPM8E2T48.130329	*Enterococcus*
LPM9E2T48.130329	*Enterococcus*
LPM10E2T48.130329	*Enterococcus*
LPM11E3T48.130403	*Cellulomonas*
LPM13E3T48.130403	*Vagococcus*
LPM14E3T48.130403	*Curtobacterium*
LPM15E3T48.130403	*Cellulomonas*
LPM16E4T48.130405	*Curtobacterium*
LPM17E4T48.130405	*Curtobacterium*
LPM18E4T48.130405	*Curtobacterium*
LPM19E4T48.130405	*Curtobacterium*
LPM20E4T48.130405	*Curtobacterium*

Table A 1 Continued

Isolate	v3-v6 GAST taxonomy (genus)
LPM21E5T48.130410	*Enterococcus*
LPM22E5T48.130410	*Enterococcus*
LPM23E5T48.130410	*Enterococcus*
LPM24E5T48.130410	*Leuconostoc*
LPM25E5T48.130410	*Enterococcus*
LPM26E6T48.130417	*Leuconostoc*
LPM27E6T48.130417	*Weissella*
LPM28E6T48.130417	*Leuconostoc*
LPM29E6T48.130417	*Leuconostoc*
LPM30E6T48.130417	*Leuconostoc*
LPM32E7T48.130419	*Leuconostoc*
LPM33E7T48.130419	*Leuconostoc*
LPM35E7T48.130419	*Leuconostoc*
LPM36E8T48.130426	*Weissella*
LPM37E8T48.130426	*Weissella*
LPM38E8T48.130426	*Weissella*
LPM39E8T48.130426	*Weissella*
LPM40E8T48.130426	*Weissella*
LPM41E10T48.130506	*Curtobacterium*
LPM42E10T48.130506	*Weissella*
LPM43E10T48.130506	*Curtobacterium*
LPM44E10T48.130506	*Curtobacterium*
LPM45E10T48.130506	*Curtobacterium*
LPM46E11T48.130508	*Weissella*
LPM48E11T48.130508	*Weissella*
LPM50E11T48.130508	*Weissella*
LPM51E12T24.130510	*Curtobacterium*
LPM52E12T24.130510	*Curtobacterium*
LPM53E12T24.130510	*Curtobacterium*
LPM54E12T24.130510	*Curtobacterium*
LPM55E12T24.130510	*Curtobacterium*
LPM56E13T24.130515	*Curtobacterium*
LPM57E13T24.130515	*Weissella*
LPM58E13T24.130515	*Weissella*
LPM59E13T24.130515	*Curtobacterium*
LPM67E16T48.130605	*Leuconostoc*
LPM68E16T48.130605	*Leuconostoc*
LPM69E16T48.130605	*Leuconostoc*
LPM70E16T48.130605	*Weissella*
LPM71E17T48.130610	*Curtobacterium*

Table A 1 Continued

Isolate	v3-v6 GAST taxonomy (genus)
LPM72E17T48.130610	*Curtobacterium*
LPM73E17T48.130610	*Curtobacterium*
LPM74E17T48.130610	*Curtobacterium*
LPM75E17T48.130610	*Curtobacterium*
LPM76E18T48.130612	*Enterococcus*
LPM77E18T48.130612	*Enterococcus*
LPM78E18T48.130612	*Enterococcus*
LPM79E18T48.130612	*Enterococcus*
LPM80E18T48.130612	*Enterococcus*
LPM82E19T24.130617	*Leuconostoc*
LPM83E19T24.130617	*Leuconostoc*
LPM85E19T24.130617	*Leuconostoc*
LPM88E19T48.130617	*Vagococcus*
LPM89E19T48.130617	*Vagococcus*
LPM90E19T48.130617	*Weissella*
LPM91E20T24.130716	*Curtobacterium*
LPM92E20T24.130716	*Enterococcus*
LPM93E20T24.130716	*Enterococcus*
LPM94E20T24.130716	*Enterococcus*
LPM95E20T24.130716	*Enterococcus*
LPM96E20T48.130716	*Enterococcus*
LPM97E20T48.130716	*Enterococcus*
LPM98E20T48.130716	*Enterococcus*
LPM99E20T48.130716	*Enterococcus*
LPM100E20T48.130716	*Enterococcus*
LPM106E21T48.130806	*Curtobacterium*
LPM107E21T48.130806	*Weissella*
LPM108E21T48.130806	*Weissella*
LPM109E21T48.130806	*Weissella*
LPM110E21T48.130806	*Curtobacterium*
LPM111E22T24.130816	*Curtobacterium*
LPM112E22T24.130816	*Curtobacterium*
LPM113E22T24.130816	*Curtobacterium*
LPM114E22T24.130816	*Curtobacterium*
LPM116E22T48.130816	*Curtobacterium*
LPM117E22T48.130816	*Listeria*
LPM118E22T48.130816	*Listeria*
LPM119E22T48.130816	*Listeria*
LPM120E22T48.130816	*Listeria*
OXA1E24T24.140718	*Curtobacterium*

Table A 1 Continued

Isolate	v3-v6 GAST taxonomy (genus)
OXA2E24T24.140718	*Curtobacterium*
OXA3E24T24.140718	*Curtobacterium*
OXA4E24T24.140718	*Curtobacterium*
OXA5E24T24.140718	*Microbacterium*
OXA6E25T24.140718	*Curtobacterium*
OXA7E25T24.140718	*Curtobacterium*
OXA8E25T24.140718	*Curtobacterium*
OXA9E25T24.140718	*Curtobacterium*
OXA10E25T24.140718	*Curtobacterium*
OXA16E27T24.140721	*Bacillus*
OXA18E27T24.140721	*Bacillus*
OXA19E27T24.140721	*Bacillus*
OXA21E26T24.140721	*Microbacterium*
OXA22E26T24.140721	*Microbacterium*
OXA23E26T24.140721	*Microbacterium*
OXA24E26T24.140721	*Microbacterium*
OXA25E26T24.140721	*Microbacterium*
OXA26E27T48.140721	*Bacillus*
OXA27E27T48.140721	*Bacillus*
OXA28E27T48.140721	*Bacillus*
OXA29E27T48.140721	*Bacillus*
OXA31E26T48.140721	*Microbacterium*
OXA32E26T48.140721	*Microbacterium*
OXA33E26T48.140721	*Microbacterium*
OXA34E26T48.140721	*Cellulomonas*
OXA35E26T48.140721	*Microbacterium*
OXA36E28T48.140723	*Microbacterium*
OXA38E28T48.140723	*Microbacterium*
OXA39E28T48.140723	*Microbacterium*
OXA40E28T48.140723	*Microbacterium*
OXA41E29T24.140724	*Curtobacterium*
OXA42E29T24.140724	*Bacillus*
OXA44E29T24.140724	*Curtobacterium*
OXA45E29T24.140724	*Curtobacterium*
OXA46E29T48.140724	*Bacillus*
OXA47E29T48.140724	*Bacillus*
OXA48E29T48.140724	*Bacillus*
OXA49E29T48.140724	*Bacillus*
OXA51E30T24.140724	*Microbacterium*
OXA52E30T24.140724	*Microbacterium*

Table A 1 Continued

Isolate	v3-v6 GAST taxonomy (genus)
OXA53E30T24.140724	*Microbacterium*
OXA54E30T24.140724	*Microbacterium*
OXA55E30T24.140724	*Microbacterium*
OXA56E30T48.140724	*Cellulomonas*
OXA57E30T48.140724	*Cellulomonas*
OXA58E30T48.140724	*Cellulomonas*
OXA59E30T48.140724	*Cellulomonas*
OXA60E30T48.140724	*Cellulomonas*
OXA61E31T24.140728	*Microbacterium*
OXA62E31T24.140728	*Microbacterium*
OXA63E31T24.140728	*Microbacterium*
OXA64E31T24.140728	*Microbacterium*
OXA65E31T24.140728	*Microbacterium*
OXA67E32T24.140728	*Microbacterium*
OXA68E32T24.140728	*Cellulomonas*
OXA69E32T24.140728	*Microbacterium*
OXA70E32T24.140728	*Microbacterium*
OXA71E31T48.140728	*Microbacterium*
OXA72E31T48.140728	*Microbacterium*
OXA73E31T48.140728	*Microbacterium*
OXA74E31T48.140728	*Microbacterium*
OXA75E31T48.140728	*Microbacterium*
OXA76E32T48.140728	*Microbacterium*
OXA77E32T48.140728	*Cellulomonas*
OXA78E32T48.140728	*Cellulomonas*
OXA79E32T48.140728	*Cellulomonas*
OXA80E32T48.140728	*Cellulomonas*
OXA81E33T48.140730	*Listeria*
OXA82E33T48.140730	*Listeria*
OXA83E33T48.140730	*Listeria*
OXA84E33T48.140730	*Listeria*
OXA85E33T48.140730	*Listeria*
OXA86E36T24.141016	*Curtobacterium*
OXA87E36T24.140804	*Microbacterium*
OXA88E36T24.140804	*Cellulomonas*
OXA90E36T24.140804	*Curtobacterium*
OXA91E36T48.140804	*Bacillus*
OXA92E36T48.140804	*Bacillus*
OXA93E36T48.140804	*Bacillus*
OXA94E36T48.140804	*Bacillus*

Table A 1 Continued

Isolate	v3-v6 GAST taxonomy (genus)
OXA95E36T48.140804	*Bacillus*
OXA96E35T48.140804	*Cellulomonas*
OXA99E35T48.140804	*Cellulomonas*
OXA100E35T48.140804	*Cellulomonas*
OXA101E37T24.140813	*Microbacterium*
OXA102E37T24.140813	*Microbacterium*
OXA103E37T24.140813	*Microbacterium*
OXA104E37T24.140813	*Microbacterium*
OXA105E37T24.140813	*Microbacterium*
OXA106E38T24.140813	*Listeria*
OXA107E38T24.140813	*Listeria*
OXA108E38T24.140813	*Listeria*
OXA109E38T24.140813	*Listeria*
OXA110E38T24.140813	*Listeria*
OXA113E37T48.140813	*Microbacterium*
OXA114E37T48.140813	*Microbacterium*
OXA115E37T48.140813	*Cellulomonas*
OXA116E38T48.140813	*Listeria*
OXA117E38T48.140813	*Listeria*
OXA118E38T48.140813	*Listeria*
OXA119E38T48.140813	*Listeria*
OXA126E40T24.140817	*Microbacterium*
OXA127E40T24.140817	*Staphylococcus*
OXA136E39T48.140817	*Staphylococcus*
OXA139E39T48.140817	*Cellulomonas*
OXA142E40T48.140817	*Staphylococcus*
OXA143E40T48.140817	*Staphylococcus*

Appendix B Type strains used in 16S rRNA phylogenic analysis

Table B 1

Type strains used in 16S rRNA phylogenic analysis
Cellulomonas uda DSM 20107 (X83801)
Cellulomonas gelida DSM 20111 (X83800)
Cellulomonas iranensis O (AF064702)
Cellulomonas composti TR7-06 (AB166887)
Cellulomonas persica I (AF064701)
Cellulomonas flavigena DSM 20109 (CP001964)
Cellulomonas phragmiteti KB23 (AM902253)
Cellulomonas soli Kc1 (AB602498)
Cellulomonas cellasea DSM 20118 (X83804)
Cellulomonas chitinilytica X.bu-b (AB268586)
Cellulomonas fimi ATCC 484 (CP002666)
Cellulomonas biazotea DSM 20112 (X83802)
Cellulomonas oligotrophica Kc5 (AB602499)
Cellulomonas terrae DB5 (AY884570)
Cellulomonas xylanilytica XIL11 (AY303668)
Cellulomonas humilata ATCC 25174 (X82449)
Cellulomonas aerilata 5420S-23 (EU560979)
Cellulomonas marina FXJ8.089 (JF346422)
Cellulomonas pakistanensis NCCP-11 (AB618146)
Cellulomonas hominis DMMZ CE40 (X82598)
Cellulomonas denverensis W6929 (AY501362)
Cellulomonas carbonis T26 (HQ702749)
Cellulomonas bogoriensis 69B4 (X92152)
Cellulosimicrobium cellulans DSM 43879 (X83809)

Appendix C Genomes used in multilocus phylogenetic analysis

Table C 1

Organism	NCBI Genome Accession
Cellulomonas fimi ATCC 484	CP002666.1
Cellulomonas flavigena DSM 20109	CP001964.1
Cellulomonas gilvis ATCC 13127	CP002665.1
Bacillus subtilis strain 168	CP010052.1
Clavibacter michiganensis subsp sepedonicus	NC 010407.1
Jonesia denitrificans DSM 20603	CP001706.1
Micrococcus luteus NCTC 2665	CP001628.1
Sanguibacter kideii DSM 10542	CP001819.1
Xylanimonas cellulosilytica DSM 15894	CP001821.1